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Beyond the Single Molecule: Multiplexed Methods in Force Spectroscopy

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Abstract

Single-molecule techniques have transformed biological research by enabling direct observation and manipulation of individual molecules. These methods overcome ensemble averaging inherent in bulk measurements and facilitate studies under physiological stresses and out-of-equilibrium conditions. They have provided valuable insights into diverse biological processes, from stepping mechanisms of molecular motors to mechanical properties of biomolecules to the dynamic strength of intermolecular bonds. Advances in multiplexed and high-throughput single-molecule force spectroscopy methods are improving throughput, capabilities, and accessibility. In this review, we detail the evolution of multiplexed force spectroscopy technologies, highlighting key advances in instrumentation, molecular engineering, and analytical techniques. We discuss diverse applications spanning molecular biophysics, biomolecular sensing, proteomics, and cellular mechanobiology. Finally, we explore ongoing challenges and future opportunities and

highlight how the impact of multiplexed single-molecule force spectroscopy can continue to grow through further developments in novel instrumentation, chemical tools, and innovative applications.

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INTRODUCTION

Single-molecule methods have significantly advanced biological research, providing insights free from the ensemble averaging inherent in bulk measurements in areas ranging from protein folding (102) to molecular motor mechanics (113, 133) to nucleic acid processing (17, 33, 150). The development of single-molecule approaches has been driven both by technological advances and by compelling biological questions. Early breakthroughs were driven largely by novel instrumentation that enabled direct observation of individual molecules and precise measurement of their mechanical responses to forces. Force has served both as a mimic of physiological stresses and as a probe to study interactions out of equilibrium.

Historically, several biological systems have underscored the importance of single-molecule force spectroscopy techniques and have motivated further technological advances. Examples include the study of molecular motors (132), mechanical characterization of cellular materials (127), and measurement of intermolecular bond strength (92). These studies fostered a virtuous cycle of technology development, where each advance in spatial and temporal resolution, or in force and torque precision, opened avenues to new biological questions, which in turn drove further technological innovation. For example, efforts to resolve and understand discrete stepping motions in molecular motors spurred progress in measurement precision and force control (132, 139), while early studies of DNA elasticity led to instrumentation capable of precisely controlling tension and twist (127, 128).

Despite significant advances, force spectroscopy techniques typically face inherent throughput constraints due to their single-molecule nature. This can limit their application in both basic and clinical contexts, making it challenging to study large molecular libraries or analyze patient samples. It has also been noted that while single-molecule studies have substantially advanced our understanding of numerous biological mechanisms, broader biological discoveries—such as identifying entirely new molecular processes—have been relatively less common, perhaps due to limitations in experimental capabilities, accessibility, and throughput (140). While single-molecule approaches hold considerable promise for detailed and comprehensive characterization of biomolecular systems, including the capture of rare events or molecular populations and the resolution of molecular heterogeneity, limited throughput remains a significant practical barrier (**Figure 1**).

The recent development of multiplexed and high-throughput single-molecule force spectroscopy methods has begun addressing these challenges. Novel platforms like the centrifuge

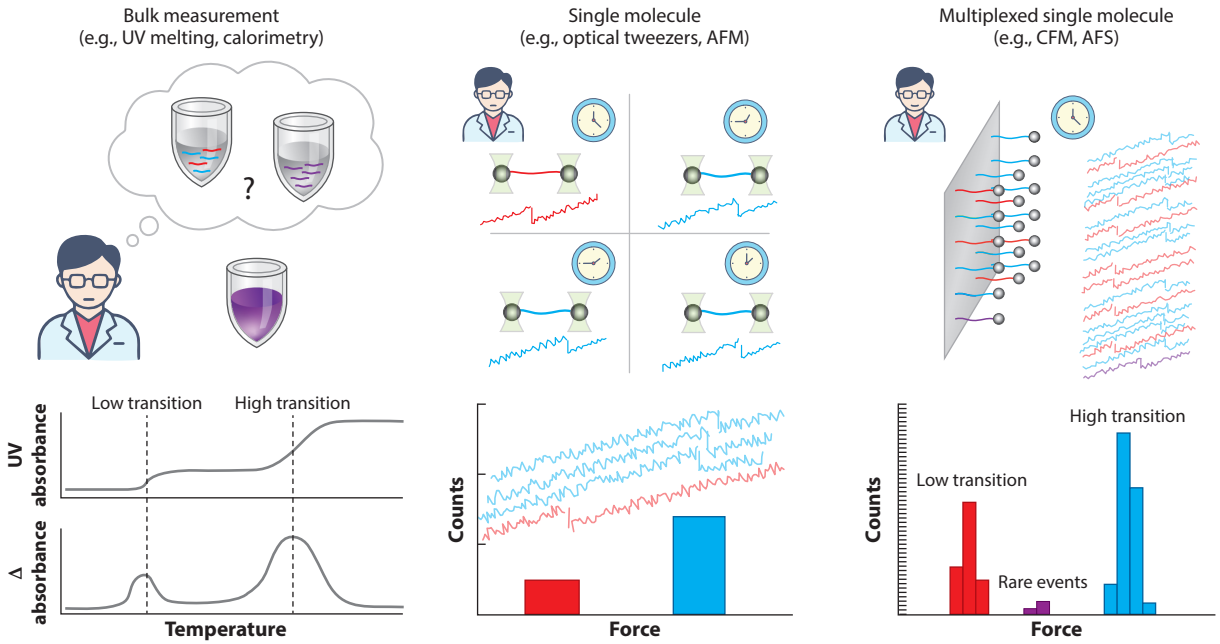


Figure 1

Overview of bulk, single-molecule, and multiplexed single-molecule methods. Bulk methods show average behavior that can be resolved in single-molecule experiments. Multiplex methods offer additional efficiencies, throughput, and opportunities for resolving molecular heterogeneity and rare events and populations. Abbreviations: AFS, acoustic force spectroscopy; CFM, centrifuge force microscope.

force microscope (CFM) and acoustic force spectroscopy (AFS) have emerged, uniquely combining high-throughput capabilities with sufficient precision for single-molecule analysis (50, 124, 146). Traditional methods, such as magnetic and optical tweezers, and flow-based methods have also expanded their throughput (6, 106, 149). Together, these multiplexed methods are transforming the range and application of single-molecule experiments, enabling large-scale screening, robust statistical analysis of rare populations and events, and comprehensive characterization of heterogeneous molecular populations (90).

