Cellular DNA is continuously engaged in a molecular dance with many accessory proteins. These proteins are responsible for orchestrating the storage, maintenance, and transfer of genetic information. Together, the genomic operations make up the various metabolic pathways of DNA, which cascade into all aspects of cellular life. Over the past two decades, optical-tweezers analyses of DNA-protein complexes have provided unique mechanistic insights into this molecular dance. Numerous assays have been devised to probe and elucidate DNA-protein interactions spanning the full range of DNA metabolic processes using optical tweezers. In this chapter, we provide an overview of the field, highlighting the unique opportunities that optical tweezers provide for DNA-protein analysis. We will describe the different experimental assays used, their limitations and advantages, and review the many contributions that optical tweezers have made to our fundamental understanding of DNA transactions.

DNA:protein interactions

DNA-processing proteins make up a multitude of molecular machineries of remarkable complexity and function. These machineries are faced with the momentous challenge to perform numerous tasks on complex macromolecular objects, namely cellular DNA. The DNA in living cells has a highly heterogeneous and dynamic structure upon which groups of DNA-processing proteins need to perform dedicated tasks. Some of these, such as the search for a damaged base, a promoter sequence, or a restriction site are specific for the base-pair sequence of the DNA. Others, such as genome replication, and protection of single-stranded DNA (ssDNA) should be relatively insensitive to sequence. Concurrently, these tasks need to be performed without error, within an acceptable time frame, and efficiently. Often, a dynamic interplay between multiple proteins is required to accomplish this. Within these given constraints, the structural and mechanistic details of DNA-protein interactions play a crucial role in balancing the functioning of the protein machinery.

Single-molecule versus bulk assays

Bulk biochemical assays have yielded a wealth of structural and mechanistic insight in DNA metabolism. Several factors, however, limit the resolving power of bulk assays. Proteins operate on an energy landscape with peaks just above the thermal bath [142]. This causes their behavior to be stochastic and the outcome of their action...
DNA:protein interactions in optical tweezers

to be heterogeneous. Moreover, due to translation errors, conformational differences and post-translational modification, individual proteins can behave differently. Bulk studies often require synchronization of protein action, which can be difficult to achieve due to the stochasticity and heterogeneity of multistep and multicomponent processes. Single-molecule methods, in contrast, do not require synchronized operation, since observations are made on individual biomolecules, avoiding population averaging of transient features. These features include rare events, heterogeneous behavior, and also the single catalytic turnovers driving the overall process. Moreover, single-molecule observation allows monitoring the activity in real time. For these reasons, single-molecule methods have been instrumental in unveiling the dynamic, structural and mechanistic nature of DNA-protein interactions. A point of caution is, however, that correct interpretation of single-molecule data crucially depends on the availability of enough statistics, which can be costly and time-consuming.

Single-molecule force measurements

In addition to the advantages discussed above, certain single-molecule techniques provide the unique ability to apply forces and to measure tension. The information that may be accessed by means of force is extremely rich: one can measure the forces generated by DNA-protein complexes to unravel their mechanochemical coupling to enzymatic reactions fueling the mechanical work. Alternatively, one can apply forces to DNA-protein complexes to analyze their mechanical response and interrogate structural properties. Moreover, applying force can be used to alter the energy landscape in which a DNA-protein complex operates, yielding unique insights into the thermodynamics and kinetics of DNA-protein interactions. Three distinct force-based techniques have been applied to DNA-protein interactions [132]: atomic force microscopy (AFM) [128], magnetic tweezers [37], and optical tweezers [126]. AFM is unique in its ability to apply forces as high as nanonewtons, but is limited in its lower force resolution (lower piconewtons) [128]. In contrast, magnetic tweezers can measure forces with femtonewton accuracy and can introduce twist to DNA-protein complexes to address the role of torque and DNA supercoiling [38]. Optical tweezers, on the other hand, cover a very useful intermediate force range from a tenth of a piconewton to a nanonewton [126]. They are characterized by an extremely high spatiotemporal resolution, providing Ångström position resolution and millisecond time resolution. In addition, optical tweezers can be readily combined with other techniques such as microfluidics and single-molecule fluorescence microscopy. The result is that optical tweezers currently represent one of the primary methods of choice for single-molecule analysis of DNA-protein complexes. Although the experimental complexity has made access to this technology relatively limited to specialized laboratories, commercial systems are now emerging on the market, just as for AFM and magnetic tweezers.

Optical tweezers

The physical principles of optical trapping were discovered by Arthur Ashkin in 1970 [6]. However, it took 25 years before optical tweezers were first applied to a DNA-protein complex [183]. In a typical optical tweezers experiment, the DNA-protein complex of
2.2 Optical tweezers analysis of DNA-protein complexes

Of interest is tethered to one or two microspheres (see Figure 2.2). These microspheres can be spatially trapped in the focus of a laser beam (optical tweezers). The trapped microsphere thus functions as a handle to hold, manipulate, and apply tension to the DNA-protein complex. Since this early study, substantial progress has been made in improving stability and resolution, culminating in the ability to resolve individual base-pair steps of molecular motor translocation along DNA [1]. For details on the technical background and instrumental details of optical tweezers setups, we refer to excellent dedicated reviews [126,131,132]. Current implementations of optical tweezers provide access to the measurement of nanometer displacements and piconewton forces on millisecond time scales. These sensitivities allow direct access to the energy range of pN·nm, which is within the regime of the thermal energy at room temperature ($k_B T$) that single biomolecules are exposed to.

This chapter

Over the last two decades, optical tweezers have been applied to many kinds of DNA transactions, including replication, transcription, recombination, repair, and organization. In this chapter, we will first provide an extensive description of the biological assays that optical tweezers enable (section 2.2), to comprehend the excellent opportunities that optical tweezers provide for studying DNA-protein complexes. We will then proceed with an overview of applications of optical tweezers to the mechanics of DNA itself (section 2.3), the molecular basis of DNA organization (section 2.4), replication (section 2.5), transcription (section 2.6), and recombination and repair (section 2.7). The discussion will be focused on a limited number of studies that compose the main research lines within each of the DNA processes. Other types of DNA-protein interactions that have been studied extensively with optical tweezers include those involving restriction endonucleases [56,93,166] and viral packaging motors [22,154,155]. These DNA transactions are not conserved over all kingdoms of life and lie outside the scope of this chapter.

2.2 Optical tweezers analysis of DNA-protein complexes

At the core of optical trapping lies the interaction between the focused laser light and the refractive microsphere. The interactions can be divided into two opposing forces: the scattering force and the gradient force. The scattering force pushes the microsphere forward, in the direction of light propagation, while the gradient force pulls the particle along the light intensity gradient toward the highest light intensity (Figure 2.1).

In the earliest optical trapping experiments, two counterpropagating laser beams were used in which the scattering force of the two beams cancel each other out to stably trap a microsphere [6]. Currently, the most common implementation of optical trapping is the single-beam optical trap [7], where the axial component of the gradient force balances the scattering force, resulting in stable trapping. In such a trap, the
Figure 2.1
Forces acting on optically trapped microspheres. (a) A microsphere in a single-beam optical trap. The scattering force, $F_{sc}$, acts in the direction of light propagation, while the gradient force, $F_{gr}$, acts along the electric field gradient toward the center of the laser focus. In the illustration, the two forces cancel each other, which leads to a stable position that is displaced by $\Delta z$ from the trap center in the axial direction. (b) An external force, $F_{ext}$, is exerted on the microsphere through a tethered DNA molecule, which causes a lateral displacement, $\Delta x$. The external force is opposed by the combination of gradient and scattering forces, leading to a net restoring force that can be described by Hooke’s law $F = k\Delta x$, where $k$ represents the effective stiffness, or spring constant, of the optical trap.
2.2 Optical tweezers analysis of DNA-protein complexes

Microsphere experiences a springlike force that varies linearly with small displacements, with a spring constant typically on the order 0.1 pN/nm. Key to quantitative optical trapping is accurate calibration of particle displacement and the spring constant, which allows calculation of the force experienced by the particle. On the basis of this general optical-tweezers scheme, a plethora of different assays, based on various trapping geometries and measurement modes, have been developed to probe DNA-protein complexes. The main aspects of these assays will be discussed in the following.

2.2.1 Optical trapping geometries

To accurately apply and measure a force in an optical tweezers assay, the biological system, for example, a DNA molecule, is tethered on two opposite ends (Figure 2.2). The three main geometries used for study of DNA-protein analysis are described below.

Single-trap optical tweezers

The first single-molecule experiments were performed using single-trap optical tweezers setups such as that depicted in Figure 2.2a [183]. In this geometry the DNA-protein complex is tethered between an optically trapped microsphere and a rigid substrate such as a glass coverslip (Figure 2.2a) or a second microsphere suctioned onto the tip of a micropipette (Figure 2.2b).

Optically, this geometry represents the simplest possible layout that facilitates straightforward implementation as well as uncomplicated force and displacement measurements. The variant of this scheme involving a micropipette has the advantage that the biological assay is positioned away from the glass surface that might interfere with the biological sample through unspecific protein or DNA binding. In addition, this implementation allows extending and stretching the DNA in a plane orthogonal to the trapping laser propagation, which is beneficial for imaging purposes. Also, a micropipette allows introducing twist to the DNA-protein complex by rotating the pipette [21]. Introducing a micropipette into the sample chamber, however, adds a substantial level of complexity, not compatible with all trapping instruments.

Dual-trap optical tweezers

The dual-trap optical tweezers were used for the first time in DNA-protein analysis in 2003 (Figure 2.2c) [149]. In this scheme, the DNA-protein complex spans two microspheres, each held by a separate optical trap. The primary advantage of this layout is that the resulting microsphere-DNA-microsphere construct (‘dumbbell’ construct) is completely suspended in solution. This effectively decouples the experiment from the sample chamber and its associated mechanical vibrations and drift, which is the most common source of noise and drift in single-trap optical tweezers [1, 125]. Furthermore, the dumbbell is suspended and not restrained by surface attachment, which allows it to be moved throughout the sample chamber. This makes near-instantaneous and complete buffer exchange possible in a multichannel laminar flow cell by moving the dumbbell from one parallel flow channel to another [167]. The implementation of
Figure 2.2
Optical trapping geometries. (a) Single-trap optical tweezers where one end of the DNA-protein complex is tethered to an optically trapped microsphere and the other to a surface. (b) Single-trap optical tweezers where the surface tether is replaced by a tether to a microsphere suctioned on the tip of a micropipette. (c) Dual-trap optical tweezers for which both microspheres are being held in two separate optical traps.
microfluidics can facilitate *in situ* dumbbell formation, which improves sample throughput. In addition, it allows the sequential steps in complex biochemical reactions to be controlled.

### 2.2.2 Resolution limits

Substantial effort has been spent in pushing the resolution of optical tweezers to allow one of the most elementary events in molecular biology, the stepping of a polymerase enzyme over a single base pair, to be resolved in real time. However, what are the ultimate limits of optical tweezers and what are the parameters that define these limits? In most cases, the performance of an optical tweezers instrument is limited by instrument drift and environmental noise. These factors can, to a large extent be suppressed by placing instruments in sound-isolated, temperature-controlled rooms on vibration isolation tables [126,131]. The ultimate noise limit of optical tweezers is set by the Brownian motion of the trapped microsphere coupled to the DNA-protein complex. Indeed, several instruments have been shown to operate near this fundamental limit [1,125,126]. For a typical trap stiffness (0.1 pN·nm) the thermal fluctuations of a microsphere are on the order of 6 nm. In a stiffer trap these fluctuations are reduced. However, this will not necessarily be of benefit, because trap stiffness often reduces the signal measured from a DNA-protein complex with an equal factor, resulting in no net gain in signal-to-noise ratio [132]. Trap stiffness can be regulated by adjusting the laser power, or microsphere properties such as diameter and refractive index [131]. What can be done to enhance the signal-to-noise ratio for measuring length changes of DNA-protein complexes? The most relevant factor is the compliance or stiffness of the DNA-protein complex itself: length changes in a floppy and soft tether will only weakly pull a trapped microsphere out of the trap center, while such changes in a stiffer construct will more effectively displace the microsphere. In high-resolution optical-trapping experiments, short DNA constructs of only few kilobase pairs are typically used, since the effective stiffness of DNA is directly proportional to its length. Even shorter constructs are less practical to tether. The stiffness of DNA is also higher when the DNA is pulled taut than when it is left slack (see section 2.3). Furthermore, time averaging can help to enhance the signal-to-noise ratio, at the cost of temporal resolution. Single base pair resolution has been obtained at a temporal resolution from 50 ms to 1 s. Conversely, at the cost of spatial resolution, the temporal resolution can be enhanced, yet to a limited extent because drag on the bead will effectively low-pass filter the signal, averaging out faster motion. Thus, temporal resolution is typically limited to the millisecond time scale. Finally, in addition to setting the limit for temporal resolution, drag will also affect the spatial signal-to-noise ratio: a smaller bead will fluctuate faster, which makes it easier to average out thermal noise on the time scale of the measurement.

### 2.2.3 Modes of measurement

How does one collect information on a DNA-protein complex? In force-measuring optical tweezers, information from the biological system can be collected using three fun-
fundamentally different measurement modes discussed below: force-extension analysis, extension clamping, or force clamping. These are illustrated schematically in Figure 2.3.

**Force-extension analysis**

Force-extension analysis is useful, for instance, for testing the mechanical response of a DNA-protein complex. An optical tweezers force-extension experiment applies either a force ramp or an extension ramp to the DNA-protein complex to retrieve information on its mechanical response. In this way, the mechanics of ssDNA and double-stranded DNA (dsDNA) have been analyzed (Figure 2.4) [23, 62], which is at the basis of every optical tweezers study of DNA-protein complexes. Force-extension curves (Figure 2.3b) can be analyzed to quantify the elastic properties of DNA or how they are affected by protein binding.
2.2 Optical tweezers analysis of DNA-protein complexes

Figure 2.4
Force-extension analysis of bare dsDNA (filled circles) and bare ssDNA (plus signs). The open circles show the stretching curve of ssDNA that was coated with a nucleoprotein filament (RecA, see 2.7.1), the polymer properties of which have been analyzed by comparison to polymer models (dashed and dashed-dotted curves). In addition, the dsDNA stretching curve exhibits a plateau near 65 pN, indicative of overstretching, which will be explained in section 2.3. Adapted with permission from ref [70].

Although the presence of proteins or occurrence of structural rearrangements is typically not detected in real time using this measurement mode, force-extension analysis does allow the mechanical nature of DNA-protein complexes in terms of, for instance, a change in extension, or flexibility etc. to be assessed (cf. change in stretching curves for bare ssDNA and protein-coated ssDNA in Figure 2.4).

