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Combining single-molecule manipulation and single-molecule detection

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Single molecule force manipulation combined with fluorescence techniques offers much promise in revealing mechanistic details of biomolecular machinery. Here, we review force-fluorescence microscopy, which combines the best features of manipulation and detection techniques. Three of the mainstay manipulation methods (optical traps, magnetic traps and atomic force microscopy) are discussed with respect to milestones in combination developments, in addition to highlight recent contributions to the field. An overview of additional strategies is discussed, including fluorescence based force sensors for force measurement *in vivo*. Armed with recent exciting demonstrations of this technology, the field of combined single-molecule manipulation and single-molecule detection is poised to provide unprecedented views of molecular machinery.

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Introduction

The ability to watch, simultaneously manipulate and control individual molecules is a powerful tool for understanding the structure-functional–mechanical working of molecular machinery. Individual behavior is typically clouded by ensemble measurement, requiring specific manipulation and detection strategies to reveal the properties of isolated molecules. Single-molecule (sm) methods have been developed over the years driven by studies such as motility of motor proteins, RNA polymerase, DNA repair enzymes, measurement of physical properties of polymers and filaments, unfolding/refolding of proteins and nucleic acid structures and general investigation of cell system machinery [1–4]. From the 8 nm step of kinesin [1], to time and spectrally resolved visualization of enzymatic reactions [5], the advent of single molecule biophysics has advanced through an impressive series of milestones. Here we review recent progress in the ability to not only 'watch', but also physically 'manipulate', individual molecules. Our ability to 'watch' with fluorescence includes fluorescence localization with spatial resolution of a few nm, angle, distance, spectral changes, time resolved studies and simultaneous tracking of multiple molecules. Our ability to 'manipulate' with an optical trap or atomic force microscope (AFM) is now measured in angstroms, enabling the ability to track moving molecules and scanning probe imaging. Application of force includes pick and place control over molecular positioning, dynamic or clamped application of stresses and forces that reveal much about the system, to control over the reaction coordinate of interest.

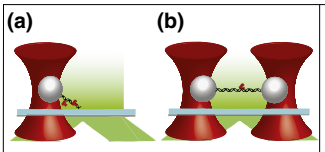
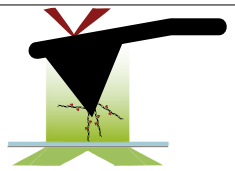
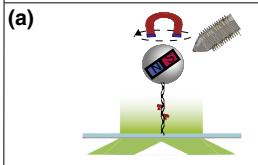
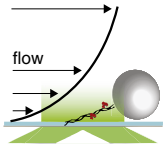
Optical trapping combined with fluorescence

Optical tweezers and single-molecule fluorescence are primary techniques in single-molecule biophysics. Given the 4.1 pN nm magnitude of thermal motions, these methods offer force and distance scales appropriate for studying biological motors and other molecular transitions. Integrating trapping and fluorescence correlates nanoscale structural changes with biomechanical transitions, pinpointing their locations, magnitudes and transition energies (Figures 1 and 2).

Early efforts in combined optical trapping and single molecule fluorescence included dual functioning microscopes and spatially separated configurations, demonstrated by Yanagida and coworkers [6–8]. In order to reduce background fluorescence, prism type single-molecule total internal reflection fluorescence (smTIRF) was employed. TIRF offers localized excitation to a $1/e$ distance range from the glass-water interface. 'Prism side' methods excite molecules on the flow cell surface opposite the objective, offering clean excitation and straightforward alignment of incident angle and wide field of view.