In this review, we discuss the history and current landscape of multiplexed single-molecule force spectroscopy, highlighting developments in instrumentation, molecular tools, advances in surface chemistry, and analytical methods that have supported these technological improvements. We also explore emerging applications enabled by high-throughput approaches in both basic biophysical research and translational and clinical applications. Finally, we outline challenges and opportunities in this evolving field, including its potential to affect areas such as diagnostics and proteomics.

DEVELOPMENT OF MULTIPLEXED INSTRUMENTATION

Since the early 1990s, single-molecule force methods have been providing a window into detailed properties and activities of biomolecules. Early studies investigated mechanical properties of DNA (22, 126, 127, 131), receptor–ligand interactions (40, 60, 92), and protein unfolding (107). These studies and the many others that followed were enabled largely by advancements in technology, particularly the ability to control force through various mechanisms and to observe microscopic movement of tethered particles. While this review is focused on methods that use force, it is worth

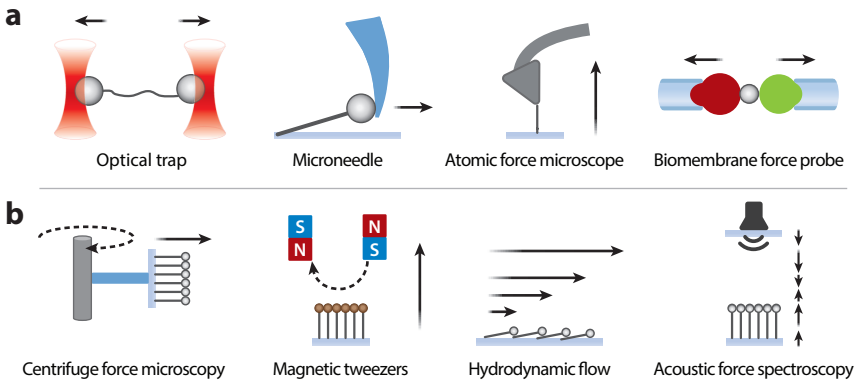


Figure 2

Overview of common geometries and force application mechanisms for single-plexed (*a*) and multiplexed (*b*) single-molecule force techniques.

noting that major advances in single-molecule microscopy techniques were also happening around this same time (93).

The main variations among techniques are the mechanisms by which force is applied and by which microscopic movements are observed. By the end of the 1990s, several viable options for force application had emerged (**Figure 2*a***), including hydrodynamic drag (19, 101, 104), magnetism (53, 127, 131), optical forces (9), cantilevers (both atomic force microscopes and microfibers) (22, 40, 60) and pressurized membranes (92). To analyze the microscopic motions, researchers often used high-speed electronics such as photodiodes and position-sensitive detectors or wide-field methods like cameras.

Going into the 2000s it had become clear that optical tweezers, magnetic tweezers, and atomic force microscopy (AFM) had emerged as the dominant tools for quantitative single-molecule force spectroscopy. These methods excelled at probing both intramolecular and intermolecular interactions, including receptor–ligand binding and other systems involving multiple distinct molecular entities interacting under force. The three dominant methods were, however, not without limitations, motivating researchers to continue exploring new technologies. One of the main shifts was the push toward multiplexing single-molecule experiments rather than being restricted to observing a single molecule at a time. There are a few inherent challenges in expanding instrumentation in this direction, including the difficulty of multiplexing a uniform force over a large area, decoupling force and distance measurements to allow a large field of observation, and achieving these two objectives while maintaining a useful force range.

Among the three major methods of the time, only magnetic tweezers were readily capable of multiplexing the force and were in fact the first to demonstrate multiplexing (25, 26, 106). As for the other techniques, AFM is poorly suited for multiplexing (though adept at high-throughput serial force probing) (70), while optical tweezers were multiplexed with holographic or time-sharing methods (47, 138), though to our knowledge have not been used for multiplexed single-molecule force measurements. Flow-based methods are generally well-suited for multiplexed force application, and several examples of these also began to arise, including flow stretching (72) and DNA curtains (39, 44). Some chip-based approaches with multiple DNA tethers were also developed (8, 56).

Another challenge is decoupling force and distance measurements. If the force application method is highly localized and springlike (such as optical tweezers or AFM), then accurate

application and measurement of forces require high-resolution positional control and detection, respectively. This creates a practical problem in multiplexing due to the conflicting goals of achieving high-force resolution (requiring high spatial resolution) and expanding the field of view to observe multiple particles (lowering spatial resolution). Highly localized fields also have difficulty maintaining a useful force range while multiplexing. In optical tweezers, multiplexing usually involves dividing the available laser power across multiple traps, causing peak force per trap to scale roughly inversely with the number of traps (i.e., 100 traps receive roughly one-hundredth of the original force). Magnetic tweezers face a similar but less severe trade-off between force range and multiplexing. High forces can be achieved with a highly localized field, while broadening the field to enable multiplexing necessarily decreases the force range and field uniformity with the same strength magnet.

To address these challenges, it is advantageous to employ methods capable of generating broadly uniform force fields across large areas of observation. Among early single-molecule methods, flow-based stretching and moderate-force magnetic tweezers inherently fit this bill by providing relatively uniform force fields that decouple precise force measurement from stringent positional tracking (72, 106).

