

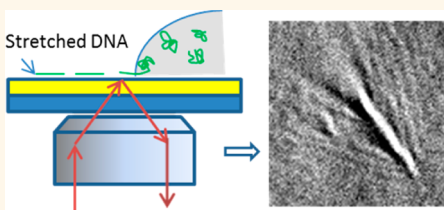
Plasmonic Imaging and Detection of Single DNA Molecules

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ABSTRACT The capability of imaging and detecting single DNA molecules is critical in the study, analysis, and applications of DNA. Fluorescence imaging is a widely used method, but it suffers from blinking and photobleaching, and fluorescence tags may block or affect binding sites on DNA. We report on label-free imaging of single DNA molecules with a differential plasmonic imaging technique. The technique produces high contrast images due to the scattering of surface plasmonic waves by the molecules and the removal of background noises and interference patterns, allowing for quantitative analysis of individual DNA molecules. Simulation of the images based on a scattering model shows good agreement with the experiment. We further demonstrate optical mapping of single DNA molecules.



KEYWORDS: single molecule analysis · surface plasmon resonance microscopy · label-free imaging · optical mapping · λ -DNA

Optical microscopy has enabled many scientific discoveries and become an indispensable tool in research, clinical, and industry laboratories.^{1–3} While powerful, traditional optical microscopy has limited contrast for imaging optically transparent samples, such as biological samples. To enhance imaging contrast, various strategies, including fluorescence labeling, have been developed.^{4–6} Fluorescence imaging has revolutionized biological research, but it requires extra sample preparation steps and may distort the native properties of the molecules. Fluorescence imaging also suffers from blinking and photobleaching, making it difficult to quantify the image intensity and study single molecules over a long time.^{7–9} In addition, because of relatively weak fluorescence emission, the fluorescence imaging speed is relatively slow, which is not suitable for imaging fast biological processes. Here we demonstrate for the first time label-free optical imaging of single biological molecules, DNA, using a differential plasmonic imaging technique.

The ability of imaging or visualizing single DNA molecules is critical for studying the biophysical and biochemical properties of DNA and for developing various applications.^{10–12} An important example is optical mapping, a technique for obtaining

high-resolution genome wide restriction maps of single DNA molecules.^{13–15} The maps serve as a “barcode” or “finger print” for sequencing the DNA of an organism. The current optical mapping technique relies on fluorescence microscopy. In addition to the limitations mentioned above, labeling DNA with fluorescent dyes, such as YOYO-1, is found to elongate and twist the native structure of DNA and affect the charge property of DNA.^{16,17} Label-free techniques could eliminate these effects and provide additional information because they measure the intrinsic physical characteristics of DNA instead of the labels. Atomic force microscope (AFM) is a powerful label-free technique for imaging single DNA molecules, but it is slow, and the scanning AFM probe may perturb the DNA samples.^{18–20} Surface plasmon resonance is a label-free technique that has been widely used to study molecular bindings.^{21–24} Recently, imaging of single viruses with high-resolution surface plasmon resonance microscopy has been demonstrated by us.²⁵ However, a capability of imaging and detecting single DNA molecules has not yet been reported.

In the present work, we demonstrate label-free imaging of single DNA molecules with a differential surface plasmon resonance technique (DSPR). Plasmons are

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excited optically at the surface of a gold film, and the associated propagating plasmonic waves are scattered by the sample molecules on the surface, creating a plasmonic image with contrast many orders of magnitude greater than the conventional bright field optical microscopy image. Noises and unwanted interference patterns from the optical system are removed with the differential method, which further enhances the image contrast and makes it possible to image and detect single DNA molecules. We study the image contrast by varying the plasmonic wave propagation direction relative to the orientation of the DNA strands stretched on the surface, and compare the DSPR images with theoretical simulated and fluorescence images. Furthermore, we demonstrate optical mapping of single DNA molecules with the differential plasmonic imaging technique.