Extension clamp

The extension clamp is the most straightforward measurement mode in optical tweezers. During an extension-clamp experiment, the optical traps are held stationary; changes in tension on the DNA-protein complex are monitored in time while the extension is held constant (Figure 2.3c). Changes in tension can be effected by protein binding or enzymatic activity that alters the compliance or length of the DNA-protein complex. This measurement mode provides the simplest qualitative real-time assessment of biomolecular presence and activity. Quantitative analysis of the force signal, however, is more complicated due to the variation in force over the course of the experiment. The rates of force-dependent processes such as DNA replication [180] are thus affected by their own stochastic activity and correct data interpretation requires corrections for the force-dependent compliance of the DNA tether.
**Force clamp**

The force-clamp mode entails an experiment where the force on the tethered DNA-protein complex is kept constant throughout the measurement. Since optical traps are not intrinsic force clamps (in contrast to magnetic tweezers [37]), force clamping is commonly achieved by maintaining constant tension on the DNA-protein complex by changing the position of the sample stage, a micropipette, or the position of an optical trap using active feedback. A particularly clever and distinct variation of a force clamp, not suffering from the loss of time resolution due to the feedback loop, is the passive force clamp introduced by Block and co-workers [1]. This implementation makes use of the fact that the force on a trapped microsphere is relatively independent of microsphere displacement in a region far away from its equilibrium position in the center of laser focus. When optical tweezers are operated in a force-clamp mode, the measurable signal is the change in extension as a function of time (Figure 2.3d). In this way, length changes of the tethered DNA-protein complex at an applied force can be monitored in real time. A key advantage here is that biomolecular activity, such as translocation of a tethered protein over DNA, can be measured directly, without the need for correcting for tether compliance.

**Force spectroscopy**

One of the main virtues of optical tweezers is their ability to accurately apply and measure forces. This ability provides a handle to analyze the energetics of DNA-protein interactions. The processes the biomolecules undergo can be represented as trajectories over an energy landscape along a particular reaction coordinate, as indicated in Figure 2.5. The application of a force that either assists or opposes the process will tilt the energy landscape (Figure 2.5, green line). The result of this tilt is that the barrier height, position, and equilibrium positions are modified, affecting transition rates between and relative occupancy of the different states. These rates and occupancies can be observed directly in single-molecule experiments, yielding detailed information on the thermodynamic parameters defining the energy landscape. In practice, the behavior of a biological system is studied while the system is subjected to a range or spectrum of forces, hence the name force spectroscopy. For force spectroscopy, use of a force clamp is most straightforward. Alternatively, dynamic force spectroscopy may be performed by subjecting a DNA-protein complex to a force ramp while monitoring events such as rupture of DNA-protein bonds [46]. In this case, the loading-rate dependence of the force at which bond rupture occurs allows investigating the interaction strengths and barrier locations.

### 2.2.4 Biological assays and measurable parameters

What information about DNA-protein interactions can be accessed using optical tweezers? Many different assays based on the optical trapping geometries and measurement modes discussed above have been devised. Such assays vary in the kind of information that may be retrieved from the biological system, the origin of the signal that is
2.2 Optical tweezers analysis of DNA-protein complexes

Figure 2.5
Force tilts the energy landscape of a biomolecular transition from state A to state B, along a reaction coordinate represented by position x. The transition state for this reaction is raised by an amount of $F \cdot \delta_{\text{trans}}$, where $\delta_{\text{trans}}$ is the distance to the transition state located between states A and B. Force also affects the thermodynamic equilibrium between states A and B, raising the relative energy of state B by an amount of $F \cdot \delta_{\text{eq}}$, where $\delta_{\text{eq}}$ is the equilibrium distance between A and B. Adapted with permission from ref [73].
measured, or the general layout of the assay. Several key assays for interrogating DNA-protein complexes are described below, highlighting the unique opportunities provided by optical tweezers.

**Protein translocation**

Translocation of proteins such as molecular motors is a process that is exceptionally well suited for study with optical tweezers: active translocation involves not only protein motion over DNA, but also force generation, both of which can be measured directly with optical tweezers. A typical assay to study protein translocation requires the protein (for example a polymerase or helicase) to be tethered on one side, while its substrate, the DNA, is tethered on the other side (cf. Figure 2.6a). During translocation, the molecular motor will effectively reel in, or reel out, the DNA that spans the tether points. When a force clamp is employed, the position signal directly reflects (the magnitude of) protein translocation. The translocation velocity, processivity and run length, as well as the duration and frequency of potential pauses can be analyzed. Furthermore, these parameters can be assessed at different applied forces, providing access to the kinetic parameters that govern translocation and the interconversion of mechanical and chemical energy, i.e. the mechanochemistry of the enzyme’s operation pathways. Great effort has been put into achieving single base-pair resolution to resolve single mechanical steps of polymerase motors that are connected to single catalytic events [1]. This has provided details of step frequencies and dwell-times that offer unprecedented mechanistic insight, by, for example, identifying rate-limiting kinetic steps, coordinated action between motor subunits, and sequence dependence of translocation [27].

**Probing structural changes of DNA-protein complexes**

Protein binding or activity on DNA can induce structural changes to the DNA or the DNA-protein complex, which can be detected by optical tweezers. Assays monitoring DNA or DNA-protein conformational changes can be used for a wide range of DNA-binding proteins without the need to tether the protein(s) to a microsphere or surface, as described in the protein translocation assays above.

**Structural changes by polymerases and helicases** Using such assays, the interconversion of ssDNA into dsDNA has been studied. This interconversion occurs during DNA replication by DNA polymerases (see Figure 2.6b) [180]. The key advantage of probing enzyme activity using this assay is that the enzyme under scrutiny need not be labeled or attached. The magnitude of the signal per polymerase step measured with this approach is, however, smaller than that of the translocation event itself. To amplify the signal, DNA-hairpin constructs have been designed to probe unzipping of the double-stranded hairpin by helicases (Figure 2.6c) [44, 84]. For each base pair the helicase unzips, the construct’s end-to-end length increases by two single-stranded bases, which conveniently enhances the signal.

**Structural changes induced by other DNA-binding proteins** Numerous DNA-binding proteins do not (actively) translocate along the DNA, but
Biological assays to interrogate DNA-protein complexes using optical tweezers. Note that any optical trapping geometry, as shown previously in Figure 2.2, can be used to perform the biological assays depicted here. Optical trapping beams have been omitted for clarity. (a) Protein translocation assay that involves a tethered enzyme translocating over the DNA. (b,c) Assays that analyze enzymatic activity by probing structural changes to the tethered DNA, without the need to tether the enzyme. Here, (b) probes protein-mediated interconversion of ssDNA and dsDNA, such as by DNA polymerases, whereas (c) probes enzymatic unwinding of a DNA hairpin, such as by helicases. (d) Assay that probes structural changes to the tethered DNA due to protein binding or activity. (e) Assay that probes DNA compaction processes, as illustrated by a single protein complex that wraps DNA. (f,g) Assays that probe how force-induced changes to DNA are affected (stabilized/destabilized) by protein binding, such as during overstretching (f) or unwinding (g) of dsDNA.
DNA:protein interactions in optical tweezers

still can induce structural changes, which can be analyzed with optical tweezers. For example, in DNA repair, protein filaments are formed on the DNA by multimerization of DNA-binding proteins (Figure 2.6d) [170]. Stretches of these nucleoprotein filaments can differ in flexibility or length from bare DNA. Such changes in polymer properties can be detected in real time by force or distance clamping, or analyzed by force-distance analysis (not in real time). A different set of proteins is involved in DNA organization and compaction. Examples are proteins that wrap DNA around them (Figure 2.6e) [31] or bridge distant sections of DNA, creating DNA loops [33]. Such proteins will change the DNA-tether length in an optical trapping assay. In particular, the magnitude and mechanism of compaction can be assessed, as well as the interaction strength.

2.2.5 Optical-tweezers instruments with additional functionalities

Most commonly used are the single-beam and dual-beam force-measuring optical tweezers described above. However, over the years, several additions or modifications have been made to this basic layout providing new opportunities for DNA-protein analysis.

Dual DNA manipulation

The optical tweezers assays described up to now focus on probing a single DNA molecule. In the cell, though, DNA segments constantly encounter each other. The proximity of two or multiple DNA sections plays an important role in, for example, protein-mediated DNA-DNA interactions that compact DNA [33], intersegmental transfer or jumping of protein complexes between nearby DNA segments during facilitated target search [166] and homology search in repair processes [51]. To allow these processes to be studied with a high level of control, dual DNA manipulation methods have been developed that allow two DNA molecules to be manipulated simultaneously and independently (Figure 2.7a). In such an assay, the layout is well defined, allowing real-time force and distance measurements [33,38,134].
2.2 Optical tweezers analysis of DNA-protein complexes

Figure 2.7  
Optical tweezers with additional functionalities. (a) Dual DNA manipulation assay. In this illustrative example, a protein complex is seen to bridge two different DNA molecules that are each held between a set of two microspheres. (b) Optical torque wrench. A polarized trapping beam is used to apply and measure torque on an optically anisotropic, birefringent particle that is tethered to a DNA molecule attached to the surface.
**Applying and measuring torque with optical tweezers**

DNA transactions are not only affected by force but also by torque and twist [21, 161]. The tool of choice for studying the effect of torque has traditionally been magnetic tweezers, for example for studying (protein-induced) winding and unwinding of DNA and DNA supercoiling. In magnetic tweezers, paramagnetic beads are used that align with the field line of the applied magnetic field. The tethered DNA can be coiled by rotating the magnetic field. In contrast, a microsphere trapped using optical tweezers is in principle free to rotate and thus cannot maintain torque. To add rotational control to optical tweezers, the "optical torque wrench" has been developed, which relies on angular trapping of optically anisotropic particles, and polarized light to apply and measure torque (Figure 2.7b) [100]. The technique has proven its use in studies of torque-related structural changes of bare DNA constructs. To date it has not been applied to DNA-protein complexes.

### 2.2.6 Optical tweezers with fluorescence microscopy

The combination of optical tweezers and fluorescence microscopy is a particularly powerful one that has gained popularity in recent years. As discussed above, optical tweezers can provide insight into the global mechanical properties of a DNA-protein complex, averaged over the entire section that spans the tether points. Fluorescence microscopy, on the other hand, can be used to directly probe the local presence, identity, spatial dynamics, and conformational dynamics of labeled entities [87]. Both methods are based on optics and, in principle, can be merged naturally. In particular, optical tweezers provide the flexibility to manipulate the DNA-protein complex in whichever plane is most suited for imaging.

**Biological assays**

In combination, optical tweezers and fluorescence microscopy are synergistic assays providing complementary information concurrently, which opens up a range of new possibilities to gain insight into complex biomolecular transactions [24, 171]. First, direct visualization of individual proteins on DNA provides information on the presence of DNA-protein complexes, even when they do not produce a detectable signal in the optical tweezers. Moreover, fluorescence microscopy allows the number of proteins present on DNA to be counted, enabling determination of, among other features, the multimeric states of proteins, [47] the number and sizes of nucleoprotein filaments on the DNA [55], or the effective footprint of DNA-bound proteins [47]. Additionally, fluorescence microscopy facilitates real-time tracking of protein location, providing direct insight into dynamic behavior such as translocation or diffusion [12, 69]. Alternatively, Förster resonance energy transfer (FRET) can be used to study the conformational dynamics of DNA-protein complexes [78]. Finally, the ability of optical tweezers to perform force-spectroscopy in combination with fluorescence microscopy provides insight into force-dependent behavior such as binding dynamics, filament formation, diffusion, and translocation by proteins on DNA [69, 170].
Implementations of optical tweezers with fluorescence microscopy

Several early optical tweezers studies of DNA and DNA-protein complexes have employed total internal reflection fluorescence (TIRF) microscopy (Figure 2.8a) [69, 103]. In TIRF, only the ~100 nm thickness of the sample closest to the glass-sample interface is illuminated, which substantially reduces background signals. However, imaging optically manipulated DNA in a very thin slice close to the sample may represent a challenge because of the steric hindrance of the coupled microsphere(s). Nevertheless, TIRF was successfully used to study RNAP promotor search [69] and DNA strand sep-

\[\text{Figure 2.8}
\]

Fluorescence microscopy of fluorescently labeled proteins on an optically trapped dumbbell construct. (a) Total internal reflection (TIRF) microscopy, accomplished by creating an evanescent wave on a glass pedestal over which the DNA dumbbell is positioned. (b) Traditional wide-field microscopy, in which a micrometers-wide excitation beam illuminates the sample chamber. (c) Confocal microscopy, where an excitation beam is focused on the DNA. Imaging requires scanning of the confocal spot over the DNA.
Traditional, epi-illuminated wide-field fluorescence microscopy, on the other hand, can be used more readily to image optically trapped DNA-dumbbell complexes, since it provides a parallel, micrometers-wide excitation beam penetrating the whole sample (Figure 2.8b) [13, 170]. This has, for example, been used to study the tension-dependent disassembly of DNA repair proteins from DNA [170], and translocation of restriction enzymes along DNA [13]. In the latter study, a tilted illumination beam was used to reduce background signal to a limited extent. Yet, more significant reduction of background signal is accomplished by using TIRF or confocal fluorescence microscopy. In confocal fluorescence microscopy, an excitation beam is focused to a diffraction-limited spot that can be positioned on the DNA (cf. Figure 2.8c). The fluorescence emitted by fluorophores that are located in this spot is detected with a point detector after spatial filtering with a pinhole to suppress out-of-focus background signal. This allows measurements of real-time binding, unbinding and activity of labeled proteins on DNA in the presence of a relatively high concentration (20-100 nM) of labeled proteins in solution [72, 152]. Additionally, the use of single-photon counting modules allows accurate counting of single photons with very high temporal resolution (submillisecond) in a single confocal spot. Imaging, however, requires scanning of the confocal spot over the DNA. Several studies have successfully combined confocal fluorescence microscopy with optical tweezers manipulation of DNA [29, 78, 152] and DNA-protein complexes [72, 190]. Finally, all fluorescence imaging modalities described above are limited by diffraction to a spatial resolution of 200-300 nm. Currently, super-resolution approaches have emerged that allow objects to be resolved below this limit [71]. In a recent study, confocal fluorescence microscopy combined with optical tweezers has been extended with stimulated emission depletion (STED) microscopy, allowing super-resolution imaging on DNA [72]. This novel approach allows visualization of the dynamics of individual DNA-bound proteins or protein-filaments on DNA, at both high densities on the DNA and relatively high concentrations in solution. Alternatively, super-resolution approaches that rely on stochastic on/off switching of fluorescent dyes [139] may be readily integrated with wide-field imaging in optical tweezers. Such technical innovations bridge the gap between idealized in vitro experiments and in vivo conditions where DNA is densely coated with proteins.

Visualizing dsDNA and ssDNA

In single-molecule experiments incorporating fluorescence imaging, it can be of great benefit to visualize the tethered DNA. Many dyes are commercially available for this purpose, and in most cases, bind to dsDNA by intercalating between adjacent base pairs. The fluorescence intensity of many of these dyes, for example YOYO, is significantly enhanced when bound to dsDNA, allowing imaging with a high signal-to-background ratio, even with free dye present in solution. Such dyes can be used, for instance, to monitor the disruption of dsDNA through unwinding and enzymatic degradation by helicases/nucleases [12], or to identify regions where the double helix has been perturbed mechanically [91]. A point of caution is that intercalating dyes affect the structure of dsDNA, altering its mechanical properties [26]. For the visualization of ssDNA, fluorescent variants of naturally occurring proteins that specifically bind ssDNA have been...
employed, such as *E. coli* single-stranded binding protein (SSB) eukaryotic replication protein A (RPA) [169]. It should be realized that these proteins are substantially larger than dsDNA intercalators (RPA binds to ∼30 nucleotides), potentially interfering with the process under study. However, in the case of RPA, it has been demonstrated that using low concentrations has a negligible effect on the structure and energetics of DNA molecules with a length of several kilobases [91]. As a result, fluorescently labeled versions of these proteins have been employed successfully for the identification and localization of ssDNA [91,169].