This combined trapping and fluorescence work used single-beam, and later dual-beam trapping with prism side smTIRF to directly visualize nucleotide turnover during kinesin walking [6], the force-generating step in myosin's mechanochemical cycle [7], and later to study

Figure 1

Manipulation method	Strategy	Challenges	Demonstrations
	-Horizontal application of force -Spatially separated trap and fluorescence beams impart controlled tension -Coincident trapping and fluoresescence (A) beams have good position resolution -Dual trap (B) assay for ultra high spatial resolution -Fluorescence excitation through TIRF, confocal, epifluorescence and superresolution	-High photon flux of trap (A) damages fluorophores, interlaced beams reduce photobleaching -Dual trap smTIRF (B) requires a pedestal, low background assays can employ confocal or Epi-illumniation	-Kinesin stepping (smFl) -DNA Hairpin unfolding (smFl, smFRET) -Holliday Junction (2 and 3 color smFRET) -Helicase driven DNA unwinding (smFl) -Myosin force generation(smFl) -DNA-protein interactions(smFl, STED, FIONA) -Force sensor calibration for in-vivo force measurements (smFRET)
	-Surface coupled vertical application of force -High spatial resolution -Large force magnitude -TIRF or confocal fluorescence	-High inherent cantilever stiffness -Scattering from cantilever reflection -Tip functionalization and tether placement	-Active assembly of nano structures (cut and paste) -Protein unfolding/refolding (smFRET) -High speed AFM for probing dynamics in real time
	-Vertical and horizontal application of force -Permanent (A) or electromagnet (B) -Surface coupled -Torque/twisting applications -Multiplexed measurements -No adverse effects on sample from magnetic field - Simpler instrumental setup	-Lacks pinpoint steering -Sub-optimal position resolution in most setups	-DNA packaging motor (polarization smFl) -Helicase activity (smFRET) -DNA streching (smFRET)
	-Horizontal applications of force -Straightforward instrumental setup -Surface proximity optimal for TIRF -Multiplexed measurements	-Slow modulation of flow -Sub-optimal position resolution -Molecule manipulation in one dimension -Larger beads required	-DNA-protein interactions (smFl, smFRET)

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Depiction of the experimental geometry for various single molecule manipulation and detection methods. Strategies, drawbacks, and achieved implementations are listed for each technique.

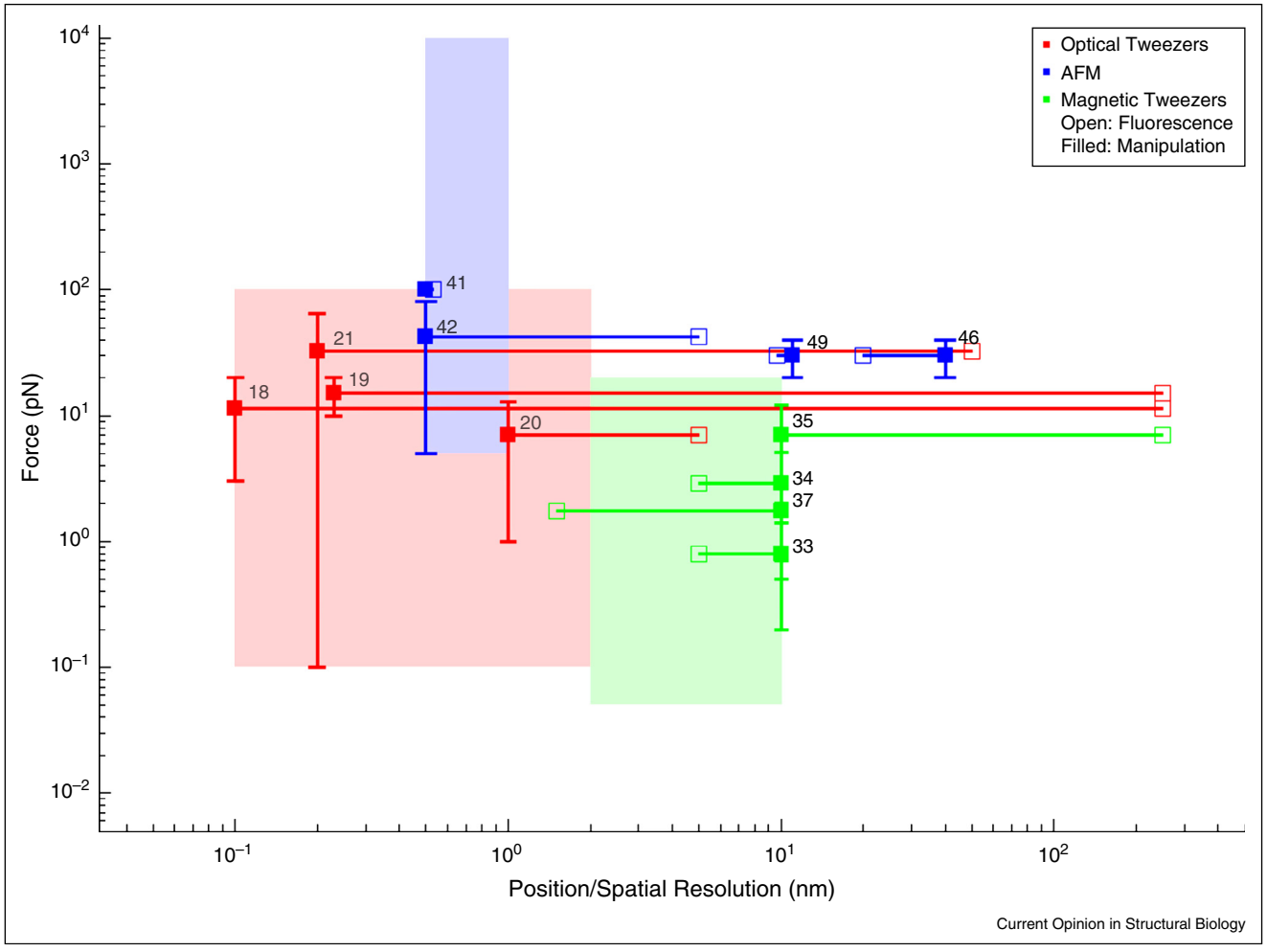
RNA polymerase binding to DNA in a suspended filament geometry. This work included a pedestal on the slide surface to permit dumbbell trapping and constrain illumination to the DNA through TIRF [8]. In these cases fluorescence was spatially separated (SS) through a pair of traps, suspending 15–16 μm of DNA, a length that introduces compliance issues and compromises the ability to sense position.