RESULTS AND DISCUSSION

Differential Surface Plasmon Resonance Imaging. Figure 1 illustrates the basic principle of DSPR imaging system (see Methods for details). Incident light is directed onto a gold film *via* an optical microscope objective, and the reflected light is imaged with the same objective. When the incident angle is tuned to a proper value, surface plasmonic waves are excited propagating along the surface. Consequently, the image of the reflected light has reached the lowest intensity as the energy of the incident light is transferred to the plasmonic waves. When an object is present on the surface, it scatters the plasmonic waves and creates a high contrast image, which has been used to successfully image viruses and nanoparticles by us.^{25,26} However, the contrast enhancement is not high enough to image a single DNA molecule. Instead of enhancing the image intensity, an alternative way to improve image contrast is to reduce background noises and

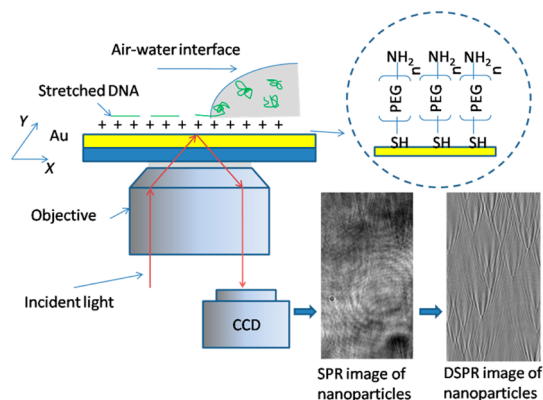


Figure 1. Schematic illustration of the differential surface plasmon resonance (DSPR) system for DNA imaging. λ -DNA molecules are stretched on a modified gold surface by the capillary force of a receding air–water interface. Images of 80 nm platinum nanoparticles on bare gold obtained with SPR and DSPR are shown to illustrate the superior contrast enhancement of the DSPR. A detailed description of the DSPR system can be found in the Methods.

interference patterns. This is achieved with a differential method.

In DSPR, the sample is translated laterally (along x or y directions) back and forth between two positions, and two images are recorded at the two positions. A differential image is obtained by subtracting one image from the other image. The differential image removes all the noises and interference patterns of the entire optical system other than the sample itself, leading to superior image contrast. The principle can be described with the equations given below. We denote the image before the translation as $I(x,y)$ as

$$I(x,y) = I_S(x,y) + I_B(x,y) \quad (1)$$

where $I_S(x,y)$ is the SPR image of the sample, and $I_B(x,y)$ is the background intensity due to all the sources unrelated to the sample. For weak scatters of the plasmonic waves, such as DNA, I_S is weak and overwhelmed by I_B . The image after the translation of Δx is

$$I'(x,y) = I_S(x + \Delta x, y) + I_B(x, y) \quad (2)$$

and the DSPR image is given by

$$D(x,y) = I'(x,y) - I(x,y) = \left(\frac{dI_S}{dx}\right)\Delta x \quad (3)$$

which is the derivative of the sample image along x -direction, and more importantly, the background intensity is removed. The contrast enhancement is illustrated in Figure 1 by comparing the images of 80 nm platinum nanoparticles on bare gold obtained with DSPR and conventional SPR. The conventional SPR cannot resolve the individual nanoparticles clearly, but the DSPR clearly reveals each nanoparticle as a bright spot with a long “tail”. We will return to the “tails” later.

DSPR Image of DNA Molecules. For optical mapping, it is necessary to stretch the individual DNA molecules. In the present work, double strand λ -DNA molecules were stretched on a gold surface modified with SH-PEG-NH₂ (MW 1000) by a receding air–water interface method.^{27–30} After imaging the DNA molecules with DSPR, the molecules were labeled with YOYO-1 and imaged with fluorescence microscopy. Figure 2A and 2B show typical DSPR and fluorescent images of individual λ -DNA molecules. Stretched DNA molecules (solid red arrows) were imaged as lines with “tails” in the DSPR image. These “tails” are due to the scattering of the surface plasmonic waves by the presence of DNA molecules. The DSPR and fluorescence images of the same sample are in excellent agreement with each other (see Figure 2), and the results are also consistent with the fluorescence images of λ -DNA published in literature.^{27–30} The contour length of single λ -DNA molecules as revealed by DSPR is $\sim 17 \mu\text{m}$, which includes the scattering “tails”. The length of the “tails” is related to the propagation length of the surface