**Bleaching and DNA damage**

Unwanted side-effects of fluorescence microscopy in combination with optical tweezers include elevated bleaching rates of the fluorophores illuminated simultaneously by fluorescence excitation and trapping beams [102]. The enhanced photobleaching is caused by excited-state absorption of the trapping light, resulting in photo ionization of the dyes and/or reactive-oxygen-species generation [41]. This enhanced photobleaching results, not only in an unwanted loss of fluorescence signal, but also in enhanced damage to the DNA, potentially severing the molecule and thus terminating the experiment. Different schemes have been devised to reduce this enhanced photobleaching. One way to achieve this is by temporal separation of trapping and fluorescence excitation beams by interlacing them [17]. Another, more simple, way is by spatially separating the DNA from the trapping lasers by either using long DNA [13, 78] or using large beads (>3 μm diameter) (Chapter 3) [72]. Alternatively, several studies have shown that the enhanced bleaching effects can be minimized by judicious choice of laser wavelengths, laser intensities, and dyes [103,170].

### 2.3 DNA Mechanics

At the heart of any study into DNA-protein complexes is an acute appreciation of the mechanics of DNA. The biochemical maintenance of the genome is influenced strongly by the mechanical properties of DNA. From wrapping and twisting in genome compaction, to unwinding and melting during transcription and replication, DNA is under constant mechanical strain *in vivo*. Single-molecule techniques have shed much light on this subject in recent years; optical tweezers, in particular, have had notable success in probing the structure of DNA under tension. Concomitant with these achievements, the myriad of experimental schemes designed to study DNA mechanics have in turn taught us about DNA-protein interactions. Here we offer a brief overview of those aspects of DNA mechanics which prove particularly amenable to study by optical tweezers, and their role in probing DNA-protein interactions.
2.3.1 Stretching dsDNA

When dsDNA is free in solution, its double-helical backbone will adopt the conformation of a random coil. In such a case, the end-to-end distance of the molecule is considerably shorter than its contour length; DNA under these conditions can be considered an entropic spring. Increasing the end-to-end length using forces \(<10\) pN straightens the DNA molecule, reduces the number of accessible three dimensional configurations, and thus lowers the entropy. The resulting force-extension behavior can be reproduced by the wormlike chain (WLC) model (Figure 2.9a) [119]. In this approximation, the polymer is treated as a semiflexible isotropic rod of length \(L\). The stiffness of the polymer is quantified using the persistence length (\(P\)), which reflects the length of the polymer chain that is aligned cooperatively in any one particular direction. For DNA under near physiological conditions, \(\sim\) pH 7-8 and \(\sim\) 50-150 monovalent salt, \(P\) is around 50 nm [23,157]. At higher forces, the end-to-end length of DNA increases beyond the contour length expected assuming the molecule to be an inextensible polymer Figure 2.9a. This indicates that, at these higher forces, the elastic response of DNA is dominated by enthalpic stretching of the double helix. Entropic plus enthalpic stretching can be modeled using a WLC approximation that is augmented by introducing the stretch modulus (\(S\)). This model is called the extensible wormlike chain (e)WLC and is valid for forces up to \(\sim\) 35 pN. However, how does the DNA change structurally when it is stretched under these forces? This question was addressed using a novel optical tweezers layout, employing rotor bead tracking, to quantify the change in twist as the DNA molecule is stretched. The experiment found that, counterintuitively, the DNA molecule overwinds when stretched with forces up to \(\sim\) 30 pN, but unwinds as the tension is increased further [58]. This coupling between stretch and twist in DNA was subsequently quantified using optical-tweezers experiments and modeled using a twistable WLC approximation which is valid up to the overstretching transition, starting around 60 pN (see section 2.3.3 for details) [62].

2.3.2 Stretching ssDNA

The base-paired double-helical structure of DNA represents a remarkably stiff and rigid architecture. In contrast, ssDNA is simply a polymer chain of interconnected nucleotides. As a result, ssDNA is considerably more flexible, and thus more contractile, than dsDNA [129,157]. Additionally, at high forces, ssDNA can be stretched to a longer length than dsDNA, due to the fact that the latter exists as a double helix. A sample force-extension curve for ssDNA, recorded under near physiological ionic strength, is presented in Figure 2.9a (blue curve). For 150 mM NaCl, the force-extension behavior of ssDNA can be reproduced well using the freely-jointed chain (FJC) model (including a stretch modulus component), and almost as well using the eWLC model. However, both approximations become less valid at high and low ionic strength. At low ionic strength, deviation from the FJC model arises due to electrostatic repulsion, and excluded volume effects. In contrast, at high salt concentrations (where electrostatic shielding is strong), the FJC model fails because the ssDNA molecule can form regions
of secondary structure. In particular, complementary bases of ssDNA can interact, forming regions of duplex DNA, known as hairpins.

### 2.3.3 The overstretching transition

The elasticity of rotationally unconstrained dsDNA changes markedly at forces of \( \sim 60-70 \) pN, (Figure 2.9a, black curve) whereby the contour length suddenly elongates by \( \sim 70\% \) over a narrow force range \([157]\). This process is termed overstretching and has received substantial interest in recent years. The structural changes occurring during overstretching tell us about the stability of the double helix under tension, revealing the fundamental mechanics of base-pairing and base-stacking interactions. The overstretching transition was first reported in a seminal study, using optical tweezers to stretch a rotationally unconstrained DNA molecule \([157]\). This early investigation suggested that overstretched DNA could adopt either a base-paired or non-base-paired structure, depending upon ionic strength. A host of single-molecule layouts have been devised recently to clarify this assertion \([16,62,91,120,169,186]\). It was confirmed that ssDNA is formed during overstretching at both low ionic strength, and at elevated temperature. Its presence can often be deduced from (i) hysteresis between forward and backward pathways in force-extension measurements (as exemplified in Figure 2.9a); (ii) permanent binding of ssDNA binding agents, such as glyoxal; and (iii) visualization the binding of fluorescently labeled ssDNA binding proteins, e.g. RPA. Under conditions that stabilize base-pairing (high ionic strength, low temperature), overstretching instead induces a double-stranded but underwound structure, typically referred to as S-DNA. The exact structure of S-DNA is still unclear, but force-extension measurements suggest that it is a helix with a pitch of \( \sim 22 \) nm and \( \sim 37.5 \) base-pairs per turn \([110]\).

Under physiological conditions, overstretching can involve a combination of melted and S-DNA, leading to a heterogeneous DNA structure. An important element in this regard is the genomic sequence. Using force-extension measurements either alone or in combination with fluorescence microscopy, it was shown that, during overstretching, base-pair breaking is favored in AT-rich sequences, whereas S-DNA has a preference for GC-rich domains \([16,91]\).

While the local environment can dictate where and when DNA will melt during overstretching, the topology of the DNA molecule determines how this melting occurs. Fluorescently labeled ssDNA-binding proteins show that melting proceeds via unzipping (unpeeling) of one strand from the other when there are free DNA ends available. In contrast, when the DNA molecule is rotationally unconstrained, but has no free ends, melting can only occur via localized bubble formation \([91,186]\). These two possibilities are illustrated schematically in Figure 2.9c. It is intriguing to note that the presence of dsDNA-binding proteins can constrain free ends, thus stabilizing melting bubbles over unpeeling during overstretching.

Combining our knowledge from different single-molecule experiments yields a complete phase diagram for DNA overstretching \([186]\), detailing the structure of over-
2.3.4 Mechanical response of DNA is dictated by microsphere-attachment geometry

The structural changes occurring in DNA under tension depend crucially on the presence of free DNA ends, which is in turn, dictated by the manner in which the DNA molecule is coupled to the microspheres [61]. Typically, one end of each strand of DNA is connected to microspheres, leaving two free ends, as shown in Figure 2.9b. If the connected ends are on the same strand, overstretching (under very low ionic strength) will induce one strand to unzip entirely from its complementary strand (Figure 2.9b, scheme (i)). This process yields a single-strand of DNA held between the microspheres, providing a homogeneous assay on which to study ssDNA-protein complexes (Chapter 3) [157].

Complete generation of ssDNA during overstretching is not permitted if the DNA molecule is connected to microspheres on opposite strand ends (Figure 2.9b, scheme (ii)). However, if a nick is present in the backbone of one strand (either deliberately, for example using a restriction enzyme), or accidentally (owing to degradation of the DNA), it is possible to melt off a fragment of ssDNA during overstretching. Upon retraction of the DNA end-to-end length, the molecule consists of a region of ssDNA juxtaposed with dsDNA. Such a construct can then be used to study the interaction of proteins at the junction of ssDNA and dsDNA (Chapter 3) [136].

By considering the effect of viscous drag on an optically trapped microsphere and the minute torque exerted by a twisted DNA molecule [21], it can be calculated that the time scale for a trapped bead to rotate around it axis fully is significantly longer than the time scale of a standard experiment. Therefore, if a DNA molecule is connected to microspheres on all four strand ends (Figure 2.9b, scheme (iii)), it is rendered rotationally constrained on the time scale of a typical experiment. The result of such restraint is that the overstretching transition increases to \(\sim 110\) pN.

It can also be useful to modify DNA such that it contains no free ends, yet is still free to rotate when stretched using single-molecule techniques. To this end, it is possible to seal the free ends of a dsDNA molecule using a hairpin oligomer which can be connected to a microsphere using a single chemical linkage, yielding a rotationally unconstrained, yet closed, construct [136]. Another way to achieve this goal is by using the emerging technology of click-chemistry [16].

stretched DNA under different conditions (topology, sequence, salt and temperature, for example). This knowledge can then be used to study DNA-binding proteins, which can often (de)stabilize double- or single-stranded DNA and therefore influence the structure of DNA under tension. Figure 2.6f illustrates such a biological assay. The result is an altered (over)stretching transition or hysteretic force-distance behavior, which can yield information on the kinetics and thermodynamics of DNA-binding by proteins as well as report on binding mode or activity [120].
2.3 DNA Mechanics

Figure 2.9

Stretching DNA. (a) Characteristic force-distance curves for DNA obtained in an optical tweezers experiment. Black curve shows the force response for increasing the end-to-end length of a single (rotationally unconstrained) dsDNA molecule, under close to physiological buffer conditions (pH 7.8 and 50 NaCl). The reverse process (retraction of the end-to-end length, red trace) reveals hysteresis between forward and backward force-distance curves. The green curve corresponds to extending a ssDNA molecule. Inset: fits of the wormlike chain and twistable wormlike chain models to experimental data for forces up to \( \sim 60 \) pN. Reprinted by permission from Macmillan Publishers Ltd: Nature Physics ref [62], copyright (2011). (b) Different attachment geometries for DNA bound between two microspheres. (c) Two different mechanisms of DNA melting under tension: unpeeling and bubble-melting. (d) Illustrative phase diagram for overstretching DNA under varying conditions of salt and temperature, for both end-opened DNA (with free ends) and end-closed DNA (with no free ends, yet rotationally unconstrained). Reprinted with permission from ref [186]. Copyright (2013) National Academy of Sciences, USA.
2.3.5 Unzipping DNA

Studies of overstretched DNA provide insight into the stability of the double helix under tension. However, the base-pairing integrity of DNA can also be disrupted by unzipping the complementary strands, for instance, by unraveling of a DNA hairpin. Optical tweezers have previously been used to establish that unzipping of a DNA hairpin occurs at forces of \( \sim 15 \) pN \([15]\). A hairpin can, however, be (de)stabilized by protein binding, which affects the local unzipping force \([93]\). This effect has been exploited to detect, for example, the presence of nucleosomes or polymerases on DNA hairpins with great positioning accuracy: DNA unzipping is affected by local sequence of the hairpin, which yields a characteristic force signature that may serve as an unzipping progress monitor for highly accurate absolute positioning of DNA-bound proteins \([151]\). In addition, the rupture force can report on the activation barrier for disruption of the DNA-protein complex \([92]\). Biological assays based on this scheme are illustrated by Figure 2.6g.

2.4 DNA organization

Organisms in all domains of life are faced with the challenge to contain their relatively long genomes within the confinement of the cell. In addition to compaction by physical mechanisms such as molecular crowding, cells employ sets of architectural proteins that mediate DNA compaction. Across life forms, the modes of protein-mediated compaction are conserved: proteins can bend DNA, enhance DNA flexibility, wrap DNA, or bridge distant sections of DNA (cf. Figure 2.10). The ability of optical tweezers to measure both force and extension allows the technique to directly probe and identify these protein-induced topological changes to DNA and quantify their effects.

In apparent conflict with the need for substantial compaction, the genetic code must remain accessible for transcription and other metabolic processes. This demands a dynamic DNA organization, but at the same time allows the proteins that are involved in genome compaction to regulate gene expression by modulating access to the genes. In vivo, decompaction of DNA is thus as ubiquitous and essential as DNA compaction. In essence, the tension applied to the DNA in an optical tweezers experiment acts to reverse the effects of DNA compaction in vitro. Force-induced decompaction by optical tweezers can therefore be used to unravel and quantify protein-induced DNA organization.

2.4.1 Wrapping DNA

Wrapping a section of DNA around a protein effectively shortens the end-to-end distance of the DNA molecule, a change which can be readily probed using optical tweezers. Besides the elementary compacting effect of DNA wrapping, what other information may optical tweezers retrieve? By applying tension to the DNA molecule, the DNA
can be unwrapped, yielding rich data, providing insights into not only the structure of the DNA-protein complex, but also on the strength, dynamics, and energetics of the DNA-protein interactions. Furthermore, a DNA wrap can act as a roadblock to protein translocation, rendering the underlying genetic code inaccessible for transcription. In vivo, proteins thus often need "plow" through or remodel sections of wrapped DNA. Force-induced unwrapping of DNA using optical tweezers mimics these processes and can provide direct information on the energetics and dynamics of remodeling in vivo.

DNA wrapping eukaryotes: nucleosomes

In eukaryotes, the basic unit of compaction is the nucleosome, consisting of 146 bp of DNA wrapped 1.65 times around a disk-shaped complex of eight histone proteins, with a diameter of 11 nm [113]. Nucleosome-nucleosome interactions generate higher order structures, forming chromatin, the universal form of the packaged DNA-protein complex across eukaryotes.

