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Current Opinion in Structural Biology

# Probing the structural dynamics of proteins and nucleic acids with optical tweezers

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Conformational changes are an essential feature of most molecular processes in biology. Optical tweezers have emerged as a powerful tool for probing conformational dynamics at the single-molecule level because of their high resolution and sensitivity, opening new windows on phenomena ranging from folding and ligand binding to enzyme function, molecular machines, and protein aggregation. By measuring conformational changes induced in a molecule by forces applied by optical tweezers, new insight has been gained into the relationship between dynamics and function. We discuss recent advances from studies of how structure forms in proteins and RNA, including non-native structures, fluctuations in disordered proteins, and interactions with chaperones assisting native folding. We also review the development of assays probing the dynamics of complex protein–nucleic acid and protein–protein assemblies that reveal the dynamic interactions between biomolecular machines and their substrates.

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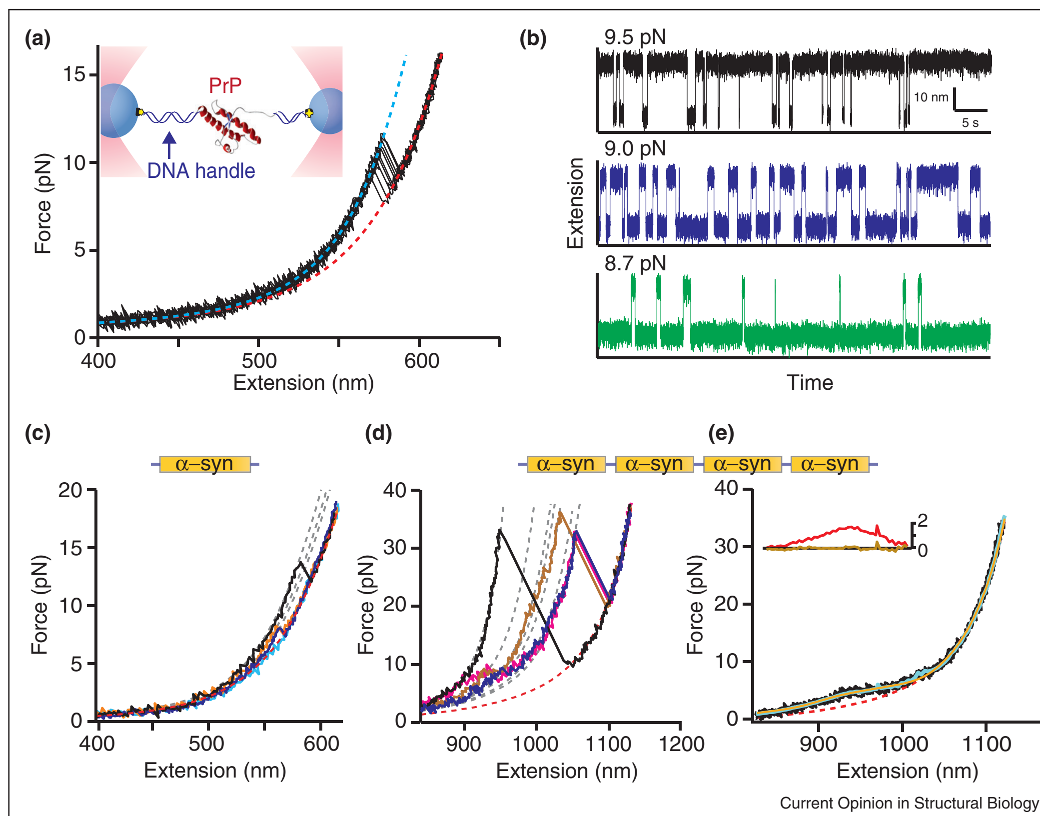
## Introduction

The structural dynamics of macromolecules like protein and RNA are critically tied to their biological function. Numerous processes, from folding to ligand binding and enzymatic catalysis, depend on conformational changes, whether they involve large-scale rearrangements or subtle fluctuations. Studies aimed at elucidating the details of protein and RNA conformational dynamics are thus essential for a full understanding of biological mechanisms. Single-molecule force spectroscopy (SMFS), whereby mechanical forces are applied to individual molecules by a force probe and the length of the molecule is measured to capture the resulting conformational changes [1,2], is a powerful tool for studying the relationship between conformational dynamics and function, owing to the very high resolution it can achieve and its high sensitivity to rare and transient events [2,3,4•]. Here we discuss recent advances made using optical tweezers to study topics such as intra-molecular and inter-molecular interactions in the folding and misfolding of proteins and RNAs and the dynamics underlying the function of molecular motors and complex macromolecular assemblies.