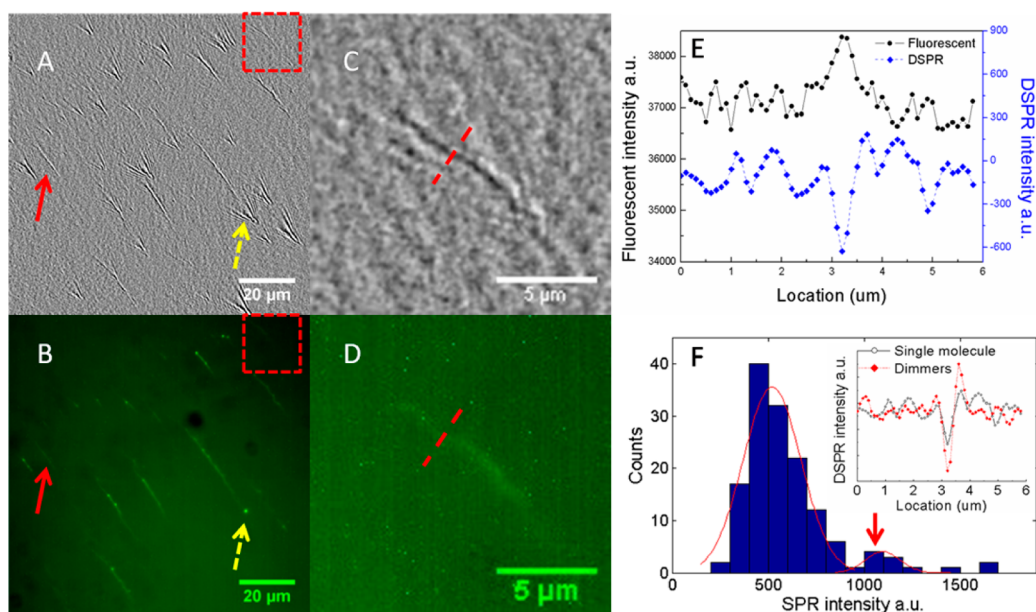


Figure 2. Images of stretched λ -DNA molecules on gold surface. (A,B) Typical DSPR and fluorescent images of DNA molecules. (C,D) Zoomed-in images of single DNA molecule in square areas. (E) Fluorescent and DSPR intensity profile at the same location on single DNA molecule in (C) and (D). (F) The histogram of the DSPR intensities and Gaussian fitting results of DNA molecules. Inset is typical DSPR intensity profile of single DNA molecule and dimers.

plasmonic wave, which can be corrected with a deconvolution method detailed later. Some of DNA molecules are coiled (dashed yellow arrows), which appeared as bright spots in the fluorescence image. In contrast, the coiled DNA molecules appeared as pairs of dark and bright spots with “tails” in the DSPR images.

The DSPR images were acquired at an exposure time of 3 ms with a $256 \times 256 \mu\text{m}$ full field of view, which are free of blinking or photobleaching effects. In contrast, an exposure time of 0.5–1 s was required to acquire a high quality fluorescence image with the same field of view. Note that the exposure time of DSPR image scan can be further shortened using faster cameras. This high temporal resolution is important for studying fast processes.

DSPR Intensity of Single DNA Molecules. Figure 2C and 2D are zoomed-in DSPR and fluorescence images of a stretched DNA molecule shown in Figure 2A and 2B. Note that the curved shape of the DNA was revealed by both the DSPR and fluorescence images. Cross-sectional intensity profiles of the DNA molecule were plotted in Figure 2E. The FWHMs (full-width at half-maximum) were found to be ~ 300 nm for both the DSPR and fluorescence image plots, which is close to the diffraction limit of the optical system (230 nm theoretically). The SNR (signal-to-noise ratios) of the DSPR image is higher than that of the fluorescent image. In addition, we observed that the fluorescent image contrast blinked and decreased over time because of photobleaching effect, while the DSPR image contrast is highly stable.