In the early 2000s we recognized the need for multiplexed single-molecule tools and conceptualized a new multiplexed single-molecule approach using centrifugal force. The basic idea was to combine a microscope and a centrifuge to allow application of a uniform, calibration-free force on many tethered particles at once while observing their individual microscopic to nanoscopic motions. Halvorsen & Wong (50) later demonstrated this idea with a proof-of-concept experiment, performing thousands of single-molecule force experiments in parallel, and coined the instrument the centrifuge force microscope.

Following our work, there was an expansion of other multiplexed single-molecule techniques (**Figure 2*b***). AFS was developed, which uses acoustic waves to produce piconewton-level forces to many tethered beads (124). There was also work on other methods, including a nanophotonic array (128), an optical pushing system (125), arrayed magnetic tweezers (28), and a chip-based approach (98), among others.

In recent years, ongoing recognition of throughput as a critical factor has driven further developments in multiplexed single-molecule force spectroscopy. Existing methods have advanced significantly, while innovative new techniques continue to emerge. The CFM has evolved with key improvements such as integration into a benchtop centrifuge (146), wireless data transmission and streaming (2, 57, 74), addition of fluorescence imaging (13, 85), and further dissemination through the publishing of detailed protocols for building and using the instrument (1, 147). AFS has similarly progressed (67), expanding into cellular microrheology and adhesion studies and becoming commercially accessible through efforts by LUMICKS. Multiplexed magnetic tweezers have also seen technical innovations, including addressing scaling challenges (27, 66) and introducing torque (78). An innovative new hybrid system combines magnetic manipulation with flow-based stretching, achieving unprecedented throughput with up to 50,000 simultaneous measurements (6).

Among the four major multiplexed techniques in **Figure 2**, each comes with distinct advantages, limitations, and quirks. Magnetic tweezers are a well-established technique with a useful force range, moderate cost, and the unique ability to apply torque. Limitations include trade-offs between field uniformity and maximum achievable force, the need for force calibration, and the requirement of magnetic particles, while quirks can include unwanted bead–bead interactions and bead magnetization variability. Flow methods are also well-established and have relatively simple instrumentation and good buffer exchange capabilities. Limitations include the need for microfluidics experience and potential surface interactions with beads. Quirks can include the potential for channels to clog or leak and variations in flow velocity that can affect the

experiments. The CFM has advantages of a uniform and calibration-free force field capable of spanning a broad force range, compatibility with any microscopic object, low hardware cost, and relative simplicity of use. Limitations include restricted temporal resolution, necessity for a custom-built device, and inaccessibility of the chamber during experimentation. AFS offers an inherently multiplexed platform capable of rapid force modulation, though it relies on nondisposable, expensive custom-fabricated chips and has a relatively complex force field that can be difficult to calibrate.

Several emerging chip-based trapping and confinement approaches also have some potential for multiplexed force spectroscopy, including dielectrophoretic tweezers (20), electrostatic trap arrays (79), optoelectronic tweezers (145), pneumatically actuated convex lens-induced confinement (12), and plasmonic tweezers (152). Recent development of the nanophotonic standing-wave array trap has already shown promise for parallel single-molecule force spectroscopy on DNA with performance approaching that of traditional optical tweezers (149). These emerging methods and continued development of more traditional methods continue to push the capabilities and throughput for single-molecule experimentation.

MOLECULAR TOOLS FOR SINGLE-MOLECULE ASSAYS

Advances in multiplexed single-molecule force spectroscopy depend not only on improving instrumentation as described above but also on developing appropriate molecular approaches to ensure precise nanoscale control. The reliability of each measurement depends on the specificity, stability, and verifiability of the molecular interactions under force. Historically, early single-molecule studies often relied on nonspecific adsorption of proteins to surfaces—a straightforward yet less controlled approach prone to randomness and limited yields (107). High-throughput multiplexed experiments benefit from rigorous molecular engineering through controlled surface chemistry, bioconjugation techniques, and advanced molecular tethers such as programmable DNA nanoswitches and other nanostructures.

Surface chemistry is critical yet notoriously challenging, as captured by Wolfgang Pauli's observation that "God made the bulk; surfaces were invented by the devil" (111, p. 230). Early surface functionalization strategies, including glass silanization protocols and self-assembled monolayers on gold substrates, introduced defined chemical functionalities for coupling reactions (92). One method to attach proteins to surfaces is by coating the glass coverslip with nitrocellulose (typically used in methods like Western blotting) to absorb proteins (86). Passivation strategies are also important to minimize nonspecific interactions. These include polymeric coatings such as polyethylene glycol or polyacrylamide, supported lipid bilayers, and the use of blocking agents such as bovine serum albumin (BSA) or casein (39, 62, 74) (**Figure 3*a***).

Precise spatial control of molecular placement on surfaces has significantly advanced multiplexed single-molecule assays. Methods such as microcontact printing, bead-templated lithography, and DNA origami–based nanoarrays have enabled the creation of defined molecular patterns with enhanced density and reduced intermolecular interference (28, 117, 125) (**Figure 3*b***). Recently, Choi et al. (21) developed a light-guided molecular patterning method that does not require conventional lithography equipment, improving accessibility and throughput (**Figure 3*c***).

Complementing surface chemistry advancements, the diversity of bioconjugation strategies has also improved. Noncovalent interactions such as biotin–streptavidin remain popular for their high affinity and simplicity. Covalent conjugation techniques, such as maleimide–thiol and *N*-hydroxysuccinimide–amine reactions, can provide mechanically robust attachments capable of sustaining higher forces over extended durations (62). The advent of bioorthogonal click