The principles and construction of optical tweezers are reviewed in detail elsewhere; with sufficient care, atomic-scale resolution can be achieved, allowing the discrimination of subtle conformational changes [3,5,6]. In a typical apparatus (Figure 1a inset), the molecule of interest is attached (often via DNA handles) to polystyrene beads, which are in turn trapped by laser beams that apply tension to the molecule [7]. Two main measurement modalities are used [8]: first, non-equilibrium measurements, such as force-ramps, where the traps are moved continuously to ramp up/down the force on the molecule (Figure 1a), or force-jumps, where the force is changed discontinuously, and second, equilibrium measurements, where the molecule is held under constant force and/or the traps are kept at a constant position and the extension is measured as the structure fluctuates at equilibrium (Figure 1b).

Conformational changes generate characteristic features in these measurements owing to the sudden change in molecular extension during cooperative transitions: sawtooth-shaped ‘rips’ in force-extension curves (FECs) (Figure 1a), and steps in equilibrium trajectories (Figure 1b). These measurements yield information such as the molecule’s contour length change (yielding the number of amino acids or nucleotides involved in structural transitions), the presence and number of intermediates on a given pathway, the existence of alternative pathways, and how each state is connected [9]. Furthermore, kinetic and thermodynamic information can be obtained including the force-dependent microscopic rates for transitions [10–12,13•], the free energy of each state [14–16], the position and height of energy barriers between states [12,17–19], the full profile of the energy landscape governing the conformational dynamics [8,18,20–24], and the diffusion coefficient setting the timescale for microscopic dynamics [8,25,26,27••]. Equilibrium and non-equilibrium measurements yield

**Figure 1**




Optical tweezers measurements of protein folding/unfolding. **(a)** DNA handles attached to each end of a protein molecule are bound to beads held under tension in optical traps (inset). Ramping up the force by moving the traps apart, the handles stretch until the protein unfolds abruptly, generating a sawtooth-like rip in the force-extension curve (FEC). Representative FECs (black) for unfolding the protein PrP are fit to wormlike chain (WLC) models for the folded (cyan) and unfolded (red) states. **(b)** Constant–force trajectories of PrP folding measured at three different forces show abrupt jumps as PrP unfolds/refolds in a two-state process. **(c)** FECs of α-synuclein monomers usually display no rips (cyan) and behave as if unfolded (red: WLC for unfolded state). Some reveal discrete transitions (black, orange, blue), with different contour lengths (gray: WLC fits). **(d,e)** FECs of α-synuclein tetramers connected in tandem by peptide linkers. **(d)** Some FECs show discrete transitions revealing many different structures with different sizes and unfolding forces (dashed lines: WLC fits; gray: folded states, red: unfolded state). **(e)** FECs without discrete rips were averaged (cyan) and compared to polymer models. Data did not fit a non-interacting WLC model (red; residuals in inset), but did fit a model incorporating rapid structural fluctuations (yellow; residuals in inset).
Adapted from Ref. [26] ((a) and (b), copyright (2012) National Academy of Sciences, USA) and Refs. [40,42•] ((c)–(e)).

similar information but from different approaches, which can be exploited for specific purposes (e.g. to select kinetically for a specific pathway [26]) or to enhance analysis reliability via independent measures of the desired information.

