

Biology, one molecule at a time

Achillefs N. Kapanidis¹ and Terence Strick²

¹ Department of Physics and Interdisciplinary Research Collaboration in Bionanotechnology, Clarendon Laboratory, University of Oxford, Parks Road, Oxford, OX1 3PU, UK

² Institut Jacques Monod, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7592 and University of Paris 7 – Paris Diderot, 4 rue M.-A. Lagroua Weill-Halle, 75205 Paris Cedex 13, France

Single-molecule techniques have moved from being a fascinating curiosity to a highlight of life science research. The single-molecule approach to biology offers distinct advantages over the conventional approach of taking bulk measurements; this additional information content usually comes at the cost of the additional complexity. Popular single-molecule methods include optical and magnetic tweezers, atomic force microscopy, tethered particle motion and single-molecule fluorescence spectroscopy; the complement of these methods offers a wide range of spatial and temporal capabilities. These approaches have been instrumental in addressing important biological questions in diverse areas such as protein–DNA interactions, protein folding and the function(s) of membrane proteins.

The era of single-molecule biophysics

New instruments and methods that help us to visualize microscopic worlds have often fuelled revolutionary advances in our understanding of nature. From the discovery of ‘cells’ and ‘animalcules’ by Robert Hooke and Antony van Leeuwenhoek by means of the first light microscopes, to the first electron micrograph of intact animal cells by Albert Claude and his colleagues, to the beautiful protein structures elucidated by X-ray crystallography, new methods have opened doors to intriguing new domains, enabling longstanding questions to be answered by direct observation.

The new methods that created much excitement in biology during the past decade belong to the family of single-molecule techniques. As with many standard methods in biosciences, most single-molecule methods were developed in physics or biophysics laboratories and subsequently became compatible with biological samples. Starting with patch-clamp and single ion-channel recordings in 1976 [1], this family grew considerably to include atomic force microscopy (AFM), optical and magnetic tweezers and single-molecule fluorescence spectroscopy. These methods have made contributions to many fields, including protein folding [2–6], transcription [7–10], replication [11,12], translation [13–15], molecular motors [16–19], membrane proteins [20] and viral biology [21], among others. In these settings, single-molecule methods either answered longstanding questions or discovered unexpected and biologically important behaviors, even in supposedly well-understood biomolecules. They also helped to shift the mindset of many biochemists away from the averaging of ensembles and the gigantic numbers

represented by moles (common in chemistry and biology) to the intuitive and unitary concept of single molecules and particles (common in physics). There are several excellent reviews on single-molecule methods [22–24] and a recent book containing detailed protocols for building instrumentation and preparing samples for single-molecule studies [25].

Even with these advances, the general consensus is that a barrier remains between the developers of single-molecule techniques and life scientists, with the latter realizing the potential of these techniques but being unsure about their use and wanting to familiarize themselves with this new technology. This review discusses instances in which a life scientist should consider using single-molecule methods, which method(s) might be most appropriate for the problem at hand, how to initiate single-molecule studies and what developments to look for in the near future.

Biomolecular heterogeneity: the single-molecule approach

What sets single-molecule methods apart from conventional ensemble-based studies? Is there new information to be mined from the single-molecule approach, or is it just an expensive and complex way to confirm existing knowledge?

A striking feature of single-molecule methods is that they report the distribution of values for a given property – and not just the mean value of the property averaged over a large molecular ensemble (Figure 1; Box 1). Such a distribution is always a more complete description of the system, given that it provides information not only on the mean behavior but also on the likelihood of fluctuations about the mean behavior; these fluctuations characterize the underlying statistical nature of the property. In addition, these distributions provide direct access to molecular heterogeneity, an intrinsic feature of complex biomolecules and their functions, and can help to uncover the ‘static’ or ‘dynamic’ origin of such heterogeneity. But what distinguishes static heterogeneity from dynamic heterogeneity?

Static heterogeneity occurs when an ensemble of molecules contains subpopulations so stable that they do not interconvert over the timescale of the observation. A common example of static heterogeneity is the presence of inactive molecules. In ensemble studies, one needs to determine the fraction of active molecules. By contrast, in single-molecule assays, inactive molecules can be ignored because they do not yield (or obscure) an experimental signal. Single-molecule methods can also identify molecular

Corresponding author: Kapanidis, A.N. (a.kapanidis1@physics.ox.ac.uk).