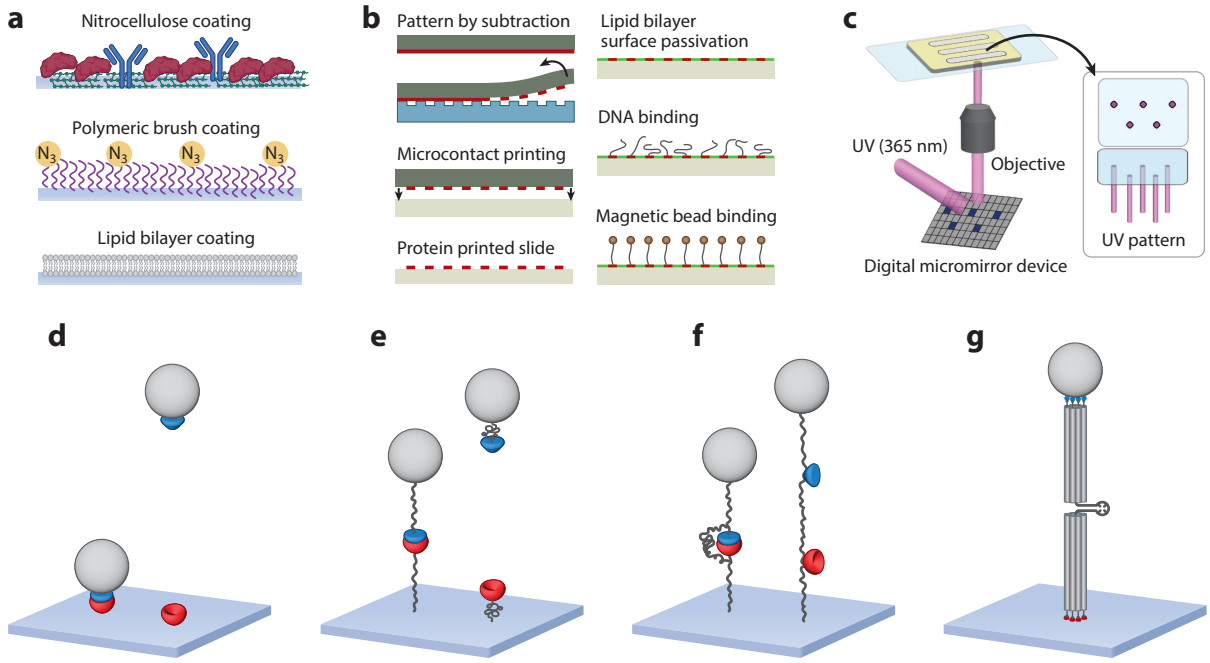


Figure 3

Surface preparation and molecular constructs. (*a*) Common methods for passivation and functionalization of surfaces include nitrocellulose coatings, polymeric brushes, and supported lipid bilayers. (*b*) Surfaces can be patterned using microcontact printing. For example, protein-coated elastomers can be patterned through subtraction by adsorption onto a silicon template. The resulting patterned elastomer is then used to transfer proteins onto a glass slide. (*c*) Light-guided surface patterning utilizes digital micromirror devices to selectively illuminate and crosslink UV-sensitive compounds, creating programmable arrays of functionalized regions on a glass slide. (*d*) Configurations lacking single-molecule signatures can make it difficult to discriminate genuine molecular binding from false positives. (*e*) Long polymeric (e.g., DNA) linkers can provide unique mechanical signatures due to their defined length and elastic properties. (*f*) Looped linkers provide characteristic length increases upon unbinding, enabling unique molecular signatures and repeated measurements of molecular pairs; these can be implemented using protein engineering (e.g., ReaLiSM constructs with polypeptide linkers between binding partners) or by using DNA self-assembly techniques as in DNA nanoswitches, which provide an addressable scaffold for attaching proteins at defined distances. (*g*) DNA origami techniques can be used to create rigid constructs that reduce thermal noise, enhancing precision. Abbreviation: dsDNA, double-stranded DNA. Panel *a* adapted from images created in BioRender by Ward, A. (2025), **https://BioRender.com/e0k8a6v**; and modeled after concepts presented in References 39, 62, 74, and 86. Panel *b* adapted with permission from Reference 28. Panel *c* adapted from Reference 21. Panels *d–f* modeled after concepts presented in Reference 49. Panel *g* modeled after concepts presented in Reference 103.

chemistries has further enhanced both the ease and the durability of covalent linkages, allowing for stable, reproducible tethering beyond 100 pN (5, 34).

For protein coupling, lysine and cysteine labeling are commonly used, with site-specific labeling approaches used when a more well-defined point of attachment is needed (87). Genetically encoded protein tags such as SNAP-tag, HaloTag, and SorTag and the SpyTag/SpyCatcher system have extended this bioconjugation toolkit (23, 68, 89, 105). SpyTag/SpyCatcher forms a covalent bond with high efficiency and stability and features a well-defined unfolding transition for verification and force calibration (151). Another approach useful for engineered proteins is incorporation of unnatural amino acids that enable placement of functional groups at almost any location in the protein (116). For antibody labeling, commercially available reagents, such as oYo-link, specifically target the fragment crystallizable (Fc) region.

Molecular handles are used in single-molecule experiments to attach and control the positioning of molecules of interest and to provide a signature when pulling (**Figure 3*d*,*e***). DNA has long served as a versatile and reliable material for single-molecule force spectroscopy, owing to its well-understood mechanical properties and the ease of bioconjugation. Early experiments using extended DNA linkers established characteristic mechanical benchmarks, such as a well-defined force extension curve and an overstretching plateau at ∼65 pN (15, 16, 52). DNA tether technology has steadily improved with robust attachment methods that can withstand sustained forces up to ∼150 pN, far beyond typical streptavidin–biotin limits (62). Dual-attachment strategies with multiple anchor points enable torsionally constrained DNA handles essential for precision magnetic torque tweezers experiments (86). The combination of modular chemical modifications, sequence-specific designs, and orthogonal attachment chemistries ensures DNA remains a powerful, reliable tether in multiplexed force spectroscopy assays.

