Recent Advances in Biological Single-Molecule Applications of Optical Tweezers and Fluorescence Microscopy


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Contents

1. Introduction 86
2. Instrumentation 88
3. Applications 95
   3.1 Single Optical Trap 95
   3.2 Dual-Trap Optical Tweezers 99
   3.3 FRET Studies With Confocal Fluorescence Microscopy 109
4. Experimental Protocol 111
   4.1 Experimental Setup 112
   4.2 Methods 112
5. Conclusion 113
Acknowledgments 114
References 114

Abstract

Over the past two decades, single-molecule techniques have evolved into robust tools to study many fundamental biological processes. The combination of optical tweezers with fluorescence microscopy and microfluidics provides a powerful single-molecule manipulation and visualization technique that has found widespread application in biology. In this combined approach, the spatial (~nm) and temporal (~ms) resolution, as well as the force scale (~pN) accessible to optical tweezers is complemented with the power of fluorescence microscopy. Thereby, it provides information on the local presence, identity, spatial dynamics, and conformational dynamics of single biomolecules. Together, these techniques allow comprehensive studies of, among others, molecular

1 These authors contributed equally.
motors, protein–protein and protein–DNA interactions, biomolecular conformational changes, and mechanotransduction pathways. In this chapter, recent applications of fluorescence microscopy in combination with optical trapping are discussed. After an introductory section, we provide a description of instrumentation together with the current capabilities and limitations of the approaches. Next we summarize recent studies that applied this combination of techniques in biological systems and highlight some representative biological assays to mark the exquisite opportunities that optical tweezers combined with fluorescence microscopy provide.

1. INTRODUCTION

The exploration of single-molecule systems and their interactions with each other in terms of mechanical forces has substantially benefitted from recent advances in the now well-established field of optical tweezers. The very high sensitivity (pN, nm, k_BT) and the wide temporal resolution (s to ms) of optical tweezers can be used to measure noninvasively the mechanics of single molecules and their interactions with other molecules. It has been about three decades since Ashkin presented the first application of optical tweezers in biology (Ashkin, Dziedzic, & Yamane, 1987), and since then, optical tweezers have become one of the most widely used single-molecule tools in biology. What makes the use of optical tweezers even more compelling is its compatibility with various types of light microscopy, such as bright field, differential interference contrast, phase contrast, and fluorescence microscopy. Not surprisingly, optical trapping was soon combined with fluorescence microscopy as a method for visualizing single molecules. In the first combination study, a long piece of DNA was labeled with ethidium bromide and stretched between two beads held in two optical traps (Chu, 1991). The relaxation of the DNA upon release of one bead could be followed by simply watching the movements of the fluorescently labeled DNA.

In the last two decades, a variety of fluorescence techniques have been successfully combined with optical tweezers. Epi-illuminated wide-field and total internal reflection fluorescence (TIRF) microscopy, being the most straightforward to implement, have been adopted first (Arai et al., 1999; Bianco et al., 2001; Funatsu et al., 1997; Saito, Aoki, & Yanagida, 1994; Sarangapani et al., 2014). More recently, confocal fluorescence microscopy and stimulated emission depletion (STED) super-resolution microscopy have been integrated with optical tweezers (Bornschlögl, Romero, Vestergaard, & Joanny, 2013;
Comstock et al., 2015; Duesterberg et al., 2015; Grashoff et al., 2010; Heller et al., 2013; Hohng et al., 2007; Wolfson et al., 2015; Zhou et al., 2011). Each of these visualization approaches has its own set of strengths and weaknesses for the analysis of protein dynamics.

Addition of fluorescence microscopy enables direct visualization of individual proteins on biomolecules such as DNA, resulting in information on the presence of DNA–protein complexes, even when they do not induce a detectable change in the signals measured with optical tweezers. Moreover, fluorescence microscopy allows counting the number of proteins bound to the DNA. In addition, dynamic processes like diffusion, translocation, and binding kinetics of proteins can be directly monitored using fluorescence imaging. Förster resonance energy transfer (FRET) allows distance measurements on the nanometer scale and can be used to study the conformational dynamics of DNA–protein complexes.

Simultaneous application of optical tweezers and fluorescence microscopy reaches further than the single-molecule field of biophysics. In recent years, optical tweezers have been applied more and more in vivo. Several research groups have used optical tweezers to hold cells at a fixed position, which was a major breakthrough in single-cell studies, allowing spatial fixing without surface interactions in a sealed device and controlled environment, while enabling continuous observation using advanced imaging techniques (Eriksson et al., 2006; Pang, Song, Kim, Ximiao, & Cheng, 2014). The application of combined optical tweezers and fluorescence microscopy has also been extended to investigate cell-scaffold adhesion (Podlipec & Strancar, 2015), immunological studies and phagocytosis (Tam et al., 2010), permeability as well as molecular partitioning associated with phase transitions in vesicles and lipid bilayers (Bendix & Oddershede, 2011; Bendix, Reihani, & Oddershede, 2010; Kyrsting, Bendix, Stamou, & Oddershede, 2011), the influence of membrane proteins (Brouwer et al., 2015; Prévost et al., 2015), and membrane–cytoskeleton interactions (Leijnse, Oddershede, & Bendix, 2015).

In this chapter, we will focus on the broad collection of in vitro single-molecule assays that have been demonstrated in recent years. We will also discuss several examples of in vivo research providing an impression of the advances in this field as well, albeit without going into detail. We will start with providing a description of the fluorescence and microfluidics techniques that have been combined successfully with optical tweezers. We will put emphasis on the advantages and disadvantages of these combinations, which could serve as guidelines for choosing the most appropriate method
when designing an experiment. We will also provide an overview of commercial instruments. Next, a collection of recent studies that have combined optical tweezers with fluorescence microscopy will be presented, categorized according to the optical trapping configuration and the fluorescence microscopy technique used. We will discuss examples drawn from across the single-molecule literature, ranging from DNA enzymes, microtubule–protein interactions, DNA/protein conformational dynamics, DNA mechanics, DNA intercalators, lipid–membrane fusion, bacterial motility, and ultimately several FRET studies on nucleosomes and DNA helicases. We also provide a brief protocol of experiments we typically perform in our lab to investigate the binding kinetics of fluorescently labeled proteins or other compounds to DNA, using a combination of dual-trap optical tweezers and wide-field fluorescence microscopy. In this chapter, we aim to highlight the excellent opportunities that the combination of optical tweezers and fluorescence microscopy provides for studying the mechanical properties of DNA (length, flexibility, and elasticity), the kinetics and mechanochemistry of motor proteins, and properties of DNA intercalators.