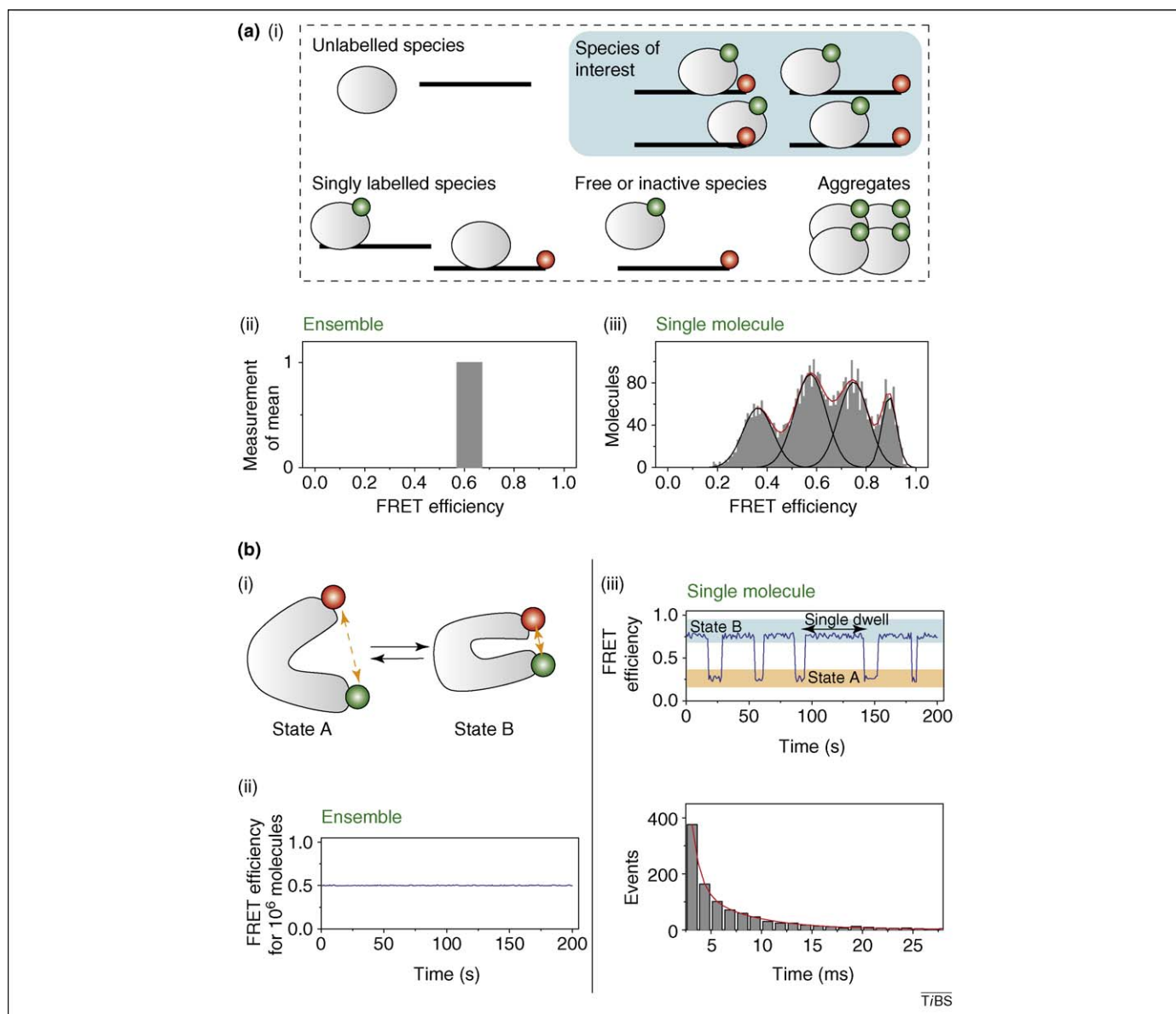


Figure 1. A comparison of ensemble and single-molecule methods. Ensemble averaging can hide the presence of heterogeneity in many important properties of a biological molecule. By contrast, the single-molecule approach can provide direct access to heterogeneous behaviors and monitor them directly to provide clues about the biomolecular dynamics, kinetics and mechanisms. **(a)** Static heterogeneity: (i) a large molecular ensemble contains several underlying subpopulations: free, inactive, unlabeled and aggregated components and doubly labeled species that constitute the species of interest; (ii) a single mean value (here, the mean FRET efficiency) characterizes the heterogeneous molecular ensemble; additional measurements and assumptions are needed to uncover the heterogeneity; (iii) a single-molecule approach enables 'zooming in' on the subpopulations of interest. **(b)** Dynamic heterogeneity: (i) a protein stochastically cycles between open (A) and a closed (B) conformational states; (ii) a mean value, constant over time, characterizes the FRET efficiency of a large molecular ensemble; (iii) monitoring a single molecule over time with adequate temporal resolution provides a direct observation of the states corresponding to each conformation. Statistical analysis of dwell times yields the lifetime of each conformation and recovers the equilibrium constant for the transition.

subpopulations with different affinities for their substrates or with different domain conformations (Figure 1a). A direct view of static heterogeneity enables one to focus on the species of interest (e.g. functional species, subpopulations) and remove free, inactive or aggregated species.

Dynamic heterogeneity occurs when a sample contains subpopulations of molecules that now can interconvert over the timescale of the observation. As an example, consider an enzyme that stochastically interconverts between two catalytically competent states, each characterized by a different affinity for a substrate (Figure 1b). If the interconversion is fast compared with the temporal resolution of the single-molecule method used, the observed

value will reflect a weighted time-average mean of the affinity of each subpopulation. If the interconversion is slow compared with the temporal resolution, one can directly observe interconversions between states (as in Figure 1b). Single-molecule methods can also provide structural (rather than catalytic) signatures that can probe conformational dynamics.

Dynamic heterogeneity is also present in enzymes that perform multi-step reactions on a substrate, with each step having a different reaction rate. Such a system is difficult to study using ensemble assays because, even with all molecules starting in the same state, the stochastic nature of individual steps will rapidly cause all molecules to react

Box 1. When can a single-molecule result be trusted?

As a first step, the time-averaged behavior of a single molecule should, in principle, be identical to the spatially averaged behavior of a large ensemble of molecules. Indeed, an ensemble measurement formally consists of taking an instantaneous 'snapshot' of the state of a large number of molecules. The average behavior of the ensemble is then obtained by summing over all of the molecules that have been frozen in place by the act of taking the snapshot; this is, therefore, known as a spatial average. By contrast, a single-molecule measurement formally consists of 'videotaping' an individual molecule as time passes. The average behavior of the single molecule is obtained by performing a temporal average over the entirety of the time trajectory for the single molecule. According to the so-called 'ergodic principle', and in agreement with common intuition, the spatial average of the ensemble and the temporal average of the individual molecule are formally identical provided that the time trajectory is long enough to enable the individual molecule to adequately sample all of its possible conformations. In practical terms, this means that the average behavior of a single molecule is a no less accurate description of the system than that derived from analysis of a large ensemble of molecules, provided that the single molecule data has been accumulated over sufficiently long timescales, typically at least 100 times the timescale of the basic phenomenon of interest. In fact, the explicit identification of molecular heterogeneity via the single-molecule approach often means that it provides a better description of the average behavior of the system than the ensemble approach.

in an asynchronous manner, blurring kinetic analysis. By contrast, single-molecule experiments can identify and analyze each step because they can track reactions in real-time 'movies' that display the kinetics of each step and the structure of intermediates. Because only one molecule is tracked, the problem of desynchronization is non-existent. Thus, the single-molecule approach recovers both mean reaction rates and the underlying statistical nature of the process and provides the opportunity for real-time observation of entire reaction pathways including rare or transient states and with multiple turnovers per single molecule. On a more practical note, single-molecule studies enable detailed analysis on minute amounts of precious samples, opening avenues for *in vivo* single-molecule imaging and ultrasensitive sensing.