## Probing folding/unfolding transitions and conformational dynamics in ordered and disordered proteins

Optical tweezers are providing significant insight into the structure formation process in proteins. Stigler *et al.* deciphered the complex network of states involved in the folding of calmodulin [10] and Yu *et al.* did the same for the prion protein [26,28], in each case characterizing the thermodynamic and kinetic properties of native and non-native intermediates in detail. Neupane *et al.* showed how the statistics of the transition paths can prove that the reaction coordinate used in SMFS is good and that 1D diffusive models of the folding are justified [29•]. Marqusee and colleagues probed the properties of different stages of folding, showing that molten globules can be distinguished from fully native states through their increased compliance [30] and extending classic phi analysis of transition states into the single-molecule regime [31]. Measurements of the unfolding and refolding of monomeric HIV-1-protease validated simulations suggesting the existence of multiple pathways [32], revealing not only two-state unfolding but also unfolding through an intermediate and an ensemble of partially folded states en route to the native state, which themselves unfolded

via multiple pathways [33]. Studies of SNARE complex assembly, which involves a stable four-helix bundle, helped clarify how it drives membrane fusion to allow transport of molecules between different membranes [34,35] and identified rare misfolded states of coiled-coils [36] that may be involved in diseases related to SNARE misfolding [37].

A notable recent advance has been the extension of optical tweezers SMFS to study intrinsically disordered proteins (IDPs), which play many important roles [38,39] but present a special challenge for conformational analysis because of their lack of stable structure. Optical tweezers are well-suited, because of their high force stability and low stiffness [3], to probing the low-energy fluctuations and marginally stable structures expected in IDPs. Studying α-synuclein, an IDP whose aggregation is associated with Parkinson's disease, Neupane *et al.* [40] captured infrequent fluctuations into a diverse set of transient metastable structures (Figure 1c). Linking copies of α-synuclein to create tandem oligomers, the complexity and diversity of the metastable structures increased with the oligomer size (Figure 1d). Not surprisingly for an IDP, however, most FECs displayed no discrete rips; nevertheless, these curves still contained significant information about the conformational dynamics. Indeed, FECs without rips deviated from the pure wormlike chain behavior expected for a non-interacting polymer [41], exhibiting a shoulder-like feature at low force (Figure 1e). This feature suggests rapid quasi-equilibrium fluctuations into structures that are only marginally stable [42•], consistent with the picture of a collapsed, molten-globule-like state for α-synuclein held together by long-range contacts that emerged from structural and computational work [43,44]. Similar shoulder-like features were seen previously in FECs of the protein villin, owing to its ultrafast dynamics which prevented resolution of discrete transitions [4•]. Through analysis of the fast kinetics of α-synuclein, the energy landscape was reconstructed and found to be flat with low barriers, but featuring slow diffusion owing to landscape roughness. Such a flat but rough landscape was expected to be a hallmark of IDPs, but had not been quantified experimentally.