Engineered molecular handles can provide additional benefits, including controlled colocalization of molecules through direct tethering, unique molecular signatures, and improvements in precision. Early examples of this include polyproteins for signatures in unfolding experiments (58) and a polypeptide linker between binding proteins that both provides a characteristic unbinding signature and enables repeated interrogation of individual molecules (71) (**Figure 3*f***). Moreover, directly tethering two or more binding partners governs their spatial arrangement, increasing local concentration through spatial confinement and precisely defining their stoichiometry at the single-molecule level. Methods in DNA nanotechnology (109, 112) have simplified construction of complex handles and extended the range of possibilities. Mechanically actuated DNA nanoswitches incorporate reversible, force-sensitive loops, providing programmable molecular signatures and enabling repeated interrogation of molecular interactions for binding and unbinding kinetics (49, 90, 146) (**Figure 3*f***). A slightly different design with the same concept was later implemented with junction DNA (75). More recent developments in this area have led to DNA nanoswitch calipers, which can achieve angstrom-level distance measurements for detailed molecular fingerprinting (119). Rigid DNA origami beams have been used to reduce mechanical noise and help enable complex measurements (69, 103) (**Figure 3*g***).

Looking ahead, continued innovations in surface functionalization, bioconjugation chemistry, and DNA nanotechnology promise even greater experimental precision, programmability, reliability, and throughput.

ANALYSIS OF MULTIPLEXED FORCE SPECTROSCOPY DATA

Multiplexed force spectroscopy involves both acquisition and theoretical analysis of experimental data, typically microscopy images acquired via digital cameras. Methods generally consist of the attachment of micron-sized colloidal particles (microspheres) to molecular tethers that are coupled to the surfaces of microscopy slides. The microspheres typically provide the means by which the force is being applied to the molecule(s) of interest (e.g., by drag, centrifugal force, or magnetic field).

Particle-tracking methods have been developed to translate digital microscopy images into physical quantities of interest such as force and distance. Several algorithms using various approaches can track the x–y position with subpixel accuracy (24, 54, 82). The z-position is more dependent on imaging method and can be obtained with interference patterns from reflected light (53), by forward scattering of the transmitted light (43), by video holographic microscopy (84), or by darkfield interferometry (108). Particle positions can be determined offline after experiments or can be analyzed in real time through the use of graphical processing units (76, 82).

Control and measurement of force can vary depending on the approach. For most methods the measurement of the applied force requires the measurement of particle position. For tethered

particles, fluctuations of the particle orthogonal to the direction of force application can be used to estimate the force (136). This is typically done in magnetic tweezers by measuring particle fluctuations at various magnet positions to estimate the force as a function of magnet position. Likewise for AFS, force is calibrated as applied voltage on the acoustic-wave-generating piezo plate is changed. For fluctuation-based methods, it is important to consider the effects of camera exposure time that result in image blur and can bias force calibrations (97, 144). For flow-based methods, a proper understanding of the geometry and flow-rate can be used to estimate the force (123). For the CFM, the force can be calculated on the basis of particle mass and rotational speed of the centrifuge, offering a unique advantage of calibration-free force without detailed knowledge of the tethered particle position.

Typical experiments either hold a constant force or have a force increased in time while the positions of the particles are tracked. For experiments using DNA tethers, the particle position gives information about the extension of the DNA, allowing for creation of a force extension profile that serves as a single-molecule signature. Unfolding or rupture events are seen as discontinuities in the particle position or as complete disappearance of the particle.

Analysis of force spectroscopy data is a rich and complex subject. The first theoretical treatment of the effect of force on protein interactions was by Bell (11) in 1978 to describe cell–cell adhesion. In the 1990s, theory was expanded further by Evans & Ritchie (38) to describe the effect of dynamically changing force load on the probability of rupture. Similar models have been used to describe atomic and molecular friction (143).

For single-bond rupture modeled as a simple two-state system, the probability of being bound, $B(t)$, can be described by the rate equation

$$\frac{\mathrm{d}B}{\mathrm{d}t} = -k_{\mathrm{off}}(t)B(t) + k_{\mathrm{on}}(t)[1 - B(t)], \qquad 1.$$

where $(1 - B(t))$ is the probability of being in the ruptured state, and k_{off} and k_{on} are the off-rate and on-rate of the interaction, respectively. Generally, k_{off} and k_{on} may be functions of force and time, but in the simplest (and common) case with effectively no rebinding, k_{on} is zero. An early model to describe the escape rate of a particle over an energy barrier was developed by Arrhenius,

$$k_{\mathrm{off}} = k_{\mathrm{att}} \exp\left(\frac{-E_{\mathrm{b}}}{k_{\mathrm{B}}T}\right). \qquad 2.$$

Here, E_{b} is the depth of the energy barrier, k_{att} is the attempt frequency, k_{B} is the Boltzmann constant, and T is the temperature. An applied force F decreases the depth of the energy barrier by $F \cdot x_{\mathrm{TS}}$, where x_{TS} is the distance to the transition state. Now the off-rate can be described by (11)

$$k_{\mathrm{off}} = k^0_{\mathrm{off}} \exp\left(\frac{F}{f_\beta}\right), \qquad 3.$$

where k^0_{off} is the zero-force off-rate and the force sensitivity of the bond, f_β, is $k_{\mathrm{B}}T/x_{\mathrm{TS}}$. Equation 3 is valid only for a single sharp barrier in 1D, and deviations from this can change the force dependence of the off-rate (38). However, for many single-molecule interactions Equation 3 works surprisingly well. These two key parameters can be obtained from most force spectroscopy experiments, and for constant force experiments Equation 3 can be used directly.

If a linear force ramp is applied, the force F in Equation 3 is a function of the loading rate, l_{r}, and the time. By substituting Equation 3 into Equation 1, the most probable rupture force, f^*, can be determined by finding the maximum in the probability density of rupture (i.e., by setting $\mathrm{d}^2B/\mathrm{d}t^2 = 0$) (38), which yields

$$f^* = f_\beta \log\left(\frac{l_{\mathrm{r}}}{f_\beta \cdot k_{\mathrm{off}}}\right). \qquad 4.$$

Here, you can see the classic result that the most probable rupture force f^* increases with the logarithm of the loading rate. Experimentally, f^* at a given loading rate can be determined by finding the maximum in a histogram of rupture forces.

