Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales
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Single-molecule spectroscopy has developed into an important method for probing protein structure and dynamics, especially in structurally heterogeneous systems. A broad range of questions in the diversifying field of protein folding have been addressed with single-molecule Förster resonance energy transfer (FRET) and photo-induced electron transfer (PET). Building on more than a decade of rapid method development, these techniques can now be used to investigate a wide span of timescales, an aspect that we focus on in this review. Important current topics range from the structure and dynamics of unfolded and intrinsically disordered proteins, including the coupling of folding and binding, to transition path times, the folding and misfolding of larger proteins, and their interactions with molecular chaperones.

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Introduction
Protein folding research has seen a large degree of diversification in recent years. Even though many of the original questions are far from being fully solved [1], the general focus has shifted away from the classic biochemical studies that had driven the field forward for decades. Instead, we currently observe a development in at least two major directions. One aims at a more detailed physical understanding of the process through an intimate combination of theory, molecular simulations, and advanced experimental methods. Especially the convergence of timescales accessible in recent computer simulations and spectroscopic experiments holds the promise of arriving at unprecedented mechanistic understanding of the folding process in the framework of statistical physics [2–7]. The other direction is based on the increasing realization that the spontaneous self-organization of polypeptide chains into three-dimensional structures has broad functional implications, for example, in the context of intrinsically disordered proteins (IDPs), which appear to be involved in many intracellular interactions and regulatory processes [8,9]. Along similar lines, a more quantitative understanding of the effect of the cellular environment on protein folding mechanisms will be important to bridge the conceptual gap between the traditionally rather separate fields of folding in vitro and in vivo.

Within the resulting multidisciplinary fields that address questions linked to protein folding, single-molecule methods have started to take an important place [10]. For both research directions outlined above, they can provide valuable and previously unavailable information. Single-molecule experiments are an ideal match for the basic concepts of statistical physics, since they can reveal the distributions and heterogeneity of the structures and dynamics underlying the ensemble average. Intermolecular interactions and the components of the cell add to this heterogeneity, and the ability to probe individual protein molecules in the context of a complex environment, for example, during their interaction with molecular chaperones, or even in an intact cell, further the appeal of single-molecule methods.

Two main types of single-molecule experiments are currently employed in biology: force-probe methods using atomic force microscopy (AFM) or optical tweezers [11], and fluorescence detection, especially in combination with Förster resonance energy transfer (FRET) [12]. AFM and optical tweezers have proven to be a remarkably versatile tool for probing the mechanical stability and folding dynamics of proteins, and the reader is referred to the article by Rief [11] and other recent reviews [10,13]. Here, we focus on the investigation of protein folding with single-molecule fluorescence spectroscopy. In particular, we will review the progress in this area over the past three to four years. During this time, the scope of single-molecule spectroscopy has expanded substantially, both in terms of questions addressed and in terms of timescales accessible. We will summarize this progress with an emphasis on dynamics by going through the relevant range of timescales and relating them to the hierarchy of structure formation in protein folding and binding with examples from the recent literature (Figure 1).

Submicrosecond dynamics: unfolded and intrinsically disordered proteins
Unfolded states have attracted considerable attention, initially because of their general importance as the
Timescales in protein folding accessible with single molecule spectroscopy. Single molecule fluorescence methods, including fluorescence correlation spectroscopy (FCS), cover more than fifteen orders of magnitude in time and allow a wide range of processes relevant for protein folding to be investigated. Essentially all timescales above the lower limit set by the photophysics of the fluorophores can be probed with the available range of experiments and analysis methods on immobilized and/or freely diffusing molecules. The approximate time ranges accessible with different techniques are indicated as horizontal bars. Recent examples for the development and application of these methods are shown at the bottom: (a) Autocorrelation and crosscorrelation functions for FRET-labeled unfolded cyclophilin at 1.5 M GdmCl obtained with ns-FCS, from which the chain reconfiguration dynamics can be determined [26,28,52]. (b) Interconversion times between species can be obtained from the analysis of transfer efficiency histograms. The solid lines are fits to simulated data according to the theory of Gopich and Szabo [30]. (c) Schematic of a microfluidic mixing device with a dead time of 200 μs for single-molecule detection designed by Gambin et al. [81]. (d) Example of a FRET efficiency trajectory of immobilized adenylate kinase, a multi-domain protein whose folding dynamics were investigated by Pirchi et al. [73]. (Data from [73].) (e) Transfer efficiency histogram of a refolded I27 tandem repeat with 5% misfolded molecules at high transfer efficiencies (red box) that convert back to the native species with a half-life of about one week [98]. (Figure adapted from [98].)