Early studies that used optical tweezers to unravel arrays of nucleosomes [11, 20, 31, 57], yielded the following insights into tension-induced nucleosome disruption (see also Figure 2.11a): (i) The force-extension behavior of chromatin fibers is characterized by an apparent persistence length below that of bare dsDNA [11, 31, 57]. (ii) Unraveling nucleosome arrays typically occurred in two phases. In the low force range, the outer turn of the DNA, which is most weakly bound in the nucleosome, unwrapped in a gradual and reversible fashion [20, 57]. Subsequently, at higher forces, the inner turn unraveled in a sudden discrete event [11, 20, 57], consistent with rupture of strong histone-DNA interactions at the ± 40 bp positions from the center (dyad) of the DNA contained in the nucleosome (Figure 2.11c) [20]. (iii) The forces required to disrupt nucleosomes were found to be lowest at high ionic strength [31, 57]. This points to the importance of electrostatic interactions between the DNA and the histones (which become screened at high ionic strength), to bend DNA with a radius (∼5 nm) that is much smaller than its persistence length (50 nm).
More recently, the location of specific DNA-histone interactions have been mapped with remarkable accuracy in a different type of optical-tweezers assay. Hall et al. used optical tweezers to unzip a DNA hairpin that was stabilized by a nucleosome (cf. Figure 2.12) [64]. As the unzipping front encountered strong DNA-protein interactions it dwelled longer before continuing to unzip the hairpin. The dwell positions thus revealed the locations of strong DNA-protein interactions with near-base-pair accuracy, whereas the dwell durations revealed the relative strength of these interactions [39, 64, 93]. In agreement with structural data and results from the previous nucleosome-stretching experiments, three regions of strong interactions were identified. The strongest interaction was at the dyad, which was flanked by two other sites of strong binding at ± 40 bp from the dyad. The entry and exit sites were particularly weakly bound, consistent with the gradual low-force unwrapping of the outer turn observed previously. Intriguingly, the maps of interaction strengths appeared to be modulated with a 5 base-pair periodicity. This was ascribed to local interactions each time either one of the DNA strands face the histone, which occurs twice every helical repeat length of ~10 bp.

Figure 2.11
Unwrapping DNA by extending nucleosome arrays. (a) Under feedback control, a nucleosomal array was stretched between the surface of a microscope coverslip and an optically trapped microsphere. (b) Force-extension curve of a fully saturated nucleosomal array. At higher force, a sawtooth pattern containing disruption peaks was observed. Force-extension characteristics of a full-length naked DNA (red dotted line) are shown for comparison. (c) A three-stage model for the mechanical disruption of the nucleosome. Adapted with permission from ref [20]. Copyright (2002) National Academy of Sciences, USA.
The mechanical unzipping of a hairpin resembles the activity of an RNA polymerase that opens up the DNA duplex as it transcribes through a nucleosome. The results of Hall et al. therefore imply that RNA polymerases can smoothly access the entry/exit DNA, but have more trouble breaking the off-dyad strong interactions leading to pausing. Up to the dyad, the zipping/unzipping signature is reversible, which implies that RNAP backtracking will result in rebinding of DNA in the nucleosome. In contrast,

Figure 2.12
DNA unzipping map of histone-DNA interactions. (a) Illustration of a DNA molecule that is mechanically unzipped through a positioned nucleosome. (b) Crystal structure of the nucleosome core particle. Dots indicate regions where interactions between DNA and one of the core histones are likely to occur. The two halves of the nucleosome are shown separately. (c) Histone-DNA interaction map constructed from the averaged dwell time histograms of the unzipping fork at constant force (~28 pN). Each peak corresponds to an individual histone-DNA interaction, and the heights of the peaks are indicative of their relative strengths. Reprinted by permission from Macmillan Publishers Ltd: Nature Structural & Molecular Biology ref [64], copyright (2009).
once a polymerase has passed the dyad, the nucleosome will be irreversibly modified and the polymerase will proceed with minimal resistance.

**Linker histones and histone tails**

In addition to the histone-DNA interactions described above, the nucleosome structure is further stabilized by linker histones and histone tails. Pope et al. addressed the energetics of nucleosome unwrapping by performing dynamic force spectroscopy, in which unwrapping was studied as function of loading rate [143]. At low loading rates, two distinct populations of unwrapping events were observed, characterized by different energy barriers. This heterogeneity was attributed to the stabilizing effect of linker histone B4, which was thought to be present in only a subpopulation of nucleosomes. After the first stretch of the nucleosomal array, most of the high-force events disappeared, most likely due to loss of weakly bound B4.

Histones possess highly positively charged tails that can stabilize nucleosomes through their interaction with the negative charge on the DNA. Acetylation of these tails neutralizes part of the charge, which can therefore be used to regulate nucleosome stability in vivo. Indeed, in vitro stretching of nucleosome arrays indicated that the outer turn of DNA was destabilized by removal or acetylation of the tails [19]. In addition, the force required to unwrap the inner turn was reduced upon tail modification. This could result from loss of nucleosome stabilization at the entry-exit points of the DNA and at the off-dyad strong interaction sites, respectively (see Figure 2.13). These results illustrate the biological function of histone tails and acetylation: acetylation opens the nucleosome array through the outer turn destabilization, resulting in partial chromatin decondensation. This in turn increases the accessibility for regulatory factors. Because acetylation decreases the energy required to disrupt the strong DNA-histone interactions, it may also facilitate translocating RNAP and remodelers.

**Nucleosome dynamics**

Chromatin remodeling and structural fluctuations of nucleosomes are important for the accessibility of proteins to the DNA. The dynamics of nucleosome wrapping and unwrapping were analyzed by stretching individual, positioned nucleosomes [124]. Low force and high force structural transitions were observed in real time and assigned to disruption of the outer and inner turns, respectively (see Figure 2.14). Constant-force experiments performed at the low-force transition showed hopping between two states, which allowed extraction of the change in free energy (~30 kJ per mol). In contrast, the irreversible high-force transition was not a simple two-state process, but involved multiple transition states and intermediates. Mihardja et al. further observed that at high salt the low-force transition was no longer bistable, but rather also exhibited many intermediates.

**SWI/SNF remodeling**

In the cell, the accessibility of chromatin is modulated by chromatin-remodeling complexes, such as SWI/SNF (SWItch/Sucrose NonFermentable). This remodeler was
Figure 2.13
Histone tails stabilize nucleosomes. (a) Locations and relative lengths of histone tails in the nucleosome. Tails are shown fully extended for the purpose of length comparison. (b) A model of the contributions of histone tails to nucleosome stability during the nucleosome disruption process. Reprinted from ref [19], copyright (2005), with permission from Elsevier.
Figure 2.14

Mononucleosome dynamics. (a) Force-extension curve of a spool model for the nucleosome. Theoretical curves for 1.5 wraps (purple), 1 wrap (blue), and 0.5 wrap (green) are plotted with experimental data for increasing (black) and decreasing (red) tension. The solid curve segment identifies that the state is thermodynamically preferred (minimum free energy), and the dashed segment indicates the state is stable but not preferred. (b) Length vs. time traces of the low-force transition at various forces. Adapted with permission from ref [124]. Copyright (2006) National Academy of Sciences, USA.
known to be an ATP-dependent transcription activator, but bulk experiments had been unable to unambiguously resolve how it remodeled chromatin. One reason might be the use of short (a few hundred bp) DNA templates in bulk experiments that amplify end-effects and introduce heterogeneity in initial nucleosome positioning and synchronization. Optical-tweezers experiments, in contrast, have the clear advantage that they allow studies of nucleosome positioning and remodeling on DNA constructs up to several kilobases long.

Shundrovsky et al. used unzipping assays to probe the locations of single nucleosomes on DNA hairpins containing a strong positioning sequence [151]. After remodeling by SWI/SNF, the canonical nucleosome disruption signature was still observed, indicating that remodeling did not result in persistent changes to the nucleosome structure. A fraction of the remodeled nucleosomes, however, were moved along the DNA, showing on average a displacement of 28 base pairs (in either direction) for a single remodeling event. Remodeling has also been observed in real time by monitoring the extension of a DNA construct during remodeling of a nucleosome by SWI/SNF and RSC [187]. In particular, Zhang et al. observed short-lived (<15 s) DNA shortening events indicative of the transient formation of loops in the DNA due to processive translocation of single remodelers at a nucleosome. These remodeling events were ATP-dependent and shortening occurred with an average velocity of 13 bp per s, reaching run lengths up to 1200 bp (at 1 mM ATP). While loop growth and loop size were independent of applied tension, the frequency of loop formation did decrease with tension. This indicates that loop initiation may involve transient bending of DNA, which is hampered by tension.

### 2.4.2 DNA bridging

Protein-mediated bridging of DNA connects distant DNA segments, creating loops that effectively compact the molecule. The compacting effect of DNA loops can be evaluated by optical tweezers experiments. In addition, the bridges can be disrupted by applying tension, which allows the energetics and kinetics of DNA bridging to be studied. Such experiments can be performed on a single DNA molecule, but interpreting DNA-loop rupture events can be difficult. Bridged structures are often poorly defined, involving multiple bridges, which are subjected to shearing and/or unzipping forces, depending on orientation. Furthermore, bridge-rupture events can often not be distinguished from interactions of DNA or proteins with microspheres. Another challenge is that the formation of bridges requires slack in the DNA, making real-time monitoring of bridge formation cumbersome. These problems are solved using well-controlled dual-DNA manipulation experiments [134], which allow monitoring of bridge formation, bridge rupture, and conformational rearrangements in the bridged structure, in real time.

**DNA bridging by *E.coli* H-NS**

Although bacteria such as *E.coli* lack a nuclear membrane, their chromosome is still folded into a compact volume (the nucleoid) smaller than the cytoplasmic space. In *E.coli*, the genome is compacted by architectural proteins that induce hundreds of
loops in the chromosome. One example is H-NS (Histone-like Nucleoid Structuring), an SMC (Structural Maintenance of Chromosomes) protein.

Figure 2.15
Rupturing protein-induced DNA bridges using a dual DNA manipulation layout. (a) Contour length change as a function of time in unzipping experiments showing rupture events of H-NS bridges. Data displayed at 1 kHz (red) including staircase fits (black). (b) Histogram of all step sizes. The curves represent a multiple-Gaussian fit (blue) and the individual Gaussians (black). Reprinted by permission from Macmillan Publishers Ltd: Nature ref [33], copyright (2006).
Dame and co-workers developed an optical-tweezers based dual-DNA manipulation scheme to study DNA bridging by H-NS (Figure 2.15) [33]. In their experimental approach, four independently steerable optical traps are formed, allowing real-time control of two DNA molecules in three dimensions. The H-NS binding configuration could thus be predefined, and after bridge formation, forces could be applied in a controlled way such that bridges are either sheared or unzipped. While unzipping H-NS bridges, step-like contour length changes were observed, indicating that individual bridges rupture sequentially (Figure 2.15). The step-size distribution could be fitted by multiple Gaussians separated by the helical pitch of DNA (3.6 nm), suggesting that bridging occurs in register with the DNA helical repeat. Moreover, the H-NS density on the DNA varied substantially, indicating that the cooperative binding that was observed previously is not caused by direct protein-protein interactions, but by the proximity of the two DNA strands. A high off rate (1.5 \text{s}^{-1}) of H-NS bridges was deduced, which might resolve the apparent conflict between DNA compaction and accessibility: on the one hand, cooperative H-NS bridging generates stable DNA loops, while on the other hand, the short binding times ensure the bridges are dynamic and allow other DNA-binding proteins to bind competitively [177].

**DNA bridging by Alba in archaea**

Alba is an abundant protein in archaea capable of bridging DNA, and thus was presumed to be involved in DNA loop formation and genome compaction. This was tested and confirmed using the dual-DNA manipulation scheme described above [107]. In this study, evidence was obtained not only for DNA bridging but also for DNA stiffening by Alba. It was found that these two effects are delicately tuned by the relative concentrations of two Alba variants, Alba1 and Alba2. Alba1, the most abundant of the two, bridges DNA at low concentrations. At high concentrations, however, cooperative side-by-side binding causes the formation of extended Alba1 filaments that stiffen DNA instead. Alba2, on the other hand, was shown to reduce the cooperativity of Alba1 binding by reducing the side-by-side binding of Alba1. The result is an altered degree of compaction of the DNA, which can change its accessibility to other DNA-processing proteins.

**2.4.3 DNA bending & enhancing DNA flexibility**

Protein-induced DNA bending reduces the radius of gyration of DNA. This effect can be described by a decrease in apparent persistence length of the bent polymer [97], which can be readily observed using force-extension analysis with optical tweezers. Whereas DNA-bending proteins introduce rigid kinks in the DNA, other proteins are known to enhance the flexibility of DNA, for example by local denaturation of the double helix. Since both effects result in DNA conformations that look kinked on average, it is hard to distinguish them in, for example, AFM images. However, this discrepancy can be resolved using optical tweezers by analyzing the load dependence of binding: proteins that rigidly bend DNA are in general more sensitive to force than proteins that enhance DNA flexibility [47, 167]. To effectively compact a long DNA molecule, the bending or
DNA:protein interactions in optical tweezers

Figure 2.16
DNA compaction by TFAM analyzed by concurrent optical trapping and fluorescence microscopy. (a) Typical force-extension curve for DNA in the absence (black trace) and presence (red trace) of 50 nM TFAM. The contour length ($L_c$) and the end-to-end distance of a DNA molecule are schematically depicted. (b) A single fluorescent TFAM bound to a DNA molecule (upper image) and a DNA molecule fully coated with TFAM (100 nM; lower image). The DNA, held between two beads in the optical tweezers, is not visible. Scale bar, 1 µm. Reprinted by permission from Macmillan Publishers Ltd: Nature Communications ref [47], copyright (2012).

DNA denaturation needs to occur throughout the DNA molecule with gaps smaller than the persistence length of dsDNA. The compacting effect of an individual protein, however, is typically smaller than in the case of DNA wrapping or bridging, which makes it hard to study this compaction mode by optical tweezers alone. Concurrent fluorescence microscopy, on the other hand, allows direct visualization of individual, or small amounts of proteins on the DNA. This provides access not only to the force-dependence of binding and unbinding, but also to the spatial dynamics of proteins on DNA, which are not accessible with optical tweezers alone.

As way of example, we highlight the case of eukaryotic mitochondria. Like prokaryotes, eukaryotic mitochondria contain circular genomes and do not have histones to compact DNA by wrapping. Yet, the mitochondrial chromosome is compacted in a ∼70 nm nucleoid. The major component of these nucleoids is TFAM (mitochondrial transcription factor A). TFAM was found to have two roles in vivo: enhancing transcription by binding to the mitochondrial DNA promoter region and organizing the mitochondrial DNA. Farge et. al. elucidated the assembly and DNA organization by this abundant protein using force-measuring optical tweezers [47]. Force-distance analysis of TFAM-coated DNA revealed a large decrease in the effective persistence length, resulting in a large reduction of the average end-to-end length at low force Figure 2.16. The authors deduced that TFAM compacts DNA by local DNA denaturation, enhancing its flexibility. Concurrent fluorescence microscopy was exploited to further quantify the TFAM-DNA interaction and its real-time spatial dynamics (Figure 2.16). By counting the number of fluorescently labeled TFAM molecules on a saturated DNA molecule, the effective footprint of TFAM was estimated to be ∼30 bp, in agreement with bulk
assays and structural data. In addition, the oligomeric state of TFAM bound to optically trapped DNA could be determined using single-molecule fluorescence microscopy, revealing that TFAM binds to DNA as monomers. The bound monomers diffuse along the DNA over several kilobase pairs and can, upon encounter, be incorporated into stably bound multimeric TFAM patches. The monomeric diffusion constant ($\sim 8 \cdot 10^4 \text{ nm}^2 \text{ s}^{-1}$) was determined to be independent of the salt concentration, which is consistent with protein sliding. The authors speculated that combination of TFAM sliding and DNA melting that allows the observed dense patch formation to compact DNA may also play a role in transcription initiation: by sliding, TFAM can find the promoter region, while local melting can enhance binding of RNAP and other transcription factors to form an open transcription bubble.

