9

Spectral Self-Interference Fluorescence Microscopy to Study Conformation of Biomolecules with Nanometer Accuracy

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CONTENTS
9.1 Introduction ............................................................... 345
9.2 Fundamentals of SSFM ........................................... 348
  9.2.1 Background and Principles ........................................ 348
  9.2.2 Physical Model of SSFM ........................................ 349
  9.2.3 White Light Reflectance Spectroscopy ....................... 353
9.3 Data Acquisition and Analysis ..................................... 355
  9.3.1 SSFM Setup ......................................................... 355
  9.3.2 Fitting Algorithm .................................................. 356
9.4 Applications ............................................................. 357
9.5 Discussions and Conclusion ......................................... 375
Acknowledgments ............................................................ 377
References ........................................................................ 377

9.1 Introduction

Despite the completion of the human genome sequencing, the revolution of personalized medicine still seems years away [1,2]. Although the Human Genome Project provides researchers with enormous amounts of genetic information, the genome is far more complex than the sequences it contains. Part of the reason is that DNA functions through critical interactions with proteins, such as genome packaging, epigenetic modifications, transcription, DNA replication, and DNA repair [3–7]. Since the idealized B-form DNA proposed more than 50 years ago by Watson and Crick, researchers have found that the conformation of DNA is naturally distorted and, depending on the particular sequence, DNA can be curved, tightly bent, or kinked [8–10]. These intrinsic variant conformations of DNA are recognized, stabilized, or even enhanced upon the formation of DNA–protein complexes
DNA–protein complexes bring distant regions of DNA together and can sharply bend or kink the DNA, resulting in conformational changes postulated to play an important role in the recognition of specific binding sites on DNA by proteins [11,13,14]. An understanding of the intrinsic conformation of DNA and deformity of DNA structure to form a specific complex in a native environment is of considerable biological significance.

Conventional tools are available to study DNA–protein binding whose critical size dimensions are on sub-nanometer scales. X-ray crystallography determines structures of biomolecules crystallized under stringent conditions at the atomic level, yet the resulting protein structure from crystallization may not be an accurate representation of its structure in its native environment [15,16]. Nuclear magnetic resonance (NMR) is one of the most precise techniques for imaging biomolecular structures and has the ability to perform dynamic protein structure measurements [17,18]. Electrophoretic mobility shift assay (EMSA) is one of the most inexpensive and easiest ways to investigate DNA conformation. Although EMSA is low throughput and does not allow measurement of protein–DNA dynamics, it is a powerful technique for accurate measurement of changes in DNA secondary structure [19,20]. Superciled, kinked, and looped DNA structures and DNA-bending angles in protein–DNA complexes can also be determined with atomic force microscopy (AFM) by scanning DNA (single- or double-stranded) that has been deposited on a very flat, smooth, and stationary substrate to obtain a 2D contour [21–25]. However, the requirement to adsorb the molecules on a surface impacts the conformation of the adsorbed biomolecules, and scanning of a surface by AFM is a slow process. Additionally, the scanning area is usually on a scale of hundreds of nanometers, limiting the throughput of AFM. With the discovery of a variety of synthetic and natural fluorophores and the advancement of specific labeling technologies, fluorescence microscopy has become an essential tool in modern biology and biomedical sciences. Although traditional fluorescence microscopy, like other conventional optical microscopy techniques, cannot provide resolution below the diffraction limit, some new fluorescence imaging techniques have broken the limit. Among these techniques, fluorescence (Förster) resonance energy transfer (FRET) allows investigation of biomolecular structure in vivo with a high temporal resolution when combined with stopped flow techniques [26,27] and provides a measurement of the distance between nearby donor and acceptor molecules in a distance range of about 10–100 Å [28]. However, the techniques previously discussed are not high throughput and therefore are not suited to study DNA sequence dependence, one of the most critical parameters influencing protein–DNA interactions. Traditionally, DNA microarray methods are used to study sequence dependence in genetic, epigenetic, transcriptional, and bioinformatics studies [29–35]. Short oligonucleotide probes, that is, 20–80 base pairs (bp), are spotted or synthesized in situ via surface engineering to allow covalent attachment of the probes to a solid surface. Detection and quantification of the binding of complementary
sequences or protein molecules (targets) are done by measurement of relative fluorescent signals from the labels of the targets. For example, protein-binding microarrays (PBMs) have demonstrated a powerful capability to investigate DNA-binding affinity of proteins at the genome scale [29]. However, there is still an unsolved challenge to accurately quantify conformations of DNA strands and DNA conformational changes induced by specific protein binding in a high-throughput manner under physiological conditions.

Another novel technique of fluorescence microscopy is spectral self-interference fluorescence microscopy (SSFM), which uses optical interference to measure the conformation of surfaced-immobilized biomolecules. From the spectral oscillations emitted by an ensemble of fluorophores located above a reflecting interface (SiO$_2$–Si interface), the average height of the fluorophores relative to the surface can be determined with sub-nanometer resolution across a broad range from a few nanometers to more than 100 nm [36–39]. Early fluorescence interference microscopy techniques, such as fluorescence interference contrast (FLIC) microscopy, rely on intensity variations of total fluorescent emission. Utilizing FLIC for a high-precision determination of axial distance of fluorophore layers requires multiple measurements and accurate fabrication of spacer steps for use as a reference [40,41]. In contrast, SSFM utilizes spectral information of fluorophore emission on a thick oxide and provides higher precision in one measurement. Measurements of SSFM are immune from factors that can affect the fluorescence intensity, such as the excitation field strength, local fluorophore density, photobleaching, or change of buffer environment. Through the integration of DNA microarray technology and SSFM, we have developed a high-throughput platform that allows precise quantification of intrinsic conformations and conformational changes of surface-immobilized DNA in a physiological-like environment. Such a high-throughput approach increases efficiency and decreases the cost (time and money) required to investigate specific protein–DNA interactions.

In this chapter, we first introduce the fundamentals, principles, and data analysis of SSFM. Then we discuss some of the applications in biosensing and bimolecular studies on the SSFM platform. First, SSFM is used to determine the distance of monolayers of fluorophores relative to a layered substrate with sub-nanometer accuracy. In the next example, SSFM is combined with white light reflectance spectroscopy (WLRS) to measure conformations of surface-immobilized oligonucleotides. The third application demonstrates the usage of SSFM for direct observation of conformational change of a 3D polymeric coating. In the fourth example, the SSFM substrate is functionalized with a novel polymeric surface to achieve control and quantify double-stranded DNA (dsDNA) orientation. In the fifth example, DNA conformational changes upon binding with proteins are measured with SSFM. In the last example, SSFM is developed to accurately measure two axial positions at one location. Finally, we will discuss the future applications of the high-throughput platform in studying DNA–protein interactions.
9.2 Fundamentals of SSFM

9.2.1 Background and Principles

Fluorescence is a process in which an electron from a molecule, atom, or some nanostructures relaxes from an excited state to a ground state by emitting a photon. Molecules that are capable of fluorescence after the electronic transition are often named as fluorescent probes, fluorochromes, dyes, or fluorophores. In this chapter, we use fluorophores to refer to these types of molecules. The fluorescence process mainly consists of three events: excitation from a ground state to an excited state by absorbing an incoming photon, which occurs in femtoseconds; internal conversion, in which the absorbed energy is converted to heat by non-radiative decay from the initial excited state to the lowest vibrational energy level of the excited states in picoseconds; and the return of the molecule to the ground state during which a photon is emitted, which happens in relatively long periods of time, that is, nanoseconds. Since the emitted photons have less energy than the absorbed photon, the fluorescence light is shifted to longer wavelengths. This phenomenon is called the Stokes shift. In fact, the emission spectrum of a fluorophore is often a mirror image of its absorption spectrum, but at lower energies.