2. INSTRUMENTATION

The combination of optical tweezers and fluorescence microscopy provides a versatile platform that opens up a range of opportunities to gain insight into complex biomolecular transactions. Optical tweezers can hold and move microscopic dielectric particles thanks to transfer of momentum from photons to the object. When forces act on the object, it is pulled out of the laser focus, which results in a deflection of the laser beam that can be detected by a position-sensitive detector. For imaging purposes, a single optical trap can be used to hold objects (Fig. 1A and B). However, in order to visualize and record the tension on a biomolecule, a second anchor is required, which can be provided by a surface or by a second optical trap. In an optical trapping system, it is relatively simple to implement a second optical trapping beam by splitting the trapping light based on polarization (Fig. 1C–F). In the last two decades, optical trapping has been widely employed in different configurations and excellent reviews have been written on the technical aspects of its implementation (Gross, Farge, Peterman, & Wuite, 2010; Moffitt, Chemla, Smith, & Bustamante, 2008; Neuman & Block, 2004).

The simultaneous application of optical tweezers and fluorescence microscopy poses several additional technical challenges. For example, the
light intensities used for the two techniques differ orders of magnitude, which requires efficient separation of trapping and fluorescence light. Moreover, the combination of optical tweezers and fluorescence microscopy is further complicated by optical trap-induced photobleaching of fluorophores (Eggeling, Widengren, Rigler, & Seidel, 1998; Heller et al., 2014; van Dijk, Kapitein, van Mameren, Schmidt, & Peterman, 2004). Despite these challenges a variety of fluorescence microscopy techniques have been successfully combined with optical tweezers (Fig. 1A–F). We categorize the most recent articles combining optical tweezers and fluorescence microscopy in Table 1, according to the optical trap configuration and the fluorescence microscopy technique employed. In this section, we will discuss the strengths and technical difficulties of combining specific fluorescence microscopy techniques with an optical tweezers system.

Epi-illuminated wide-field fluorescence microscopy is the simplest fluorescence microscopy technique that has been combined with optical
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<th>Table 1</th>
<th>Summary of the Most Recent Literature That Describes Various Combinations of Optical Tweezers and Fluorescence Microscopy</th>
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The articles in each column of the table are organized according to their optical trap configuration (vertically) and the fluorescence microscopy (horizontally) techniques that are used. The articles within a cell of the table are organized alphabetically based on the last name of the first author. In this chapter, we specifically focus on those combinations that are highlighted with double-line borders. The tick marked articles are discussed in more detail, both the biological assay used as well as a short summary of the pros and cons of the combination of techniques used.

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<th>Single Trap</th>
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<td>1 Trap (Hold)</td>
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| Confocal       | - Brenrix et al. (2010)  
- Bendix and Odershede (2011)  
- Kyrsting et al. (2011)  
- Leijne, Odershede, and Bendix (2015)  
- Podlipec and Strancar (2015)  
✓ Bornschlögl et al. (2013)  
✓ Grashoff et al. (2010)  
✓ Holng et al. (2007)  
✓ Maffeo, Ngo, Ha, and Aksimentiev (2014)  
✓ Ngo, Zhang, Zhou, Yodh, and Ha (2015)  
✓ Duesterberg et al. (2015)  
✓ Wolfson et al. (2015) | | |
| STED           | ✓ Heller et al. (2013) | | | | | |

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tweezers (Fig. 1C). It allows for direct visualization of a whole field of view. Moreover, modern electron-multiplying charge-coupled device (EMCCD) or sCMOS cameras with improved signal-to-noise ratios can image single molecules with a temporal resolution in the order of milliseconds (Heller et al., 2014). Illumination of the sample with a relatively wide, parallel light beam often results in high background fluorescence. This limits the concentration of fluorophores in solution at which it is still possible to detect single fluorophores to about 1 nM (Heller et al., 2014).

TIRF microscopy significantly reduces the background fluorescent signal. The reflection of the excitation beam on the sample—cover glass interface causes an evanescent field of excitation light that decays exponentially away from the interface, with a typical depth of only a few hundred nanometers inside the sample volume. This substantially reduces background fluorescence, but at the same time requires the experiments to be carried out close to the surface. TIRF is therefore often combined with a trapping configuration where the biomolecule is tethered between a surface and an optically trapped bead or in between two beads that are brought close to a pedestal in the flow chamber (Fig. 1A and D). A disadvantage of the surface-tethered configuration is that the biomolecule is not homogeneously illuminated due to the angle it makes with the interface. Furthermore, measuring close to a surface can sometimes interfere with the biological processes under investigation. Alignment of this fluorescence microscopy technique is only slightly more challenging than an epi-illuminated wide-field fluorescence microscope and commercial implementations in inverted microscopes exist (see later).

Confocal fluorescence microscopy has the advantage that background fluorescence can be substantially reduced by spatial filtering of the fluorescence signal using a pinhole, reducing the out-of-focus fluorescence background. In this way, single molecules can be detected in a solution containing about 100 nM fluorophores. This technique, however, requires much more precise alignment and is therefore substantially more challenging to combine with optical tweezers. Visualizing the whole sample requires scanning, since fluorescence is only detected in a very small volume of the sample. Without implementing scanning, confocal fluorescence microscopy has been applied mostly to measure FRET from a single molecule at a fixed position (Fig. 1B and E). Such FRET measurements have a sub-nanometer spatial resolution (Hohng et al., 2007) and are therefore very suitable to measure conformational changes of biomolecules.
Scanning confocal fluorescence microscopy (also including STED microscopy) has also been combined with optical tweezers. Scanning the confocal spot over the sample is required to generate an image of part of the sample, which prevents continuous visualization of the whole sample. When detecting single molecules, this scanning approach is typically slower than using camera-based wide-field fluorescence approaches. To overcome this problem, Heller et al. developed a one-dimensional scanning approach, where the two optical traps are being used to linearize the biomolecule (in this case DNA), such that only one-dimensional scanning is necessary (Heller et al., 2013). Implementation of one-dimensional STED allowed for a fivefold enhancement of the diffraction-limited spatial resolution (to ~50 nm). This higher spatial resolution allows STED to measure at higher fluorophore concentrations, both in solution and on the DNA itself. A drawback of the 1D-STED approach is the high laser intensity required to generate stimulated emission, substantially increasing photobleaching.