## Probing interactions that influence protein misfolding and aggregation

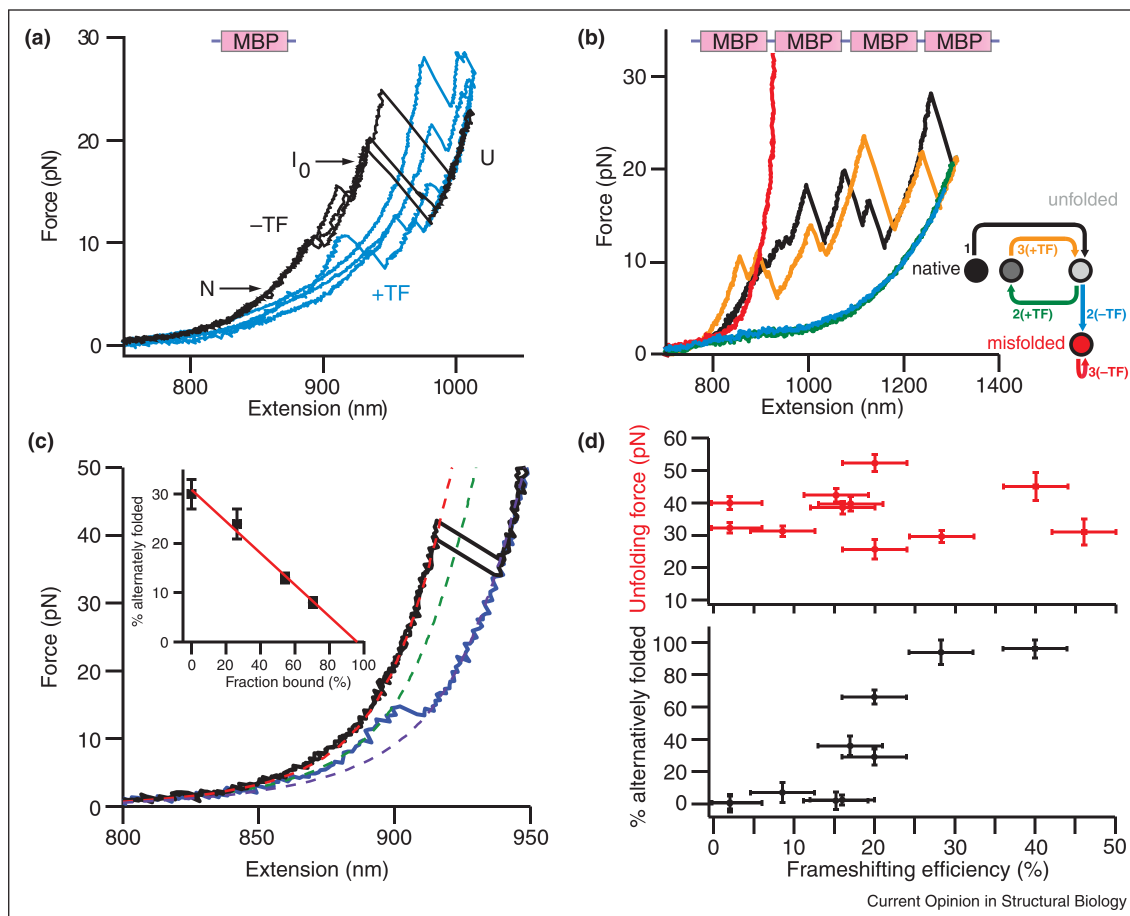
Another area of particular interest is protein misfolding and its relation to disease [45], which is well-suited for study by single-molecule methods because of their ability to distinguish and characterize even transient components of heterogeneous mixtures [46], as shown by recent work with optical tweezers. For example, dimeric prion protein was found to misfold into a stable aggregated state via multiple intermediates, with much slower diffusion than for native folding indicating a rougher energy landscape for misfolding [27••]. The slow refolding of the enzyme luciferase and its propensity to aggregate were linked to the formation of a misfolded state [47]. The effects of calcium concentration on misfolding of a calcium sensor, neuronal calcium sensor-1 (NCS-1) [48•] suggested the missing link between $Ca^{2+}$ dysregulation, misfolding, and a NCS protein involved in neurodegenerative disorders [49,50].

Of course optical tweezers' utility extends beyond studying proteins in isolation to probing the relationship between dynamics and function under conditions that more closely recapitulate key aspects of the cellular environment. Kim *et al.* studied how the binding of von Willebrand factor (VWF) to platelets is regulated by hydrodynamic forces in the vasculature, revealing that force activates platelet binding and increases the effects of disease-related mutations [51]. Especially fascinating is recent work probing the activity of molecular chaperones, which are required for the folding of many proteins but whose mechanisms remain poorly understood at the molecular level [52]. Full understanding of chaperone activity must also be tied to insights into protein misfolding in the absence of chaperones. Tans and colleagues [53••] investigated how the general bacterial chaperone trigger factor (TF) influences the folding of maltose-binding protein (MBP), which folds efficiently in isolation, and a MBP tandem-repeat oligomer ($MBP_4$), which has a high tendency to misfold and aggregate. Unfolding curves of MBP typically showed a single intermediate state without TF present, whereas they contained additional intermediate states with TF present (Figure 2a). The $MBP_4$ oligomer tended to form a stable aggregate that could not be unfolded in the absence of TF, but adding TF dramatically changed the picture: although misfolding interactions generating non-native structures were still common, they were typically much weaker, and most of the protein chain was now natively folded (Figure 2b). Taken together, these results suggest that TF promotes native folding by protecting partially folded states from long-range interactions driving stable misfolded states [53••].