![Figure 2.17](image)

**Figure 2.17**
**Mechanical compression of equilibrated chromosomes.** (a) A polystyrene microbead held by optical tweezers was used to compress the chromosome against the closed channel end. The residual membrane of the cell after lysis was used as a gasket to prevent leakage of the chromosome. (b) (Inset) Raw force-compression data show two groups of curves that represent one and two nucleoids. When rescaled, all data collapse onto a single master curve. Adapted with permission from ref [140]. Copyright (2012) National Academy of Sciences, USA.
2.4.4 Genome compaction in vivo

In vivo, the protein-mediated compaction mechanisms described above work together with physical effects like molecular crowding to compact the genome. In contrast to the microscopic analysis of single DNA-protein interactions, very little is known about the global physical forces that act to shape chromosomes. Pelletier and co-workers devised a unique optical tweezers, microfluidics, and fluorescence approach to probe the micromechanical properties of entire *E.coli* chromosomes in confinement [140]. Individual *E.coli* cells were lysed in micrometer-sized channels. Fluorescence microscopy was used to reveal that, upon lysis, the chromosome expands about 10-fold, like a loaded entropic spring. Subsequently, an optically trapped microsphere was used as a micropiston to compress the chromosome and probe its micromechanical properties (Figure 2.17). Force-compression curves confirmed the loaded entropic spring model and revealed that a force of 100 pN and $10^5 k_B T$ of mechanical work are required to compress *in vitro* the chromosome to its *in vivo* size. This pressure is ~1000-fold lower than the turgor inside the cell. The authors estimated the number of DNA loops (for example induced by H-NS) to be 60-280 per cell. In addition, the authors demonstrated, by addition of PEG, that molecular crowding can play a major role in chromosome compaction. This large effect of crowding agents demonstrates that the bacterial chromosome is of surprisingly soft nature.

2.4.5 DNA organization in hindsight and foresight

Over the past two decades, optical tweezers have contributed significantly to our understanding of DNA compaction, providing information on the structure of compacted DNA-protein complexes, as well as on interaction strengths. Optical-tweezers studies of protein-induced DNA compaction are particularly helped by concurrent fluorescence imaging of the proteins, to determine the number of proteins bound, cooperativity, spatial dynamics and how local topological effects lead to global compaction. Although the study of mitochondrial DNA compaction by TFAM is a good example of such an investigation, exploitation of this combination of techniques in the field of DNA organization is still in its infancy.

With the molecular mechanisms of DNA compaction being understood better, an important next step is to understand how DNA-processing proteins interact with compacted DNA. This is, in fact, the substrate DNA-processing enzymes face *in vivo*, not the idealized bare DNA that is typically used *in vitro*. Although some studies have addressed the problem of polymerases "plowing" through nucleosomes (see section 2.6), we have only begun to scratch the surface of understanding how DNA metabolism functions in compacted chromosomes such as eukaryotic chromatin. In our outlook in section 2.8 we will further elaborate on how optical tweezers could serve to unravel DNA metabolism in the context of higher-order compacted DNA.
2.5 Replication

Before a cell can divide, it needs to replicate its full genome. Four protein-mediated processes play a central role in replication. First, the two complementary DNA strands are unwound. Unwinding is typically performed by helicases. The unwound strands serve as templates for the synthesis of the two new DNA strands. Second, short primers are laid down on an unwound strand by a primase. Third, complementary strands are synthesized by DNA polymerases (DNAP’s) that catalyze the incorporation of complementary nucleotides into the growing primer. In addition, single-stranded binding proteins (SSB), bind and protect the ssDNA generated by helicase-induced DNA unwinding.

Both DNAP and helicase alter the structure of DNA: helicases convert dsDNA to ssDNA by unwinding; DNAP does the opposite by synthesizing a complementary DNA strand. Such structural changes to the DNA can be readily identified as well as monitored in real-time using force-measuring optical-tweezers assays. Moreover, by inferring enzymatic activity from the structural changes to the tethered DNA, elegant single-enzyme activity assays can be performed without the need to actually label or tether the enzyme itself. As described in 2.2.6, SSB can be well detected by fluorescence microscopy in optical tweezers experiments. In this section we will emphasize the contribution of optical-tweezers assays to our understanding of DNAP, helicases, and DNA replication in general.

2.5.1 DNA polymerase

Replicative DNAP catalyzes the addition of nucleotides to the 3'-end of a growing DNA terminus at a low error rate \(10^{-5} \text{ to } 10^{-7} \text{ errors per nucleotide}\) \([85,98]\). The polymerization activity of DNAP converts an ssDNA template to dsDNA, requiring a 3'-DNA or RNA end as primer. Many DNAP’s involved in replication contain, beside a polymerization active site (pol), a second catalytic center: the exonucleolysis (exo) active site. This active site catalyzes the excision of erroneously incorporated nucleotides as part of a proofreading mechanism that enhances DNAP fidelity. From a biological point of view a central question in understanding this complex enzyme is how DNAP balances the two opposing activities, polymerization and exonuclease, to swiftly produce an accurate copy of DNA. Optical-tweezers studies are an excellent means to address such a question, providing insight in its kinetic mechanism and the underlying mechanochemistry.

Polymerization and exonuclease lead to length changes that can be measured in real time with optical tweezers. The magnitude of the length changes depends on the applied tension (Figure 2.18a). For example, at 30 pN tension, ssDNA has a length of \(\sim0.56 \text{ nm per nucleotide}\), while for dsDNA this is \(\sim0.34 \text{ nm per base pair}\). As a result, the DNA is shortened, or lengthened by \(\sim0.22 \text{ nm per nucleotide incorporated or excised, respectively}\). In contrast to RNAP (see below), single-nucleotide steps have
not (yet) been resolved. In a typical optical-tweezers assay, a DNA molecule, with one free 3’-end that can serve as a primer for DNAp, is tethered between two optically trapped microspheres. In force-clamping mode, length changes of the DNA tether can be measured, from which the number of incorporated nucleotides can be calculated. This relatively straightforward, label-free assay thus allows extraction of DNAp processivity, the duration of activity and the rate of polymerization.

First experiments on DNAp using optical tweezers were performed on bacteriophage T7 DNAp [180]. At constant tension, polymerization was observed to occur in bursts of activity, resulting in a decrease of tether length, with pauses in between (Figure 2.18b). Pauses were attributed to dissociation of the enzyme from the DNA, bursts of activity to an enzyme binding from solution and catalyzing polymerization until dissociation from the DNA. The polymerization rates obtained were heterogeneous, varying between individual DNAp molecules. In addition, the polymerization rate decreased strongly with DNA tension, the enzyme effectively stalling at $\sim 35$ pN. Interestingly, higher tensions induced exonucleolysis activity, in bursts interspersed with pauses. It was proposed that fraying of dsDNA, due to tension on the DNA tether, promotes DNAp binding with its $\text{exo}$ active site to the primer. Lowering of the tension, below the stalling force led to resumption of polymerization activity. A similar tension-dependent activity was observed for DNAp for bacteriophage $\Phi_{29}$ [79].

A more detailed analysis of pausing and exonucleolysis activity using high-resolution optical tweezers provides deeper insight into the kinetic pathways of DNAp during replication, see Figure 2.18c. For T7 DNAp, the main proofreading pathway was shown to involve dissociation of DNAp from the DNA, followed by rebinding with the $\text{exo}$ active site 4. In contrast, $\Phi_{29}$ DNAp was shown to transfer the primer to the exo active site without unbinding from the DNA [79]. From the T7 DNAp measurements a stochastic picture is emerging in which DNAp very frequently removes correctly incorporated nucleotides to reduce failure in removing erroneously incorporated ones (Chapter 4). This can be justified, when taking into account the asymmetry between the cost of failure to rectify an error and the cost of the removal of correctly incorporated nucleotides: missing an error will have a deleterious effect on future generations, while excision of a correct nucleotide only carries a marginal cost in time and energy.

Optical tweezers have revealed that different kinds of DNAp react qualitatively similar to tension on the DNA, although the exact kinetics differ substantially. A general observation was that the rate of polymerization by DNAp decreases drastically with increased force on the DNA, indicating that the rate-limiting step in polymerization is directly affected by DNA tension. The second observation was that exonucleolysis can be triggered by applying tension on DNA. This allowed studying the dynamics of DNAp and observation of its switching between polymerization and proofreading activity.

### 2.5.2 Helicases

Helicase-catalyzed separation of dsDNA strands provides replicating DNAp access to ssDNA. The mechanism and kinetics of helicase activity can be revealed by studying the unwinding of DNA at different tensions, using optical tweezers. Such experiments al-
Figure 2.18
DNA replication by T7 DNA polymerase. (a) DNAP activity can be observed in real time as it results in the conversion from ssDNA to dsDNA or vice versa. (b) Activity versus time shows both polymerization and exonucleolysis by T7 DNAP at 45 pN. (c) The current kinetic model of DNA replication by T7 DNA polymerase. Adapted from Chapter 4.
allowed discrimination between active and passive DNA unwinding models (Figure 2.19a). In passive models, thermal-fluctuation-induced DNA fraying results in the temporary opening of several base pairs, which can be stabilized by a translocating helicase. In active models, the helicase itself destabilizes the base pairing actively.

Helicases are grouped by their oligomeric appearance: some are hexameric rings, other do not form rings, but still can form oligomers. So far only one hexameric DNA helicase, bacteriophage T7 helicase (gene 4 protein) has been studied with optical tweezers [84]. T7 helicase forms a ring around ssDNA, can translocate along it and can unwind dsDNA upon encounter of a ssDNA-dsDNA junction by displacing the other strand. In optical-tweezers studies of T7 helicase, an assay was used that involved the unwinding of a hairpin in a DNA tether, which substantially enhanced the length changes caused by helicase activity: in this geometry, unwinding of a single base pair leads to a lengthening of the DNA tether by two ssDNA nucleotides, corresponding to ~1.1 nm (Figure 2.6c). The rate of translocation on ssDNA was determined as follows: as soon as the helicase was detected to start unwinding the hairpin, the distance between the microspheres was increased by moving one of the optical traps. This is opening up a known part of the hairpin and creating an ssDNA template for the helicase to translocate on. The time the helicase needed to translocate to the remainder of the hairpin and start unwinding was determined to be 320 nt s$^{-1}$, independent of tension on the DNA.

Next, the force dependence of the DNA unwinding process was determined to increase in a nonlinear fashion with tension on the hairpin [84]. At high tensions (>10 pN) unwinding approached the rate of translocation on ssDNA. These results indicate that T7 helicase actively unwinds DNA, fueled by the free energy obtained from dTTP hydrolysis. Remarkably, T7 helicase activity was found to be sensitive to the DNA sequence, which can be understood by considering that the free energy needed to open a base pair varies between 1.1 and 3.4 k$_{B}$T, depending on sequence, while the amount of energy with which the helicase destabilizes the dsDNA is estimated to be 1-2 k$_{B}$T per base pair (Figure 2.19b). Interestingly, Φ29 DNAp does not require a separate helicase, since it harbors strand-displacement functionality itself. Similar optical-tweezers experiments involving DNA-hairpin opening demonstrated that Φ29 DNAp actively destabilizes dsDNA with ~2 k$_{B}$T per base pair [127], similar to T7 helicase.

In a subsequent optical-tweezers study, T7 helicase was found to unwind dsDNA faster with ATP present instead of dTTP, at the cost of enhanced slippage (Figure 2.19c) [162]. Slippage is caused by the helicase losing its grip on the DNA and sliding backward due to the reannealing of unwound DNA, resulting in loss of helicase efficiency. Remarkably, addition of only a small amount of dTTP increased the processivity threefold, with minimal slippage, while helicase velocity occurred at the enhanced speed observed in the presence of ATP alone.

### 2.5.3 Single-stranded DNA binding proteins

SSB is an essential component in replication of all organisms. The directionality of DNA synthesis by DNA polymerase results in so-called leading and lagging strand synthesis. The leading strand can be synthesized continuously by DNAp following the
2.5 Replication

Figure 2.19
Helicase unwinding of DNA. (a) In a passive model of DNA unwinding, the helicase can only translocate when the DNA frays by thermal fluctuations. In the active model, the helicase destabilizes dsDNA ahead of the fork. (b) The rate of helicase unwinding is dependent on the sequence. (c) Fast unwinding of T7 helicase by hydrolysis of ATP induces slippage (top), and addition of dTTP reduces slippage (bottom). (a,b) Reprinted from ref [84], copyright (2007), with permission from Elsevier. (c) Reprinted by permission from Macmillan Publishers Ltd: Nature ref [162], copyright (2011).
helicase. The lagging strand, however, is synthesized in the direction opposite of helicase progression—in short, so-called Okazaki, fragments (∼2000 bp) that are later annealed to form one continuous strand. The ssDNA generated by the helicase needs to be protected, preventing hairpins from forming, a task fulfilled by SSB binding specifically to ssDNA. SSB can be readily visualized on ssDNA that is held by optical tweezers using concurrent fluorescence microscopy. *E. coli* SSB wraps ∼65 nt of ssDNA, but can still diffuse along the ssDNA, which might be important for redistribution of the protein after initial binding [146]. The mechanism of diffusion was elucidated using optical tweezers combined with FRET microscopy [190]. FRET pairs were created by placing donor and acceptor fluorophores on SSB and/or DNA. From these experiments it was concluded that SSB cannot "roll" over ssDNA, but actually slides by forming a bulge in the DNA through thermal fluctuations.

2.5.4 DNA replication in hindsight and foresight

In conclusion, studies of enzymes that are active in DNA replication have benefited extensively from the fact that these enzymes induce structural transitions to the DNA that can be probed well using optical tweezers. This thus enabled elegant, label-free enzymatic activity assays, which have started to reveal the basis of faithful DNA replication by DNA polymerases, and active DNA unwinding by helicases. In addition, an innovative combination of optical tweezers with single-molecule FRET revealed how SSB slides over ssDNA, which showcased the great potential of this approach to correlate conformational changes in DNA-protein complexes to force spectroscopy measurements.