Fluorescence microscopy is one of the most powerful tools in modern biological research. The potential of fluorescence as a contrast agent for microscopy was realized by August Köhler in 1904 while he was working on improving the resolution of microscopes by illuminating with short-wavelength light. He realized that biological materials fluoresce when illuminated with ultraviolet (UV) light [42]. The application of a variety of fluorophores has made it possible to identify labeled subcellular components noninvasively and with a high degree of specificity. Like other conventional light microscopy, fluorescence microscopy is constrained by the limitation of spatial resolution due to diffraction. The minimum lateral resolution is proportional to wavelength $\lambda$ and inversely proportional to numerical aperture (NA), i.e., $NA = n \sin(\theta)$, where $n$ is the refractive index in the object space and $\theta$ is the half angle of the largest cone of rays that can enter or leave the optical system. The spatial resolution of conventional high-resolution optical microscopes is not better than about 200 nm laterally and about 600 nm longitudinally. Several methods have achieved spatial resolution beyond the diffraction limit utilizing the specific nature of fluorescence, such as increasing the effective NA (as in 4Pi confocal microscopy) [43–46], introducing spatial variation in the excitation light creating finer spatial features in the image (as in standing wave microscopy) [47–49], using multiple-photon fluorescence absorption or emission mechanisms that lead to nonlinear effects in the light field (as in two-photon microscopy) [50], and selectively quenching the fluorescence from a focal spot to obtain a very small fluorescing volume (as in stimulated emission...
depletion microscopy) [51–53]. Localization of fluorescent molecules with high accuracy is also of great interest and may provide valuable information not accessible even by high-resolution imaging. Two techniques, stochastic optical reconstruction microscopy (STORM) [54] and photoactivated localization microscopy (PALM) [55], exploit parallel localization of sparse emitters by estimating the center of the emission point spread function of the emitters. Fluorescence emission can also be localized in the axial dimension, and nanometer-scale precision can be achieved by utilizing self-interference of light.

It has been known for more than a century that fluorescence emission is modified by nearby dielectric or metal surfaces [56–58]. In the early 1960s, Drexhage determined the fluorescence decay of an organic dye embedded into a lipid layer as a function of its distance above a silver mirror [59]. Later, Lambacher and Fromherz [41] developed a fluorescent localization technique, FLIC microscopy, based on the change in the total emission intensity as a function of distance from a nearby reflecting substrate. The fluorescently labeled object is within an \( \lambda \) of vertical distance from a reflecting surface, so there is little difference between standing waves for different wavelengths within the emission range. This causes the entire emission spectrum to oscillate as the direct and reflected emitted light undergoes constructive or destructive interference depending on the vertical distance. Careful calibration of fluorescence intensity as a function of monolayer distance from the surface is required to achieve nanometer accuracy [40,60] and to measure cellular membranes [61] and molecular motors [62].

When the separation between a fluorophore and the mirror is larger than \( 10\lambda \), even at the same height, the interference between the direct optical path of fluorescent emission and the reflected path from the \( \text{SiO}_2-\text{Si} \) interface results in several oscillations or fringes of interference within the fluorophore emission spectrum. The oscillation in the emission spectrum is a unique signature of the optical distance of the fluorophore to the interface. SSFM utilizes this interference-based modulation of the emission spectrum to determine the precise location of fluorophores above a layered surface (\( \text{SiO}_2-\text{Si} \)) with sub-nanometer accuracy. Small height differences produce shifts in the fringes and changes in the period of oscillation, although the latter are less apparent (Figure 9.1). Here, we note again that SSFM determines the axial location of fluorophores from the spectral oscillations, not from fluorescence intensity variations; therefore, experimental conditions that can influence fluorescence intensity do not affect the result.

### 9.2.2 Physical Model of SSFM

We can model an emitting fluorophore as an oscillating dipole with a random orientation above the \( \text{SiO}_2-\text{Si} \) substrate [36]. All three vectors, the emitter
μ-dipole moment, the wave vector $k$, and the electric field vector $E$, lie in the same plane; this is the plane of polarization of the emitted light (Figure 9.2a). If the environmental factors remain constant, the far-field amplitude of the electric field of a fluorescently emitted wave is proportional to the sine of the angle between the dipole and the wave vector. The emission is therefore nonuniform; a 3D picture of the emission profile of a classical dipole has a donut shape (Figure 9.2b).

To describe the emission pattern of a dipole above a reflecting surface, we consider the intensity and polarization of both the direct and reflected waves. Two coherent waves are radiated from each dipole: one goes directly to the observation point (detector) and the other is incident on the mirror and reflected, propagates in parallel with the direct wave in the far field, and arrives at the same spot on the detector. We further describe the model based on two assumptions: First, we ignore near-field radiation because the SiO$_2$–Si reflecting interface is far away from the dipole. Second, the observation point is in the far field. Therefore, calculation of electrical

FIGURE 9.1
fields is sufficient to calculate total intensity, and only one significant plane wave component is used to describe the electrical field in any direction. Reflections of plane waves at the interface are sensitive to polarization; therefore Fresnel reflection coefficients are used to describe reflection for each polarization.

Thus, the direct and reflected fields are decomposed into two orthogonal components: transverse electric field $E_{TE}$ and transverse magnetic field $E_{TM}$. We find the angular dependence of each field by decomposing the dipole moment into three orthogonal components such that each component lies either within or perpendicular to the plane of incidence. $E_{TE}$ is generated by the perpendicular component and $E_{TM}$ is generated by the two in-plane orthogonal components for both direct and incident fields. The reflected fields are incident fields modified by Fresnel reflection coefficients $R_{TE}$ and $R_{TM}$ at the interface.

Figure 9.3 illustrates the angle of the dipole $\theta$ as well as the plane of incidence defined by the polar angle $\theta_{em}$ and azimuthal angle $\varphi$ of the observation point and z-axis. The propagation directions of the direct, incident, and reflected waves all lie in the plane of incidence. Therefore, the angular dependence of $E_{TE}$ and $E_{TM}$ can be presented as follows:

$$E_{TE}^{\text{dir}} = E_{inc}^{\text{dir}} \propto \sin \theta \sin \varphi,$$

$$E_{TM}^{\text{dir}} = E_{TM}^{\text{inc}} R_{TM} e^{2i\varphi},$$

$$E_{TM}^{\text{dir}} \propto \cos \theta_{em} \sin \theta \cos \varphi - \sin \theta_{em} \cos \theta,$$
Nanoscale Spectroscopy with Applications

$E_{TM}^{inc} \propto \cos \theta_{em} \sin \theta \cos \phi - \sin \theta_{em} \cos \theta$,

$E_{TM}^{refl} = E_{TM}^{inc} R_{TM} e^{i2\phi}$,

where

$\phi = \left(2\pi n/\lambda\right)hc \cos \theta_{em}$

$h$ is the axial position of the dipole

$R_{TE}$ and $R_{TM}$ are Fresnel coefficients for $E_{TE}$ and $E_{TM}$ that take into account the thickness of the SiO$_2$ spacer layer and the wavelength-dependent refractive indices of Si and SiO$_2$. In SSFM, since it is the spectral shape of fluorescent emission that is important rather than absolute emission intensity, we only consider the angular dependence of the electrical fields and use the proportionality sign ($\propto$). The total intensity can be represented by the absolute square of the total electrical fields at the observation or detection point in the far field. The intensity is given as

$I = |E_{TE}|^2 + |E_{TM}|^2$,

where

$E_{TM} = E_{TM}^{dir} + E_{TM}^{refl}$.