Single-molecule versus ensemble approaches: kinetics, structures and forces

As the single-molecule approach differs considerably from the ensemble approach, one might legitimately wonder about the types of information that can be extracted from single-molecule measurements and the way this information is recorded and analyzed.

For kinetic studies of a reaction pathway, single-molecule methods do not provide smooth transitions from one mean value at time 0 to another mean value at time t , as would be expected, for example, for single-exponential kinetics. Rather, kinetic analysis uses dwell times of a single molecule at certain states along a pathway, which is a more intuitive and direct representation. To understand this concept better, one can consider a molecule that fluctuates between two structural states A and B (e.g. global conformational states that persist for milliseconds) tracked by a single-molecule observable (Figure 1b[iii], top). In the case of an ensemble experiment, the asynchronous and stochastic nature of the dynamic interconversion

completely hides the presence of dynamics. Hence, for a single-molecule assay with sufficient temporal resolution to observe the two states, one can measure the dwell time in each state and plot a frequency histogram of dwell times. For a single-step process, this histogram obeys a single-exponential decay law that yields one characteristic lifetime (Figure 1b[iii], bottom). Intriguingly, 2- or 3-step processes result in dwell-time histograms distinguishable from a single-step process; from these histograms it is possible to infer 'fast' steps indirectly [16,26]. Apart from conformational dynamics, dwell-time distributions are used to study enzyme-substrate interactions.

This example also illustrates the important ability of single-molecule approaches to identify distinct structural states of a biological macromolecule, even for transient or rare states. Although structural information from single molecules cannot rival that derived from X-ray crystallography, it is possible to obtain nanometer-scale information on key structural features. Considering that this information is time-resolved, one can envisage how single-molecule techniques can 'bridge' classic biochemical experiments and X-ray crystallography.

The use of force-based methods can also manipulate single molecules to interrogate and alter the energy landscape of reactions, thus directly probing elusive structural features such as transition states [4,27–29]. Such approaches bring to mind the proposals of Linus Pauling and Daniel Koshland [30] concerning the nature and origin of transition states in biomolecules: in the 1950s; Koshland proposed the 'induced-fit' mechanism for enzyme catalysis, suggesting that for a reaction to occur both enzyme and substrate undergo coupled conformational changes. These changes 'pre-stress' the substrate (by straining chemical bonds through mechanical flexing of the substrate), thus bringing it structurally and energetically closer to the transition state. In line with these proposals, single-molecule manipulation approaches apply gentle external forces to single molecules to mimic this molecular strain. Resulting changes in reaction rates can be directly measured from kinetic analysis. Using this approach, known as 'force spectroscopy', conformational changes during rate-limiting features of the interaction cycle can be studied and molecular deformations at the transition state can be measured [4]. The use of force spectroscopy has enabled the observation that complex and coupled conformational changes are particularly slow steps of reaction pathways [31,32] and has prompted special interest in understanding reaction mechanisms. Once the transition state forms, the relevant chemistry occurs rapidly. Single-molecule methods can identify and characterize central rate-limiting mechanical deformations in proteins and their substrates and yield structural insight into ephemeral conformations.

The caveat for studying individual reactions is that many such reactions should be observed and careful statistical analysis should first be performed before drawing conclusions (Box 1). A 'single-molecule' observation refers to observations of many discrete single molecules (either sequentially or in parallel). The exact number of accumulated events depends on many factors (e.g. statistical nature of underlying process, instrument resolution and

stability, reaction stability and implementation speed) and ranges from 25 to 50 events for slower methods (e.g. AFM or particle tracking) to hundreds or thousands with rapid methods (e.g. fluorescence). Multiplexing of single-molecule fluorescence detection in a microfluidic chip has been demonstrated for single-molecule DNA sequencing [33] and might provide a model for large-scale screening at single-molecule resolution. Nevertheless, it is useful to build 'self-regenerating' experiments, in which the same reaction can be observed multiple times during the same run in the absence of user intervention. An index of activity (e.g. catalytic rate, translocational capability) at the single-molecule level must also be measured to ensure that experimental conditions (e.g. surface immobilization or protein modification) do not perturb the activity. Deviations from the mean values might be instructive, reflecting shortcomings of either the ensemble or the single-molecule measurement. Most often, an experimental format that maintains a large fraction of active molecules is used and they are studied as subpopulations or as a function of time.

Main single-molecule methods

The arsenal of single-molecule methods continues to grow. After humble beginnings marked by the development of the first true single-molecule technique (single ion-channel recordings), various innovations pioneered by physicists and biologists were converted and modified to assemble the single-molecule toolbox. Different single-molecule methods afford different time resolutions (Table 1), from fractions of

a millisecond (for some fluorescence techniques) to seconds (for some AFM images). Transient states or interactions can be best captured with shorter time resolution. Different spans of observation are also possible (Table 1), ranging from hundreds of microseconds (for fluorescence detection of diffusing molecules) to hours (for tethered molecules). As the interaction is read in real time, it is even possible to adjust reaction conditions in real time, saving considerable time relative to the off-line analysis required in classic biochemistry.