## Conformational dynamics in RNA

SMFS studies of RNA have helped to connect dynamics in complex folding pathways — manifested for example by the tendency of some RNAs to form alternative structures — to function. Measurements of the *E. coli rpsO* gene operator transcript, which folds into either a pseudoknot or double-hairpin conformation but binds the gene product only in the pseudoknot conformation, showed that the two transcript structures can interchange spontaneously [54], which is unusual because conformational switching in RNA typically requires regulatory factors such as metabolites, non-coding RNAs, or proteins [55,56]. Studies of RNA kissing-loops have led to better understanding of the structural determinants of these complexes, including roles for flanking nucleotides

**Figure 2**




Effects of chaperones and small-molecule ligands on protein and RNA folding. **(a)** In the absence of TF, MBP monomers (black) unfold via an intermediate state ($I_0$) between the native (N) and unfolded (U) states. In the presence of 1 μM TF (blue), MBP frequently occupies a variety of intermediate states. **(b)** FECs of a tandem MBP tetramer show more complex behavior. Initial unfolding (black) shows features corresponding to four natively folded MBP monomers. When refolded without TF (blue), subsequent pulling (red) reveals no unfolding, indicating the tetramer misfolded with tight inter-domain interactions. When refolded with 1 μM TF (green), subsequent pulling (orange) showed extension changes characteristic of native interactions (e.g. at ~1230 nm) and weak non-native interactions (at ~1100 nm), but no tight ones. Inset: sequence of pulling curves and states observed. **(c)** Most FECs for the SARS frameshifting stimulatory pseudoknot showed a length change, found from WLC fits (dashed lines), consistent with the native structure (black), but some revealed a smaller, alternative structure (blue). Inset: the extent of alternative structure formation decreased linearly with the fraction of ligand-bound pseudoknots and extrapolated to zero at 96 ± 8% binding, indicating that ligand binding effectively eliminates the formation of alternative structures, mirroring the suppression of −1 PRF to near-background levels caused by the ligand [67]. **(d)** The average unfolding force of pseudoknots is uncorrelated with efficiency of −1 PRF stimulation (upper panel), indicating that mechanical stability is not a primary determinant of −1 PRF efficiency. Higher frameshifting efficiency is instead correlated with increased tendency to form alternate structures (lower panel).
Adapted from Ref. [53••] ((a) and (b), by permission from Macmillan publishers Ltd: Nature, copyright (2013)), Ref. [68] ((c), copyright (2014) American Chemical Society) and Ref. [65•] ((d), copyright (2012) National Academy of Sciences, USA).

[57]. Riboswitches are an especially interesting class of RNAs that undergo large-scale, functional conformational changes upon ligand binding [58]. Anthony *et al.* deciphered the ligand-dependent and ligand-independent steps for the metabolite-sensing domain of the TPP riboswitch [59], and Neupane *et al.* showed how structural changes in the metabolite-sensing domain of the *add* riboswitch are communicated to alter the structure of the neighboring domain controlling gene expression [60]. By studying *pbuE* riboswitch dynamics while the RNA was being transcribed, Frieda *et al.* were able to demonstrate kinetically controlled folding directly and predict the regulatory outcome — transcription termination or run-through — from the initial RNA dynamics [61•].