Smart and creative application of microfluidic chips that contain micrometer-to-millimeter-sized channels has also led to a wealth of new experimental possibilities. Two main experimental schemes can be distinguished (Fig. 2). In the first approach, buffers are inserted into the micro-channels in a sequential manner (Fig. 2A), which allows to construct complex structures such as biomolecules that are attached with one end to a surface and, subsequently, with the other to a bead. In the second approach, buffers are inserted in a flow cell side by side without barriers in between (Fig. 2B). Because the flow is laminar, mixing of buffer flows does not occur. This implementation of microfluidic chips is particularly powerful in optical tweezers configurations without surface tethers, since it allows rapid switching of the buffer composition simply by moving the trap(s) to another laminar “lane.” This rapid switching can, for example, be exploited to suppress high background-fluorescence signals by imaging in a flow channel containing no fluorophores, after incubating in another channel containing the fluorophore.

The unique power provided by these sophisticated single-molecule instruments makes studying biological processes almost straightforward. It can, however, be daunting to decide which technique or equipment to use and to know how to deal with technical complexities and maintenance of the instruments. Access to commercial alternatives will undoubtedly make this powerful and versatile combination of techniques accessible to a broad range of researchers in biophysics, biochemistry, drug discovery,
toxicology, and many other fields. One of the few instruments combining both optical tweezers and fluorescence microscopy techniques and being commercially available is the JPK Nano Tracker™. The optical tweezers system from JPK instruments is designed around a standard inverted microscope and can be combined with various fluorescent techniques. More recently, LUMICKS introduced the C-Trap™. This instrument combines optical tweezers, confocal fluorescence microscopy or STED nanoscopy, and an advanced microfluidics system in a fully integrated configuration. With the development of commercial fluorescence techniques, it is to be expected that single-molecule methods will find wider application in the life and biomedical sciences, providing access to a novel way of studying biomolecular processes in a quantitative way.

Fig. 2 Illustration of different geometries of microfluidic chips used in optical tweezers. (A) Sequential delivery of buffers into the microchannel. This configuration allows for the construction of complex structures such as biomolecule attachment to a surface with one end and subsequently attachment to a bead with the other end. Arrows indicate the direction of flow. (B) Insertion of buffers in a parallel fashion. This geometry allows for rapid switching of buffer composition by moving the traps to another laminar “lane.” This configuration enables imaging both in the same lane with labeled entities still present (arrow 1) and in a different lane where no fluorophores are present in the solution (arrow 2).
3. APPLICATIONS

In the following we will discuss applications of different combinations of optical tweezers and fluorescence microscopy and highlight their capabilities and key practical aspects.

3.1 Single Optical Trap

One of the main optical trapping geometries used to study biomolecular interactions is single-trap optical tweezers. Over the past two decades, several studies have employed different configurations of fluorescence microscopy combined with a single-trap geometry, which has yielded valuable knowledge about biomolecular transactions (Table 1). Here, we will first describe some of these studies in which a single trap was used in combination with flow stretching and wide-field microscopy. Next, we will focus on studies in which one end of the biomolecule was tethered to an optically trapped microsphere and the other to a surface, while being visualized using wide-field and TIRF microscopy, a geometry that provides the simplest possible layout that facilitates force and displacement measurements. Finally, we will highlight how confocal microscopy in combination with the single-trap/surface geometry can advance our knowledge of DNA/protein conformational dynamics by enabling FRET measurements.

3.1.1 DNA Enzymes

A tremendous amount of new insights in DNA repair enzymes has been obtained using a DNA attached from one side to an optically trapped bead and stretched by buffer flow (see Table 1). Handa et al. described a procedure to track directly DNA translocation by the *Escherichia coli* RecBCD helicase enzyme (Handa et al., 2005). To visualize a rapidly moving RecBCD molecule directly, a 40-nm streptavidin-coated fluorescent bead (nanoparticle) was attached to RecBCD that had been biotinylated *in vivo* at a unique site on RecD. The biotinylated DNA was separately bound to a streptavidin-coated polystyrene bead and then mixed with the RecBCD that had been tagged with the fluorescent bead. The bead–DNA–RecBCD–nanoparticle complex was captured by an optical trap and then moved to a second channel of a flow cell containing ATP to start translocation. In their optical trap a Nd:YLF infrared laser (wavelength 1047 nm, 500 mW; Spectra-Physics) was used, which was focused through an oil-immersion
objective lens (Plan Fluor 100×, 1.3 N.A.; Nikon), to a position 10 ± 15 μm below the upper surface of the flow cell. The trapping was performed in a 4000 μm wide flow cell. The fluorescent nanoparticle–RecBCD complex was excited with an appropriate filter set (Ethidium Bromide set 41006; Chroma Technology Corp.) and the images were captured in real time by an electron bombardment CCD camera (EB-CCD C7190-23; Hamamatsu Photonics, Hamamatsu, Japan). The position of the nanoparticle relative to the microsphere in their experiments was determined from individual video frames and velocities were determined. This assay allowed determination of the fast-moving rate of RecBCD (up to 1835 bp/s; 0.6 μm/s). Furthermore, the translocation rate of RecBCD was determined on a DNA containing two χ-loci, a recombination hot spot recognized by RecBCD, which was known to reduce the translocation speed of RecBCD. Using this substrate, RecBCD pauses were observed at specific locations, indicating that RecD did not dissociate but underwent conformational modification upon interacting with a χ-locus. This approach can be generally applied to other rapidly translocating motor proteins on DNA.