In future studies, a more complete understanding of the fidelity and pausing behavior of DNA polymerases could be obtained if the resolution of optical tweezers assays were to be pushed to the limit of detecting the single catalytic cycles of DNA polymerases. Furthermore, great challenges lie in experiments that approach the complexity of DNA replication *in vivo*, including the concerted action of the different proteins making up the replisome. For example, the concerted action of a helicase and polymerase could be studied as they open up and partially replicate a DNA hairpin that is being held in optical tweezers. Alternatively, leading and lagging strand synthesis could be studied by attaching optically trapped microspheres to all three arms of the DNA that make up a replication fork. Finally, super-resolution fluorescence imaging techniques could be used in conjunction with optical tweezers to observe, for example, SSB binding to Okazaki fragments.

2.6 Transcription

Transcription is the process in which genes on DNA are read and transcribed to RNA by RNA polymerase (RNAP). RNAP activity can be divided into three stages. First, initiation involves the binding of RNAP to a promoter sequence mediated by transcription factors (Figure 2.20, top panel). This stage results in an open promoter complex with local disruption of dsDNA base pairing and formation of a transcription bubble
Figure 2.20
Cartoon model of initiation, elongation, and termination. Initiation (top) encompasses several intermediates before a stable elongation complex is formed. In the stage of elongation (middle) RNAP adds nucleotides to nascent RNA. Activity is interspersed with pauses. Termination occurs either by an intrinsic signal encoded in the DNA or by an external factor, like the Rho protein. Adapted with permission from ref [73].

with bases exposed to RNAP. After a process called abortive initiation, an extremely stable transcription elongation complex is formed (Figure 2.20, middle panel right). In this second stage, RNAP elongates the nascent RNA, base by base, while the transcription bubble moves along the gene. Elongation is interspersed with pausing of the transcription bubble (Figure 2.20, middle panel left). At the final stage, transcription is terminated and the transcript is released (Figure 2.20, bottom panel).

Over the past two decades, *E.coli* RNAP has been one of the most studied DNA enzymes using optical tweezers. This can in part be attributed to the extreme stability of elongating RNAP-DNA complexes. Due to this stability, it is possible to stall the RNAP on the DNA for a long time by withholding the proper nucleotide and restarting the elongation again by providing all four nucleotides. The stalled complexes can be tethered straightforwardly on beads, to apply and measure forces using optical tweezers. This approach makes RNAP highly suitable for single-molecule experiments. In
a typical experiment, RNAP is tethered on one end, while one of the DNA strands or the protruding RNA is tethered on the other end. In this configuration, stepping of RNAP along the DNA or RNA tether can be directly observed by changes in tether length. In addition, optical tweezers can be used to apply load that either opposes or assists transcription, by tethering either the upstream DNA, downstream DNA, or the synthesized RNA. These assays allow investigation of the mechanochemistry of the RNAP elongation in great detail. In this section we will provide an overview of optical-tweezers studies of RNAP, addressing the three stages of transcription and how they are regulated.

2.6.1 Initiation

To initiate transcription, RNAP has to locate and bind specific promoter sequences on the DNA and form a transcription bubble by locally unwinding the DNA. Subsequently, a stable, processive transcription elongation complex (TEC) can be formed, capable of transcribing thousands of nucleotides. Promoter search has been studied by a combination of fluorescence microscopy with optical tweezers [69]. In this study RNAP was fluorescently labeled on a specific cysteine residue on the α-subunit, and TIRF-illumination was used to suppress background fluorescence due to RNAP free in solution. The experiments showed that the RNAP can diffuse along the DNA. RNAP was shown to remain bound longer to promoter sites compared to nonspecific DNA.

In a recent study a DNA molecule, tethered between two microspheres, was brought into proximity of a third, T7 RNAP-coated microsphere on the surface [153]. One of the DNA-attached microspheres was oscillated, resulting in coupled motion of the second microsphere. Upon T7 RNAP binding to DNA, which introduces an additional tether point of the DNA, the motion of the second microsphere was decoupled, yielding a distinct signal. In this way, the lifetime and force-dependence of initiation events could be measured. The rate of stable complex formation was shown to decrease with increasing force. It was speculated that the formation of a stable elongation complex requires a scrunched intermediate, where the DNA is shortened by as much as 2.5 ± 1.4 nm, and which is restricted by applying force to the DNA. Tension could thus provide a control parameter to the cell for transcription regulation.

2.6.2 Elongation

Elongation is the stage that is most suited for study with optical tweezers since it involves translocation of a protein over long distances along the DNA. In initial experiments, RNAP was nonspecifically attached to the surface of a glass flow cell while the DNA was attached downstream to a trapped microsphere [183]. During transcription RNAP reeled in the DNA, displacing the microsphere from the trap center until elongation stalled due to the opposing trapping force. These first experiments demonstrated that the rate-limiting step of RNAP translocation does not depend on the tension on the DNA, up to 30 pN [174], and thus suggested that the rate-limiting step does not gen-
erate the movement. A remarkable observation was that the velocity during bursts of activity was constant for each RNAP enzyme, but the variance in velocity over the population exceeded the measurement uncertainty. This velocity heterogeneity was also observed when RNAP was attached through another subunit and for T7 RNA polymerase, confirming that there is molecule-to-molecule variation in velocity for RNAP enzymes [2, 164].

Many of the complexes that stalled during elongation due to an opposing load were found to resume elongation upon lowering the applied force; some, however, arrested permanently. The forces required to temporarily pause or permanently arrest elongation were indistinguishable, suggesting that pauses are precursors to arrest. Analysis of the pauses and arrest as a function of force suggested that pausing kinetically competes with elongation and is an intermediate to complete arrest of RNAP activity. Support for this mechanism was obtained by applying an assisting load by pulling on DNA upstream of RNAP, reducing the frequencies of pausing and arrest while leaving the velocity unaffected [50]. These observations indicate that force does not affect the main elongation pathway but does directly act on off-pathway transitions. The elongation and pausing behavior of RNAP has been studied in great detail with optical tweezers [105, 189]. Single-molecule approaches to studying RNAP elongation behavior have a major advantage over bulk studies: due to the stochastic nature of transcriptional pausing and a heterogeneous elongation rate, RNA polymerases will quickly move out of register with each other. In bulk these effects will be averaged out, or can only be studied over short distances.

**Mechanism of translocation: a Brownian ratchet model**

Two microscopic models have been put forward to describe RNAP translocation over DNA: the Brownian ratchet model and the power-stroke model. In the Brownian ratchet model, the enzyme thermally fluctuates between pre- and post-translocation states and NTP binding and hydrolysis in the post-translocation state leads to directed motion. The power-stroke model, on the other hand, predicts that translocation is directly driven by NTP hydrolysis. The Brownian ratchet model predicts a strong dependency of the stepping rate on force only at low NTP concentrations, while in the power-stroke model the rates of NTP binding and translocation are decoupled, resulting in a stepping rate that is sensitive to force at high NTP concentrations. Unambiguous discrimination between these two models required the single-base-pair steps of RNAP translocation to be resolved.

Block and co-workers succeeded in detecting such single base-pair stepping of RNAP during elongation at NTP concentrations low enough to drastically slow down translocation (Figure 2.21a) [1]. The force-velocity relationship was measured at different NTP concentrations, ranging over two orders of magnitude. The single-base-pair accuracy of the experiment allowed removal of all pausing and backtracking events to obtain accurate pause-free forward velocities. The elongation velocity was found to be more sensitive at low NTP concentrations, confirming the Brownian ratchet model. Another study of T7 RNAP showed that opposing loads at low nucleotide concentration reduce the transcription rate [163]. Further insight into the translocation of RNAP was
obtained by examining the function of the so-called trigger loop in eukaryotic RNA polymerase from budding yeast (PoIII), which undergoes conformational changes upon NTP binding [106]. Experiments on enzymes with mutations in this loop confirmed that NTP can bind in the pre- or post-translocated state of PolII and that translocation takes place before hydrolysis, consistent with the Brownian ratchet model (Figure 2.21b).

Figure 2.21
Base-pair resolution of RNAP translocation reveals a Brownian ratchet model. (a) High-resolution data show single steps of 0.34 nm. Reprinted by permission from Macmillan Publishers Ltd: Nature ref [1], copyright (2005) (b) Binding of an incoming NTP can occur either before or after translocation. Adapted with permission from ref [106]. Copyright (2012) National Academy of Sciences, USA.

Pausing

The studies of the RNAP mechanism discussed above have shown that bursts of transcription activity are interspersed with pauses. A systematic analysis of pausing demonstrated that the vast majority (95%) of pauses are short lived (in the seconds range) and do not occur at known pause sequences. Neither the duration of such ubiquitous pauses or their occurrence depends on tension [130]. Longer-lived pauses (>20 seconds) [36] become dramatically less frequent when an assisting load is applied, indicating that
they are the result of backward motion of RNAP along the DNA [149]. During such a backtrack, the 3′-end of nascent RNA is displaced on average 5 bp from the catalytic site of RNAP [135]. A thermal excursion (helped by assisting load) is required to realign the 3′-end of RNA with the RNAP catalytic site, ending the pause.

The relative spatial resolution of optical tweezers in resolving individual steps is remarkably high, less than a base pair (∼0.34 nm). In contrast, absolute localization of RNAP on the DNA tether is more challenging, with a typical accuracy of only 10-100 bp, due to variations in microsphere sizes, instrument drift, and calibration errors. To relate pause events to the underlying DNA sequence, a far better absolute accuracy is required, which has been obtained using reference points along the DNA. In one study, the end of the DNA template was used as a fiducial marker [150], allowing substantial enhancement of absolute resolution to ∼5 bp, however, only during the last ∼200 bp of the template. In another study, a DNA template was used containing eight repeats of sequences that were known to be regulatory sites inducing RNAP pausing [74]. These preprogrammed pause events allowed accurate alignment of traces, providing localization of other pauses with base-pair accuracy. Using this approach, ubiquitous pauses were found to be linked with DNA sequences similar to ones well known to induce backtracking. This led to the conclusion that ubiquitous pauses are states branched off from the elongation pathway, acting as intermediates toward backtracking. In contrast, a study of PolII showed that in fact all pauses involve backtracking of the enzyme, and that the distribution of pause durations follows a $t^{-3/2}$ power law [54]. No backtracking was observed in the short pauses, which may simply reflect the limited resolution. These results could be explained in a model in which all observed pauses, including short, ubiquitous ones are caused by the polymerase diffusing between different backtracked positions and only resuming elongation when the 3′-end of the RNA realigns with the RNAP catalytic site (Figure 2.22) [40]. In this model, short pauses would be insensitive to force because of the small and short backtracks that cause them. Also the pause distribution of *E.coli* RNAP could be fitted by a $t^{-3/2}$ power law [121]. It is, however, not generally accepted that all short pauses are the result of backtracking, since this has not been observed experimentally [73].

**Regulation of transcription elongation**

Regulation of transcription is of utmost importance for controlling gene expression. Transcription is regulated at the RNAP level, by factors altering, for example, initiation or the overall rate of transcription. The suitability of optical-tweezers assays to study and quantify elongation (as discussed above) has so far restricted single-molecule studies of transcription regulators to factors influencing elongation. A central question in these studies has been whether these factors directly affect the elongation rate or pausing.

An example is NusG, an *E.coli* transcription factor that enhances the overall rate of transcription. NusG was shown to increase the elongation rate itself, but also eliminated the occurrence of long pauses (durations >20 sec) [75]. Both observations suggest that NusG has an effect on the translocation step of RNAP, assisting RNAP in reaching the post-translocation state, thereby not only increasing the translocation rate but
Figure 2.22
**Backtracking of RNAP.** RNAP can backtrack on the RNA, displacing the 3'-end from the catalytic center. Shown here are the first two backtracked states. Elongation can continue when RNAP has diffused back and the 3'-end is realigned with the active site.
also inhibiting backtracking. In contrast to NusG, NusA was shown to decrease the elongation rate and enhance pausing \[188\]. In particular, NusA was observed to enhance sequence-specific pausing, similar to applying an opposing load of \(~20\) pN. Zhou et al. speculated that NusA does not influence backtracking directly, but increases the rate of entry into ubiquitous pauses, which can cause backtracking. These results demonstrate that the pausing of RNAP plays an important role in transcriptional regulation. It thus appears that changing RNAP pause propensity is a more efficient way of controlling gene expression than altering the intrinsic rate of nucleotide addition. Further support for this notion came from studies of the effect of the antibacterial peptide MccJ25 on transcription elongation, which showed that it has no effect on the pause-free velocity, but instead increases the occurrence of pauses \[3\].

Figure 2.23
Transcription of PolII through nucleosomes. Single-molecule traces of a PolII enzyme passing the nucleosome positioning sequence (NPS) with and without a nucleosome (top panel). Modified nucleosomes alter the efficiency of passage (middle and bottom panels). On the right are the passing percentages. Reprinted from ref \[14\], copyright (2012), with permission from Elsevier.
Transcription through nucleosomes

In the cell, RNAP often encounters obstacles that control or hinder transcription, such as DNA polymerases. In particular, eukaryotic RNAP must overcome nucleosomes when transcribing genes. Bulk biochemical studies have demonstrated that RNAP elongation slows down substantially or is even halted when a nucleosome is encountered. From the optical-tweezers studies addressing the single-molecule behavior of RNAP described above, it is known that RNAP activity is highly stochastic, involving pausing and backtracking, processes to a large extent averaged out in bulk studies. To fully grasp the mechanistic details of translation through nucleosomes, single-molecule measurements using optical tweezers are essential. In an example of such a study, a nucleosome was bound to a specific nucleosome positioning sequence (NPS) on the DNA downstream of a PolII attached to a microsphere. The elongating PolII was monitored as it transcribed through the NPS [77]. At low ionic strengths, most polymerases arrested in proximity of the NPS. Only at high ionic strength (300 mM KCl) were histone-DNA interactions destabilized enough to allow the majority of PolII to pass the NPS. Many polymerases that passed, paused at the NPS, with durations longer than sequence-independent pauses on bare DNA, indicating that PolII does not actively detach histones from the DNA, but pauses until the nucleosome-bound DNA unbinds by thermal fluctuations. Nucleosomes can rewrap DNA when PolII backtracks, stabilizing backtracked pauses and slowing down pause recovery. In an effort to further elucidate the role of nucleosomes in transcriptional pausing, Bintu et al. studied PolII transcription across modified nucleosomes [14]. Tailless nucleosomes, acetylated nucleosomes and nucleosomes with a mutation in the core histones, all resulted in an increased efficiency of NPS passage (Figure 2.23). Nucleosomes appeared to amplify the occurrence and duration of sequence-dependent pausing and the addition of RNase increased the arrest probability at the NPS. These effects highlight the impact of secondary-structure formation in the nascent RNA, which reduces the distance over which the enzyme can backtrack [184]. The model previously proposed by Hodges et al. was extended with sequence effects, resulting in an RNA-structure dependent backward stepping rate.