There are two broad groups of single-molecule methods: force-based detection and manipulation (Figure 2), and fluorescence imaging and spectroscopy (Figure 3). A third family that combines force and fluorescence capabilities is also emerging. Each method has features that make it more appropriate for certain type of studies (Table 1).

Atomic force microscopy

AFM (Figure 2a) was first developed as a tool for topographical imaging of molecules on an atomically flat surface. Imaging is performed by scanning an ultra-sharp tip along the sample surface and measuring the tip deflection using a laser and a quadrant photodetector. Imaging can be performed on dry samples and samples in solution, although the temporal resolution of the latter is considerably worse. Because this approach requires substantial time to raster scan the surface and form an image (compared with, for example, single-molecule fluorescence imaging), early AFM studies focused on generating essentially static images of biomolecules. Recent improvements in the

Table 1. Capabilities and applications of the main single-molecule methods in biology

| Attributes | Force AFM | Optical tweezers | Magnetic tweezers | Fluorescence | | |
|--|--|---|---|--|---|--|
| | | | | FRET | Fluorescence intensity | Tracking and localization |
| Temporal resolution^a | 10 ms | 10 ms | 30 ms | 50 ms (TIRF) 1 ms (confocal) ns (populations) | 50 ms (TIRF) 1 ms (confocal) ns (populations) | 10 ms (TIRF) 1 ms (special probes) |
| Spatial resolution^a | 5 Å (routine) 1 Å (high end) | 1 nm (routine) 1 Å (high end) | 10 nm | 1–10 nm | 1–10 Å | 1–10 nm (localization) 50 nm (resolution) |
| Range of applied forces^a | 10–2000 pN | 1–200 pN | 0.01–200 pN | None | None | None |
| Main applications^b | Force-extension analysis [2] Transition-state analysis [20] Protein and nucleic acid folding and unfolding [2] Membrane protein dynamics [30] Static and/or dynamic structure of large complexes [20,34] | Force-extension analysis [3,28,29] Transition-state analysis [7,28,29] Protein and nucleic acid folding and unfolding [3,28,29] Motion of molecular motors [7] | Force-extension analysis [66] Transition-state analysis [33] Protein and nucleic acid folding and unfolding [66] Molecular interactions involving DNA or RNA [10] DNA topology [35] | Static and/or dynamic structure of small proteins and protein complexes [66,67] Timescales of protein dynamics [68] Timescales of DNA interactions involving or RNA dynamics [28,69] Molecular interactions involving DNA or RNA [70] Molecular interactions not involving DNA or RNA [71] | Membrane protein dynamics [72] Protein dynamics [73] Reaction kinetics [74] Measuring subunit [19] or binding stoichiometry [75] | Motion of molecular motors [76] Studies in living cells (e.g. diffusion, interactions) [77] |

^aNumbers quoted are the 'routine' value for that parameter under typical experimental conditions (AFM on protein chain at ~100 pN [1 p = 10⁻¹² N]; optical or magnetic trapping of DNA at ~20 pN). Temporal resolution, spatial resolution, force and tether stiffness are interrelated. As applied force increases, temporal and spatial resolutions improve. At any given force, temporal resolution must be sacrificed to improve spatial resolution, and vice versa. Typically, the 'high end' spatial values are obtained by decreasing temporal resolution to ~1 s.

^bThis table serves as a general guide for a large set of applications and chemistries and, as such, cannot list all of the applications in which the listed single-molecule techniques have been used.