Using a slightly different approach, Bianco et al. (2007) visualized in real time the DNA network formation and cross-bridging activity of RAD54 oligomers, a DNA repair and recombination enzyme in eukaryotes. This was achieved by manipulating two fluorescently labeled (fluorescent dye YOYO-1) DNA–bead complexes trapped side by side, using two optical tweezers in a flow cell. Preformed RAD51–RAD54 nucleoprotein filaments and ATP were introduced by moving the two DNA tethers to a second channel, initiating pairing of the two DNA molecules. These measurements revealed that RAD54 oligomers possess a unique ability to cross bridge or bind double-stranded DNA molecules positioned in close proximity, stimulating the formation of DNA networks, and priming the DNA for rapid and efficient DNA-strand exchange by the recombinase RAD51. In their experiment, optical traps were formed by passing an Nd:YVO4 infrared laser (wavelength 1064 nm, 5 W, Spectra-Physics) through a polarizing beam splitter, which results in fixed and mobile optical traps in the focal plane of the microscope. The resulting, independent laser beams were focused through an oil-immersion objective lens (Plan Apo 100×, 1.4 NA, Leica) to a position 20 μm above the lower surface of the flow cell. Fluorescent DNA–bead complexes were excited using an XCite120 LED (XFO) in combination with a GFP-endow fluorescence cube bandpass filter (Chroma Technology, VT). Finally, fluorescence
images were captured at video rate by an EB-CCD camera (Hamamatsu) and recorded on digital videotape (DV184) using a digital VCR (Sony DSR-11).

Hilario et al. (2009) also used a fluorescent RAD51 to visualize nucleation, assembly, and disassembly of individual nucleoprotein filaments directly. Using this approach, they revealed that the rate of RAD51–nucleoprotein growth increases with a third-order dependence on RAD51 concentration. They determined that a minimum of two to three monomers of RAD51 is required to form a stable nucleus, based on the RAD51-concentration dependence of nucleation.

3.1.2 Interactions of Proteins With Microtubules

Combinations of optical trapping with fluorescence microscopy have made important contributions to mitosis and meiosis fields (Akiyoshi et al., 2010; Sarangapani et al., 2014), and in particular to how kinetochores—the molecular machines that drive chromosome separation—are attached to microtubules. To this end, a combination of the single-trap/surface geometry and TIRF microscopy was used by Umbreit et al. (2014) to investigate how Dam1, one of the kinetochore subcomplexes, influences kinetochore–microtubule attachment. They quantified the residence time \((5.3 \pm 0.5 \text{ s})\) and diffusion \((0.021 \pm 0.001 \text{ mm}^2\text{s}^{-1})\) of Dam1 complex on microtubules using TIRF microscopy. In addition, they determined the strength of microtubule attachments \((7.4 \pm 0.4 \text{ pN})\) using an optical trap-based rupture force assay, in which they decorated a polystyrene bead with the Dam1 and allowed it to interact with one side of a microtubule that was attached to the surface with the other side.

In a similar approach, Kudalkar et al. (2015) aimed to understand force transmission throughout the kinetochore and to discern the precise role of two other kinetochore subcomplexes, MIND and Ndc80. To this end, they used a two-color TIRF setup with a far-red laser (FTEC-635-0-25-PFQ, Blue Sky Research, Milpitas, CA, USA) that excites either Cy5 or Alexa-647, and a blue laser (473-30, LaserPath Technologies, Oviedo, FL, USA; or, more recently, Sapphire 488-75, Coherent, Santa Clara, CA, USA) for the excitation of GFP-tagged kinetochore subcomplexes. They found that MIND dramatically reduced the microtubule-binding time of Ndc80 (Fig. 3). In contrast, MIND alone did not interact with microtubules, even when added at high concentrations, indicating that MIND activates the microtubule-binding activity of the Ndc80 complex. By means of a rupture force assay with a constant loading rate, the strength of the MIND–Ndc80
linkage was probed. By performing a dual-label TIRF experiment, Kudalkar and coworkers demonstrated that MIND does not enhance Ndc80 oligomerization, but induces a conformational change in the Ndc80 complex, activating the microtubule-binding domains.

Fig. 3 Top panel: Effect of MIND subcomplex on microtubule-binding time of Ndc80. Left: Representative TIRF kymographs of Ndc80c-GFP, Ndc80c-GFP + 2.5 nM Dam1c, MIND-GFP/Ndc80c, and MIND-GFP/Ndc80c + 2.5 nM Dam1c. Right: Diagrams denote each GFP-tagged complex (ovals) and untagged (circles) complex binding to microtubules in kymograph on left. Bottom panel: Tolerance of the MIND/Ndc80c linkage for substantial load. (A) Representative traces of bead position versus time for 20 nM MIND-His/Ndc80c-FLAG beads under 1.7–2.5 pN of force applied in the direction of microtubule assembly. (B) Survival probability as a function of force is shown for Ndc80c-His, MIND-His/Ndc80c-FLAG, and MIND-His beads with Ndc80c-FLAG and Dam1c-FLAG. The average rupture forces derived from the distributions of survival probability versus force are indicated. Ndc80c-His and MIND-His/Ndc80c-FLAG were not significantly different. Adapted with permission from Kudalkar, E. M., Scarborough, E. A., Umbreit, N. T., Zelter, A., Gestaut, D. R., & Riffle, M. (2015). Regulation of outer kinetochore Ndc80 complex-based microtubule attachments by the central kinetochore Mis12/MIND complex. Proceedings of the National Academy of Sciences of the United States of America, 112, E5583–E5589.
3.1.3 DNA Helicases

The configuration of a single-trap optical tweezers and TIRF discussed above has also been used by Lee et al. (2013) to study dynamics of the *E. coli* helicase/translocase, UvrD, on a long ssDNA substrate. For this experiment, an oil-immersion objective lens (100×, N.A. 1.40, Olympus) was used for objective-type TIRF microscopy and optical trapping. Fluorescence excitation was only provided close to the cover glass, sample interface using a 532-nm diode-pumped solid-state laser (Spectra-Physics). Fluorescence was detected with an EMCCD camera (Andor iXon). Optical trapping light was provided by a 1064-nm Nd: YAG laser (Spectra-Physics). Using this instrument, they demonstrated UvrD binding to and translocation along ssDNA by tracking the position of individual UvrD fluorescent spots and determined the number of UvrD monomers from fluorescence intensity and counting the number of photobleaching steps. In addition, using the force measured from the optical tweezers, they monitored the unwinding activity. They determined that the processivity of UvrD translocation along ssDNA is 1260 (±60) nt, with a velocity of 193 (±2) nts⁻¹. UvrD monomer translocation stopped at an ssDNA/dsDNA junction, indicating that the translocating UvrD monomers do not make a transition to unwinding duplex DNA.