starting point of the protein folding reaction [14], later in the context of the ‘speed limit’ of folding: structure formation cannot be faster than the diffusive encounter of the parts of the polypeptide chain forming interactions [15–17]. Renewed interest in the properties of unfolded proteins has come with the identification of intrinsic disorder in large parts of eukaryotic proteomes and the investigation of its functional importance [8,9]. Single-molecule spectroscopy is ideally suited for probing such structurally heterogeneous and dynamic systems [18,19] and for complementing the information available from methods such as NMR [8,20].

Single-molecule FRET, which allows distances and distance dynamics to be probed in a range from about 2 nm to 10 nm (Box 1), has been used very successfully to investigate unfolded proteins [21,22] and IDPs [18,23]. Advances in methodology, in particular the use of fluorescence lifetimes and anisotropies in addition to fluorescence count rates from donor and acceptor [24,25*], rigorous data analysis, especially based on the development of comprehensive theoretical concepts [24,26–28,29*,30,31*,32,33] now enables distances [24], distance distributions [34,35], and interconversion dynamics [36*,37] to be obtained very accurately [21]. An
### Box 1 Comparison of FRET and PET

In its classical description, Förster resonance energy transfer (FRET) originates from the resonance of the transition dipole moments of donor (D) and acceptor (A) fluorophores. The rate constant of the resulting energy transfer \( k_{\text{FRET}} \) from D to A depends on the inverse 6th power of the D-A distance \( \langle r \rangle \), the fluorescence lifetime of the donor \( \tau_D \), and the characteristic distance \( R_0 \) that can be calculated from the spectroscopic properties of D and A. The typical values of \( R_0 \) make single-molecule FRET suitable for obtaining distance information in the range of ~2 nm to ~10 nm. By contrast, photo-induced electron transfer (PET) requires much closer contact between fluorophore (F) and quencher (Q, typically tryptophan). The rate constant of quenching \( k_{\text{PET}} \) depends on the contact radius \( \langle a \rangle \), a characteristic distance \( r_s \) in the subnanometer range, and the quenching rate constant at the distance of closest approach \( k_s \). The on/off behavior resulting from the steep distance dependence of the fluorescence emission in PET can in principle be used to probe any process that leads to a change in quenching dynamics, but it does not afford direct distance information. As an example, both PET and FRET, when combined with ns-FCS, can provide information on the dynamics of unfolded and disordered proteins in the nanosecond to microsecond time regime. FRET-FCS can be used to determine the reconfiguration time of the chain; in addition, the distance or distance distribution between D and A can be obtained from the analysis of transfer efficiencies and/or fluorescence lifetimes. PET-FCS allows the determination of loop-closure rates between two points within a polypeptide chain. The complementarity of the two methods is expected to be increasingly useful for probing biomolecular dynamics over a wide range of distances and times (Figure I).

**Figure I**

![FRET and PET](current_opinion_in_structural_biology_image)

The important strength of single-molecule FRET is the separation of folded and unfolded subpopulations [38,39]. As a result, the properties of the unfolded state can be quantified even under conditions where the majority of molecules are folded and would thus dominate the signal in an ensemble experiment. This separation of subpopulations led to the identification of the continuous compaction, or collapse, of the unfolded state with decreasing concentration of denaturant [39], a rather generic behavior [21,22] (and references therein) that reflects the change in solvent quality for the polypeptide chain [14,40,41]. Most recently, this approach was used to quantify the effect of sequence composition on the length scaling of unfolded proteins and IDPs within the framework of polymer theory [41].