In a DNA-hairpin unzipping experiment, the influence of a nucleosome on the position of RNAP was also monitored [82]. In this study, E.coli RNAP was used, which does not encounter nucleosomes in vivo, but is a good model for eukaryotic RNAP because of its homology. RNAP could be localized accurately using the characteristic force signature of the transcription bubble in a hairpin unzipping experiment. Locations of RNAP pausing were found to occur with a periodicity of 10 bp along the DNA, similar to the 10 bp periodicity of nucleosome-DNA interactions. Upon encountering a nucleosome, RNAP backtracked 10-15 bp. When RNase was added, no backtracking was observed, as expected since RNase degrades the RNA track on which RNAP can backtrack. A trailing RNAP could function in a similar fashion, limiting the range of backtracking and pushing a leading RNAP through a nucleosome.
2.6.3 Termination

The RNAP elongation complex is extremely stable and therefore needs a termination signal to dissociate from the DNA and release the nascent RNA. It is unlikely that force alone induces termination, since tensions up to 30 pN on the nascent RNA did not terminate elongation [32]. In bacteria, termination signals can be intrinsic, encoded in the DNA, or extrinsic, such as the protein Rho. Larson et al. investigated the effect of force on different terminator sequences [104]. One terminator was found to be sensitive to tension on the DNA, while the others were insensitive to force. When tension was applied to the nascent RNA instead, the termination efficiency of the latter two increased with increasing force. A quantitative model was presented, taking into account the stability of the terminator RNA hairpin and RNA:DNA hybrid inside the RNAP. The authors concluded that termination occurs either by forward translocation of RNAP or by shearing of the DNA:RNA hybrid by the RNA hairpin when the termination signal is encountered.

Termination factor Rho is a hexameric helicase that binds nascent RNA at a specific sequence. It terminates transcription by translocating along the RNA and pushing off RNAP. In a study of the mechanism of transcription termination by Rho, Rho was bound to the nascent RNA and transcription was allowed to proceed up to a roadblock [95]. The roadblock hindered RNAP ejection, allowing the mechanism of Rho binding to be probed. Force-extension curves showed force rips, indicating that Rho remains on its initial binding site, while translocating along the RNA. In this tethered-tracking model, Rho forms a loop with the intervening downstream RNA and terminates transcription by displacing RNAP from the DNA template. Optical tweezers studies have thus provided a better insight in the mechanisms of transcription termination, not only by applying controlled loads to RNA and DNA in the context of specific terminator sequences, but also by allowing direct observation of the activity and translocation of a terminator protein such as Rho.

2.6.4 DNA transcription in hindsight and foresight

Optical tweezers studies have provided an unprecedented picture of all three stages of transcription. In particular elongation has been studied extensively, providing substantial insight into the mechanochemistry of this enzyme. The high sensitivity of optical tweezers allowed direct observation of single base-pair stepping by RNAP and revealed a rich and biologically relevant pausing behavior. Loads could be applied to each of the DNA or RNA strands protruding from the enzyme which allowed extensive unraveling of the elongation process, transcription termination, and demonstrated that backtracking plays an important role in transcriptional pausing and regulation.

Further research will be required to resolve the different pausing mechanisms proposed. In the eukaryotic cell, many cofactors regulate transcription in an unknown way. In addition, a structural understanding of the mechanisms of initiation, nucleotide binding, hydrolysis, translocation and termination requires resolving the conformational
changes involved. To address these, the combination of optical tweezers and fluorescence microscopy, in particular incorporating FRET, will be of great value. For example, conformational changes of the RNAP enzyme, observed through FRET, could be related to sequence-dependent pauses, the locations and durations of which can be detected using optical tweezers, to obtain a better understanding of how transcription is affected by the underlying sequence.

2.7 DNA repair

During a cell’s life, its DNA is continuously corrupted by DNA synthesis errors and damaged by, for example, ultraviolet light, free radicals, and carcinogenic intercalators. To maintain the integrity of the genome, cells have adopted several mechanisms to repair damaged DNA. An extensive protein machinery is devoted to scanning the DNA for these damages, recruiting the necessary repair proteins, and initiating a series of biochemical steps that result in repair of the damaged DNA molecule. Examples of DNA-repair pathways are nonhomologous end-joining, homologous recombination, base excision repair and mismatch repair. Below we will briefly address mismatch repair, but focus primarily on the pathway that has been studied most extensively using optical tweezers, homologous recombination.

2.7.1 Homologous recombination

Homologous recombination concerns repair of, arguably, the most severe damage to DNA: a double-stranded break. During a double-stranded break event, bases can be lost. This can result in corruption of the genetic information in case the broken ends are ligated, such as happens in nonhomologous end-joining. Homologous recombination, however, exploits the sequence documented in the sister chromatid as a template to restore the DNA molecule without loss of genetic information (Figure 2.24). The intricate scheme of homologous recombination involves the partial resection of one of the strands of the broken DNA to create a long 3’-single-stranded overhang. Next, nucleoprotein filaments, which catalyze the search and invasion of the homologous sequence on the sister chromatid, form on such overhangs. Finally, polymerases restore the missing DNA sections, followed by resolution and separation of the two sister chromatids. The key processes in homologous recombination involve widely varying physical characteristics. Yet, the versatility of optical tweezers permits investigating these processes. For example, mechanical probing by optical tweezers allows measurement of protein translocation, nucleoprotein-filament formation, and protein-mediated DNA-DNA interactions. Moreover, optical tweezers can be combined with other modalities, often essential to study homologous recombination. For example, microfluidics can be used to control of the sequential steps in biological assays, and fluorescence microscopy can be used to observe, distinguish, and quantify local effects such as nucleation and growth of protein filaments on DNA.
2.7 DNA repair

Figure 2.24 Homologous recombination. This simplified scheme shows the process of repairing a double-stranded break (black DNA) using information documented in the sister chromatid (red DNA). The proteins involved in this process are not shown. (1) End resection to create 3’-single-stranded overhangs on which nucleoprotein filaments form. (2) Homologous pairing with the sister chromatid. (3) Polymerization of the complementary strands. (4) Resolving the two chromatids, which can result in redistribution of the genetic material.
2.7.2 End resection to create a ssDNA overhang

In the initial phase of homologous recombination in *E.coli*, the enzyme RecBCD is responsible for binding to the blunt end of a broken dsDNA molecule, for creating 3’-single-stranded overhangs, and for loading filaments of the recombinase RecA (see ). RecBCD is a remarkably sophisticated molecular machine that exhibits both helicase and nuclease activities. It consists of three subunits (RecB, RecC, and RecD), of which two (RecB and RecD) are known to drive translocation over DNA. Processive nuclease activity of RecBCD allows the enzyme to destroy foreign DNA, for example from bacteriophages, a secondary function of this enzyme. However, degradation of the bacterium’s own DNA is limited by switching its activity after encountering a specific DNA sequence called $\chi$ [159]. At this sequence, RecBCD switches activity from degrading both ssDNA strands to degrading only the 5’-strand, leaving a 3’-single-stranded overhang. Crucial aspects of RecBCD’s sophisticated mechanism have remained unclear: what is the molecular basis of the different activities and how are they coordinated and regulated?

RecBCD degrades DNA, which complicates study of its helicase/translocase activity using label-free translocation assays (Figure 2.6b and c), since this requires intact DNA that can be tethered on two sides. Alternatively, either translocation assays that tether both the enzyme and the DNA (Figure 2.6a), or assays that monitor enzymatic activity using fluorescence microscopy (Figure 2.8) have been used. In the latter assay, the enzymatic activity was visualized on a DNA molecule that is tethered on one end to an optically trapped microsphere and subsequently extended in a buffer flow. Fluorescence imaging of the YOYO-1-stained DNA being degraded by the enzyme’s activity revealed a translocation rate of at most 1000 bp s$^{-1}$, with high variability between helicase molecules [12]. The enzyme was also found to be highly processive, degrading on average 30 kb before unbinding. In a force-measuring optical tweezers assay, which provided higher spatial resolution than possible using the flow-stretched DNA assay [141], Perkins et al. observed smooth movement of RecBCD against applied forces up to 8 pN. Moreover, at opposing loads of 5-8 pN, backward sliding was observed without loss of contact with the DNA. The observed ability of the enzyme to restart after backsliding was suggested to help overcoming mechanical blockades in vivo.

Several studies, employing the single-bead assay in combination with fluorescence microscopy mentioned above, have addressed the $\chi$-induced activity switch, which had proven elusive in bulk studies. Spies and co-workers found that RecBCD consistently paused at $\chi$ for several seconds, followed by translocation at half the velocity (Figure 2.25) [159]. This slower velocity might facilitate the loading of RecA on the 3’-strand.

Fluorescent nanoparticle labeling of RecD demonstrated that the switch of RecBCD activity at the $\chi$ sequence did not involve ejection of the RecD subunit as previously thought [68]. Subsequent studies revealed that RecBCD enzymes that contained a defective RecD subunit are slower than the wild type, but this velocity was not altered after the pause at $\chi$ [158]. This was interpreted to indicate that in wild-type RecBCD, RecD is the faster motor subunit, setting the pace of the whole complex before encountering $\chi$. After the pause at $\chi$, RecD continues to translocate on DNA, but its velocity is attenuated and RecB sets the pace (see model in Figure 2.26).
Figure 2.25
Monitoring RecBCD activity using fluorescence imaging of DNA flow-stretched from an optically trapped microsphere. (a) Analysis of a representative video visualizing RecBCD-mediated unwinding of a single DNA molecule (illustrated at the right of the graph) containing χ-sequences. The length of the dsDNA molecule was measured for each frame and presented as a time trace (red squares). (b) Schematic depiction of the phases of RecBCD-mediated unwinding of χ-containing dsDNA. (I) RecBCD-DNA-bead complex. (II) dsDNA unwinding by RecBCD enzyme results in a linear decrease in the dsDNA length in presence of ATP. (III) interaction with χ results in a pause in RecBCD enzyme translocation. (IV) the χ-modified enzyme continues to unwind the DNA, but at a reduced rate. (V) RecBCD enzyme dissociates from the DNA. (c) Representative frames from the movie analyzed in (A). the green arrow in frames I-III indicates the end of the dsDNA molecule, where RecBCD enzyme acts, while the white arrow in frames IV-V points to the putative ssDNA. Reprinted from ref [159], copyright (2003), with permission from Elsevier.
RecBCD is shown as a bipolar helicase with its two motor subunits translocating on the opposite strands of the DNA molecule. Before encountering the IG sequence, the RecD subunit is shown as the leading motor subunit. Such an arrangement of the two motor subunits moving with different rates generates an ssDNA loop in front of the RecB subunit. Upon χ-recognition, the RecD motor is controlled and the RecB subunit becomes the driving motor of the enzyme. Arrows indicate the directions and relative rates of translocation by the motor subunits. Reprinted from ref [158], copyright (2007), with permission from Elsevier.
Nucleoprotein-filament formation and disassembly

In homologous recombination, nucleoprotein filaments are formed on the resected single-stranded overhangs of a double-stranded break. These nucleoprotein filaments on ssDNA are functional in the search for and pairing with the homologous sequence in the DNA of the sister chromatid (Figure 2.24). In prokaryotes, filaments are formed by RecA, while in eukaryotes the filaments consist of RAD51 or Dmc1. Since filament formation typically involves changes in flexibility and extension of the DNA-protein complex, optical tweezers are excellent tools to probe, identify, and quantify these changes. Furthermore, tension applied to the DNA-protein complex can be used to modulate the free-energy landscape of filament assembly and disassembly. Nucleoprotein filaments can form on both ssDNA and dsDNA. To study filaments on ssDNA, force-induced melting of dsDNA using optical tweezers can be used to generate long ssDNA constructs (see also 2.3.4 and Chapter 3). Additionally, local phenomena, such as the nucleation and growth of nucleoprotein filaments on DNA, can be well studied and distinguished using fluorescence microscopy applied to DNA held in optical traps.

RecA filament assembly A force-measuring optical tweezers study by Hegner et al. revealed the mechanical properties of filaments of the prokaryotic recombinase RecA on ssDNA and dsDNA [70]. It was found that RecA filaments substantially stiffen the DNA and increase its length (cf. Figure 2.4). RecA-ssDNA and RecA-dsDNA filaments were of similar length, about 1.5 times longer than bare dsDNA, which was proposed to facilitate homologous pairing. Moreover, the dependence of mechanical properties on the type of nucleotide bound (ATP/ADP/ATP-γ-S) highlights the role of ATP hydrolysis in filament conformation. Particularly for ADP-RecA, the application of force was found to affect the transition between an extended filament conformation, the conformation that is active in strand exchange, and a compressed filament conformation thought to be inactive. However, the dynamics and energetics of the nucleotide-dependent and force-dependent conformations and their role in homologous pairing remain unclear. In another approach, the assembly dynamics of RecA filaments were visualized by fluorescence imaging of dsDNA tethered on one end to an optically trapped microsphere and stretched in buffer flow [55]. Snapshots of the assembly process revealed the formation of multiple RecA nuclei on dsDNA, followed by bidirectional growth. In vivo, RecA competes with SSB for binding to ssDNA. SSB has a higher affinity for ssDNA than RecA and thus inhibits RecA-filament formation. Fluorescence microscopy on SSB-coated ssDNA confirmed that under these conditions, RecA filament formation was indeed very slow (few nuclei per hour) [10].

RAD51 filament-binding dynamics Hilario and co-workers monitored the assembly of RAD51 on dsDNA in real time by quantifying the extension of a DNA molecule with a fluorescently labeled free end that was flow-stretched from a single trapped microsphere [76]. Similar to RecA, ATP-dependent RAD51 binding extends dsDNA about 1.5 times. Through a series of experiments involving nucleation of both wild-type and labeled RAD51 on dsDNA, the authors concluded that, in contrast to RecA, filament assembly was dominated by nucleation of many short filaments along DNA. Disassembly of RAD51 filaments occurs upon ATP hydrolysis and was found
DNA:protein interactions in optical tweezers

to be surprisingly slow and incomplete [76]. Disassembly caused a shortening of the DNA over time which could be fitted with a double exponential time dependence. The authors assigned this to a two-step disassembly process: the faster step was assigned to hydrolysis of ATP, compacting the filament, and the slower step was assigned to the dissociation of the ADP-bound RAD51. Although the changes in filament extension during (dis)assembly implicate an important role of tension, the flow-stretching assay does not allow precise control of measurement of the tension on the DNA.

Figure 2.27
Quantifying Rad51 filament disassembly under tension. (a) Kymograph (upper panel), tension trace (blue) and intensity trace (red, lower panel) of a RAD51-dsDNA complex held in an optical tweezers. Tension-stalled disassembly is reinitiated by tension release (orange dashes) (b) Kymograph (upper panel) and intensity traces (red, lower panels) showing disassembly of isolated, short RAD51 patches in bursts interspersed by pauses in the order of minutes. Blue lines represent a step fitting routine. (c) RAD51 disassembly pathway: (1) All RAD51 (red) starts out ATP- (green) and DNA- (blue) bound; (2) ATP hydrolysis is triggered, filaments remain stable as long as terminal RAD51s have ATP bound; (3) ATP hydrolysis at terminal monomer.; (4) Monomers dissociate until next ATP-bound monomer is terminal (arrow); (5) Disassembly pauses until terminal ATP hydrolyzes. (6) Disassembly relaxes DNA. Reprinted by permission from Macmillan Publishers Ltd: Nature ref [170], copyright (2009).