As high numerical aperture objectives became available, 'objective side' smTIRF became possible. A simultaneous and spatially coincident optical trapping and single molecule fluorescence microscope was developed by Block and coworkers to monitor strand separation of a dye labeled 15 base pair region of dsDNA [9,10]. They witnessed the mechanical transitions corresponding to DNA hybrid rupture occurs simultaneously with changes in fluorescence emission. In spatially coincident experiments, enhanced photobleaching blocked use of favored single molecule dyes, which showed shortened lifetimes in the presence of the high photon flux of an optical trap [11]. A solution to this problem was provided by interlacing the trapping and fluorescence lasers fast enough that the trapped bead behaved as if the trap were always on [12]. With interlaced methods, coincident trapping and single molecule fluorescence was possible for a range of dyes [13]. Chu and coworkers used combined optical trapping and fluorescence with an actively stabilized imaging system to resolve different colored dyes (Cy3 and Alexa 647) bound to optically stretched DNA with subnanometer resolution [14].

The next major combination incorporated fluorescence resonance energy transfer (smFRET), another powerful tool capable of revealing conformational/structural changes on the length scale of 2–8 nm (Figure 1). Combining manipulation through force with short distance smFRET techniques enables direct localization of conformational dynamics of loaded biomolecules providing unprecedented mechanistic details of molecular machinery in real time. Tarsa et al. developed this combination through an interlaced trap and fluorescence method [15]. Their coincident trapping and sm-FRET technique allowed simultaneous observation of the mechanical transition of bead position with smFRET changes between a donor (Cy3) and acceptor (Alexa 647), during un/re-folding of a DNA hairpin. This hairpin may act as a binary fluorescence based force sensor.