This type of experiments can also be used to investigate the effect of other changes in conditions on unfolded proteins. In response to an increase in temperature, for example, Nettels *et al.* [42] observed a collapse of an unfolded cold shock protein and the intrinsically disordered protein prothymosin α, suggesting an increase in intramolecular interactions upon heating. Molecular dynamics simulations indicated an important role of solvation and the possible involvement of secondary structure formation [42,43] in this process. For IDPs, which frequently contain a large fraction of charged amino acids, a contribution of particular importance is the interaction between the charges in the polypeptide chain. Single-molecule FRET experiments in combination with an analysis based on polypeptide theory showed that charge repulsion can in fact dominate the chain dimensions of IDPs and lead to a pronounced chain expansion at low ionic strength [44,45], in agreement with results from simulations [46]. Surprisingly, however, attraction between opposite charges within polypeptide chains with low net charge can lead to an additional compaction, as predicted by polypeptide theory [44]. Structural and dynamic properties of more complex IDPs are also starting to be investigated with single-molecule spectroscopy [47–49].

The quantitative description of experimentally derived distance distributions and intramolecular interactions in terms of polymer models has been very successful [35,44,50,51], and the corresponding distance information provides the first important piece of information for understanding the dynamics of unfolded proteins and IDPs. The second piece of information, the timescale of chain dynamics, can be obtained from nanosecond correlation spectroscopy (ns-FCS) [28,52,53] (Figure 1a), which can be readily implemented in confocal instruments with the latest generation of counting electronics [54]. In ns-FCS experiments based on FRET, the long-range distance fluctuations between the donor and the acceptor fluorophore report on the characteristic timescale of...
interconversion between different configurations of the polypeptide chain. The resulting relaxation times are in the range of tens of nanoseconds to ~200 ns for the segment lengths from ~30 to ~200 amino acid residues investigated to date [25*,28,52,53]. These times are remarkably close to the reconfiguration times expected for ideal polymers [55], indicating that the underlying dynamics approach chain diffusion in the absence of persistent interactions within the polypeptide.

However, at low denaturant concentrations, the reconfiguration time can increase substantially above the values expected for an ideal chain [25*,52]. This behavior had been ascribed to ‘internal friction’ caused by dissipative mechanisms within the polypeptide, such as dihedral angle rotations or transient side chain or backbone interactions [52], but it had eluded a quantitative description. The recent work of Soranno et al. [25*] (Figure 2) illustrates the use of polymer-physical principles in combination with single-molecule FRET and ns-FCS for quantifying internal friction in unfolded proteins and IDPs. Polymer dynamics models, such as the Rouse model with internal friction [56], show that the reconfiguration time, \( \tau_n \), of a polymer can be decomposed into a sum of two relaxation times, that of the ideal chain, \( \tau_0 \), and a contribution from internal friction, \( \tau_i \), with \( \tau_r = \tau_0 + \tau_i \). Based on this relationship and solvent-viscosity-dependent and position-dependent measurements of unfolded state dynamics, Soranno et al. [25*] showed for a number of proteins that internal friction is close to zero at very high concentrations of denaturant, where the chains are very expanded and thus behave similar to an ideal polymer. For very compact chains, however, \( \tau_i \) can dominate the chain dynamics and increase \( \tau_r \) up to an order of magnitude above the value expected for an ideal chain. Highly charged IDPs do not exhibit this compaction but are expanded owing to charge repulsion [44,46], which reduces internal friction and may affect the dynamics of their interactions with cellular binding partners [25*,57].

In summary, single-molecule FRET in combination with ns-FCS provides an opportunity to quantify the properties of unfolded and disordered proteins even on submicrosecond timescales and rationalize them in terms of polymer-physical concepts. Interestingly, the estimate of the pre-exponential factor or ‘speed limit’ of the folding reaction based on these results and a simple Kramers model is in the range of 0.5 \( \mu \)s [52], which is surprisingly close to the folding times of the fastest folding proteins [16] and their transition path times [58**], suggesting that changes in unfolded state dynamics can affect folding rates [59] (see also Microsecond dynamics: barriers, transition paths, and folding at the speed limit).