3.2 Dual-Trap Optical Tweezers

The use of two optical traps has several advantages over the single optical trap configurations. First, since there is no need for surface tethering, measurements can be performed far from possibly interfering surfaces. Second, a microfluidic device can be used to change buffer conditions rapidly (Beebe, Mensing, & Walker, 2002; Squires, 2005). Third, since both traps can be positioned independently, the user has three-dimensional control over the biomolecular construct that is trapped. Fourth, the dual-trap configuration also has advantages for imaging. Since the traps can be positioned horizontally with respect to the imaging plane, homogeneous illumination is more easily obtained. This makes it possible to perform epi-illuminated wide-field, TIRF, (scanning) confocal as well as STED experiments. These different configurations have been adopted widely (Table 1). Here we will focus on several applications of the dual-trap optical tweezers approach in combinations with fluorescence microscopy.
3.2.1 DNA Mechanics

The dual-trap configuration has been broadly adopted to study the mechanics of biomolecules tethered between two optically trapped beads. Arai et al. used this approach to tie knots in actin and DNA in a high viscosity medium that prevented polymers relaxations (Arai et al., 1999). They were able to estimate knot diameters by comparing the fluorescence intensity of a knot with that of the unknotted polymer. In a similar assay, Bao et al. studied knot behavior in DNA (Bao et al., 2003). They were able to tie several types of knots in the DNA and observed and quantified the diffusion of knots along the DNA.

Also other aspects of dsDNA mechanics have been studied extensively. In particular, force–extension behavior has been studied using optical tweezers alone (Gross et al., 2011; Moffitt et al., 2008; Neuman & Block, 2004; Smith, Cui, & Bustamante, 1996), revealing that DNA acts as an entropic spring at forces below 2 pN, followed by an enthalpic regime where the DNA is extended. At forces above 65 pN, DNA overstretches and only a small rise in force is required to extend the DNA to 1.7 times its length. The molecular nature of this overstretching transition had been fiercely debated. Combinations of fluorescence microscopy and optical tweezers have helped settling the debate, thanks to the local information provided by fluorescence microscopy (King et al., 2016; Le, Liu, Lim, & Yan, 2016).

In particular, to study the local DNA configuration during overstretching, van Mameren et al. used dual-color epi-illuminated wide-field fluorescence microscopy to image intercalating dyes specific for dsDNA, and ssDNA-binding proteins for ssDNA (van Mameren, Gross, et al., 2009). The DNA construct was attached to the beads with the two opposite strands allowing the DNA to rotate freely with respect to the trapped beads. The intercalator-labeled fraction of the DNA, usually forming a single, continuous patch in the DNA, decreased linearly with extension. The remainder of the DNA was labeled by ssDNA-binding protein, indicating that overstretching can be due to force-induced melting of the dsDNA, resulting in unpeeling of the DNA from the DNA ends. By applying a gentle flow perpendicular to the DNA, they could also observe the ssDNA being flow stretched, thereby confirming their hypothesis. In a follow-up study, King et al. performed experiments on torsionally unconstrained dsDNA without free ends, using fluorescent reporters for ssDNA and dsDNA (King et al., 2013). Under high-salt conditions, during overstretching, part of the
DNA was labeled by neither reporter, indicating that a third form of DNA, S-DNA, is involved. S-DNA is a DNA conformation proposed before, in which base pairing is still intact but the helicity is largely lost. Under low-salt conditions, patches of the DNA (corresponding to regions with low AT content) could be stained by the ssDNA probe, indicating that local melting bubbles formed. These results directly show that the overstretching of dsDNA can have three different products, peeled ssDNA, ssDNA melting bubbles and S-DNA, and that the ratio between these three depends on sequence, the presence or absence of free DNA ends, and salt concentration. More recently, King et al. determined the mechanics of torsionally constrained dsDNA (King et al., 2016). They found evidence for an additional, previously predicted DNA conformation, P-DNA, with the phosphate backbone facing inwards and the bases outwards, and which is substantially overwound. From the fluorescence data, the percentage of DNA in the P-DNA form could be estimated to be 20–30% which is in good agreement with a previous prediction (Léger et al., 1999).

### 3.2.2 DNA Intercalators

DNA intercalators, which bind between adjacent base pairs of dsDNA, are often used as a fluorescent marker for DNA visualization. It is, however, well known that intercalators perturb the structure and mechanical properties of DNA. To elucidate the effect of intercalators on DNA and to test under what conditions they can be used as DNA probes, Murade et al. and Biebricher et al. combined dual-trap optical tweezers and epi-illuminated wide-field fluorescence microscopy (Biebricher et al., 2015; Murade et al., 2009). Both studies showed that there is a linear correlation between DNA extension and total intercalator fluorescence intensity, indicating that intercalator binding increases with tension. Biebricher et al. quantified the dsDNA-binding and –unbinding kinetics of several intercalators (Biebricher et al., 2015). A combination of force and fluorescence measurements allowed determination of intercalator coverage over an unprecedented four-orders-of-magnitude range. They showed that the force dependence of intercalator binding is mostly governed by the strongly force-dependent unbinding rate (Fig. 4). The unbinding rate varies over seven orders of magnitude, depending on intercalator species, salt conditions, and DNA tension. This detailed quantification of intercalator–binding and –unbinding kinetics provides the insights needed for researchers to select the optimal intercalator species and conditions for a given application.
3.2.3 DNA Enzymes

Dual-trap optical tweezers also allow quantitative analysis of DNA-binding proteins. One such protein that has frequently been studied this way is RAD51, which forms ATP-dependent filaments on dsDNA and ssDNA. van Mameren et al. used a combination of optical tweezers and epi-illuminated wide-field fluorescence microscopy to study the mechanics and kinetics of RAD51 filaments on dsDNA (van Mameren et al., 2006). They showed that, in the absence of ATP hydrolysis, RAD51 forms immobile filaments that remain stably bound to DNA even at high forces. Moreover, the measurements allowed estimation of the dsDNA length increase upon binding RAD51 to be 150%. Notably, the filaments of RAD51 on DNA are very rigid and could hardly be stretched when the DNA molecule is pulled (Fig. 5).