Using force-measuring dual-trap optical tweezers, however, this has proven possible [169]. Since RAD51 disassembly shortens the DNA-protein complex by about 50%, tension builds up in a disassembling DNA-protein complex held by optical tweezers in a distance clamp. Van Mameren et al. used this change in tension to study disassembly, while concurrently visualizing fluorescently labeled RAD51 using fluorescence microscopy (Figure 2.27). Disassembly depended strongly on tension: at forces above 50 pN, disassembly stalled. Upon relaxing the tension, however, disassembly continued. Analysis of the force dependence of the disassembly rate provided support for the idea that disassembly occurs monomer by monomer. The fluorescence intensity of individual filaments was found to decrease in bursts, due to a fast sequential disassembly of, on average, 5-10 monomers (Figure 2.27). This followed an exponentially distributed waiting time, consistent with a single rate-limiting step. The waiting step was identified to be ATP hydrolysis on the terminal monomer. (Figure 2.27). Thus, when the terminal monomer hydrolyses ATP and dissociates, all neighboring monomers with their ATP hydrolyzed will dissociate consecutively until another ATP-bound monomer is encoun-
2.7 DNA repair

Figure 2.28
DNA conformation and nucleoprotein filament length contribute to the homology search. (a) Effect of DNA end-to-end distance on the fraction of molecules that is observed to be homologously paired. (b) Model for RecA homology search by intersegmental contact sampling. Reprinted by permission from Macmillan Publishers Ltd: Nature ref [51], copyright (2012).

tered, which will form the new filament end until its ATP is hydrolyzed. While tension was found to impede dissociation, ATP hydrolysis was not substantially affected by tension.

REGULATORS OF NUCLEOPROTEIN FILAMENTS  In vivo, the specificity and speed of nucleoprotein-filament assembly is regulated by mediator proteins. The need for regulators is, for example, illustrated by the excessively slow assembly rate of RecA filaments on SSB-bound ssDNA in the absence of mediators [10]. Indeed, Bell et al. confirmed that addition of mediator proteins RecOR and RecF enhanced the nucleation and/or growth rates of RecA filaments on SSB-ssDNA [10]. Similarly, the slow rate of disassembly from the ends of RAD51 filaments on dsDNA could explain the need for mediator proteins to prevent filament formation on dsDNA, which would hamper DNA repair. In support of this, optical-tweezers studies have shown a suppression of RAD51-filament formation on dsDNA in the presence of fragments of the breast cancer susceptibility gene BRCA2 [25]. Other regulatory roles have been assigned to RAD54 and Tid1, which interact with RAD51 and Dmc1, respectively. Optical-tweezers studies have demonstrated that RAD54 and Tid1 are translocating ATPases [5, 133], but their dynamic interaction with DNA-bound RAD51 and Dmc1 remains to be observed and quantified on the single-molecule level.

Homology search

After decades of debate and study, it is still unclear how a nucleoprotein filament can find a homologous sequence quickly in the large background of the whole genome. Forget and Kowalczykowski used concurrent dual-trap optical tweezers and fluorescence microscopy
experiments to demonstrate the importance of the DNA conformation for an effective homology search by RecA nucleoprotein filaments [51]. The rate of homologous pairing was found to increase with the amount of slack in the DNA, which increases the number of accessible DNA conformations (Figure 2.28). This effect is similar to the enhanced target localization by restriction enzymes that was observed when DNA was coiled instead of stretched [111, 166]. The observations by Forget and Kowalczykowski are consistent with a ‘intersegmental contact sampling’ model, where different regions of a long ssDNA-RecA complex can sample several distant sections of DNA simultaneously (Figure 2.28). Multiple nonspecific and weak localized interactions facilitate efficient and rapid sampling for homology within a genomic context.

**HOMOLOGY SAMPLING INTERMEDIATES** Other relatively unexplored areas of homologous repair involve the structure and kinetics of transient homology-search intermediates and the homologously paired structure itself. De Vlaminck et al. demonstrated the important role of the secondary DNA binding site of RecA, which resides on the outer surface of the nucleoprotein filament [38]. Using an innovative force-measuring dual-DNA manipulation assay combining optical with magnetic tweezers (see Figure 2.29), the authors found that the secondary binding site has an affinity for ssDNA that is at least two orders of magnitude higher than that for dsDNA. In vivo, however, RecA filaments interact with double-stranded sections of the sister chromatid, not the kinetically preferred ssDNA. An enhanced interaction strength with dsDNA was found when RecA filaments engaged dsDNA that was underwound using magnetic tweezers, such that it transiently exposes ssDNA (Figure 2.29). This interaction was, however, still too weak to form a stable complex at nonhomologous DNA. At a homologous site, on the other hand, stable complexes could be formed with underwound dsDNA, most likely due to base-pair formation between one of the dsDNA strands and the homologous ssDNA in the RecA filament (Figure 2.29). It was suggested that the weak and short-lived interactions with nonhomologous DNA allow rapid homology sampling.

### 2.7.3 Mismatch repair

Repair of mismatched nucleotides in DNA that are caused by errors in DNA replication has not been studied extensively using optical tweezers. Nevertheless, optical tweezers can prove powerful in this regard, as exemplified by the study of the MutS system by Jiang et al. [81]. The MutS-family protein MSH2-MSH6 recognizes single-base mismatches and small loop mismatches and is involved in recruiting other proteins that perform the actual mismatch repair. Mismatch repair *in vivo* is highly specific for mismatches and improves the overall fidelity of replication 1000-fold. Bulk assays, however, fail to demonstrate a high specificity of the enzymes over intact DNA (only 10-30 fold specificity). This might be caused by the use of relatively short linear DNA substrates in bulk assays, which might amplify the effect of end-binding on specificity. Jiang and co-workers used unzipping-force analysis to locate positions of MutS-family proteins on DNA hairpins [81]. The recorded force signatures allowed accurate localization of single base mismatches, as well as of proteins bound to the hairpin. Using this approach it was found that the affinity of MSH2-MSH6 to a mismatch site is at least 3 orders of
2.7 DNA repair

Figure 2.29
Homology sampling intermediates. (a) Side view of dual-molecule assay where a RecA filament that is held in the optical tweezers interacts with a coiled dsDNA that is tethered in a magnetic tweezers configuration. Inset: schematic of the RecA filament represented with primary (1) and secondary (2) DNA-binding sites. (b) Push-probe experiments reveal binding (red arrow) of a RecA-ssDNA filament to homologous, negatively supercoiled dsDNA (bottom) and absence of binding to positively supercoiled dsDNA (top). (c) Binding probability as function of supercoil density. Underwinding of dsDNA strongly promotes the efficiency of pairing. (d) Comparison of binding probability as function of supercoil density for RecA-dsDNA (red) and RecA-ssDNA filaments (blue). Reprinted from ref [38], copyright (2012), with permission from Elsevier.
magnitude higher than for undamaged DNA, in better accordance with in vivo data. Moreover, by unzipping from both sides of the mismatch, the size and location of the binding site could be measured with 2-3 bp accuracy, yielding a footprint of MSH2-MSH6 of 20 bp that was positioned asymmetrically around the mismatch site. Several models have been proposed to explain how MSH2-MSH6 recruits repair proteins. In one of these, MSH2-MSH6 switches in an ATP-dependent way to a sliding clamp mode of DNA binding. The sliding clamp model was tested by blocking the end of the hairpin with a lac repressor, preventing it from sliding off the end. Indeed, in the presence of ATP, multiple MSH2-MSH6 were observed to be trapped between the mismatch and the lac repressor, providing support for the sliding clamp mode.

2.7.4 DNA repair in hindsight and foresight

Optical tweezers have provided many new insights into the mechanisms of DNA repair, which have illustrated the large versatility of the technique: using DNA stretching experiments, protein translocation as well as filament formation has been detected. In addition, combined optical trapping, fluorescence microscopy, and microfluidics proved very useful in quantifying various aspects of repair processes. Furthermore, using DNA-unzipping assays, the location and extent of mismatch-repair proteins have been probed. Finally, force-induced melting of dsDNA and precise control of the conformation of (multiple) DNA molecules have been instrumental in studying homologous recombination, which involves interactions of protein-coated ssDNA with segments of dsDNA. Further analysis of DNA-protein complexes in homologous recombination using optical tweezers can shed more light on numerous unresolved issues. For example, the dynamics and energetics of the extended and compressed conformations of nucleoprotein filaments can be probed, and the effects of mediator proteins such as RAD54 and BRCA2 on filament assembly and disassembly can be studied. Concurrent (multicolor) fluorescence microscopy will further enhance the ability of optical-tweezers experiments to address open questions in (multicomponent) DNA repair processes: the spatial dynamics of mismatch-repair proteins and subsequent recruitment of downstream repair proteins and the importance of 1D and 3D search processes can be directly visualized on optically manipulated DNA. Finally, utilizing more advanced fluorescence techniques such as FRET may allow correlation of the conformational dynamics of enzymes such as RecBCD with their translocation activity and regulation.

2.8 Concluding remarks and outlook

Optical tweezers have grown to be one of the most powerful and versatile single-molecule methods for analysis of DNA-protein complexes. The power of optical tweezers lies primarily in its extremely high sensitivity (pN, nm, k_BT) and bandwidth (ms-s) in combination with a wide and biologically relevant force range (pN-nN). However, just as important are the limited number of restrictions that render optical tweezers highly flexible and versatile. DNA-protein complexes can be manipulated at will in three
dimensions and far away from any surface. Not only does this turn out to be crucial for suppressing environmental noise, but it also facilitates interfacing optical tweezers with microfluidics and fluorescence microscopy. It is this versatility of optical tweezers, which allows incorporating multiple measurement modalities, that may prove to be the most important feature for future investigations that aim to unravel cellular life in its full complexity.

**Biological Assays in DNA Metabolism** In this chapter, we have tried to convey that optical tweezers—like microscopic hands that are both flexible and sensitive at the same time—have enabled a wide range of biological assays, each one cleverly devised to analyze or mimic the plethora of DNA-protein interactions that occur in major DNA transactions. For example, in the field of DNA organization, mechanical stretching was shown to be highly successful in probing DNA compaction and mimicking DNA decompaction processes. In replication, elegant label-free protein translocation assays have provided clarifying insights into faithful DNA replication by DNA polymerases. Furthermore, extreme refinement of optical tweezers sensitivity has led to unprecedented mechanistic insight into the single base-pair stepping of RNA polymerases during DNA transcription. Finally, experiments on DNA repair, which involves the orchestrated action of many proteins, each with different physical characteristics, have showcased the versatility of optical tweezers assays and also illustrated the large advantages of concurrent visualization by fluorescence microscopy.

**Uncharted Areas** Despite the progress made, our understanding of protein-mediated DNA transactions is far from complete. There is ample room, and indeed need, for optical tweezers to further explore the mechanisms of DNA metabolism at the single-molecule level. Examples of relatively uncharted areas where optical tweezers can still make large contributions include the mechanisms of remodeling compacted DNA, which is even less understood in prokaryotes than in eukaryotes. In addition, whereas our understanding of transcription has greatly benefited from studies with single-base pair resolution, such high-resolution studies have not yet been applied to replication. However, high-resolution studies are urgently needed to understand the fidelity of DNA replication and investigate possible sequence dependencies of replicational pausing. Regarding transcription, optical tweezers studies can still greatly contribute to our understanding of transcription initiation and regulation. Finally, DNA repair processes such as nonhomologous end-joining, mismatch repair, and also the final stages of homologous repair, have received little attention, even though there is great potential for their exploration by optical tweezers.

**Methodological Innovations** The history of optical tweezers is marked by astonishing methodological advancements enabling innovative experiments that have deepened our understanding of life at the single-molecule level. It is to be expected that further innovations will keep advancing the field. One technological advancement may address a current limitation of optical tweezers: that DNA molecules are studied only one at a time. This limits efficiency and throughput, which is crucial for obtaining the statistics required in single-molecule investigations. While the handling of multiple
DNA molecules is already possible (section 2.4.2, for instance), accurate and rapid force measurements on more than two beads still require innovations in array-based force detection, such as by high-speed cameras.

Even more exciting are innovations, some of which are already under way, that will alter the level at which biomolecular research into DNA-protein complexes takes place. Currently, this level is mostly limited to studying the activity of single proteins. The combination of optical tweezers with fluorescence microscopy has the potential to unlock two new types of investigations; either reaching a more fundamental mechanistic level by interrogating the dynamics of individual protein domains rather than that of entire proteins; or rising well above the single-protein level, by moving toward in vivo complexity.

The first type of investigation is made possible by combining FRET and force spectroscopy [78, 190], which enables direct observation of the conformational dynamics of DNA-bound proteins that are at the core of enzymatic activity. The observed conformational dynamics can be correlated to the applied tension or to the observed protein activity to provide unprecedented mechanistic insights into, for instance, transcribing or replicating polymerases. Confocal fluorescence microscopy of DNA-bound proteins, which has already been established [29, 72, 78, 152], appears most suited for this type of study.

The second type of investigation would be enabled by (multicolor) fluorescence imaging on DNA [91, 169] that is held in optical tweezers. This allows increasing biological complexity to study the orchestrated action of, for example, the many proteins involved in the replisome or in DNA repair processes. This approach will bring in vitro studies closer to reproducing and studying the in vivo situation. Nevertheless, to distinguish and study individual DNA-bound proteins using diffraction-limited imaging methods, artificially low densities of proteins are still needed in vitro. Super-resolution fluorescence microscopy, on the other hand, will allow this gap to be further bridged by enabling study of DNA metabolism at densities much closer to those found in vivo, yet it will still be able to analyze the role of individual proteins [72].

**TOWARDS IN VIVO COMPLEXITY** This chapter illustrates that we are beginning to comprehend the molecular mechanisms of DNA metabolism under idealized in vitro conditions. Feeble though is our knowledge of how DNA metabolism occurs in the context of compacted DNA. However, in due course, we envision that optical tweezers may function as a highly versatile platform for multimodal studies aimed at unraveling the role of individual DNA-processing proteins within the full complexity of cellular DNA. For example, optical tweezers could be used to hold eukaryotic chromatin and accurately apply tension to maintain and manipulate its compacted higher order structure. Subsequently, multichannel laminar buffer flows can be used to control and step through the different phases of, for instance, DNA repair, DNA transcription, or DNA replication. In the latter case, for example, fluorescence imaging can be used to visualize a fluorescently labeled helicase as it unwinds the compacted DNA in the replisome, while simultaneously leading- and lagging-strand synthesis by fluorescently labeled DNA polymerases can be observed. Complementary magnetic tweezers can be used to introduce and study the role of supercoiling on replication in chromatin.
At the same time, super-resolution imaging can provide the means to resolve Okazaki fragments sequestered by single-stranded binding proteins or resolve the motion of the replication forks that progress through the chromatin. Only in such a complex, yet controlled, environment can we start to comprehend the full concerted action of the processes that lie at the heart of cellular life. With developments in biochemistry, optical tweezers technology, and concurrent super-resolution visualization moving at a rapid pace, this enthralling vision might not lie too far ahead of us.