A single-molecule method complementary to FRET that has started to provide important dynamic information on the submicrosecond timescale is photo-induced electron transfer (PET) combined with ns-FCS, pioneered by Sauer and colleagues [60]. In this case, static quenching of a fluorophore by tryptophan is exploited for obtaining dynamics based on contact formation within proteins or peptides, conceptually related to some ensemble quenching methods [15,17,61] and complementary to the long-range dynamics accessible with FRET (Box 1). Recent applications include studies of model peptides [60,62], for example, in the context of molecular crowding [63], and fast-folding proteins [64,65*]. Neuweiler et al. [65*] used PET to monitor the 10 \( \mu \)s-folding time and the submicrosecond contact rate in the denatured state of the small protein BBL, including the presence of internal friction. The same method was applied to investigate the surprisingly slow dynamics in a trapped intermediate of enzylized homeodomain [64], which the authors assigned to the presence of nonnative interactions that lead to interconversion on a timescale similar to that of the overall folding process.

**Microsecond dynamics: barriers, transition paths, and folding at the speed limit**

Folding dynamics on the microsecond timescale have been a particularly attractive research topic in recent years. Many proteins that fold in microseconds still exhibit the key structural properties of folded proteins, with specific tertiary structure and a hydrophobic core [42], and their kinetics are starting to become accessible to all-atom
Experimental determination of the folding transition path time for a two-state protein [58**]. (a, top) Schematic of the kinetics of protein folding represented by diffusion on a one-dimensional free-energy surface with an order parameter (x) as a reaction coordinate. The unfolded molecule spends the vast majority of time visiting a large number of conformations in the free energy well of the unfolded state. A transition path is the part of the trajectory that crosses the point $x_0$ on the reaction coordinate and reaches $x_1$ on the other side of the barrier without recrossing $x_0$. The duration of this process is the transition path time. (a, bottom) Sketch of a corresponding FRET efficiency trajectory showing transitions between the folded state (high FRET efficiency) and the unfolded state (low FRET efficiency). The duration of the jump in the trajectory corresponds to the transition path time. (b) Example of donor (green) and acceptor (red) photons recorded from an immobilized WW-domain molecule, showing a transition from unfolded to folded. (c) The difference between the log likelihoods of a two-state model with a finite transition path time and a two-state model with an instantaneous transition, $\Delta \ln L = \ln L(t_T) - \ln L(0)$, is plotted as a function of the transition path time $t_T$. The value for $t_T$ at the maximum of $\Delta \ln L$ identifies the most likely average transition path time, $\langle t_T \rangle$. (Figure adapted from [58**].)

simulations [4,6]. The convergence between experiments and simulations on this timescale has thus become a very promising avenue towards a detailed understanding of protein folding mechanisms. The use of single-molecule fluorescence is challenging on the microsecond timescale and below, because the maximum average photon count rate from an individual molecule is only about $10^5$ Hz to $10^6$ Hz owing to the limited photon collection efficiencies of current instruments and the photophysical properties of the fluorophores. However, the broad distribution of interphoton times results in a finite probability of observing shorter interphoton times, and rapid correlation methods as described in Submicrosecond dynamics: unfolded and intrinsically disordered proteins allow access to this time range, both in FRET and PET experiments [36*,37*,60,64,65*,66].

A different approach to probing the microsecond range is illustrated by the remarkable recent use of single-molecule FRET for measuring transition path times of protein folding (Figure 3). While the chemical kinetics of protein folding contain the information on the frequency of the folding and unfolding transitions, the information on the mechanism, that is, the sequence of microscopic events that take the protein from the unfolded to the folded state, is contained in the actual process of barrier crossing. An ultimate goal of single-molecule experiments will be to resolve these transitions [21,67], which is currently only possible in computer simulations. The distribution of transition paths may in fact only be experimentally accessible with single-molecule observations. A first important step towards this goal is to determine the time it takes a molecule to cross the barrier. Building on earlier work [68], Chung et al. [58**] have recently succeeded in measuring the average transition path time for the FBP28 WW domain (folding time $\sim 100 \mu$s) and an upper bound for protein GB1 (folding time $\sim 1$s) by analyzing the photon statistics of a very large number of fluorescence trajectories from immobilized molecules with a maximum likelihood method developed by Gopich and Szabo [29*]. In spite of the very different folding times of the two proteins, their transition path times are remarkably similar, with $\sim 2 \mu$s for the WW domain and $<10 \mu$s for GB1 [58**]. From all-atom explicit solvent simulations on the similar Fip35 WW domain, Shaw et al. [4] found a very similar average transition path time of $\sim 1.5 \mu$s (after correcting the observed 0.5 $\mu$s for the 3-fold smaller viscosity of the water model used in the simulations), which is also expected to be closely related to the $\sim 1.5 \mu$s ‘molecular phase’ observed in ensemble
laser temperature jump experiments on FiP35 WW domain [69]. These times are comparable to the protein folding ‘speed limit’ in the range of ~1 μs previously estimated from experimental and theoretical approaches [16], and remarkably close to the unfolded state dynamics from nsFCS [25*,52,65*] (see Submicrosecond dynamics: unfolded and intrinsically disordered proteins). It will also be interesting to see whether a correlation exists between transition path times and the presence of internal friction at the transition state, for example, for the spectrin domains, some of which were recently found to exhibit unusually high internal friction in their folding reactions [70].