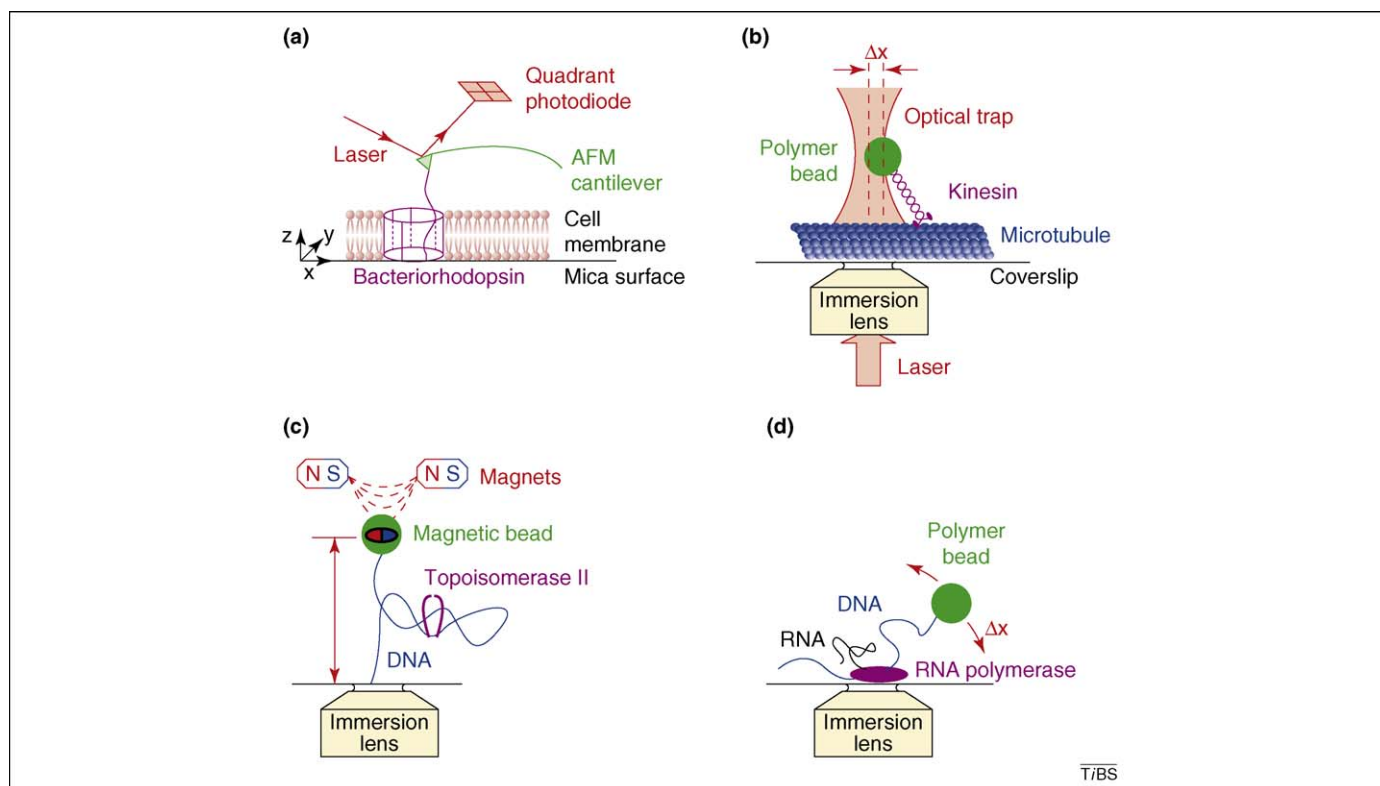


Figure 2. Commonly used single-molecule force-based methods. Force-based methods exert forces on a biomolecule and monitor the effect of force on the structure, dynamics and mechanism of the manipulated biomolecule. Such methods can also evaluate the effect of a second biomolecule on the conformation of the biomolecule that experiences the force in the first place, for example, see panel (c). In the case of the tethered particle motion method (d), the force exerted on the bead is due to thermal energy. **(a)** Atomic force microscopy (AFM). A small lever (AFM cantilever) with a sharp, \sim nm-scale tip is attached to one end of a biomolecule (here, bacteriorhodopsin). The surface to which the biomolecule is adsorbed can be scanned in the three spatial directions to \AA -scale resolution using piezoelectric positioning. The position of the tip is measured by deflecting a laser beam off its surface and onto a position-sensitive photodetector (quadrant photodiode). The lever is, in effect, a linear spring, and when the sample is moved relative to the tip the lever flexes and applies a force to the molecule. The high rigidity of the lever enables large forces to be applied; these are particularly useful for measuring the structural properties of folded proteins and chemical bonds. **(b)** Optical tweezers. A tightly focused infrared laser beam traps a micron-sized bead at its focal spot, enabling the position of the bead to be controlled. The trap acts like a spring, exerting a higher force on the bead the farther it is moved away from the laser axis. By measuring the position of the bead relative to the focal spot (Δx), the force applied by the trap can be determined; this is usually accomplished with a quadrant photodetector as in part (a). Either the trap or the surface can be displaced (the former using scanning mirrors, the latter using piezoelectric positioning). In the example shown, the force exerted by kinesin as it tracks along a microtubule is measured. **(c)** Magnetic tweezers. The magnetic field gradient generated by a pair of magnets imposes a constant vertical force on the micron-sized magnetic bead, extending the ~ 2 kb DNA tether away from the surface. The force can be increased or decreased by moving the magnets closer to or farther away from the sample. Rotating the magnets causes the magnetic bead to rotate in a synchronous manner, as would a compass needle in an external magnetic field, enabling the DNA to be quantitatively twisted and supercoiled. Tether extension (l) is measured using optical videomicroscopy to track the bead position in real time. In the example shown here, DNA disentangling by topoisomerase II causes DNA extension to increase as loops are topologically removed by the enzyme. A single catalytic turnover can be detected in this manner. **(d)** Tethered particle motion. This approach is as in part (b) or (c) but in the absence of a net external force. Shortening of the tether length causes the Brownian motion of the bead to decrease as observed using optical video microscopy. In this example, RNA polymerase reels in the DNA, causing the Brownian motion (Δx) of the tethered bead to become more restricted and enabling real-time measurement of the rate of elongation. Discontinuous changes in DNA extension (e.g. occurring during DNA looping, hybridization or bending) can also be detected. The spatiotemporal resolution depends on the tether length and bead size, ranging for a ~ 1 μm -size bead from \sim nm accuracy with ms time averaging (for ~ 100 bp DNA tether) to ~ 50 nm accuracy with second-scale time averaging (for 1 kb DNA tether). In the presence of applied force, as in parts (b) and (c), spatiotemporal resolution is dramatically increased (Table 1).

temporal (~ 100 ms per frame) [34] and spatial resolution (sub-nanometer) of AFM indicate that it also will be useful for real-time imaging of conformational changes on complex samples. In parallel, AFM has also been an extremely powerful tool for force spectroscopy of protein structure [2–4,29] and chemical bonds [30].

Optical tweezers

Optical tweezers were first used as an improvement to an earlier single-molecule technique, the tethered-particle motion assay [35]. In an optical tweezers setup (Figure 2b), a high-power infrared laser is focused tightly owing to a high-end microscope objective. Small glass or plastic beads are trapped at the focus, enabling the position of the bead to be imposed in the sample. The trap acts as a spring, generating a restoring force (a force that

brings the bead back to the trap center) that grows linearly with the bead distance from the trap center. Pulling the bead out of the trap is counteracted by the restoring force, which tends to move the bead back toward the trap center. Optical tweezers are used in two main modes: ‘constant force’ and ‘constant position’. In the constant-force mode, a feedback loop leads to displacement of either the optical trap or the sample coverslip surface to keep the position of the bead constant within the trapping zone and, hence, maintain a constant trap force. In the constant-position mode, the center of the trap position is held stationary and as the bead is pulled out of the trap it experiences a progressively growing force. Recent improvements in trap configuration have yielded ultra-stable instrumentation capable of resolving \AA -scale displacements of biomolecules as they interact [7].