Later, Hohng et al. combined smFRET with a SS optical trap to probe conformational dynamics of Holiday Junction (HJ) molecules [16]. Here, the trap was used to

Figure 2



Clusters of combined methods for optical tweezers (red), AFM (blue) and magnetic tweezers (green) with fluorescence. Numbers adjacent to the squares represent the reference number in the text. Filled squares represent the position resolution for the manipulation techniques; open refers to the spatial resolution for fluorescence detection. Light colored regions are typical force ranges and spatial resolutions of the three manipulation techniques.

apply precise loads through a long (>10 μm) strand of DNA. The spatial separation uncouples fluorophores from the proximity of the laser trap at the expense of decreased mechanical position resolution. Recently, expansion upon this system has been achieved through three-color smFRET implementation with a SS trap configuration [17•].

While surface bound assays are well suited for combinations with smTIRF detection, an advantage of the suspended configuration of optical tweezers is the ability to remove common mode drift of the trap laser and decouple the system from the sample stage leading to ultra high position resolution optical trapping (Figure 1) [2]. Recent advances have embraced this common mode strategy for high position resolution, including design with interlaced trapping and fluorescence. Comstock et al. developed an interlaced, ultra-high resolution, dual trap instrument combined with confocal smFluorescence [18]. Achievable position resolution was below 1 nm, a single base pair, similar to recent advances by other groups [19]. Several other studies successfully combined confocal fluorescence microscopy with optical tweezers for manipulation and detection of DNA–Protein interactions [20,21••].

Position sensing has also advanced through adoption of 'super-resolution' fluorescence techniques in combined instrumentation. As in tracking a bead, the ability to watch an individual object and repeatedly measure its centroid enables resolving the position below the diffraction limit [22]. A combination of a dual-beam trap with fluorescence imaging with nanometer accuracy (FIONA) was used to track quantum dots on actin filaments [23].

Further combinations with stimulated emission depletion (STED) using dual trap tweezers was achieved by Heller et al., where proteins bound to suspended DNA were resolved to 50 nm [21••].

Other combinations include epifluorescence microscopy with optical trapping. Van Memeren et al. studied tension induced disassembly kinetics of fluorescently labeled DNA binding protein, RAD51, in a SS geometry [24]. Similarly, Kowalczykowski and coworkers, studied RecA homology search and nucleation growth on ssDNA using dual trap tweezers combined with fluorescence microscopy and flow from microfluidics [25••,26]. Presently, many hurdles in combined instrumentation have been worked out. Advances in surface-bound, dual-bead and multi-color configurations described above provide a firm foundation for future work in combined trapping and fluorescence.

Magnetic tweezers and fluorescence

While optical traps are versatile in pinpoint application of force, the high flux of photons necessary to generate a trap and spectral congestion of multiple wavelengths pose hurdles when combined with single molecule fluorescence experiments (Figure 2). Permanent magnetic tweezers remotely impart loads on paramagnetic beads using fields capable of generating up to ~200 pN of force [27]. In addition to force range advantages and avoiding trap-induced photo-damage, magnetic fields apply forces to multiple objects simultaneously for high-throughput measurement. Field directions are configurable for horizontal, vertical and even rotational application. Electromagnetic tweezers are capable of exerting force approaching 1 nN for a 1 μm bead and can be used to manipulate and rotate magnetic beads in three-dimensional configurations [28,29]. Electromagnets feature fast control of the force and rotation of the magnetic bead by changing only current. Magnetic tweezers have been widely used in studying DNA mechanics and various molecular machines, including DNA topoisomerases and the F_0F_1 ATPase [3,30,31].

Recent progress has been made in combining magnetic tweezers with fluorescence detection techniques. Given the typical surface-tethered geometry of magnetic tweezer experiments (Figure 1), smTIRF is commonly used. A combination with smFRET was first developed as a force sensor during ssDNA stretching [32], and later used to study B–Z DNA transitions [33]. Recently, smFRET was used to visualize mechanical un/re-folding of a G-quadruplex [34]. Similarly, combination with smTIRF provided direct observation of force dependent binding of single-fluorophore labeled vinculin to talin rods [35]. Combined polarization/angle sensitive smTIRF and magnetic tweezers enabled observation of connector rotation during phi-29 DNA packaging motor operation [36].