A key benefit of single-molecule experiments is the possibility to extract dynamic information even from equilibrium measurements, which avoids the necessity for a synchronization of the system by perturbation methods, as frequently employed in ensemble experiments. For protein folding, equilibrium dynamics have been obtained, for example, from correlation functions [25*,52,65*,66,71] (Figure 1a), the analysis of broadening and exchange between subpopulations in FRET efficiency histograms [30,36*,37*,39] (Figure 1b), and from fluorescence trajectories of immobilized molecules [36*,58**,66–68,72,73*,74] (Figure 1d). In this way, both the equilibrium distributions and the kinetics can sometimes be obtained from the same measurement.

A recent example for which this possibility has been of great interest is the distinction of two-state and downhill (or ‘one-state’) protein folding. In single-molecule FRET experiments on freely diffusing molecules, two-state behavior will result in two separate subpopulations in the FRET efficiency histograms if the interconversion kinetics are slower than the observation time [27], which is determined by the ~1 ms diffusion time of the molecules through the confocal volume, and by the photon count rate during this time, which limits the minimal binning time that can be used for the analysis. In the case of much faster exchange or for a one-state scenario, a single peak that shifts continuously with denaturant concentration is expected. For BBL, a small protein previously suggested to be a one-state folder by Muñoz and colleagues [75], Huang et al. [76] reported a separation of folded and unfolded subpopulations at an observation time of 50 μs, and, from ensemble temperature-jump experiments, a relaxation time for their interconversion at 4 М guanidinium chloride (GdmCl) of ~340 μs, suggesting barrier-limited folding kinetics. Recently, this finding was challenged by Liu et al. [77], who reported their single-molecule FRET experiments on a slightly different variant of BBL to be incompatible with two-state behavior. They obtained a relaxation time of ~200 μs from a correlation analysis of their single-molecule data at 6.1 М urea and ensemble temperature-jump experiments at 2.2 М GdmCl. To clarify the origin of this discrepancy, further experiments will be required, ideally with instrumentation that enables single-photon counting, to obtain additional observables, such as fluorescence lifetimes and anisotropies, and to allow a more detailed analysis of the photon statistics.

### Millisecond dynamics: microfluidics coming of age

Even though it is often possible to obtain information on kinetics from equilibrium single-molecule experiments, in many cases it is still required to probe nonequilibrium dynamics, especially if the reaction of interest is essentially irreversible during the observation time accessible at equilibrium. A method that lends itself very well to the combination with single-molecule detection optics is microfluidic mixing, and since the first application to protein folding [78], several different implementations have been reported [79,80,81*,82]. The basic idea of such devices is to mix solutions in continuous laminar flow by reducing the dimensions such that the components of the solutions that are combined exchange very quickly, solely by diffusion [83]. Since the typical length scale over which small molecules such as denaturants diffuse in a millisecond is about a micrometer, structures in this size range are required to obtain dead times comparable to established methods based on turbulent mixing, such as stopped-flow. After mixing, the confocal observation volume is placed at different points in the observation channel, corresponding to different times after mixing. In a recent mixer design (Figure 1c), Gambin et al. [81*] achieved a dead time of ~200 μs, very close to the limits of time resolution dictated by the minimum residence time that is required to observe a sufficient number of photons from a single molecule flowing through the confocal volume. They used the device to investigate the kinetics of the structural transitions of the IDP α-synuclein upon interaction with sodium dodecyl sulfate (SDS) and were able to resolve the formation of an intermediate that they assigned to a helix–turn–helix structure preceding the final extended helix bound to micelles [81*].