The previously mentioned calculation of a dipole emission above a reflecting surface is for a specific direction characterized by $\theta_{em}$ and $\phi$. In the applications of SSFM, samples contain thin layers of fluorophores that are
assumed to be randomly distributed. Thus, the total emission of a mono-
layer of random-oriented dipoles should also be integrated over all possible
angles of $\phi$ and $\theta$. However, the range of polar tilt angles can sometimes be
restricted. We should also integrate over $\sin \theta d\theta$ for light collected by a micro-
scope objective with the maximum collection angle $\theta_{em}^{\text{max}}$. As a result, the total
emission of a monolayer of random dipoles measured with an objective with
maximum collection angle $\theta_{em}^{\text{max}}$ is

$$I_{\text{total}} = \int_{\phi=0}^{\pi/2} \int_{\theta=0}^{\theta_{em}^{\text{max}}} I(\theta, \phi, \theta_{em}) \sin \theta_{em} d\theta_{em} d\phi d\theta.$$  

The model is further modified if there are additional layers between the
dipole and the microscope objective. The dipole can be considered as resid-
ing in a cavity, affecting both the direct and reflected fields in the same
way, and the emission of a dipole in such an environment may be modeled
in two steps: first, we calculate the total fields without the overhead lay-
ers as $E_{0} = E_{dir} + E_{refl}$, and then, the resulting field should be multiplied by the
"cavity coefficient" caused by multiple reflections from the top and bottom
interfaces inside the cavity and the transmission to the objective. The total
field at the objective is

$$E = \frac{E_{0}T'}{1-R'R'^{2}} \cdot \phi = \frac{4\pi n}{\lambda} D \cos \theta,$$

where $R'$ and $T'$ are the generalized reflection and transmission coefficients
for the layers above the fluorophore in the direction toward the objective, $R$ is
the generalized reflection coefficient below the fluorophore, and $D$ and $n$ are
the thickness and refractive index of the cavity.

The previously mentioned expression needs to be calculated for each
wavelength to express the spectral oscillations. The final SSFM spectrum is
composed of the envelope of the free-space fluorescence spectrum and the
oscillatory modulation determined by the axial position. We will discuss the
interpretation of self-interference spectra of SSFM and the fitting algorithm
in Section 9.3.

### 9.2.3 White Light Reflectance Spectroscopy

WLRS offers great precision for determination of the thickness of a trans-
parent spacer layer on a reflecting surface [63–69]. A broadband light source
is focused onto the surface and reflected light is collected. The interference
fringes in the collected spectrum are created by waves reflecting from the
top and bottom interfaces in contrast to the direct and reflected waves in
SSFM (Figure 9.4). The resulting oscillations are based on the total reflectiv-
ity of the stack of dielectric layers. With WLRS, 10–20 $\lambda$ thick films of trans-
parent materials can be measured with 1–2 $\text{Å}$ precision.
WLRS is added to the SSFM to determine the amounts of biomass on the surface by measuring the change of the thickness of the spacer layer. For both SSFM and WLRS, we can only determine optical path length, which is the product of refractive index and physical path length. Deposition of DNA and protein molecules on the surface could change the refractive index, and we should regard the change of thickness with caution. Often lower surface density of molecules results in lower refractive index, and for our cases we can assume the refractive index of the biomaterial to be close to that of SiO$_2$ [70–74]. However, the additional amount of biomaterial on the surface can always be quantified accurately by the change of optical path length.

It needs to be noted that in SSFM, the amplitude of the direct wave and the reflected wave from the SiO$_2$–Si interface is comparable. However, for WLRS, the reflection from air–SiO$_2$ or buffer–SiO$_2$ interface is much less than that of the SiO$_2$–Si interface, resulting in a reduction of fringe contrasts (Figure 9.4). Since the white light source is external, we can increase light intensity to achieve sufficient spectral fringe contrast not to affect the precision of optical thickness determination. We can quantify the amount of biomass and the
height of a monolayer of fluorophores from one spectral measurement by looking at different spectral windows for WLRS and SSFM. For example, we use a broadband LED that is passed through a band-pass filter and a thick spacer layer that results in enough periods of oscillation in each spectrum for data fitting (Figure 9.5).

9.3 Data Acquisition and Analysis

9.3.1 SSFM Setup

SSFM measurements were performed with a system that combines an upright Leica DM/LM microscope and a Renishaw 100B micro-Raman spectrometer. Figure 9.6 is a schematic illustration of the recent SSFM setup built in-house with purchased optical components and instruments. A helium–neon laser with center wavelength of 632.8 nm is used to excite red fluorophores, such as Alexa Fluor 647 and Atto647n, and a diode-pumped solid-state green laser with center wavelength of 532 nm was used to excite green fluorophores, such as Atto532. Dichroics are used in place of beam splitters to maximize excitation and collection efficiency of fluorescence. A 2-axis positioning micro-stage is used to scan samples with a minimum step

FIGURE 9.5
Interference spectra of white light and fluorescence measured at the same time. Dashed line is spectrum of filtered broadband LED, whereas the dotted line is emission spectrum of Atto647n given from the manufacturer.
size of 190 nm and a scanning range of 2.54 cm for each axis. The emission from the fluorophores is collected with a 5× (NA = 0.13) objective and transmitted through notch filters, which block the excitation laser’s wavelength, and is then focused into the slit of the spectrometer, which has an air-cooled CCD camera as the detector. For WLRS measurements, we used normal Köhler illumination with a halogen lamp. This has been replaced with a combination of high-power green–yellow LED and a band-pass filter to allow simultaneous acquisition. Data can be collected manually or automatically using a custom-built application, which controls the lasers, LED, the microstep stage, and the spectrometer.

9.3.2 Fitting Algorithm

Typical SSFM spectra are composed of three parts: the spectral envelope represented by the fluorophore emission profile, the oscillatory interference component, and the noise from the spectrometer. The envelope can be described by a low-order polynomial. Both WLRS and SSFM spectra are fitted with custom algorithms executed with MATLAB®. The algorithms take into account the variation of refractive index of SiO$_2$ within the wavelength span and the complex reflectivity of the underlying Si. Dipole orientation
can also be set as a parameter for fitting. In the fitting procedure, an estimated initial axial position is used to generate an oscillatory curve using the described physical model. Then, the spectrum is divided by the generated curve resulting in the envelope curve, which is fit to a low-order polynomial. If the position is not accurate, the envelope curve is not decoupled from oscillations. The final position is determined iteratively until the envelope spectrum is smooth, free from oscillations, and can be fit to a low-order polynomial with minimum least square error.