Confocal fluorescence has also been combined with magnetic tweezers to develop a single molecule 'FRET-enconder' capable of tracking helicase motor activity [37]. While not as widely used as optical trapping and fluorescence, recent advances in combined magnetic tweezers and smFluorescence, along with the relative simplicity and low cost associated with this method, make it a promising technique. We anticipate increased use of magnetic based systems as the resolution limit is improved to rival high-resolution optical traps.

AFM combined with fluorescence

Early development of the AFM was driven by the desire to image surfaces with atomic resolution. Other applications, including manipulation, functional imaging and single molecule force spectroscopy, expanded the role of the AFM as a manipulation tool useful for biological applications as a molecular force probe (Figure 2). An AFM cantilever can apply ~10 pN to nN loads in the vertical direction on a single molecule bound between the probe tip and surface sample [38]. The advent of high speed AFM enabled observation of molecules with sub-100 ms temporal resolution in real time [39].

Early work relevant to combined instrumentation employed TIRF strategies to optically manipulate the length of an azobenzene-integrated polymer [40]. Later, Sarkar et al. measured the position of a fluorescently labeled cantilever within the evanescent wave to resolve protein unfolding events without the need to track laser deflection by the cantilever [41]. Single molecule detection was later achieved by combining AFM with smFRET to monitor conformational changes during force-driven protein unfolding [42••]. In a sequential dual functioning system, Gaub and coworkers developed an AFM-based technique called single molecule cut and paste (SMCP), which they used to assemble split nucleotide-based aptamers individually [43–45]. This was later combined with a super resolution technique termed Blink Microscopy to view reconstructed images of assembled structures below the diffraction limit [46].

Freely diffusing fluorescent molecules have traditionally been maintained at dilute concentrations in single-molecule fluorescence experiments in order to minimize background fluorescence. Zero-mode waveguides (ZMW) circumvent this problem by confining the excitation waves to atto-liter wells on the surface of the sample [47]. When combined with AFM, ZMWs also help minimize background signal, created by light reflection from the large cantilever [48,49]. Heucke et al. employed simultaneous and coincident smTIRF with ZMWs to detect fluorescently tagged nucleotide binding upon mechanical activation of titin kinase by an AFM cantilever [48].

Despite broad applications and commercial availability, AFM based systems are limited by the larger size and the high stiffness of the cantilever, which energetically are far from equilibrium and dominate many biomolecular systems. Combined with fluorescence detection, the size and proximity of the probe to sample surface (contact method) introduces unwanted scattering, yet strategies that leverage field confinement and enhancement may benefit from such contact methods.

Combined flow-fluorescence

Hydrodynamic flow represents another method for applying load on single molecules [50]. Laminar flow is established in a channel to exert force (typically ~4 pN) on beads tethered to long strands (>10 kbp) of surface-bound DNA [51]. Flow aligns the strands by pushing them close to the cover glass surface (Figure 1). Thus, TIRF has advantages in visualizing smFluorescence from DNA associated molecules/motors.

Early work in this area, demonstrated by Chu and co-workers, used flow to extend single molecule DNA bound to a trapped bead and observed their relaxation kinetics when the flow stopped, using fluorescently labeled DNA molecules [52]. Graneli et al. visualized arrays of single DNA molecules tethered on one end to the surface thereby generating a DNA 'curtain' when the array was extended by flow [53]. Recent work using smFlow-Fluorescence focused on translocase mediated protein removal from DNA and RNA tracks [54]. Cho et al. showed a smFlow-FRET system outlining MutS, an ATPase motor involved in DNA repair, activity [55•]. Although ultra-high position sensing is difficult in flow, and the method is difficult to achieve and actively modulate high forces, it is increasingly becoming a strategy for manipulation and detection with the advent of advances in microfluidics.

Fluorescence based force sensor

Single molecule force and detection techniques enable manipulation and quantification of accessible biological interactions, typically isolated to a surface or suspended filament. Fluorescence based force sensors provide a window for measurement of forces and stresses on molecular systems and potentially within the complex network of the cellular environment. Fluorescence based force sensors can be particularly useful for quantifying interactions of single molecules *in vivo* [56].