A key step towards the usability required for a broader application of microfluidic mixing in protein folding and binding studies has been the utilization of replica-molding in elastomers such as polydimethylsiloxane (PDMS)[84], which greatly simplifies the production of the devices from a microfabricated mold in large numbers and increases reproducibility [80,85]. A mixer design optimized along these lines for single-molecule fluorescence experiments [80] has recently been employed to investigate internal friction in unfolded proteins [25*] and protein folding inside the GroEL/ES chaperonin cavity [86*]. Soranno et al. [25*] used the device in combination with ns-FCS to quantify internal friction in unfolded proteins (see Submicrosecond dynamics: unfolded and intrinsically disordered proteins). The
microfluidic mixer allowed the unfolded state of a protein to be transiently populated at low denaturant concentrations (where it is folded at equilibrium); in this way, the reconfiguration time could be determined under conditions where the unfolded state is most compact (Figure 2). Owing to the continuous-flow mixing employed, even correlation experiments with long acquisition times can thus be performed on such non-equilibrium states. Microfluidic mixing also provides new opportunities for studying protein folding and binding in systems involving multiple components and greater structural complexity. Hofmann et al. [86] used it for probing the folding of the chaperone substrate protein rhodanese upon encapsulation in the GroEL/ES chaperonin cavity. The absence of obvious changes in the transfer efficiency histograms of FRET-labeled rhodanese bound to GroEL in the first few seconds after binding of ATP and the co-chaperone GroES suggested that the forced unfolding proposed for other substrates [87,88] does not occur in the case of rhodanese.

**Seconds, minutes, and beyond: fluorescence trajectories and free diffusion**

Observing one individual molecule at equilibrium for more than a few milliseconds usually requires immobilization. In contrast to experiments on freely diffusing molecules, where confocal detection is used predominantly, two detection schemes are commonly employed for immobilized molecules. Confocal detection in combination with avalanche photodiodes has the advantage of subnanosecond time resolution, and provides fluorescence lifetime information and anisotropy decays if combined with time-correlated single photon counting (TCSPC) [24], but the molecules need to be interrogated sequentially. For monitoring slower dynamics of many immobilized molecules in parallel in a larger field of view, sensitive CCD cameras with their millisecond frame rates, combined with total internal reflection fluorescence (TIRF) are becoming increasingly popular. Building on earlier studies [72,74,89], many of the original limitations of immobilization experiments have been overcome. An important factor is the use of photoprotectants [42,90–92] to minimize the photochemistry that leads to blinking (which can easily be misinterpreted as conformational dynamics [93], and to reduce bleaching, which limits the length of fluorescence trajectories from single molecules.

Two interesting examples of equilibrium folding dynamics from single-molecule FRET experiments on the timescale of seconds are recent reports on immobilized adenylate kinase [73] and on several IDPs [94]. Based on a Markov-state analysis of thousands of fluorescence trajectories from confocal measurements of the three-domain protein adenylate kinase encapsulated in lipid vesicles (Figure 1d), Pirchi et al. [73] reported evidence for about six distinct interconverting states whose connectivity depends on the denaturant concentration. This work illustrates that even the folding dynamics of larger multi-domain proteins are starting to become accessible with single-molecule experiments. Using a camera-based approach, Choi et al. [94] made a surprising observation on some proteins previously categorized as intrinsically disordered. For three of them, the transfer efficiency values were observed to be constant on the millisecond integration time of the camera, as expected from the rapid conformational dynamics expected for IDPs (see Submicrosecond dynamics: unfolded and intrinsically disordered proteins). But neuroligin and the NMDAR-2B glutamate receptor exhibited ‘conformational switching’ on the time scale of seconds, indicating the presence of unexpectedly large free energy barriers. In both examples [73,94], the structural identity of the transient states is currently unclear, but future experiments with multiple dye pairs, complementary methods, or the combination with simulations might facilitate a structural assignment.