The fitting procedure is very fast, which enables real-time feedback within a second during an experiment and allows thousands of spectral measurements to be fit in a short time. In most of our applications, the only variable of interest is the height of a monolayer of fluorophores with respect to the surface. In order to increase the processing speed, the spectral envelope is independently fit with low-order polynomials using linear algebra, leaving the height as the only variable. This procedure dramatically expedited the fitting process and makes the fitting algorithm immune to spectral modifications and any potential quenching of the spectrum.

9.4 Applications

Example 9.1: Determine the Axial Locations of Monolayers of Fluorophores on SiO$_2$–Si Substrates [36]

To demonstrate the validity of SSFM, the positions of monolayers of fluorescein and quantum dots (QDs) above a silicon mirror were measured. Fluorescein isothiocyanate was immobilized on an aminosilane aminopropyltriethoxysilane (APTES)-covered SiO$_2$–Si substrate with the thickness of SiO$_2$ spacer layer being approximately 5 μm. A monolayer of QDs was also prepared on another chip by first silanizing the surface with APTES. QDs were then treated with mercaptoacetic acid to make them hydrophilic and negatively charged at neutral pH, which enables them to electrostatically attach to the aminated surface [75]. The optical thickness of the oxide layer was determined by WLRS. Axial locations of the fluorophores are measured by SSFM regardless of their density. Dipole orientations are assumed to be isotropic in the fitting algorithm. Figure 9.7 shows the schematics of the samples. The WLRS measurement of the chip shows only an additional few angstrom indicating a sparse layer, while SSFM shows the average height of the emitters above the surface to be 3 nm, about half the size of a QD. Compared with smaller molecules such as fluorescein, SSFM determined its position to be within 1 nm from the surface. Hence, axial location of a monolayer of fluorophores can be determined relative to an interface within a few angstrom, even for a sparse layer of fluorophores.
DNA microarrays have been widely used in gene expression profiling, biomarker detection, drug discovery, single-nucleotide polymorphism (SNP) detection, and sequencing, all benefiting from the high-throughput capacity of the technology [76–78]. A routinely used detection process of DNA microarrays and other biosensing technologies is the hybridization of surface-immobilized single-stranded DNA (ssDNA) with solution-phase complementary strands or the binding of dsDNA to target protein molecules. The distance of the probe to the solid surface, the surface hydrophobicity and charge, and physical steric hindrance of the probes influence the accessibility of surface-immobilized molecules for contact with target molecules in solution. The closer the probe molecules are to the solid support, the less likely for the target to reach the probe diffusively. Hydrophobic surfaces can act like a shield for bound molecules positioned close to it because of the associated steric factors and lack of diffusion of the bound molecules [79–83]. A charged surface can either repel or attract molecules through electrostatic interactions [84–86]. It has been known that high DNA probe density does affect hybridization kinetics and efficiency, largely due to electrostatic repulsion between DNA strands and steric hindrance [87–89]. Thus, understanding the conformation of surface-bound DNA probes is of great value not only for the future development of DNA microarrays but also for other nanotechnologies that utilize surface-immobilized DNA oligomers as sensing or actuation agents [90–92].

Some methods were proposed to characterize surface-immobilized DNA probes, such as ellipsometry, optical reflectivity, neutron reflectivity, x-ray photoelectron spectroscopy, FRET, surface plasmon resonance (SPR), and AFM [39]. Most of these experimental techniques characterized the DNA layer by studying its thickness or density. Previous studies helped visualize the conformation of surface-immobilized DNA depending on its density and surface charge. The advantage of SSFM
combined with WLRS measurements is that it not only measures the optical thickness or the bound mass of DNA layers, but it can also provide insight into the conformations of DNA by measuring axial positions of fluorophores that are tagged to a certain position of the DNA strands.

We studied the conformation of surface-immobilized ssDNA and dsDNA by using 50 and 21 bp long oligonucleotides. The 5' end of the first strand of the DNA is amine modified and is covalently linked to the SiO\textsubscript{2} surface via a homobifunctional cross-linker. Fluorophores were tagged either at the 3' end of the immobilized first strand or at the 3' or 5' end of the second complementary strand.

WLRS measurements showed that immobilization of 21 and 50 bp long DNA results in additional optical thickness of 1.0–1.5 and 2.0–2.5 nm, respectively, assuming a refractive index of 1.46 for the DNA [70–74]. As we discussed previously, although the absolute thickness depends on the refractive index, WLRS provides an accurate relative measure of the additional biomass by detecting the difference of optical path length, from which we can monitor DNA hybridization. Adding complementary second strands to 50 bp ssDNA results in an increase in the film thickness by approximately 1.0 nm corresponding to a hybridization efficiency of approximately 50%. The optical thicknesses of the layers are used in SSFM measurements to determine the axial location or the height of the fluorophores. Here, we note that the axial locations of the fluorophores are described as the heights of fluorophores above the surface in SSFM data analysis. The average height values measured by SSFM are an indication of different distributions of fluorophore heights within the microscope focal spot. Figure 9.8a shows the incomplete hybridization for the 50 bp ssDNA with the complementary strands labeled either at the 5' (distal) end or at the 3' (proximal) end. In principle, the maximum heights of the fluorophores are constrained by the contour length of the dsDNA, which are approximately 7 and 17 nm for 21 and 50 bp long dsDNA fragments, respectively. The average heights of the distal end fluorophores are 5.5 nm for 21 bp and 10.5 nm for 50 bp dsDNA, whereas those of the proximal end are 1 nm for 21 bp and 2.5 nm for 50 bp dsDNA [39].

The persistence length of dsDNA is 50 nm in physiological conditions [93–95], so the short dsDNA fragments in our experiments are considered as rigid rods hinged to the surface. Assuming that these rods can freely rotate on hinges, the average angle of the rods to the surface can be calculated from the average distal height and the length of the rods in this case. A more detailed analysis of the calculation of average angle will be discussed in the next example, where the DNA orientation is additionally influenced by external electrostatic forces. Thus, the angles of the 21 and 50 bp long dsDNA to the surface are approximately 40° and 50°, respectively. We also measured the fluorophore heights tagged on the surface-immobilized strand at the 3' (distal) end to study the conformation of ssDNA (Figure 9.8b). Unlike dsDNA, ssDNA is very flexible, often described as random coils [96–99], and little is known about its conformation on surfaces. The height of the fluorophore is about 1 nm for 21 bp ssDNA and 5.5 nm for 50 bp ssDNA, which implies a considerably more extended conformation for the 50 bp ssDNA. When a second
unlabeled complementary strand is hybridized, the surface DNA molecules are composed of two species, unhybridized ssDNA and hybridized dsDNA, both with labels at the distal ends, the average height of which should be somewhere between the distal fluorophore heights of ssDNA and dsDNA. The hybridization efficiency can also be estimated from comparing the average height of the first strand or the second strand both labeled at the distal end, which is around 30%–50% in this experiment and is consistent with WLRS measurements. The results demonstrate that SSFM can be used to estimate the conformation of surface-bound ssDNA and dsDNA: ssDNA can be flexibly coiled on the surface, whereas dsDNA is rigid and angled from the surface.