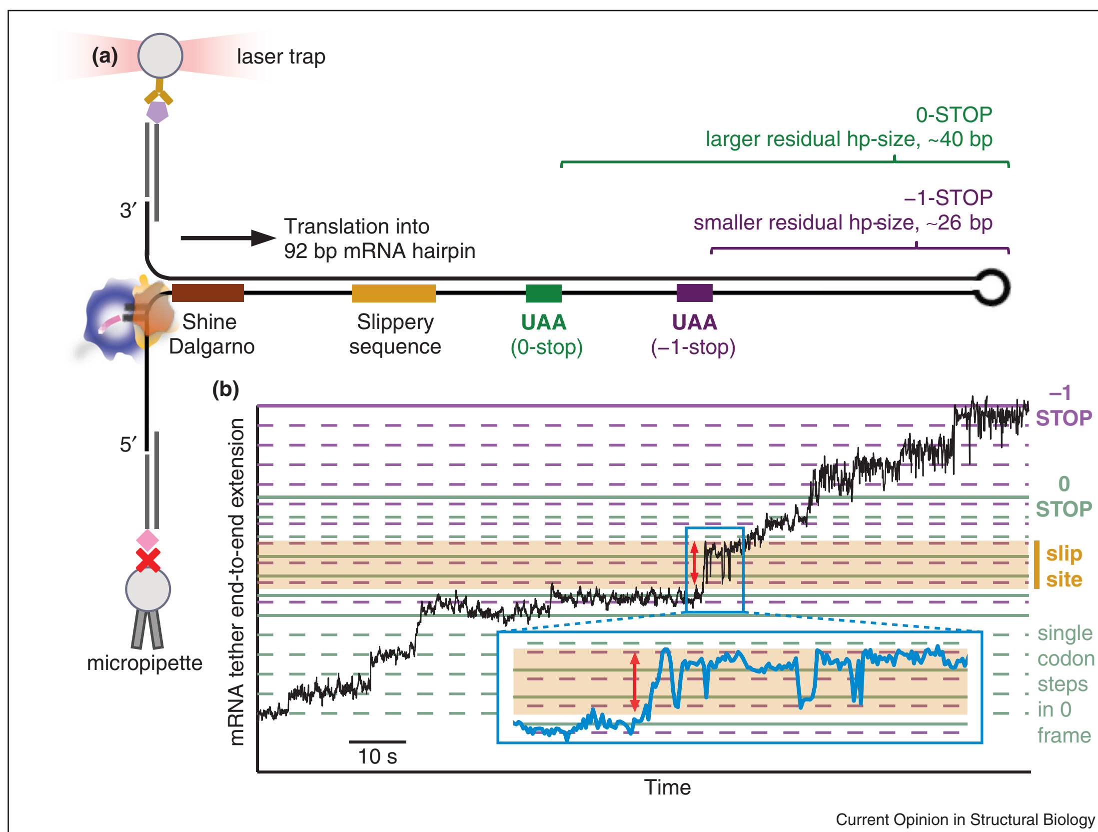
Recent work with optical tweezers also highlighted the unexpected importance of conformational dynamics for the function of RNA pseudoknots stimulating −1

programmed ribosomal frameshifting (−1 PRF) [62]. Early work identifying a correlation between the pseudoknot unfolding force and −1 PRF stimulation efficiency [63,64] was shown not to extend to a larger panel of viral pseudoknots; instead, −1 PRF efficiency was correlated with pseudoknot conformational plasticity [65•], reflected in the tendency of the RNA to refold into alternative structures (Figure 2c,d). The importance of pseudoknot dynamics in stimulating frameshifting was corroborated by studies of the frameshift signal from human CCR5 mRNA, showing that it manifests several distinct unfolding pathways when mechanically destabilized [66], and by measurements of the effect of a ligand that abolishes −1 PRF upon binding specifically to the pseudoknot from the SARS coronavirus [67], showing that the ligand reduced the conformational plasticity of the pseudoknot proportional to the amount of ligand bound (Figure 2c inset) [68]. While such studies provide important insights, they investigate only one part of the −1 PRF mechanism, which involves interactions between many different elements. A more complete picture emerges from experiments probing the full translation complex, like those described below.

## Complex assemblies in motion: dynamics tied to function

In recent years, optical tweezers have increasingly been applied to reveal the functional dynamics of complex macromolecular assemblies, especially those of protein–nucleic acid complexes. Recent work on nucleosomes, the basic unit of DNA compaction consisting of DNA wrapped around a histone core, elegantly showed how local conformational transitions in single nucleosomes govern DNA unwrapping [69••], demonstrated alternate