Fig. 4 Force-dependent DNA intercalation of a mono- (SbG, open symbols) and a bis-intercalator (YOYO, solid symbols) at 1000 mM NaCl. (A) DNA elongation and representative fluorescence images as a function of tension. (B) Binding constant as a function of tension, calculated using elongation data. Adapted with permission from Biebricher, A. S., Heller, I., Roijmans, R. F. H., Hoekstra, T. P., Peterman, E. J. G., & Wuite, G. J. L. (2015). The impact of DNA intercalators on DNA and DNA-processing enzymes elucidated through force-dependent binding kinetics. Nature Communications, 6, 1–12.
Using a similar approach, Candelli et al. focused on RAD51-filament nucleation and growth on both dsDNA and ssDNA (Candelli et al., 2014). First, by counting the number of nuclei that formed in a given time, they determined the nucleation rate, which increased with RAD51 concentration. From the fluorescence intensity of individual nuclei, they determined the number of RAD51 monomers per nucleus and found a wide distribution of nucleus sizes. In addition, filament growth was studied with a single-molecule fluorescence recovery after photobleaching assay, in which nuclei were first completely photobleached, followed by incubation of the DNA in a RAD51-containing buffer. When a new fluorescent spot occurred at the same location as a previously photobleached one, this was interpreted as a growth event. From such experiments the filament growth rate was determined. Remarkably, both nucleation and growth rates are force dependent for RAD51 binding to dsDNA, but not for binding to ssDNA.

Fig. 5 Elastic properties of a single 48 kbp λ-DsDNA molecule partly coated with fluorescent RAD51. (A) Fluorescence image (left) of such an assembly, tethered between two streptavidin-coated polystyrene beads. Kymograph (right) generated from the successive frames of the movie recorded during extension of the construct. The corresponding force time trace is depicted in gray scales (top bar; white corresponds to 90 pN). (B) Force–extension curve corresponding to the construct in (A). The gray trace shows a bare λ-DNA reference curve. (C–E) Force–extension curves of the bare zone (i), the continuous fluorescent zone (ii), and the composite fluorescent zone (iii) as indicated on the right of the kymograph. Adapted with permission from van Mameren, J., Modesti, M., Kanaar, R., Wyman, C., Wuite, G. J. L., & Peterman, E. J. G. (2006). Dissecting elastic heterogeneity along DNA molecules coated partly with Rad51 using concurrent fluorescence microscopy and optical tweezers. Biophysical Journal, 91(8), L78–L80.
van Mameren et al. looked at the disassembly of RAD51 filaments from dsDNA (van Mameren, Modesti, et al., 2009). Preformed RAD51-nucleoprotein filaments were rapidly brought from a nonhydrolyzing to an ATP-hydrolyzing condition by moving them to another channel in the flow cell. While holding the traps at a fixed distance, tension was observed to increase, while the RAD51 fluorescence intensity decreased, consistent with RAD51 disassembling from the DNA (Fig. 6). Remarkably, a sudden release of the tension resulted in an enhanced disassembly rate. This result was interpreted to indicate that the force-dependent event was not ATP hydrolysis, but the actual detachment of a RAD51 monomer from the filament. Dissociation occurred in bursts interspersed

![Fig. 6 RAD51 disassembly rate is reversibly reduced by DNA tension. Top: Kymograph of a RAD51–dsDNA complex, held at fixed length. Bottom: Intensity trace (red, descending line from top left) and tension trace (blue, ascending line from bottom left) of a RAD51–dsDNA complex. Tension-stalled disassembly is reinitiated by tension release (orange dashes). Adapted with permission from van Mameren, J., Gross, P., Farge, G., Hooijman, P., Modesti, M., Falkenberg, M., et al. (2009a). Unraveling the structure of DNA during overstretching by using multicolor, single-molecule fluorescence imaging. PNAS, 106(43), 18231–18236; van Mameren, J., Modesti, M., Kanaar, R., Wyman, C., Peterman, E. J. G., & Wuite, G. J. L. (2009b). Counting RAD51 proteins disassembling from nucleoprotein filaments under tension. Nature, 457(7230), 745–748.](image-url)
with pauses comparable to the typical RAD51-catalyzed ATP hydrolysis time, providing support for a model in which RAD51 disassembly only takes place from filaments ends after ATP hydrolysis in the RAD51 monomer on the end.

Another DNA-binding protein that has been studied using optical tweezers and wide-field fluorescence microscopy is TFAM, a key protein in mitochondrial DNA transcription and compaction (Farge et al., 2012). Fluorescence intensity measurements of dsDNA saturated with fluorescently labeled TFAM allowed determination of the footprint of the protein. The intensity of individual fluorescent spots observed at low TFAM concentrations indicated that TFAM binds as a monomer to dsDNA. These monomers diffused along the DNA and with single-molecule tracking the diffusion coefficient was determined. Force–extension measurements at different TFAM concentrations showed that TFAM binds to DNA in a cooperative way, decreasing the persistence length. In a follow-up study, it was shown how compaction by TFAM blocks unwinding of duplex DNA resulting in inhibition of mitochondrial DNA transcription and replication (Farge et al., 2014).

To obtain even higher-resolution insights in TFAM diffusion and DNA binding, Heller et al. applied optical tweezers in combination with STED super-resolution microscopy (Heller et al., 2013). The advantage of using STED was that TFAM could be studied at higher, more physiologically relevant concentrations in solution and on the DNA. It was observed that TFAM monomers can cluster and form multimers, diffusing with a lower diffusion constant (Fig. 7).

### 3.2.4 Lipid–Membrane Fusion

Using a very different dual-trap optical tweezers assay, Brouwer et al. studied lipid–protein interactions (Brouwer et al., 2015). To this end, they coated the two trapped beads with lipid bilayers and repeatedly brought them together and separated them. In the presence of Doc2b, a protein that is involved in SNARE-mediated membrane fusion during neurotransmitter-vesicle release, high rupture forces were observed. These high rupture forces indicated Doc2b-mediated membrane fusion. When the membranes were fused, fluorescently labeled lipids on one bead could diffuse to the other bead, while dyes in the lumen between membrane and bead did not, indicating that Doc2b mediates fusion of only outer leaflet of the lipid bilayer, known as hemifusion (Fig. 8).
3.2.5 Cytoskeletal Motor Proteins