Unlike fluorescent images, the DSPR image contrast measures the intrinsic mass density distribution of DNA

molecules, which provides quantitative information about the sample molecules. We measured the DSPR image intensities of the individual DNA molecules and constructed a histogram (Figure 2F). The histogram can be approximately fit with Gaussian distributions with a mean intensity of 520 au, which measures the average mass of a single DNA molecule. A small peak appears at an intensity twice of the main peak (mean intensity: 1097 au) in the histogram, which is consistent with the formation of DNA dimers as DSPR image intensity is basically proportional to the mass of molecules.²⁵ Intensity profiles of typical single DNA molecule (black line) and dimers (red line) were plotted as the inset in Figure 2F.

Scattering Pattern in DSPR Imaging. Different from the isotropic nanoparticles,²⁵ DNA is anisotropic. Consequently, the scattering of the propagating plasmonic wave by a DNA molecule depends on the orientation of the DNA molecule relative to the plasmonic wave propagation direction. Figure 3A, 3B, and 3C show the DSPR images of the same molecules with surface plasmonic wave (indicated by red solid arrows) propagating along three different directions, parallel, 45° tilted, and perpendicular to the linear direction of a DNA molecule (indicated by yellow dashed arrow), respectively. The “tail”-shape scattering patterns rotated with the change in the plasmonic wave propagation direction.

To better understand the scattering pattern, we modeled the DSPR images of stretched DNA molecules by the superposition of a planar plasmonic wave and cylindrical scattered waves (see “simulation of DSPR image” in Supporting Information for details).

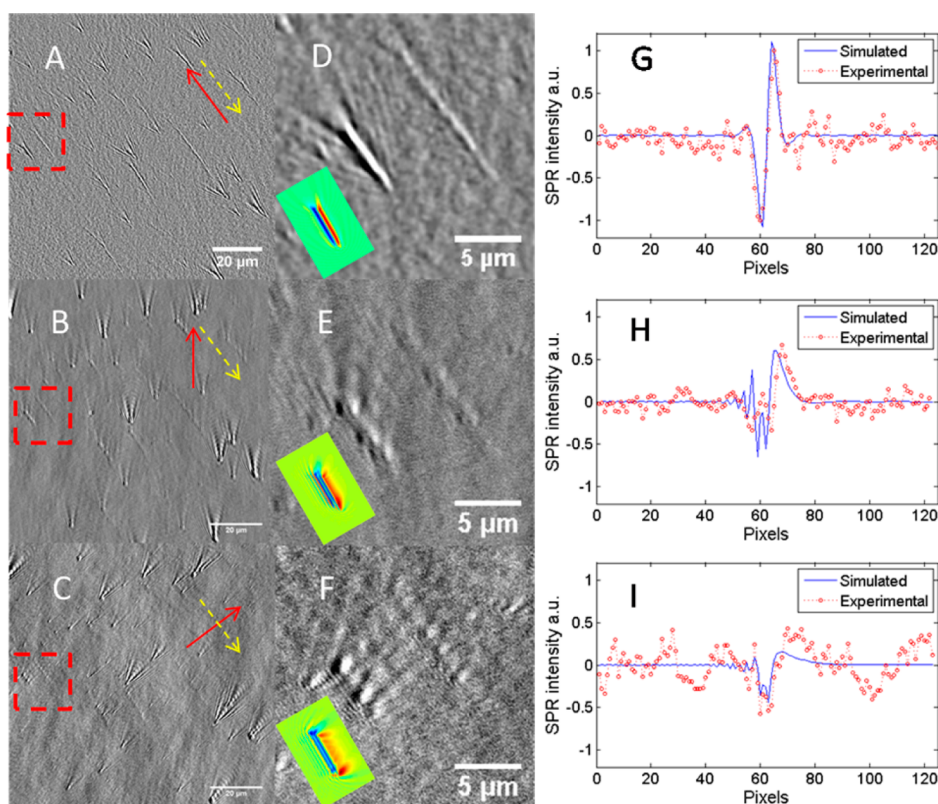


Figure 3. DSPR images taken under different surface plasmonic wave propagation directions: (A) parallel, (B) 45° tilted, and (C) perpendicular to stretching direction. (D,E,F) Zoomed in images of square areas. (G,H,I) The cross sectional intensity profiles of simulated and experimental SPR images. The dashed yellow arrows and solid red arrows indicate the stretching direction and surface plasmonic wave propagation direction.