**Figure 3**



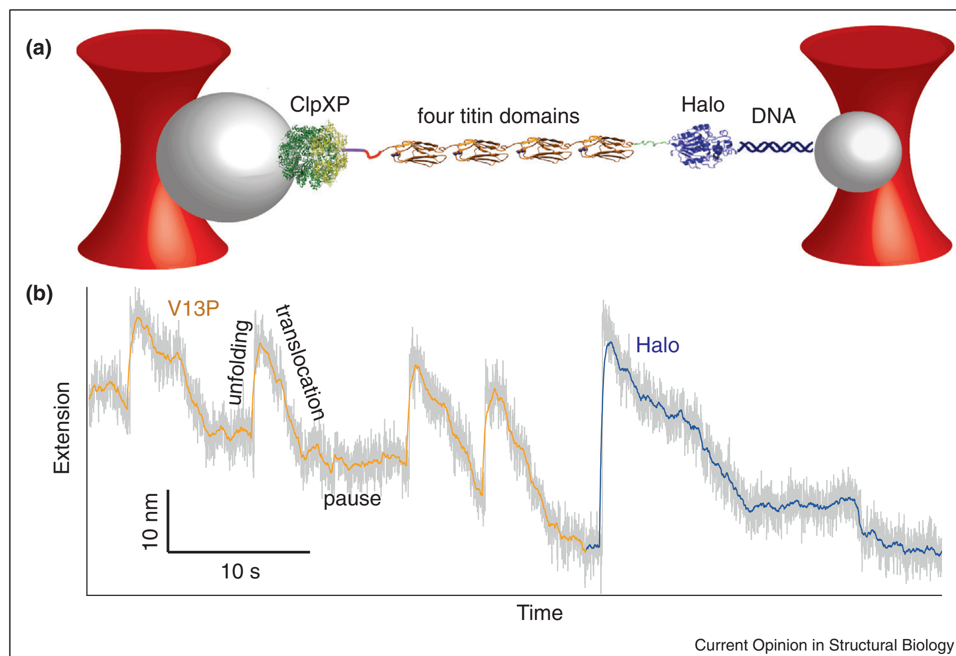

Translocation dynamics in programmed ribosomal frameshifting. **(a)** An mRNA hairpin molecule containing the frameshift signal from *dnaX* (internal Shine–Dalgarno sequence, slippery site, stimulatory hairpin) and bound with a single ribosome is held under tension. The hairpin unzips by 3 basepairs/codon as the ribosome translocates, increasing the tether length. When the first 0-frame codon in the slippery site resides in the ribosome P site, a 55-basepair hairpin remains downstream. To determine the ribosome location on the mRNA at the end of each trajectory, hairpin portions not unwound by the ribosome are mechanically unfolded. Ribosome termination at the −1 stop codon leaves a smaller residual hairpin than at the 0 stop. **(b)** A single-ribosome translation trajectory along the mRNA construct shows individual translocation steps (green and purple lines: single-codon steps in 0 and −1 frames, respectively). The ribosome continually translocates against a hairpin, but characteristic fluctuations in mRNA extension (red arrow) occur around the slippery sequence region (orange-shaded area), reflecting dynamics in the ribosome induced by the frameshift signal.Adapted from Ref. [78••], copyright (2015), with permission from Elsevier.

pathways for nucleosome unwinding [70], and showed through the use of torque-wrench tweezers [71] that torque can modulate nucleosome stability in a way that may regulate histone exchange during transcription and replication [72]. These studies have important implications for how DNA accessibility to enzymes might be regulated at the level of DNA sequence and modifications. In a similar vein, Ma *et al.* investigated the effects of torsion from DNA supercoiling on transcription by RNA polymerase, finding that torsion modulates the transcription rate but RNA polymerase can generate sufficient torque to melt DNA of arbitrary sequence [73]. Turning to translation, the mechanics of translation were studied by Bustamante, Tinoco, and colleagues: after demonstrating that individual codon steps could be discerned and showing that ribosomes apply force actively to unfold structures in the mRNA during translation [74,75], they investigated how the ribosome affects nascent polypeptide folding [76] and showed that nascent chain folding near the surface of the exit tunnel exerts a force that can rescue sequence-induced ribosome stalling [77•].