**Example 9.3: Direct Observation of Conformational Change of a 3D Polymeric Coating**

Surface functionalization is one of the most important components of a solid surface-based biosensor, such as DNA microarrays and SPR biosensors. The direct contact of molecules to the interface as well as other local steric conditions will affect the conformations of the biomolecules,
which potentially influence their functions in binding. The ideal surface chemistry for the functionalization of the solid support should have functional groups for probe attachment, minimal nonspecific adsorption, and stability to environmental changes, and for practical applications, it should be low cost, robust, and easily prepared. More importantly, the surface chemistry should keep probe functionality after immobilization for efficient target capture. Among the existing surface chemistries, 3D polymeric surface coatings are the most promising in meeting these criteria. One such 3D polymeric coating, copoly(DMA–NAS–MAPS), has been used in DNA and protein microarray studies [100] and is now commercially available. Copoly(DMA–NAS–MAPS) has improved performance in terms of probe density and hybridization efficiency of DNA microarrays than organosilanization-based microarray surface chemistries [101]. The understanding of the conformation of the surface-adsorbed copoly in both air and solution is extremely important for interpretation and optimization of the observed superior performance.

SSFM was successfully used to quantify the conformation, specifically the swelling of the surface-adsorbed polymer upon hydration [102]. Short DNA oligonucleotides (23 mer), both ssDNA and dsDNA, were used as probes to measure the swelling of the polymer in the axial direction. ssDNA and dsDNA are labeled with fluorophores at one end and modified with amine groups at the other end, which covalently link to the functional groups (NHS esters) in the polymer. An increase in the axial position of the fluorophores measured by SSFM indicates an increase in the length of polymer chains due to hydration (swelling). SSFM and WLRS measurements are performed before and after hydration of the surface. For labeled ssDNA immobilized on the polymer surface, the measured fluorophore height increase upon hydration (Figure 9.9, Case A) is ~7.5 nm, comparing to no significant change (~0.5 nm) for the same probes on non-swelling epoxysilanized surface, indicating that the polymer swells axially upon hydration. For labeled dsDNA immobilized on the surface (Figure 9.9, Case C), a higher difference in averaged fluorophore height (~14 nm) before and after hydration was seen compared to that of labeled ssDNA (Case A, ~7.5 nm). This indicates that before hydration, the immobilized dsDNA is collapsed onto the surface. After hydration, dsDNA is oriented on the surface, and, therefore, the fluorophore height is higher than that of flexible, randomly coiled ssDNA in Case A. When the labeled ssDNA probes are hybridized with complementary strands on surface, the measured average fluorophore height increase upon hydration becomes ~8 nm (Figure 9.9, Case B). Furthermore, unlabeled ssDNA are hybridized with labeled complementary strand on surface (Figure 9.9, Case D). The measured ~17 nm fluorophore height increase in this case is much higher than that of the hybridization of labeled ssDNA (Case B, 8 nm) and also higher than that of the immobilized dsDNA (Case C, ~14 nm). The difference of the fluorophore heights between Case B and Case D indicates that not all ssDNA are hybridized in the experimental condition. In Case B, the signal originates from the spotted labeled ssDNA and the measured fluorophore height is an ensemble average of the ssDNA and hybridized dsDNA. In Case D, fluorescence signal is from the labeled complementary strands, and the average fluorophore height is only of the hybridized
dsDNA. The difference of the measured fluorophore heights between Case C vs. Case D and Case B vs. Case D suggests that the likelihood of hybridization to probes further away from the surface is higher than to the probes located closer to the surface. WLRS technique shows no significant change of the optical thickness upon hydration of the polymer. This is expected because there is no additional mass introduced to the surface during the swelling of the polymeric surface. The information from this example suggests that the control and manipulation of the conformation of the polymeric coating will have a significant impact in improving the hybridization efficiency of microarrays. These results also indicate that the probes are distributed within the hydrated 3D polymer structure in the axial direction. The study of the conformation of 3D polymeric coatings helped understand and optimize the copolymer and strengthened the earlier analysis of this polymeric coating as a high-quality microarray surface chemistry. Characterization of existing surface chemistries and developing new ones with the help of SSFM will enable designing platforms of optimized surface chemistry, probe immobilization, and target detection for DNA microarray and biosensing applications.
Example 9.4: Manipulation and Quantification of dsDNA Orientation

In recent years, researchers have been exploiting surface-immobilized DNA oligomers for a variety of applications in nanotechnology, such as biosensing and DNA switches and motors [90–92], taking advantage of its sequence designability, chemical stability, and automatic complementary base-pairing property. For biosensing and DNA-based molecular devices, sensitive detection and accurate actuation requires ordering and cooperation of the molecules, such as directed movements and simultaneous response of the molecules [90–92,103,104]. In Example 9.2, we discussed the measurement of surface-immobilized dsDNA conformation, in which the DNA is almost randomly oriented to the surface due to stochastic thermal motions. If we want to detect protein-induced conformational changes of dsDNA, such as the binding of integration host factor (IHF) protein, which causes a \(\sim 160^\circ\) kink of the dsDNA at the binding site [105], such disordering of dsDNA molecules on the surface will make it very difficult to accurately quantify conformational changes specific to IHF. Other platforms, such as “switchable DNA” [106], induce ordering of DNA monolayers immobilized on gold electrodes by applying alternating electric fields. On the switched DNA platform, negatively charged short oligonucleotides are switched between lying and upright positions on oscillations of positive and negative surface potentials. The switching dynamics are sensitive to the intrinsic molecular properties of oligonucleotides, such as structural flexibility, and can be characterized and used as a kinetic signature for detection of hybridization and binding events [105,107].

A similar concept of electric-field-induced ordering is adopted with the SSFM platform, but we utilize a novel electromechanical approach to orient dsDNA on the SiO\(_2\) surface. We functionalize the surface with a highly amphoteric polymer that adopts a net negative or positive charge depending on the buffer pH [108–110]. The polymer also has N-hydroxysuccinimide ester (NHS ester) groups that covalently bond with amine-modified oligonucleotides. The isoelectric point of the polymer was tested to be around pH 6 by electroosmotic flow in a capillary coated by a polymer with identical composition. Lower buffer pH results in a net positively charged surface, attracting dsDNA to the surface, whereas higher buffer pH results in a net negatively charged surface, repelling dsDNA to a higher orientation to the surface [111]. An overview of the manipulation of dsDNA orientation on a charged polymer surface through adjustment of buffer pH and ionic strength is shown in Figure 9.10a.