The zero-force off-rate obtained from the fit can be compared with measurements of the bulk zero-force off-rate (e.g., by biolayer interferometry or surface plasmon resonance) as a consistency check. Deviations between these two measurements could indicate that the model being used is incorrect or oversimplified. Such an indication could be due to effects such as the formation of multiple bonds, the occurrence of rebinding, catch bond behavior, multiple pathways to dissociation, or other complex kinetic mechanisms (29, 36, 94). Additionally, examining the full distributions of rupture forces at different loading rates and comparing them to predicted distributions from the model can act as an additional quality control of both the experiment and the subsequent analysis.

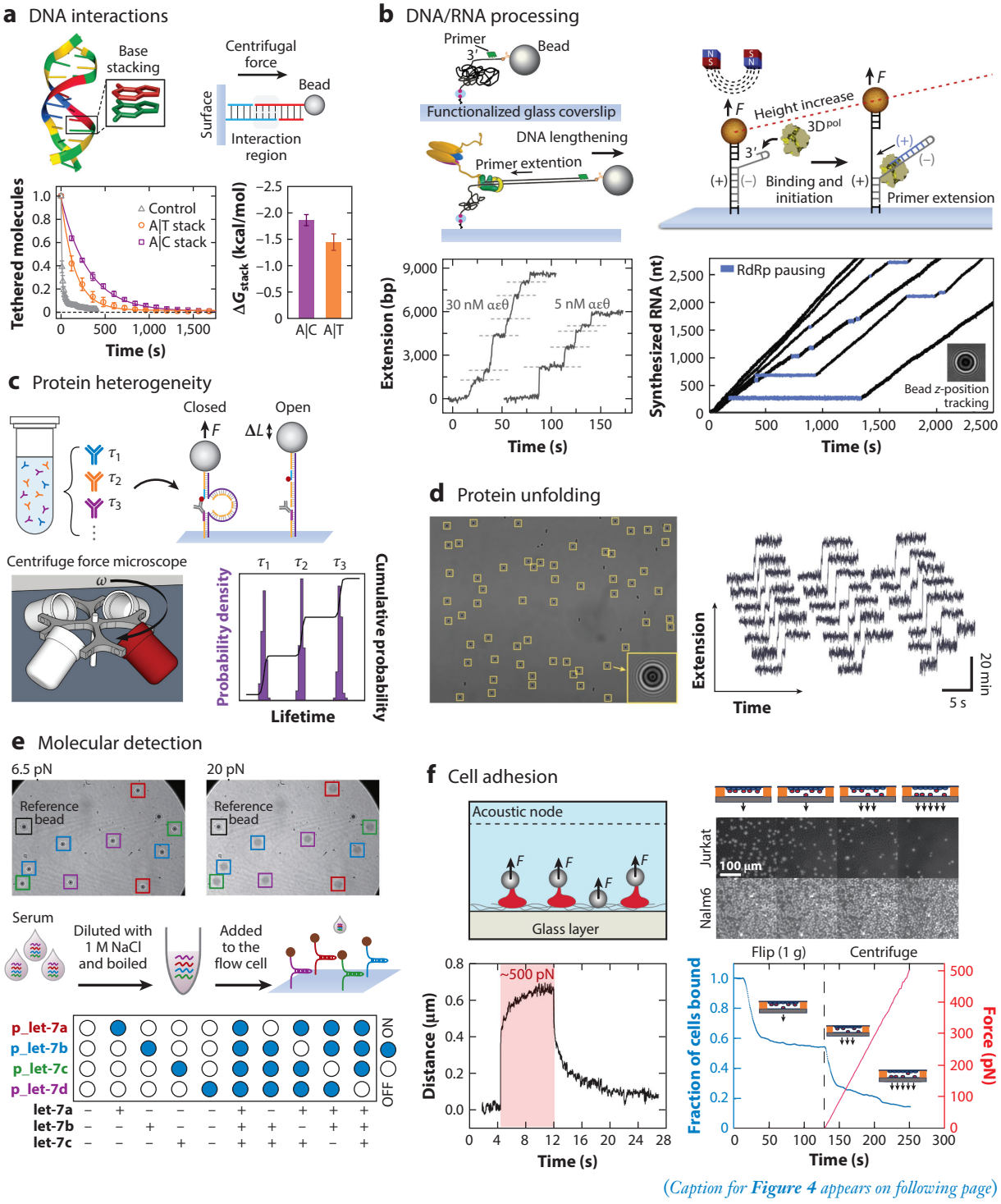
Recent advances have broadened the analytical approaches available for single-molecule force spectroscopy. Model-free techniques facilitate conversion of force-ramp data into equivalent constant-force measurements, simplifying analysis and reducing assumptions inherent in traditional methods (32, 37). Additionally, nonparametric Bayesian inference has emerged as a particularly powerful technique for analyzing single-molecule data and resolving molecular heterogeneity with fewer predefined state assumptions (55, 90, 135). Emerging machine learning techniques, including neural network and simulation-based inference, are also increasingly used to extract information such as potential energy landscapes from rupture data, expanding analytical capabilities beyond traditional parametric methods (30, 31). Collectively, these advanced analytical tools enhance our ability to interpret single-molecule experiments, providing deeper, more nuanced insights into complex biological mechanisms.

APPLICATIONS OF MULTIPLEXED FORCE SPECTROSCOPY

Multiplexed single-molecule methods offer a set of advantages and trade-offs distinct from those of traditional single-molecule techniques. Multiplexing offers observation of more molecular interactions at a given time, which can be used either to shorten the collection time of a certain number of statistics or to observe variation of behavior among a population. This expanded scope of observation tends to come at the cost of some spatial and/or temporal resolution. Most current applications of these multiplexed methods have sought to exploit these benefits while minimizing impact of the trade-offs. In this section we discuss application examples and contextualize the ways in which the applications benefit from multiplexing, with a focus on molecular biophysics, including intramolecular and intermolecular interactions.