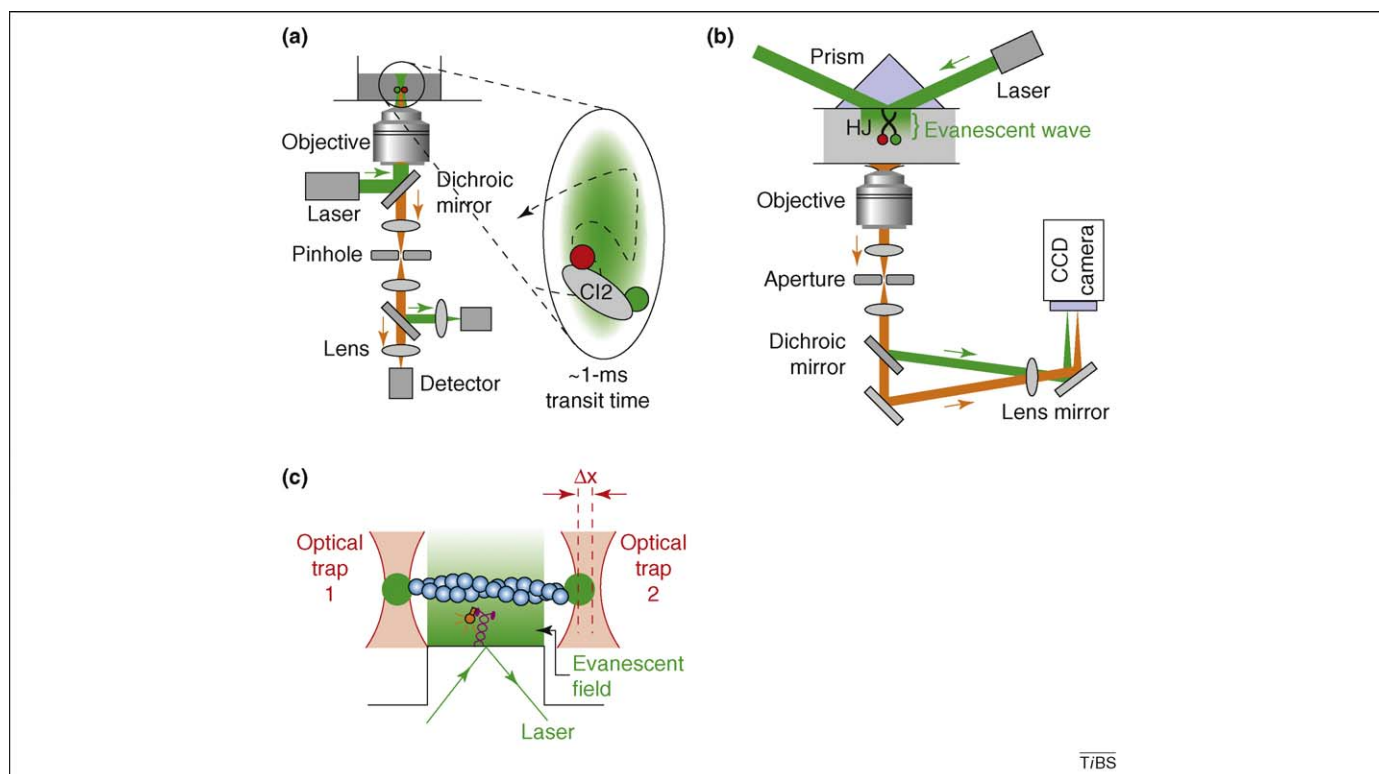


Figure 3. Commonly used single-molecule fluorescence-based methods. Single-molecule fluorescence methods involve detection of fluorescence photons either from a single point in a sample (a diffraction-limited spot with sub- μm dimensions) or from a large area on a surface (e.g. with dimensions of $30 \times 30 \mu\text{m}^2$). Combinations of single-molecule fluorescence methods with forced-based methods have also been described. **(a)** Confocal microscopy. A microscope for single-molecule FRET detection within diffusing molecules. This technique detects fluorescence emitted from a small volume element during each $\sim 1\text{-ms}$ -long molecular transit and builds FRET distributions for thousands of individual diffusing molecules. In the example, the biomolecule is a doubly labeled (with red and green fluorophores) protein and chymotrypsin inhibitor 2 (CI2; gray) (see also Ref. [78]). **(b)** Total-internal-reflection fluorescence (TIRF) imaging of single immobilized molecules. This technique detects fluorescence emitted from a thin slice close to the interface between the top of the reaction chamber (a quartz coverslip) and the solution. The use of two fluorophores permits real-time monitoring of conformational changes through changes in FRET. In this example, the biomolecule is a doubly labeled (with red and green fluorophores) Holliday junction (HJ) (i.e. a 4-way DNA junction; see also Ref. [69]). **(c)** Combination of optical tweezers and TIRF microscopy. A dual optical trap holds a molecular track (an actin filament; blue) within the evanescent wave generated up to $\sim 100 \text{ nm}$ from a pedestal where a myosin molecule (purple) is immobilized. Movement of the bead (green; Δx) out of optical trap 2 reports on force generation by myosin, whereas simultaneous imaging of fluorescence (indicated by an orange light bulb) detects the presence or absence of ligand (ATP; see also Ref. [79]). CCD, charged-coupled device.

Magnetic tweezers

In a magnetic tweezers setup (Figure 2c), the small bead used for optical trapping is replaced by a small magnetic bead that is controlled using a pair of magnets located close to the sample. This enables a force to be generated on the tethered biomolecule and a rotation to be easily imposed. This approach is particularly well suited for studying the structural properties of DNA and the mechanical and topological interactions involved in protein–DNA transactions [32]. Although the method has so far been unable to achieve the exquisite spatial sensitivity of the optical trap, it is easier to implement and is compatible with long-term (i.e. hours, days, and even weeks) tracking of individual biomolecules. Magnetic tweezers naturally function in constant-force mode, which is important because force is frequently the crucial control parameter for the interaction of interest. The zero-force equivalent of magnetic or optical trapping experiments is known as the ‘tethered particle motion’ configuration (Figure 2d).