Figure 3D, 3E, and 3F are the zoomed in images of the DNA molecule marked by dashed squares in Figure 3A, 3B, and 3C, and the inset shows the corresponding simulation results. The simulation reproduced the unique scattering patterns of the experimental images. The close agreement between the experimental and simulated images is more clearly shown in the cross-sectional intensity profiles (Figure 3G, 3H, and 3I). The best image contrast was obtained with the plasmonic waves propagating parallel to the DNA orientation, and the worst contrast occurred in the perpendicular direction.

DNA Measurement. The unique scattering pattern or “tails” of the plasmonic waves helps distinguish DNA molecules from other features on the surface, but it affects the accuracy of measuring the length. In order to improve the accuracy, we developed an image deconvolution algorithm to correct the scattering pattern from the DNA images. In the deconvolution algorithm, the measured DSPR image of a DNA is a convolution of the “true” DNA image with the DSPR image of a point scatterer (as the point spread function, or PSF). In the present work we used the DSPR image of a 40 nm-polystyrene nanoparticle as the PSF of imaging system. This approximation is reasonable because the size of the nanoparticle is much smaller than the wavelength of the surface plasmonic wave

(400–500 nm). Smaller nanoparticles could also be used, but the images would be noisier.

DNA images free of scattering patterns were obtained by performing deconvolution of the original DNA image with the PSF describe above (see Figure S2 in Supporting Information for details).³¹ Figure 4A shows the deconvolved image of Figure 2B. By comparing the images obtained after (Figure 4B) and before (Figure 4C) deconvolution, it is clear that the deconvolution algorithm effectively removed the scattering patterns. The “ends” of DNA were defined to at the half-maximum intensity of the line profile along the DNA of the “tail”-corrected image, with which the lengths of stretched DNA molecules were measured from the deconvolved images.

We measured the length of multiple stretched DNA molecules and constructed a histogram in Figure 4D. By fitting the histogram to Gaussian functions, an average length of about 14.6 μm was observed for single stretched λ -DNA molecules, which is 15% shorter than the theoretical full contour length of the molecules. This histogram is consistent with the intensity histogram in Figure 2F, both showing mainly single λ -DNA molecules stretched on surface. Compared with the fluorescent method, the DNA length measured by DSPR is free of unwanted elongation due to intercalation of dye molecules. Furthermore, since

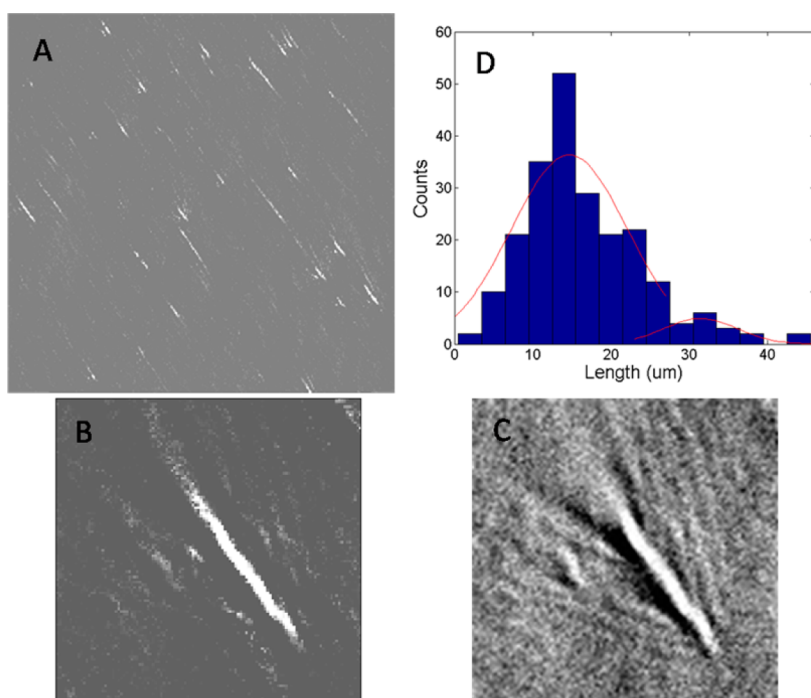


Figure 4. Image deconvolution and length measurement of λ -DNA molecules. (A) Deconvolved DSPR image of DNA molecules. Image of the same DNA molecule, after (B) and before (C) deconvolution. (D) Length distribution of stretched λ -DNA molecules measured by DSPR imaging, and the Gaussian fitting results.