While immobilization is required for observing long fluorescence trajectories from individual molecules, the temporal evolution of population distributions can also be monitored with confocal experiments on freely diffusing molecules, which can be of interest especially for nonequilibrium experiments on very slow processes. Simple manual mixing can be used to extend the observation time to any range that sample stability permits. By
combining microfluidic and manual mixing experiments, Hofmann et al. [86] were able to monitor the folding of rhodanese, an aggregation-prone chaperone substrate, in the GroEL/ES chaperonin cavity from the encapsulation (see Millisecond dynamics: microfluidics coming of age) to the completion of the folding reaction over about one hour. Since substrate protein aggregation is negligible at the picomolar concentrations in these experiments, the authors could exclude the contribution of this side reaction and thus rigorously compare the folding rates of rhodanese in the cavity to those free in solution, outside the chaperone. The surprising observation was that the C-terminal domain of rhodanese folds up to a factor of eight more slowly inside the chaperonin cavity than in free solution (Figure 4), while the N-terminal domain and the sequence of folding events are unaffected. This reduction in folding rate was suggested to be a result of interactions with the cavity walls or confinement-induced internal friction, as previously proposed based on simulations [95–97].

A case of an extremely slow process monitored by single-molecule FRET was reported by Borgia et al. in their studies of misfolding in tandem repeats of Ig-like domains from titin [98] (Figure 1e). They demonstrated that 5% of I27-I27 repeats form a misfolded structure upon dilution from denaturant. In the misfolded molecules, the swapped arrangement of the terminal β-strands of the two domains is identical to that within a single domain in the properly folded structure, which explains the mechanical stability of the misfolded state [99]. Interestingly, the probability of misfolding is lower for tandem repeats with lower sequence similarity, supporting the hypothesis that low sequence similarity of neighboring domains in multi-domain proteins is a result of evolutionary pressure to avoid misfolding [100]. The clear separation of folded, unfolded, and misfolded subpopulations also allowed the kinetics of all subpopulations to be monitored as a function of denaturant. Based on molecular simulations and the slow unfolding kinetics of the misfolded state, the authors concluded that in the misfolded state, two stably folded domains are formed. However, over longer times, the misfolded subpopulation spontaneously converts back to the folded state, indicating that misfolding is a result of kinetic partitioning, but that the natively folded structure corresponds to the free energy minimum. In the absence of denaturant, the conversion from the misfolded to the folded state exhibits a relaxation time of about one week [98]. This example also illustrates the possibility to obtain structural and kinetic information even on very low populated states that would be inaccessible with ensemble experiments.

**Conclusions**

Single-molecule spectroscopy has progressed remarkably over the past few years, and we have seen a transition from feasibility studies and proof-of-principle experiments to an important tool for gaining conceptual insight in protein structure and dynamics that would be difficult to obtain otherwise. Particularly remarkable is the broad range of timescales now accessible with single-molecule spectroscopy. Already the examples reviewed here cover 13 orders of magnitude, essentially all timescales relevant for protein folding dynamics (Figure 1).

In spite of these advances, single-molecule methods are still developing rapidly, and many of the experimental techniques and analysis methods introduced only a few years ago are now an established part of the state-of-the-art toolbox. Aside from further developments in the directions mentioned above, we expect a particularly important role on protein folding and binding from several newly emerging methods. Three-color FRET is clearly one of them: monitoring up to three distances at a time allows correlations to be established between conformational changes in different parts of a protein or between folding and binding events in IDPs [101]. Related goals might be attainable with a direct combination of FRET and PET. The continued development of methods to analyze single molecule experiments and to model photon statistics [24,26–33,62,102,103] will play an important role for dissecting systems of increasing complexity. The biochemically very demanding specific labeling of proteins with three chromophores will benefit from improved labeling and chromophore incorporation strategies [104,105].