Fluorescence-based approaches

Fluorescence spectroscopy and microscopy takes advantage of several properties of fluorescent biomolecules to monitor their location, structure and dynamics. There are two main

formats for single-molecule fluorescence: confocal microscopy [36,37] for point detection (which collects fluorescence emitted by a diffraction-limited volume of ~ 1 femtoliter) and wide-field imaging for area detection. For the latter, a popular geometry employs total-internal-reflection fluorescence (TIRF) microscopy [38], which uses evanescent-wave excitation within a thin layer just above a surface and imaging on an ultrasensitive camera to observe hundreds of surface-immobilized molecules for extended periods.

If a single fluorophore or light-scattering particle (such as a small gold particle) is attached to the molecule of interest, one can identify the presence of a molecule, track it as it moves on molecular tracks *in vitro*, or track its diffusion *in vivo*. Tracking motion has reached the remarkable precision of 1 nm (through FIONA [fluorescence imaging with one nanometer accuracy]), revealing how molecular motors such as kinesin and myosin move on their tracks [16,17,39]. The concept of high-precision localization, combined with discoveries of complex ways to switch on and off the fluorescence of some probes, is also at the heart of photoactivated localization microscopy (PALM [40]) and stochastic optical reconstruction microscopy (STORM [41,42]); these are methods that shattered

the diffraction limit of optical microscopy to obtain the characterization of 'super-resolution microscopies' because they achieve spatial resolutions better than 50 nm.

Other methods that use singly labeled biomolecules examine conformational changes by probing changes in the orientation of molecular domains by monitoring the orientation of a fluorophore rigidly attached to a mobile domain; these methods can use either point detection [43] or imaging (DOPI [defocused orientation and position imaging] [44]).

If two probes are attached to a molecule, new capabilities emerge. The distance dependence of fluorescence resonance energy transfer (FRET) [45] can be exploited to measure nanometer distances (2–10 nm) and distance changes within single molecules [46,47]. In FRET, the first probe acts as the FRET donor and is fluorescent, whereas the second probe (FRET acceptor) can quench the donor in a distance-dependent manner and can also be fluorescent. In this way, movements that change the donor–acceptor separation change their fluorescence; such changes are used to study the extent and kinetics of conformational changes or molecular association and dissociation [26,48–50]. Single-molecule FRET is not only affected by distance changes but also can be influenced by the relative orientation and rotational freedom of the fluorophores and by fluorophore photophysics that lead to FRET changes that can be mistaken for conformational changes. Such complications can be examined using advanced single-molecule FRET methods such as multi-parameter fluorescence detection (MFD) [50–52], a powerful method that can report on many fluorescence properties of single molecules including fluorescence intensity, anisotropy and lifetime at several wavelength ranges; alternating laser excitation (ALEX) [8,53,54], a method that uses two alternating lasers to measure FRET and relative probe stoichiometries; and two-color coincidence analysis [55]. Apart from FRET measurements, these advanced methods can report on molecular stoichiometries and interactions. Finally, some advanced methods use pulsed-laser illumination of fluorophores and time-correlated single-photon counting to measure the time required for de-excitation to occur [52,56–59]. This can serve as an effective metric for local probe environment and can be used to determine the conformation of the probe relative to the biomolecule to which it is attached; such approaches also provide access to ultrafast conformational changes with time resolution to the level of picoseconds (for subpopulation analyses).

Methods that feature combinations of approaches

An essential direction in single-molecule analysis that is being pursued by many single-molecule groups consists of combined platforms that enable simultaneous manipulation and visualization of single molecules as they react or interact [27,60,61]. This format can sense mechanical changes during biochemical reactions simultaneously with fluorescence-based monitoring of conformational changes within the molecule of interest (Figure 3c). The force- and fluorescence-based approaches are highly complementary; whereas nanomanipulation can achieve timescales of 50–100 ms under the appropriate force conditions, fluorescence approaches can be much faster and are not

Box 2. Physical challenges in single-molecule studies

Successful implementation of single-molecule methods requires overcoming various issues related to the construction and operation of high-end optical instruments and issues related to rigorous data analysis of complex images and noisy time-dependent signals.

Instrumentation issues

Single-molecule instruments are often custom built from high-end components and are sensitive not only to minuscule signals but also to noise sources such as stray light, mechanical vibrations and temperature fluctuations. For example, temperature changes of <1 °C can cause mechanical drifts that 'mimic' the displacements observed in typical single-molecule assays. Furthermore, specialized software is required for their operation. In addition, some high-end components have not necessarily been extensively tested over their full lifetimes, creating the potential for artifacts. Finally, some instruments are very complex and require substantial time for alignment and maintenance, which is a non-ideal feature when unstable biomolecules are to be measured.

Data analysis issues

Once the appropriate hardware is purchased, special software for data acquisition and analysis is required because commercial software can only perform basic processing tasks. Available high-level programming languages can simplify acquisition and analysis and deal with the conflicting requirements of high temporal resolution and acquisition of large datasets, but their development translates into an important time investment. Thankfully, some software is available as freeware and several laboratories share some of their code (although without full-time support). Newcomers must also deal with new ways of processing and presenting the data; the need for statistically significant sets of data and proper statistical analysis cannot be overemphasized. Finally, it is necessary to ensure that the appropriate statistical weight is given to the observed subpopulations (e.g. by performing control experiments) and that objective and rigorous criteria have been used to select subpopulations for further analysis.

constrained by applied force. Whereas force spectroscopy reports on more global structural and mechanical rearrangements in biomolecules, fluorescence measures local conformational changes. Being able to simultaneously pursue both forms of inquiry will provide future generations of researchers with many exciting experiments to undertake, provided that the considerable challenges surrounding the set up of single-molecule experiments are overcome (Box 2).