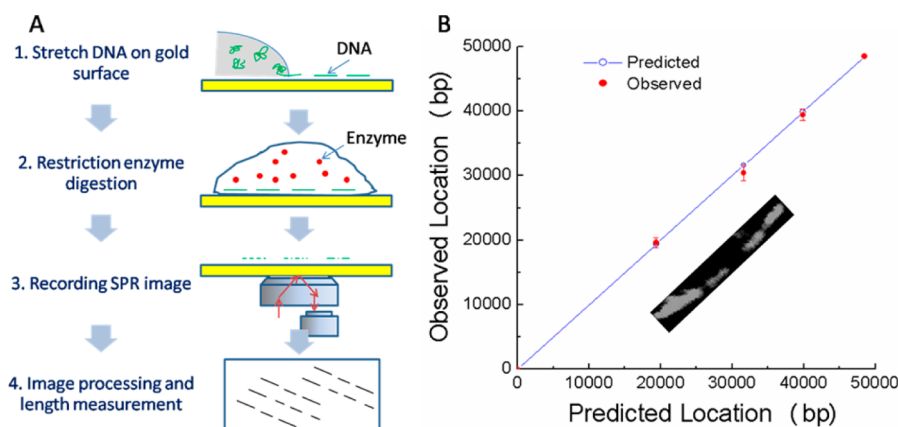


Figure 5. Optical mapping with DSPR imaging. (A) Procedures in optical mapping with DSPR imaging. (B) Restriction map of λ -DNA by *Sma*I restriction endonuclease and DSPR image of DNA fragments after digestion.

no pretreatment or modification of DNA molecule is required for DSPR imaging, the native structure of DNA is preserved, which is important for the study of DNA interactions with other molecules, such as proteins.

Restriction Map of DNA with DSPR Imaging. We anticipate that the capability of label-free imaging and detection of single DNA molecules have many applications. We demonstrate here optical mapping of DNA using *Sma*I restriction endonuclease as an example. Figure 5A illustrates the procedures for obtaining restriction maps with DSPR. The DNA molecules are cut into small DNA fragments by the enzyme, which can be directly imaged with DSPR without further labeling or treatments. From the images, lengths of the DNA fragments

were measured, and consequently, cutting sites were determined and aligned according to the length ratio among DNA fragments.

Figure 5B shows the observed cutting sites after *Sma*I digestion, and the predicted cutting sites obtained from the manufacturer. The inset shows four DNA fragments after digestion, as expected for the enzyme cutting. However, some molecules were cut into only 2 or 3 fragments, depending on the cutting sites activity after DNA stretching. The cutting sites were determined by assembling the ratio of fragment lengths from different molecules. The observed cutting sites fit well with the predicted ones. The DSPR imaging resolution is about 300 nm, and thus the mapping

resolution is about 1000 bp (assume a 0.34 nm/bp ratio for stretched DNA molecule without fluorescence labeling). The DSPR imaging technique is fast and compatible with micro- and nanofluidic devices, which are attractive for high throughput optical mapping of DNA.^{13–15}

CONCLUSIONS

We have developed a differential plasmonic imaging technique to achieve high image contrast of optically transparent molecules. Using the technique, we have demonstrated for the first time label-free imaging and accurate length measurement of single DNA

molecules. The DSPR images of DNA molecules exhibit unique scattering patterns, arising from the scattering of the surface plasmonic waves by DNA molecules. Both experiment and simulation showed that the contrast and scattering patterns of the DSPR image of DNA depend on the orientation of DNA molecules relative to the propagation direction of the plasmonic waves. The capability of label-free imaging of single DNA molecules has been used to create restriction maps of DNA. DSPR imaging of DNA molecules is label-free, fast, and quantitative, making it suitable for single molecule DNA analysis.