Nucleic acids biomechanics are a major area of study and have been probed with several multiplexed methods. CFM-based approaches have been used to determine force-dependent DNA shearing (2, 57) and unzipping (146) and most recently to resolve individual base-stacking interactions (3, 4) (**Figure 4*a***). These latter examples highlight some strengths of multiplexing, in particular the collection of large amounts of data on minutes-to-hours timescales that would be impractical with traditional single-plexed methods. Multiplexed magnetic tweezers have also been used to investigate DNA biomechanics such as force–extension behavior (28, 106) and torsional stiffness (78).

Nucleic acid interactions with proteins such as enzymes and other cofactors have also been an area of interest. Flow-based approaches and multiplexed magnetic tweezers have been used heavily in these areas (**Figure 4*b***). One of the earliest studies was of exonuclease activity in converting double-stranded DNA (dsDNA) to single-stranded DNA by observing changes in tether extension (137). Similar experimental setups by the same laboratory have been used to investigate various



*(Caption for **Figure 4** appears on following page)*

Figure 4 (*Figure appears on preceding page*)

Diverse applications of multiplexed force spectroscopy. (*a*) Measurement of base-stacking interactions in DNA using the CFM, with representative data comparing A|C (adenine stacked with cytosine) and A|T (adenine stacked with thymine) pairs. (*b*) Enzymatic processing of nucleic acids using flow to measure primer extension with 30 nM and 5 nM of enzyme (*left*) or magnetic tweezers to investigate pausing during primer extension (*right*). (*c*) Resolving molecular heterogeneity in antibody interactions using the CFM combined with DNA nanoswitches for repeated interrogation of individual binding pairs. (*d*) Forced unfolding of VWF protein using magnetic tweezers. (*e*) Sensing of microRNAs using magnetic tweezers and cyclic hairpin unfolding. (*f*) Studies of cellular adhesion using AFS (*left*) and CFM (*right*). Abbreviations: AFS, acoustic force spectroscopy; CFM, centrifuge force microscope; RdRp, RNA-dependent RNA polymerase; VWF, von Willebrand factor. Panel *a* adapted from Reference 4 (CC BY 4.0). Panel *b*, *left* adapted with permission from Reference 134; panel *b*, *right* adapted with permission from Reference 63. Panel *c* adapted from Reference 90 (CC BY-NC-ND 4.0). Panel *d* adapted with permission from Reference 88 (CC BY-NC-ND 4.0). Panel *e* adapted with permission from Reference 148 (CC BY-NC 4.0). Panel *f*, *left* adapted with permission from Reference 129 (CC BY-NC-SA 3.0); panel *f*, *right* adapted from Reference 13.

aspects of DNA replication (51, 83, 134). Other laboratories have used multiplexed flow assays to investigate dynamics and mechanics of DNA repair (64, 99), proofreading by polymerases (100), and replication termination (35). Multiplexed magnetic tweezers have been used in several studies of viral RNA-dependent RNA polymerases (63, 78, 80, 114). An interesting combination of flow and magnetic tweezers was also recently introduced and used to investigate drug-induced dsDNA breaks with topoisomerases (6). AFS has also been used to measure DNA mechanics with and without RecA protein (124).

Intramolecular and intermolecular protein interactions have also been extensively studied with various multiplexed single-molecule methods (**Figure 4*c*,*d***). For example, AFS has been used to measure protein–protein interactions (142), as well as protein–carbohydrate interactions (48). Multiplexed magnetic tweezers have been applied to study protein unfolding dynamics in von Willebrand factor (VWF) (88), while multiplexed flow-based assays have characterized tension-dependent interactions involving VWF, such as its interaction with platelets (42, 65) and its self-association (41). Additionally, multiplexed assays using the CFM have facilitated studies of single-molecule protein proteolysis (73). Other notable examples include investigations of the mechanical stability of SARS-CoV-2 with its cellular receptor (10). Recent efforts from our laboratory combined multiplexed force measurements via the CFM, repeated interrogation of individual molecular interactions using DNA nanoswitches, and nonparametric Bayesian inference to characterize molecular heterogeneity of binding strength within antibody populations (90).

Single-molecule biomolecular sensing can also be enabled by multiplexed methods, using, for example, molecular tethers that change their mechanical pulling signature upon binding to extrinsic biomolecules (91). A recent study applied this idea to multiplexed magnetic tweezers, showing mutation-sensitive detection of nucleic acids, including microRNAs and viral RNAs by repeated pulling of an array of single-molecule hairpins (148) (**Figure 4*e***). To address situations where the molecules of interest are not known a priori, Shrestha et al. (118–120) have developed a mechanical fingerprinting approach to measure multiple intramolecular distances within single biomolecular complexes and have demonstrated this using multiplexed methods. As discussed in the next section, this approach could have significant impact on the emerging field of single-molecule proteomics.

Beyond individual molecules, some of these tools have been used for measurements of larger structures, including colloids and cells. Such measurements benefit from multiplexed force methods, especially using the CFM or AFS, both of which can directly apply physiologically relevant forces without specialized probes (**Figure 4*f***). One such application used the CFM to measure the physical interactions between colloids and surfaces in the nanonewton range (85). AFS has investigated cell adhesion forces between CD4 and fibronectin (67), mechanical stretching of red blood cells (130), endothelial cell mechanics under shear stress (122), microrheology of cells (95),

and various cells and biomaterials (14). A recent CFM study also investigated immune cell avidity with T cell and B cell lines (13).

Collectively, these examples illustrate how multiplexed single-molecule force spectroscopy enables detailed interrogation of biological systems at scale, revealing insights otherwise inaccessible. Yet substantial challenges—and corresponding opportunities—remain as the field expands toward broader biological and clinical applications.