The controlled orientation of dsDNA on the charged polymer can be interpreted by considering the electrical DNA switching on a gold surface previously mentioned, which was studied extensively by Rant and coworkers [112–115]. A diffuse double layer of counterions accumulates at the interface of a charged surface and an electrolyte solution. An intense electric field (\(\sim 100 \text{ kV/cm}\)) results near the ionic buffer–polymer interface due to the high concentration gradient of mobile charges that accumulate. The characteristic length of this electric field (on the scale of nanometers) is inversely proportional to the square root of buffer salt
FIGURE 9.10
(a) SSFM measurements of 60 bp dsDNA on charged polymer surface. Surface-proximal labeled dsDNA indicate polymer swelling; positively charged polymer collapses onto the negatively charged oxide surface, and negatively charged polymer is repelled from the oxide surface. Fixed charges within the polymer also repel each other and contribute to polymer swelling. The surface-distal and surface-proximal fluorophore heights allow precise quantification of dsDNA orientation: the dsDNA is oriented in a lying position by the positively charged polymer and in a standing position by the negatively charged polymer. (b) Calculated orientations at each pH for NaCl concentrations at 40 and 300 mM. A low salt concentration allows the electric field to penetrate far from the charged polymer to more effectively orient immobilized dsDNA.
concentration. At low ionic strength (10 mM), for instance, the thickness of the double layer is around 3 nm, whereas at high ionic strength (300 mM), it is about 0.6 nm. Thus, the electric field does not cover the entire length of the dsDNA probes, and the electrostatic interactions are confined to the base of the probes. As a result, the charged polymer surface orients the immobilized dsDNA more effectively in low ionic buffers because the electrostatic force is applied to a larger proportion of the dsDNA (Figure 9.10b). To give a more quantitative view of the control of the orientation of surface-immobilized dsDNA by charged polymer surface, we refer to the model proposed by Rant and coworkers [112,113]. The adaptation of the model helps us understand the relationship between the measured average height and average orientation on the SSFM platform. The model also presents a quantitative analysis of the dsDNA probe orientation over a wide range of salt concentrations, which gives further insight into the behavior of the polymeric system.

First, we regard the polymer surface as a charged plane, the surface potential of which varies with buffer pH. Since dsDNA is inherently negatively charged in electrolyte solution, a negatively charged surface repels dsDNA and a positively charged surface attracts dsDNA. This is the principle of induced ordering of oligonucleotides for both gold and charged polymer surfaces. In electrolyte solution, the surface electrostatic potential is screened by redistributed ions and, according to the Gouy–Chapman theory, reduces as a function of distance to the surface with a characteristic Debye length. The Debye length $l_D$ is defined as

$$l_D^2 = \frac{\varepsilon \varepsilon_0 kT}{2nne^2}$$

where
- $\varepsilon$ is the dielectric constant of ionic solution
- $\varepsilon_0$ is the permittivity of vacuum
- $k$ is the Boltzmann constant
- $T$ is the temperature
- $e$ is the elementary charge
- $n$ is the ion density

The Gouy–Chapman equation describes the diffusive potential distribution along the $z$-axis:

$$\Phi(z) = \frac{2kT}{e} \ln \left( \frac{1 + \gamma e^{-\frac{z}{l_D}}}{1 - \gamma e^{-\frac{z}{l_D}}} \right), \quad \gamma = \tanh \left( \frac{e\Phi_0}{4kT} \right)$$

where $\Phi_0$ is the surface potential. Since the contour length of short (<80 bp) hybridized oligonucleotides is much shorter than the persistent length of dsDNA (~50 nm) [93–95], they are modeled as rigid rods with equally spaced (0.34 nm) negative point charges, which can freely rotate around the anchor point on the surface (Figure 9.11). A charge of $\sim 0.24 e$ [116] is assumed per point charge along dsDNA to account for the counterion condensation effect [117]. The electrostatic energy of charges along
Nanoscale Spectroscopy with Applications

The dsDNA depends on their axial location relative to the surface, which is defined as \( h = r \sin(\theta) \), where \( \theta \) is the orientation of dsDNA to the surface and \( r \) is the root-mean-square end-to-end length of the DNA fragment, 

\[
r = \sqrt{2l^2((L/1) - 1 + e^{-l^2/4})},
\]

where \( l \) is the persistent length of dsDNA and \( L \) is the contour length of the dsDNA [118].

Thus, the electrostatic energy of each rod is a function of \( \theta \) and is calculated as the sum of the electrostatic energy of all the charges [113]:

\[
E(\theta) = E_{\Phi_{\psi,\lambda}}(\theta) = \sum_i q_{eff} \Phi(z_i)
\]

The average orientation of dsDNA is a balance between thermal stochastic 3D rotations and the electrostatic force on the dsDNA.

To calculate the average orientation \( \langle \theta \rangle \), without any external electrostatic forces, considering a dsDNA as a rigid rod that rotates freely around the anchor point, we have

\[
\langle \theta \rangle = \frac{\pi}{2} \int_0^{\pi/2} \theta f(\theta) d\theta = 33^\circ
\]

where \( f(\theta) \) is the probability distribution function (PDF) of \( \theta \)

\[
f(\theta) = \frac{\cos(\theta)}{\frac{\pi}{2} \int_0^{\frac{\pi}{2}} \cos(\theta) d\theta} = \cos(\theta), \quad 0 \leq \theta \leq \frac{\pi}{2}
\]

In the presence of varying electric potential, the average orientation is calculated by taking the Boltzmann distribution to calculate the probability density of all the energy levels [113]; thus

FIGURE 9.11

dsDNA is modeled as a rigid rod with an orientation \( \theta \) to the surface. \( r \) is the root-mean-square of the end-to-end distance of dsDNA under experimental conditions. \( h \) is the distance of surface distal end of dsDNA to the surface along \( z \)-axis. The half sphere is drawn to illustrate all the possible states occupied by the distal end of dsDNA by rotating around the anchor point on the surface.
Spectral Self-Interference Fluorescence Microscopy

\[ \int_0^{\pi/2} \theta \cos(\theta) e^{-\Delta E(\theta)/kT} d\theta \]

\[ \int_0^{\pi/2} \cos(\theta) e^{-\Delta E(\theta)/kT} d\theta \]

\( \cos(\theta) \) is the degeneracy factor, representing the stochastic rotations as degenerate microstates, which occupy the same energy level with the same orientation. The potential of a rod for a given orientation is calculated as \( \Delta E(\theta) = E(\theta) - E(90^\circ) \), where the standing rod (\( \theta = 90^\circ \)) is taken as the reference state. Figure 9.12 shows the PDFs of dsDNA orientations for different surface potentials, \( \Phi_0 \). We can see that when \( \Phi_0 \) is positive, most of the dsDNA are at lower orientations, and when surface potential is above a critical positive value, almost all of the dsDNA will be pulled down within 2° from the surface with the higher orientation energy states less accessible and unpopulated. When \( \Phi_0 \) goes from 0 to negative potentials, the PDFs shift to higher degrees with narrower distributions with the low orientation energy states being less probable.

In the experimental data of SSFM, we approximate the mean of dsDNA orientation, \( \langle \theta \rangle \), as \( a \sin \left( \langle h \rangle / r \right) \). To determine the validity of the approximation, we calculate the average of axial height of the dsDNA:

\[ \langle h \rangle = \int_0^{\pi/2} r \sin(\theta) \cos(\theta) e^{-\Delta E(\theta)/kT} d\theta \]

\[ \int_0^{\pi/2} \cos(\theta) e^{-\Delta E(\theta)/kT} d\theta \]
Figure 9.13a and b plot both theoretical values of $\langle \theta \rangle$, calculated $\theta_{cal}$, from average height $\theta_{cal} = a \sin(\langle h \rangle / r)$ as a function of surface potential for 60 bp dsDNA. When the surface potential equals 0 V, $\langle \theta \rangle$ is 33° corresponding to a stochastically rotating rod model. When the positive surface potential exceeds a particular value, most of dsDNA will be pulled down to the surface with the average orientation of less than 2°. The PDF shifts less as the surface potential goes further negative and the mean of $\theta$ plateaus.