Dual-trap optical tweezers in combination with fluorescence microscopy have also been used to study the interaction of myosin motor proteins with filamentous actin. To this end, myosin motors were attached to a pedestal in the flow cell, while an actin filament was held between two optically trapped beads. Using this assay, interactions and force generation
Fig. 8 Investigation of lipid–protein interaction using dual-trap optical tweezers combined with epi-illuminated wide-field fluorescence microscopy. (A) In the presence of fluorescent NBD-PE in a single membrane-coated bead, membrane-stalk formation was accompanied by a strong fluorescence increase in the unlabeled membrane and a concurrent decrease in the labeled membrane, indicative of either hemifusion or full membrane fusion. (B) To distinguish between hemifusion and complete fusion, content mixing was tested by coating the beads with nonfluorescent PC/PS liposomes, while one of the beads was loaded with fluorescein in the liposomal lumen. On a timescale of 1000 s, fluorescence increase in the unlabeled bead or membrane stalk was not observed, indicative for hemifusion. (C) Schematic representation of the hemifused configuration with Doc2b bound to the membrane surface. (D) Fluorescence images of bead pair in (A) at three different time points. Scale bar: 1 μm. (E) Fluorescence images of bead pair in (B) at three different time points. Scale bar: 1 μm. Adapted with permission from Brouwer, I., Giniatullina, A., Laurens, N., Weering, J. R. T. Van, Bald, D., Wuite, G. J. L., et al. (2015). Direct quantitative detection of Doc2b-induced hemifusion in optically trapped membranes. Nature Communications, 6, 1–8.
can be measured with the tweezers, while binding and release of fluorescent ATP analogues to the motor proteins can be followed with TIRF microscopy. With this approach, step sizes, processivity, and ATPase activity of several myosin motors were characterized (Ishijima et al., 1998; Iwane et al., 2005; Komori et al., 2009; Tanaka et al., 1998, 2008; Watanabe et al., 2004).

### 3.2.6 Bacterial Motility

Min et al. and Mears et al. held a living bacterium fixed with two optical traps, while flagellum position was monitored using fluorescence and cell-body displacement with the tweezers (Mears et al., 2014; Min et al., 2009). Fourier analysis of the cell-body displacements revealed two frequencies, one corresponding to rotation of the flagellum and the other with the resulting rotation of the bacterium. The bacterial rotation signal contained indications of periods of constant, directional movement, and tumbling, consistent with two different kinds of appearances of the flagellum fluorescence images (Min et al., 2009). Improvements in fluorescence imaging allowed Mears et al. to distinguish multiple flagella at the same time and determining their sense of rotation (Mears et al., 2014). They demonstrated that only a single flagellum rotating clockwise was required to switch the bacterium into tumbling mode (Fig. 9). They also showed that flagella do not switch their sense of rotation independently (as was assumed before),

![Fig. 9](image_url)  
**Fig. 9** Assessment of bacterial motility. *Top:* Representative images from a trapped cell with three flagella. The approximate location of the unlabeled cell body is indicated by a dashed line. Flagella rotating CW (purple) and CCW (underlined-white) are numbered in frames in which they appear distinct. *Bottom:* corresponding cell-body rotation signal for the same cell as detected from deflections of the trapping laser. Tumbles (shaded area) were determined from the erratic cell-body rotation signal. Adapted with permission from Mears, P. J., Koirala, S., Rao, C. V, Golding, I., & Chemla, Y. R. (2014). Escherichia coli swimming is robust against variations in flagellar number. eLIFE, 3, e01916.
but do so in correlated fashion, which has important implications for understanding bacterial motility and chemotaxis.

### 3.3 FRET Studies With Confocal Fluorescence Microscopy

Force-measuring tools like atomic force microscopy or optical tweezers cannot detect small-scale conformational changes, unless a relatively strong force is applied. This limitation has been overcome by combining single-trap optical tweezers with FRET confocal microscopy, providing access to measurements of conformational dynamics. Hohng et al. used this approach to investigate the dynamics of Holiday Junction structures (HJ) (Hohng et al., 2007). An HJ is a four-stranded DNA structure that is an intermediate state during homologous recombination. A trapping beam of 1064 nm was fixed in the field of view of the microscope, while forces were applied by moving the surface–tethered HJ using a piezoelectric sample stage. The confocal laser focus (532 nm) was scanned to follow the motion of the molecule in response to the moving optical trap. By specifically labeling the HJ at different locations a 2D reaction landscape could be obtained, by probing the HJ dynamics in response to pulling forces in three different directions. In this way, global structural information could be obtained on transient species involved in HJ conformational changes.

The application of combined optical tweezers and FRET microscopy has also been extended to investigate DNA–protein interactions. For example, Ngo et al. (2015) employed single-trap optical tweezers and FRET microscopy to manipulate an individual nucleosome under force and simultaneously probe its local conformational changes. They anchored a DNA molecule with a nucleosome-binding site with one end to a glass surface and with the other end to an optically trapped microsphere. The DNA contained FRET dye pairs at various locations. They found that the DNA, which is wrapped around the nucleosome, unwraps asymmetrically from a nucleosome: from one side unwrapping occurs at low forces (3–5 pN), while from the other side, substantially higher forces are required for unwrapping (12–15 pN). These results have important implications for chromosome remodelers, which move or expel nucleosomes from DNA.

In their setup, they applied an infrared laser (1064 nm, 800 mW, EXLSR–1064–800–CDRH, Spectra-Physics) to form the optical trap through the back port of a commercial microscope (Olympus) and applied forces on the sample tethers by moving the microscope slide using a piezoelectric stage (Physik Instrumente). The position of the tethered bead with
respect to the trap was determined using a quadrant photodiode (SPOT/9DMI, UDT). Excitation light for confocal excitation (wavelength: 532 nm, World Star Tech) was coupled into the side port of the microscope and was scanned by a piezo-controlled steering mirror (S-334K.2SL, Physik Instrumente). Fluorescence was filtered using a bandpass filter (HQ580/60m, Chroma) and separated from the excitation light using a dichroic mirror (HQ680/60m, Chroma) before detection by two avalanche photodiodes (APDs).