Technical developments that are expected to have a major impact (in particular on experiments based on fluorescence trajectory analysis) include zero-mode waveguide arrays for increased throughput [106], single photon avalanche diode arrays [107] for the combined advantages of area detectors with time-correlated single photon counting and full correlation analysis, and methods that allow individual molecules to be monitored for extended times without requiring immobilization [103], to name but a few. A key requirement for improving the time resolution of single-molecule fluorescence trajectories will be to increase the photon emission rate by further optimization of photoprotective additives and related methods [92].

Advances of this kind will help to further expand the accessible timescales and the scope of single-molecule spectroscopy for addressing the diverse range of questions related to protein folding and dynamics. For all levels of complexity, from fundamental aspects such as unfolded state/IDP dynamics [23,25] and transition paths [58] to the role of misfolding and alternative native structures [98,108], molecular chaperones [86,87], and experiments *in vivo* [109], a quantitative approach will be essential for bridging the divide between these areas, often in combination with simulation and theory. Even
though current evidence indicates no fundamental differences between folding and binding processes in vitro and in vivo [10,11], even moderate modulations of rates and equilibria might be relevant in vivo, and understanding the influence of cellular factors in more mechanistic detail will certainly be essential. The potential of single-molecule spectroscopy for investigating heterogeneous systems in the context of interactions with a broad range of environmental factors thus provides many opportunities for reaching a more comprehensive picture of the mechanisms and functional roles of protein folding processes.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest
• of outstanding interest


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diffusion through the confocal volume. A global analysis of transfer efficiency distributions and fluorescence lifetimes allows static and dynamic heterogeneity to be discriminated.


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The authors investigated a tryptophan-containing variant of the small protein BBL with ns-PET-FCS. In the correlation functions in the absence of denaturant, they observed two submillisecond contributions, one with a decay time of ~10 μs and the other with ~0.5 μs to ~1 μs, which are assigned to folding kinetics and loop closure dynamics in the denatured state, respectively. The solvent viscosity dependence of the loop closure rates provided evidence for internal friction in the denatured state. This work demonstrates the potential of ns-PET-FCS for investigating dynamics occurring at different timescales.


The authors investigated the folding of the multi-domain protein adenylate kinase by analyzing thousands of single-molecule trajectories with Markov-state models. The results suggest a complex energy landscape for adenylate kinase folding, with about six meta-stable states whose connectivity is strongly denaturant-dependent.


An elegant microfluidic mixing device for non-equilibrium single-molecule kinetics was designed with a dead time of 200 μs. The authors used this device to investigate the SDS-induced folding of α-synuclein, an intrinsically disordered protein, and observed the rapid population of transient structures in the formation of the α-synuclein-SDS complex.


The advantage of single-molecule spectroscopy to work under conditions of nearly infinite dilution was used together with microfluidics to investigate the folding of a highly aggregation-prone enzyme, rhodanese, in free solution and inside the confining cavity formed by the chaperonin GroEL/ES from millisecond to hours. The surprising finding of a deceleration of folding inside the confined space of the GroEL/ES cavity challenges the concept that confinement universally accelerates folding in vivo.


Using camera-based TIRF, the authors investigated five different IDPs with different sequence composition and found that two of them exhibit conformational dynamics with time constants in the seconds range. Even though the underlying mechanism is unclear, this is the first reported case of slow dynamics in IDPs in the absence of stabilizing ligands (compared to the submicrosecond dynamics of unfolded proteins [29, 32]).


In a combination of protein engineering and single-molecule spectroscopy, the authors provide evidence for an evolutionary mechanism to prevent misfolding in multi-domain proteins. Tandem arrays of immunglobulin-like domains only showed a misfolded population if adjacent domains had a high sequence identity. The misfolded species was shown to convert to folded proteins with a half-life of 100 hours, demonstrating that single-molecule FRET can be used to investigate very slow conformational changes.


109. Sakon JJ, Weninger KR: Detecting the conformation of individual proteins in live cells. Nat Methods 2010, 7:203-206. The authors present the first application of single-molecule FRET to conformational changes of proteins inside living cells. Using objective-type TIRF in combination with single-particle tracking and microinjection, they monitored individual proteins incorporating into folded complexes at the cell membrane. This work provides a first important step towards investigating protein folding with single-molecule spectroscopy in vivo.