(b) Average height of distal fluorophore of 60 bp dsDNA is plotted as a function of surface potential. The proximal end height is assumed to be 5 nm. Since $h$ equals to $r \sin(\theta)$, it follows the same trend as $\theta$, but plateaus earlier because $\sin(\theta)$ varies less when $\theta$ is at higher degrees.

To give a further insight into the working mechanism of the charged polymer surface, we discuss a quantitative analysis and the experimental results of the dsDNA probe orientation over a wide range of salt concentrations. At pH 7.6, when the charged polymer is negatively charged, the measured orientation matches well with the calculated results at low ionic strength, with the maximum orientation exceeding 70° (Figure 9.14). At high ionic strength the effects of negative charge on the polymer become negligible, but the average dsDNA orientation approaches 45°, while the expected average orientation is 33° as calculated previously. The 45° orientation of probes at high ionic strength indicates steric repulsion between the polymer and the base of the dsDNA and between the negatively charged dsDNA themselves. At pH 4.4, when the polymer surface is positively charged, a nearly lying position of dsDNA is induced at low ionic strengths, whereas the orientation once again approaches 45° for high ionic buffer strengths.

In the theoretical model, the steric effects between the polymer scaffold and the dsDNA are not included in the calculations. Thus, the calculated orientations deviate from experimental results and approach 0° for a positively charged surface at low ionic strength and 33° at high ionic strengths. However, the model accurately predicts the ionic strength at which the dsDNA sharply transits from a random orientation to a lying orientation when the polymer is positively charged. This relationship
FIGURE 9.14
(a) Calculated orientations of 60 bp dsDNA probes on a charged planar surface as a function of ionic strength. The steric effects between the polymer scaffold and the dsDNA are not included. The surface potential is taken as −100 mV. (b) The measured orientation of 60 bp dsDNA probes for negatively charged (pH 7.6) and positively charged (pH 4.4) polymeric surfaces at a wide range of concentrations of NaCl.
between the probe orientation and the ionic strength reflects the different mechanisms by which DNA are attracted to a positively charged surface as opposed to repelled from a negatively charged surface [115]. As discussed previously, the average orientation measured by SSFM is a balance between the electrostatic force and stochastic thermal motion. On the positively charged polymer surface, when buffer ionic strength transits from high to low, the Debye length is increased and the electrostatic energy is higher for all dsDNA orientations. In the beginning, the attracting electrostatic force does not exceed the thermal motions of dsDNA for most of the orientational states. As the buffer ionic strength goes down further, the dsDNA, whose orientation passes a threshold where the attracting electrostatic force dominates over the thermal motion are pulled down and captured [115]. For SSFM steady-state ensemble measurements, the measured orientation is a weighted average of the orientations of the captured and un-captured probes. As the ionic strength decreases beneath the threshold value, the electrostatic force is large enough to effectively capture a majority of the dsDNA probes. Therefore, the calculated and experimental average orientations are both seen to switch from freely rotating to a horizontal orientation.

Example 9.5: Detection of Protein-Induced Conformational Changes of dsDNA

The ability to control and quantify surface-immobilized dsDNA conformation makes it possible for us to detect specific conformational changes of dsDNA upon binding with proteins in their native environment on SSFM. The advantages of SSFM over other conventional techniques were reviewed in the beginning of this chapter. Here, we use the protein IHF of Escherichia coli (E. coli) to demonstrate the methodology of SSFM for detecting protein-binding-induced DNA structure distortions. IHF, a ~20 kDa heterodimeric DNA-binding protein, has been shown to induce a specific bend of dsDNA of more than 140° when estimated by gel mobility assays [119] and approximately 160° by x-ray crystallography [10]. IHF is not only required for the integrative recombination of lambda phage DNA into the bacteria genome, it is also involved in diverse cellular functions, such as DNA replication, transcription regulation, and genome packaging [120–122]. IHF recognizes its binding sequence through recognizing the intrinsic conformation (narrowed minor groove) of dsDNA (“indirect readout”) [10,123–125] and functions by causing a sharp bending of dsDNA, bringing separated sequences together [126,127]. It has been shown that IHF binds to its specific sequence with $10^3$–$10^4$ times higher affinity than with nonspecific sequences [125,128,129]. Figure 9.15 shows schematically the detection mechanism on SSFM. We performed a titration experiment and measured the average height of distal fluorophores on 60 bp dsDNA probes with sequentially increasing concentrations of wild-type IHF (Figure 9.16a). Little conformational change was detected for the negative control sequence, and a larger height change was observed in the sequence “IHF 34” versus sequence “IHF 44” (sequences are named by
the position where the IHF consensus binding sequence starts from the surface-proximal end of dsDNA). This is expected because the lower the binding sequence, the larger the change of the fluorophore heights upon binding. Figure 9.16b shows the sequence dependence of distal fluorophore height change after IHF binding, where we can distinguish a two-bp shift of binding locations. Since the charged polymer surfaces are used to covalently link dsDNA and anchor them to the sensor surface and the laser local spot of SSFM is about 10 μm, we can spot or synthesize thousands of different DNA sequences in an array format to study sequence specificity in parallel.

Example 9.6: Apply Dual-Color SSFM to Determine Two Axial Locations of Surface-Immobilized DNA

SSFM utilizes spectral oscillations to locate the distance of a monolayer of fluorophore above a mirror. If the fluorophores are distributed along the z-axis, the measured fluorophore height is the average of all the fluorophores within the focal spot. Theoretically, SSFM can be extended to multiple tags in different spectral windows to determine the axial locations of multilayers of fluorophores, each with a different color. The period of spectral oscillations is a function of the thickness of the spacer layer between the fluorophore and the mirror. Thus, we can engineer the thickness of the spacer so that we get sufficient periods of oscillations in
FIGURE 9.16
(a) SSFM measurements of distal fluorophore heights in a titration experiment for two different 60 bp dsDNA. dsDNA is named by the start location of consensus IHF-binding sequence from the bottom of the dsDNA. For example, dsDNA “IHF 34,” the IHF-binding sequence, starts at 34 bp away from the bottom. “IHF control” does not have IHF-binding sequence. (b) Distal fluorophore height changes after binding to IHF. Each column is the average height change of distal end of 20 spots of dsDNA spotted at 7.5 μM by Bio-Rad MiniArrayer. The lower localized binding sequence results in a larger height change. SSFM is able to distinguish 2 bp shifts in the binding location.
Spectral Self-Interference Fluorescence Microscopy

each spectral window for accurate fitting. Practical application is limited by the availability of thicknesses of SiO$_2$ (thick thermally grown SiO$_2$ layers can take months to grow), spectrally separated fluorophores, and multiband dichroics that correspond to the fluorophore spectral bands. As a proof of concept, we demonstrate co-localization of two axial positions of dsDNA using dual-color SSFM.