METHODS

Materials. λ -DNA (clind 1 ts857 Sam 7) (48 502 bp), TE buffer (1 \times , pH 8.0), and *Sma*I digestion enzyme were purchased from Invitrogen. YOYO-1 dye (1 mM in DMSO) was purchased from Molecular Probes. Thiol-PEG-Amine (HS-PEG-NH₂, MW 1000) was purchased from Nanocs (Boston, MA). 2-Mercaptoethanol was purchased from Gibco.

Surface Modification. Coating SH-PEG-NH₂ Self-Assembled Monolayer on Gold Surface. The SPR chips were BK7 (from VWR, www.vwr.com) glass coverslips coated with 2 nm chromium and then 47 nm gold. Each chip was rinsed with deionized water and ethanol, and then blown dry with nitrogen gas. The chip was then further cleaned with hydrogen flame and immediately submerged in 0.5 mM HS-PEG-NH₂ water/ethanol (1:1) solution. After being left in the solution for 24 h in the dark, the chip was taken out of the solution and rinsed with deionized water and ethanol and then blown dry with nitrogen gas.

DNA Fixation. Stretching DNA on Coated Gold Surface. Before stretching, λ -DNA was restored to its linear form by heating to 65 °C for 10 min followed by quick cooling in ice–water bath, according to the manufacturer's instruction. λ -DNA was stretched by the capillary force of a moving air–water interface, using a similar method as molecular combing.^{27–30} A 2 μ L drop of λ -DNA (5–50 ng/mL, in 1 \times TE buffer, pH 8.0) solution was deposited onto the edge of a clean glass coverslip and carefully placed onto the top of the SH-PEG-NH₂ modified gold surface. The drop spread immediately as the cover glass and the gold sealed together. After waiting for 2 min, the cover glass was slid away, and the gold surface was rinsed with deionized water thoroughly and blown dry with nitrogen gas.

DSPR Setup. The DSPR was based on the Kretschmann configuration³² using a high numerical aperture oil immersion objective (NA = 1.49) and an inverted microscope (Olympus IX81), an approach similar to that by Huang *et al.*³³ SPR chip was placed on the objective with index-matching liquid. A 680 nm 15 mW p-polarized free-space super luminescence diode (from SUPERLUM, Ireland) was used as the light source. The incident angle of the light was adjustable by a motorized translation stage (Thorlabs, Newton, NJ). A CMOS camera (ORCA-Flash4.0 from Hamamatsu, Japan) was used with the system for recording the SPR image. A motorized XY stage (Ludl Electronic Products, Ltd., Hawthorne, NY) was incorporated on the microscope to translate the sensor chip. The system can obtain high-resolution distortion-free images with diffraction-limited spatial resolution in the transverse direction and near diffraction-limited spatial resolution along the plasmonic wave propagation direction.

DSPR Imaging. The incident angle of the light beam was adjusted to the surface plasmon resonance angle at which the reflection imaged by the camera reached a minimum intensity. Surface plasmonic wave propagating direction was tuned by changing the direction of incident light. Sampling rate was set at 3 ms/frame with the Hamamatsu camera. Two SPR images were recorded before and after laterally moving the sensor chip by a small distance (~100 nm) with the motorized

XY stage, and the differential image was used as the resulting DSPR image to increase the image contrast (sensitivity).

Fluorescence Imaging. After being imaged by DSPR, stretched λ -DNA was stained with YOYO-1 fluorochrome (300 nM in TE buffer containing 20% 2-mercaptoethanol, pH 8.0) for 2 h in the dark. Fluorescent images were taken in imaging buffer (100 nM in TE buffer containing 20% 2-mercaptoethanol, pH 8.0), using the mercury lamp with a filter set of 450 \pm 50 nm illumination band and 510 \pm 50 nm emission band. Exposure time was chosen to be 0.5–1 s to maximize the signal noise ratio.

Restriction Endonuclease Digestion of DNA. Surface stretched DNA molecules were digested by 40 μ L of 1 \times restriction buffer (manufacturer recommended) containing 10–20 units *Sma*I restriction endonuclease. Surfaces were incubated in a humidified chamber for 1 h at 30 °C. After digestion, the overlying buffer was removed, and the chip was rinsed with deionized water.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Simulation of DSPR image and deconvolution algorithms for removing interference patterns. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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