Tinoco and colleagues also applied a single-ribosome translocation assay to probe −1 PRF stimulated by a structured RNA hairpin from the *E. coli dnaX* gene [78••]. Tight regulation of −1 PRF efficiency is achieved by three elements in the mRNA: a 7-nt slippery sequence where −1 PRF occurs, and an internal Shine–Dalgarno sequence and downstream hairpin flanking the slippery sequence. Codon-by-codon translation was monitored along a mRNA template embedding this frameshift signal within a 92-bp hairpin held under tension by optical tweezers (Figure 3a). Back-and-forth motions of the ribosome around the slippery sequence (Figure 3b), previously detected by single-molecule fluorescence measurements [79,80], were corroborated and shown to occur regardless of the presence of a frameshift, suggesting that they are a property of the mechanics of the frameshift signal rather than a feature of −1 PRF itself. Notably, the fluctuation timescale was similar to that for the dynamics of the ribosome 30S body and head during regular translation [81], suggesting that these fluctuations reflect conformational excursions of the 30S head during multiple forward translocation attempts [78••].

The ability of optical tweezers to track substrate movement through molecular machines has been applied to other systems, too, such as elegant work investigating how the bacterial protease ClpXP unfolds and translocates a wide variety of substrates [82,83••]. To probe the

**Figure 4**




Unfolding and translocation of single proteins by the protease ClpXP. **(a)** ClpXP is attached to one laser-trapped bead and engaged with a multidomain substrate consisting of four titin domains separated by short peptide linkers and a Halo-tag domain, which is attached to a second laser-trapped bead via a DNA linker. **(b)** A trajectory for ClpXP unfolding and translocation of the V13P titin mutant (gray, unaveraged; gold, averaged data for V13P domains; purple, averaged data for Halo domain), in which the titin domain is destabilized to increase ClpXP degradation, shows three main features. Unfolding of individual domains by ClpXP causes abrupt increases in the construct extension under tension (upward movement), whereas translocation decreases the extension gradually as the protease reels in the unfolded polypeptide chain (downward movement). After each domain is translocated, ClpXP pauses for a variable dwell time before unfolding the next domain.Figure adapted from Ref. [83••], copyright (2014), with permission from Elsevier.

interactions between protease and substrate, Cordova *et al.* [83••] engaged ClpXP with a substrate consisting of four titin domains (Figure 4a). Measurements under constant force displayed three signatures of ClpXP mechanical function: first, abrupt extension increases due to protein unfolding; second, gradual extension decreases due to translocation of the unfolded polypeptide; and third, unchanged extension representing a pre-unfolding dwell between completed translocation of an unfolded domain and denaturation of the next native domain (Figure 4b). As expected, titin mutants with lower stability were less resistant to degradation. These measurements led to a model of how ClpXP uses ATP to move along its substrate: ClpXP takes steps of variable sizes (1–4 nm), in no defined order but with steps of similar sizes clustering together, indicating interplay between stochastic and deterministic behavior of the subunits. Incorporating hydrolysis-incompetent mutant subunits into ClpXP showed that the largest steps required no more than two subunits. This study also illustrates nicely how a complete mechanistic understanding of complex biological processes requires fitting single-molecule data into a wider framework provided by other techniques: here, the degradation rate measured by optical tweezers was consistently higher than in ensemble experiments, suggesting that the rate-limiting step in solution is the threading of the substrate into the protease, a step done before the tweezers measurements started.

## Outlook

Models describing the activity of proteins and RNAs have not always included the role of conformational dynamics in determining function. Optical tweezers provide a powerful tool for probing the dynamics of biomolecules at the single-molecule level, providing new information about the relationship between dynamics and function. Studying the dynamics of molecules in isolation continues to yield valuable new insights, but it is the application of optical tweezers to more complex systems that is particularly exciting. Sensitive and high-resolution measurements with optical tweezers hold promise for elucidating the conformational dynamics underlying the complicated mechanisms of phenomena such as ribosomal translocation and frameshifting, co-transcriptional and co-translational folding, protein misfolding and chaperone action, and protein degradation by proteases. With a constant flow of new information from such experiments to complement results from other single-molecule and ensemble techniques, it will be exciting to see how mechanistic models evolve in the coming years.

## Conflict of interest statement

Nothing to declare.

## Acknowledgement

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- • of special interest
- •• of outstanding interest


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