Concluding remarks: challenges, opportunities and frontiers

The unprecedented access to the underlying kinetic and structural features of biochemical reactions offered by single-molecule methods creates many exciting prospects, but it also poses substantial experimental challenges. Here, we discuss such challenges along with prospects for improvements in instrumentation for *in vitro* and *in vivo* analysis, in addition to training opportunities for young scientists in the life sciences.

To master the single-molecule approach, one must establish an interdisciplinary team or collaboration and tackle challenges such as complex instrumentation, the need for specialized software (Box 2) and complex biomolecule labeling and surface immobilization (Box 3). Some of these challenges are being addressed by commercially available single-molecule instruments and protein-modification methods of increased specificity and efficiency.

Box 3. Biochemical challenges in single-molecule studies

Detecting single molecules often requires their chemical modification, which becomes complex when using large proteins and macromolecular assemblies. It, therefore, is necessary to test that these modifications do not result in loss or perturbation of the activity in question.

For force spectroscopy, the molecule of interest must be, directly or indirectly, tethered to a surface, and it must be ensured that the attachment does not perturb the behavior of the molecule. Similar issues arise in single-molecule fluorescence experiments, in which site-specific labeling is required to study conformational changes using single-dipole techniques or FRET. Albeit straightforward for nucleic acids (especially DNA) and small proteins with a single surface-exposed cysteine, labeling of large proteins (with multiple surface-exposed cysteines) can be challenging. It is also non-trivial to doubly label proteins in a site-specific manner (for co-localization or FRET experiments) because it is difficult to control the site and extent of labeling to produce a pure labeled protein. After labeling, it must still be verified that the modification did not perturb the native behavior of the protein. To simultaneously tether and fluorescently label a biomolecule, then issues of background fluorescence on the tethering surface arise. In addition, fluorophore photophysics and photochemistry can be complex and can lead to artifactual signals that resemble conformational changes.

Finally, although single-molecule experiments detect individual molecules, some methods require considerable amounts of pure starting material for characterization purposes; for example, the polymerisation or ATPase activity of a certain protein might have to be tested under different conditions at the ensemble level to establish that the labeling procedure or the buffer conditions of the single-molecule experiment do not perturb the protein activity. Moreover, the low concentrations needed for single-molecule detection (e.g. ~100 pM for single-molecule fluorescence in solution) can lead to dissociation of unstable complexes or nonspecific absorption to the surfaces of reaction vessels.

These issues connect to a deeper problem: to identify and eliminate potential artifacts in the activity of a single molecule, bulk biochemical data against which to compare the single-molecule data must be available. Similarly, for rational protein modification (e.g. site-specific labeling or tethering), structural insight into the biomolecule, preferably to atomic resolution, is necessary. Thus, single-molecule methods are not ideal for poorly characterized or purified biological systems. What then is their contribution once these issues have been resolved? We note that their essential contribution lies in the identification and characterization of rare, transient or heterogeneous conformations and intermediates in addition to conformations and intermediates that are biochemically difficult to characterize (e.g. DNA looping) and, finally, in studying rate-limiting features and biomechanical coupling in well-established biochemical reactions. Note, however, that these methods will not be useful in the first assault against a protein of unknown function.

Moreover, detailed protocols, dedicated single-molecule meetings and courses and a growing community of trained doctoral and postdoctoral graduates have reduced the barriers to entering the single-molecule field.

There is also ample room for improving the throughput and information content of single-molecule methods. A clear path for improving throughput requires the development of ways for parallel tracking of many individual molecules (as in TIRF), either by exploiting the capabilities of newer computers and CPUs to achieve simultaneous real-time tracking of multiple particles or by carrying out parallel reactions in multi-well microfluidic systems, such as those for high-throughput single-molecule sequencing [33,62]. It is likely that the increase in throughput will appeal to the pharmaceutical industry, which so far has not

shown strong interest in adapting these approaches for drug discovery and characterization and ultra-sensitive diagnostics.

There is also great potential for combined force- and fluorescence-based analysis. However, despite proof-of-principle demonstrations, few biological questions have been addressed using such methodologies, mainly owing to technical difficulties and the strict requirements set by the fragile biomolecules under study. Collaborations between single-molecule groups and improvements in instrument automation, surface chemistries and fluorescence labeling should bring this group of techniques to the limelight in the near future.

Although most single-molecule methods are performed *in vitro*, *in vivo* single-molecule assays (which present a new set of challenges for the single-molecule community) are being pursued by many groups and have generated much enthusiasm [21,23]. These developments mostly involve fluorescence methods, although innovative force-based approaches also are being developed. *In vivo* fluorescence detection of a single molecule will be most applicable to molecular species with low abundance, precisely the species for which stochasticity and fluctuations are most crucial in regulating biological outcomes [23]. Advances in single-molecule cellular imaging are also linked to the exciting field of super-resolution imaging [63], which is rapidly moving from the study of static samples to dynamic measurements in living cells [64].

In academia [65], these approaches bring together multiple fields of life science (e.g. biochemistry, genetics and X-ray crystallography) with fields of physical sciences (e.g. chemistry, physics, mathematics and computer science). For undergraduate and graduate students, these assays provide a useful vehicle for explaining and illustrating difficult theoretical concepts, including the structural nature of transition states and intermediates, structural and kinetic heterogeneity, stochasticity and the role of thermal fluctuations in driving reactions and their net outcomes. At the experimental level, they provide students with opportunities to challenge themselves with experimental methods that will only become more pertinent in the future, notably, computer programming, optics and imaging, statistics and advanced biochemistry. In this regard, the single-molecule approach represents a 'melting pot' for modern biology and an exciting field that promises to unveil new discoveries for many years to come.

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