CHALLENGES AND OPPORTUNITIES

Significant progress has been made in multiplexed single-molecule assays, yet opportunities for further methodological improvement remain. Here, we outline key areas for technological advancement and highlight emerging biological and clinical applications that can both benefit from multiplexed assays and motivate continued innovation.

Major technical challenges remain in molecular preparation, data analysis, and automation. Instrumentation for multiplexing single-molecule experiments has progressed rapidly, but many experimental and analytical approaches initially developed for single-plexed methods have not kept pace. Scaling up requires improved efficiency and control in molecular tethering to reliably form large numbers of single-molecule tethers, development of confirmatory molecular tethers (49, 71), and advanced patterning approaches (21) toward precisely localizing and verifying distinct molecular interactions. Data analysis similarly poses substantial challenges due to increasingly large datasets and the unique types of data in different experiments. Although robust methods for data analysis already exist, workflows within and between laboratories are often still fragmented across different platforms (e.g., MATLAB, Python, C++, LabVIEW) and different programs or subroutines. Greater standardization of analysis tools and software, similar to advances in genomics, could strongly benefit the field. In addition to multiplexing, automation offers a complementary route to higher throughput. Near-term opportunities include improved software-driven automation, such as more general and flexible control scripts; fully automated data acquisition allowing for unattended operation; and reproducible, largely hands-off data analysis pipelines. Integration of software control with specialized hardware, including automated fluid handling and fast scanning stages, could further improve experimental efficiency. Commercial implementations, such as the LUMICKS optical tweezers platform, illustrate how hardware–software integration can streamline workflows, enhancing throughput for a given measurement modality. Further automation across sample preparation, data collection, and computational analysis could maximize the throughput and reproducibility of existing approaches.

Another key technological challenge and opportunity is increasing molecular diversity. Current multiplexed methods often focus on collecting extensive statistics from many copies of a single molecular species, but broader biological insights could be gained by simultaneously measuring interactions across diverse molecular species, potentially enabling omics-scale single-molecule force studies. Achieving this goal requires balancing complex trade-offs between spatial and temporal resolution, throughput, force range, and experimental costs. Technologies such as engineered tethers with integrated barcodes (120) and molecular patterning approaches capable of creating spatially indexed molecular arrays (21) will likely be critical for increasing multiplexing diversity. Another promising route comes from sequencing-based methods such as MUSCLE (7) and SPARKX (115), which repurpose Illumina sequencing platforms to index thousands of distinct species and correlate single-molecule behavior with sequencing information. Adapting this sequence-derived spatial indexing approach to force spectroscopy could substantially enhance molecular diversity by enabling parallel force measurements of sequence-barcoded molecular interactions.

Clinical sample analysis presents another substantial opportunity for single-molecule methods, though accompanied by challenges of sample complexity, heterogeneity, and limited abundance. Recent developments, such as DNA nanoswitch calipers, offer single-molecule mechanical fingerprinting suited for single-molecule proteomics, including protein identification, geometric analysis, and post-translational modification mapping (119). A few multiplexed single-molecule biomolecular sensing techniques, some of which are using force-based approaches, have begun demonstrating biological detection directly from clinical samples (148). Collectively, applying multiplexed single-molecule approaches to clinical samples could help uncover novel biomarkers for disease, enable sensitive diagnostics, and aid in drug discovery.

Another compelling opportunity is to combine multiplexed force spectroscopy with complementary measurement modalities to increase the depth of information obtained from single-molecule assays. Single-molecule fluorescence integration is now relatively common in optical tweezers setups (18, 59, 81), magnetic tweezers (61, 121), and commercially available systems such as the LUMICKS C-Trap. However, its adoption remains limited in highly multiplexed force assays, aside from specialized flow-based examples (42, 46). More broadly integrating optical spectroscopy methods, including fluorescence and fluorescence resonance energy transfer (FRET), into multiplexed platforms, such as magnetic tweezers, centrifugal force microscopy (13, 85), and AFS, could substantially enrich our ability to dissect molecular heterogeneity by simultaneously tracking force, conformation, and potentially chemical state.

Perhaps one of the most interesting opportunities from recent developments is to enable multiplexed force spectroscopy with engineered nanostructures by using the structure to measure or apply force. DNA nanotechnology-based tools such as tension gauge tethers enable scalable mapping of forces at multiple cell-attachment sites (121, 141). Programmable DNA structures capable of applying forces to single molecules include early loop-based force actuators (153) and autonomous DNA origami force clamps (77, 96).

Multiplexed force spectroscopy can also open new possibilities for biological applications; extending force studies to living cells is one compelling direction. Next-generation CFM platforms recently enabled high-throughput quantification of cell–cell adhesion and avidity (13), and various AFS studies have probed cells as well (67, 129, 122, 95). Genetically encoded FRET-based tension sensors have already enabled in situ measurements of molecular-scale forces inside living cells (45). Integrating these sensors with advances in fluorescence microscopy, such as high-speed lattice light-sheet imaging, could further improve multiplexing and enable mapping of force propagation across cellular structures. Combining such measurement with active mechanical control inside cells using engineered, optically controllable molecular motors (110) could enable sophisticated, closed-loop experiments to probe cellular mechanotransduction pathways.

Finally, democratizing multiplexed single-molecule force spectroscopy is essential for broadening its impact and enabling a wider range of meaningful applications. Increasing accessibility through open-source, low-cost platforms such as the CFM (1, 50, 146, 147) or commercially available systems can accelerate its adoption across biological and biomedical research communities. Together, increased accessibility, new capabilities, and higher throughput will enable more researchers to address broader biological questions, unlocking discoveries that fully harness the potential of single-molecule methods.

DISCLOSURE STATEMENT

The authors have filed patent applications (K.H., A.W., and W.P.W.) and have received patent royalties (K.H. and W.P.W.) on various technologies related to this review. A.W. is on patents for the DNA nanoswitch and centrifuge force microscope (CFM). K.H. is on patents and has filed patent applications related to the CFM and has received royalties from CFM patents.

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