Fluorescence based force-sensors are being adapted for measuring focal adhesion, signal transduction, receptor-ligand and DNA–protein interactions in vivo. Fluorescent protein variants such as green fluorescent protein (GFP) and others have been used as force sensing probes including a demonstration by Iwai et al. to visualize interactions between myosin II and F-actin in living cells [57]. Here, GFP was expressed as a fusion protein covalently linked to the protein of interest. In a similar experiment, Grashoff et al. genetically encoded FRET pairs on vinculin for quantifying mechanical force in fibroblast and endothelial cells [58].

Recently, using a quenched to unquenched strategy, Salaita and coworkers developed a molecular tension sensor for probing mechanical strain exerted by a cell surface receptor protein called EGFR [59••]. They covalently attached the fluorophore to the EGF ligand and a quencher to surface bound streptavidin. Forces from EGFR binding lengthen the linker from a relaxed state (quenched) to an extended unquenched state. Force probes consisting of a single strand DNA loop flanked by fluorescent donor and acceptor dyes, developed by Shroff et al., employ a complementary strand interacting with the DNA loop to change donor–acceptor separation and FRET efficiency [60]. Engineering of the loop adjusts force sensitivity.

Combined instrumentation such as those detailed in the sections above is critical for calibration of fluorescence based force sensors. Interpretation of such results must also be done with care. Many sensors are binary, either closed or open and thus report that a force of a certain threshold or greater has been achieved. Signals from sensors that are reported as continuous, having been calibrated directly or by proxy using single molecule methods, must be interpreted with care when originating from multiple sensors in the same imaging voxel. For example, a population of half fully open and half fully closed sensors might appear as a population with all half open. Finally, the relationship between transitions states leading from folded to unfolded depends on the loading rate, amount of time and force magnitude. Systems such as titin may unfold at high forces ~100 pN when pulled on with a stiff probe at high loading rates, whereas forces as low as 5–10 pN can unfold this system, given enough time.

Perspectives

A number of common themes emerge when one dives into the details of combined instrumentation design. Although commercial solutions to single-molecule methods are on the horizon, most capabilities are in the hands of a few labs. Instruments are physically congested with optics, hardware and electronics tightly packed around the sample location. Computer automation is central to these experiments, which simultaneously juggle many tasks. Most instruments are built by modifying a commercial high-end microscope, reducing the design challenge, but presenting access issues to critical locations around the sample. Removing the microscope in some completely home built rigs adds flexibility. Many designs zone the upper and lower regions of the microscope to merge the technologies. Microscopes themselves are

housed in specialized rooms or chambers featuring low vibration, acoustically quiet, dark environments.

Spectral congestion is also an issue, even in more advanced AFM and magnetic systems where laser subsystems are included for better position detection. For optical trapping, 10 orders of magnitude separate the photon flux of the trap versus those emitted from typical fluorophores. Devoting the visible spectrum to fluorescence and infrared to trapping/position sensing helps with this issue. Use of specialized filters that isolate signal and block unwanted light sources are critical. Still the fluorophore itself needs to be able to survive trap and other photon flux sources. Complex transitions among excited states, driven by these photon sources, can lock fluorophores in non-emitting states. Such turning off of fluorophores has been exploited in 'super resolution' schemes.

Another design challenge is engineering the molecular system itself, generally requiring two physical handles and one or more spectral handles. Physical connectivity is typically achieved through orthogonal systems such as biotin–streptavidin, dig–antidig or other standard linkages such as nucleotide hybridization. More advanced methods are being developed to covalently link structures and 'build' the connectivity. Combined instrumentation is critical in testing these attachments and dialing-in assay conditions. Method advances such as buffer cocktails are also central to the success of combined measurements.

Despite these challenges, we are armed with wonderful designs on both instrument and biological–chemical fronts. Perhaps the most exciting development of combined instrumentation is the interdisciplinary collaboration between the instrumentation builders and the chemical and biological engineering required in designing the molecular system of interest.

Conflict of interest statements

The authors declare no conflict of interest.

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- • of special interest
- •• of outstanding interest

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