Figure 9.17a shows the spectra of two fluorophores: Atto532 and Atto647n. With the help of notch filters and multiband dichroics, we can measure the two spectra at the same time and do fitting with same initial estimation of the distance. One problem is that the tail of the spectrum Atto532 falls in the spectral window of Atto647n (shown in Figure 9.17a), potentially affecting the fitting results. More reliable dual-color measurements are taken sequentially. Due to intensity insensitivity, SSFM measurements are not subject to energy transfer and quenching of fluorophores.

Figure 9.17b shows the measurements of height difference between distal and proximal fluorophore heights on dsDNA. Previously, we have used the height difference between surface-distal fluorophore and surface-proximal fluorophore on dsDNA to quantify DNA conformation on polymer surfaces. Due to the swelling of the polymer upon hydration [102], DNA becomes elevated and fluorophores labeled at the proximal end are an indication of the average height of the polymer surface. Therefore, to determine the orientation of dsDNA, we measure fluorophore heights of two separate spots: one with distal fluorophores and the other with proximal fluorophores. Since the conformation of DNA is affected by surface density, if the two spots have different immobilization densities, the results may not be precise. Using dual-color SSFM, we determine the surface-proximal fluorophore height from the spectrum of Atto647n and surface-distal fluorophore height from the spectrum of Atto532. Thus, we can quantify DNA orientation from measurements of one spot. We measured the orientation of dsDNA spots with different immobilization densities on a neutral polymeric surface with dual-color SSFM (Figure 9.18). When the density of dsDNA layer is too high, negatively charged dsDNA molecules repel each other, resulting in higher orientations. At very low surface densities, the measured average orientation is lower than expected for random rotations. This phenomenon on this neutral charged polymer may be due to a slight positive charge resulting from the hydrolysis of some monomers in the polymer, but needs to be investigated further.

It has been well characterized that high surface density in DNA layers results in reduced efficiency for complementary sequences to penetrate the layer to hybridize with probe sequences [130,131]. However, little is known regarding the effect of dsDNA probe density on the binding rates with proteins. SSFM will be used to study the surface density effects on protein binding to surface-immobilized DNA layers, the results of which will be important for all surface-based biosensors. Moreover, the ability to determine the positions of two different fluorophores also extends the application of SSFM in studies of DNA–protein interactions, as we can label the protein with a fluorophore and determine the binding location of the protein on the DNA sequence as well as the conformational change of DNA from a different fluorophore tagged on the distal end of the DNA.
FIGURE 9.17
(a) SSFM measurements of fluorescence spectra of fluorophores Atto532 (green) and Atto647n (red) on a 17.5 μm thick SiO₂. Dotted lines are spectral envelopes given by manufacture. (b) The height difference between distal (d) end and proximal (p) end of 60-bp dsDNA measured by single-color SSFM and dual-color SSFM. For single-color SSFM, the height difference is obtained by measuring the distal fluorophore height (red (d)) of one dsDNA spot and proximal fluorophore (red (p)) height of another dsDNA. For dual-color SSFM, the height difference is obtained by measuring one dsDNA spot that is labeled with different fluorophores at each end, either the distal end with green fluorophores or the proximal end with red fluorophores, or vice versa. Each height difference is the average measurement of 15 dsDNA spots.
Discussions and Conclusion

This chapter presents a novel self-interference method for determining the axial position of fluorescent markers with high precision. In practical applications, the most suitable systems for analysis with SSFM are monolayers of fluorophores on layered surfaces, such as surface-immobilized DNA monolayers tagged with fluorescent labels or monolayers of fluorescein in a lipid membrane or polymer scaffold on a solid substrate [38,39,127,128,132]. For example, in recent years, DNA hairpins and G-quadruplex have been used as nanoswitches for biosensing and molecular motors [133–139], and they are used for the detection of DNA in low concentrations and cancer biomarkers [103,134]. SSFM can be used to confirm the formation of hairpins of DNA microarrays or the detection of its conformational changes due to the occurrence of binding events. G-quadruplex is found to be formed in G-rich telomere repeats, can inhibit telomerase activity [140–143], and is an active target for drug discovery [144–149]. By careful design of the labeling locations of fluorophores, SSFM can be utilized to detect sequence dependence of ssDNA folding into G-quadruplex or the formation of G-quadruplex through binding with small molecules and proteins [150,151]. It should be noted that when labeled DNA is used as probe for detection on SSFM, no labeling is required for the target, which is still in its native state.
DNA conformational changes induced by DNA-binding proteins can be measured noninvasively in their native environment with SSFM. We give another example of a protein that can induce conformational changes in DNA, TATA-binding protein (TBP). TBP is an essential protein required for transcription initiation of all three eukaryote RNA polymerases [152–156]. TBP recognizes its target binding sites (TATA boxes) also by indirect read-out of the DNA conformation or deformation upon forming a DNA–protein complex [157,158]. In co-crystal structures, TBP was found to unwind the bound double helix by about 120° and cause the DNA to bend toward its major groove by 80° [156,159]. The bent angle is sequence dependent, ranging from <34° to 106° for variant “TATA-box” sequences [160], which is correlated with the stability of the complex and transcription activity [160,161]. Some other studies suggest that the flanking sequences of the TATA box also affect the stability of TBP binding to its target sites [158,162,163]. SSFM, for example, can be used to study the different factors that increase or reduce the stability of TBP–DNA complexes by measuring conformational changes of DNA in a high-throughput way. Combining WLRS and SSFM, we can also advance the study of TBP and its cellular function by studying the relationship between binding affinity and specific bending angles for numerous combinations of TATA-box sequences and its flanking sequences. As mentioned in the SSFM setup section, WLRS and SSFM measurements can be taken at the same time. Combined with custom-designed flow cells, SSFM can be used to perform dynamic measurements of both biomass accumulation and DNA conformational changes, making it possible to detect binding and bending simultaneously.

The versatility of the presented platform makes the system an advanced tool to study more complex DNA–protein interactions. For example, in DNA base excision repair (BER) [164–167], a damaged base is specifically recognized and removed by DNA glycosylate to generate an abasic site. Depending on the initial events, the repair patches may be single nucleotide (short patch) or 2–10 nucleotides (long patch). For the short patch repair, the phosphodiester bond at 3′ of the abasic site is cleaved by glycosylate, and the 5′ bond is incised by APE1 endonuclease, which then recruits DNA polymerase to fill the gap that is ligated by a DNA–ligase complex. During the process of BER, the conformation and flexibility of DNA changes with the binding of different enzymes, which kinks the DNA to different angles [168–175]. Through careful design of experiments, the SSFM platform can potentially detect DNA BER through direct detection of DNA conformational changes and protein-binding events. Monitoring DNA repair is an example of applying the SSFM platform to study more challenging DNA–protein interaction processes. The application of polymer-functionalized surfaces and microarray sensor formats adds the capacity of parallel detection of thousands of sequences. SSFM provides critical information required to move DNA interfacial applications forward. What’s more, the quantification of DNA conformations and conformational changes, through integration with new
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surface functionalization techniques and label-free detection, provides critical information to understand DNA–protein interactions in their native environment, allowing the SSFM platform to play a unique and productive role in emerging biotechnological fields.

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