In a different approach, Suksombat et al. studied the interaction of ssDNA-binding protein, SSB, with a short stretch (70 nucleotides) of ssDNA held between two optical traps with dsDNA handles (Suksombat et al., 2015). In their experiments, they used two high-resolution optical traps, formed by timesharing a single IR laser (a 5-W, 1064-nm diode-pumped solid-state laser, YLR-5-1064-LP; IPG Photonics), by intermittently deflecting the laser in two directions with an acousto-optic modulator (IntraAction). Constant-force experiments were performed with a PID controller loop that monitored the trapped bead positions and controlled the trap separation to maintain a constant tension on a tethered DNA molecule. Fluorescence probes were excited by a 532-nm 5-mW laser (DPGL-05S, World Star Tech) interlaced with the trapping IR laser at a rate of 66 kHz and imaged onto two APDs (Perkin Elmer). Using a force clamp they observed step-wise length switches of the DNA tether, due to partial wrapping and unwrapping of the DNA around SSB (Fig. 10). This switching could also be observed using FRET between dyes on the DNA and SSB. They also observed changes in FRET efficiency uncorrelated to changes in DNA extension, indicative of SSB diffusing along the DNA.

With a similar assay, Comstock et al. studied UvrD helicase unwinding a DNA hairpin (Comstock et al., 2015). Changes in DNA extension revealed unwinding and rezipping of the DNA. Concomitant fluorescence measurements allowed discrimination of activity of UvrD monomers and dimers. For monomers, frequent, repetitive switching between unwinding (of at most 20 bp) and rezipping was observed, while dimers processively unwound the DNA over lengths of 70 bp. FRET experiments showed that the conformation of the protein, open or closed, correlated to unwinding or rezipping activity.

Furthermore, the subnanometer sensitivity of FRET in measuring distances, together with piconewton force sensitivity of optical tweezers enables studies of mechanotransduction. With the help of this capability, Brenner et al. used this combination to determine how spider-silk
flagelliform repeat peptides react to force (Brenner et al., 2016). They first showed that, at zero force, each peptide has a conformation stable on the timescale of one second to minutes. Next, they showed, by measuring FRET efficiency as a function of force, that the peptides behave as linear springs. This was unexpected since disordered proteins act as nonlinear springs. The results indicate that these peptides are highly compact and in an ordered, rod-like coil structure. Brenner et al. also used these peptides as intracellular force sensors, by integrating them in the focal-adhesion protein vinculin and measuring FRET using fluorescence lifetime imaging.

4. EXPERIMENTAL PROTOCOL

In this section, we describe a general scheme to perform experiments such as those described in the previous section using a dual-trap optical tweezers setup combined with wide-field epi-fluorescence microscopy, as we typically perform them in our laboratory (Biebricher et al., 2015; Farge et al., 2012; King et al., 2016).
4.1 Experimental Setup

A detailed description of our dual-trap optical tweezers setup combined with epi-fluorescence microscopy has been presented elsewhere (Gross et al., 2010). In brief, two perpendicularly polarized trapping beams are generated by splitting a 1064-nm trapping laser (YLR-LP, IPG photonics) with a half-wave plate and a polarizing beam splitter. These beams can be manipulated independently using steerable mirrors. Displacement of the beads with respect to the trap centers are measured by back-focal-plane interferometry using two position-sensitive detectors (DL100-7-PCBA3, Pacific Silicon Sensor); the position of the beads is determined using bright-field imaging, using blue LED illumination and a CCD camera (both Thorlabs). Fluorescence excitation is provided, for example, by a 532-nm diode-pumped solid-state laser (Samba, Cobolt) into the microscope and the fluorescence is detected using an EMCCD camera (Ixon3 897, Andor).

A microfluidic chip containing up to six channels, forming parallel flow lanes (Fig. 2B), is mounted on the microscope stage and connected to a flow system based on pressurized air (u-Flux, LUMICKS). Valves are used to control independently the buffer flow in separate channels. The DNA construct is obtained by binding of biotinylated nucleotides to the 3'-recessive ends of Bacteriophage Lambda (λ) DNA (~48.5 kb) (Roche) according to protocols described before (Gross et al., 2010).

4.2 Methods

- Cleaning of the microfluidic flow cell: rinse the flow cell thoroughly with bleach (a sodium hypochlorite solution) to clean the surface. Rinse afterward with sodium thiosulfate solution to neutralize remaining bleach and subsequently with water.
- Passivation of flow cell to prevent sticking of proteins to surface: flush the flow cell with casein or BSA solution, followed by a Pluronic® F127 solution.
- Introduction of buffer solutions: flush a dilution of streptavidin-coated 4.5-μm microspheres (Spherotech) through the first microfluidic channel. Flush a buffered solution of the biotinylated DNA construct through the second channel and buffer through the third channel. Flush your fluorescently labeled protein or compound of interest through the fourth and/or the fifth channel.
- Catching DNA: trap, in the presence of flow, two beads by moving the traps to the first channel. Subsequently, move the traps to the second
flow channel and monitor jumps in force that indicate binding of DNA to one of the beads. Move the beads to the third channel and approach and withdraw the downstream bead from the upstream bead until a change in force indicates the formation of a DNA dumbbell construct.

- Protein incubation: move DNA construct to the channel that contains your fluorescently labeled protein or compound of interest and allow it to bind to the DNA. Depending on the binding kinetics, this will take seconds up to several minutes. Note that for some compounds, binding is facilitated by application of a pulling force on the DNA.
- Single-molecule fluorescence: move the incubated DNA back to the third channel containing only buffer to reduce the background fluorescence. Illuminate with fluorescence excitation laser and measure fluorescence images with the EMCCD camera. In this way the unbinding kinetics of the compound or protein of interest can be followed, for example, as a function of force.

5. CONCLUSION

The state-of-the-art combination of optical tweezers with fluorescence microscopy and microfluidics techniques provides a valuable addition to the single-molecule toolkit. It has enabled scientists to study biological systems with previously unobtainable precision and clarity on a vast number of different biological interactions as illustrated in this chapter. Some of the most recent advancements such as STED microscopy promise to give rise to even more exciting new possibilities by pushing the experimental limits toward single-molecule experiments in conditions closer to the physiological reality. Moreover, the current state of maturity of the technology and the concurrent development of their application in biological assays, as is reviewed in this chapter, paves the road for broadening the application of this combined technique from the realm of fundamental research in a specialized biophysics laboratory to biochemical, biological, and pharmaceutical laboratories. Finally, commercial solutions are becoming increasingly available, which makes this powerful combined approach accessible to a broad range of researchers from different backgrounds. When this technology spreads we expect to see more innovative and exciting single-molecule experiments which can revolutionize research in biophysics, biochemistry, drug discovery, toxicology, and many other fields.
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