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Dual Order Snapshot Spectral Imaging of Plasmonic Nanoparticles

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Abstract

The development of truly scalable, multiplexed optical microarrays requires a detection platform capable of simultaneous detection of multiple independent signals in real-time. We present a technique we term dual order snapshot spectroscopic imaging (DOSSI) and demonstrate that it can be effectively used to collect spectrally-resolved images of a full field of view of sparsely located spots in real-time. Resonant peaks of plasmonic gold nanoparticles were tracked as a function of their surrounding refractive index. Measurement uncertainty analysis indicated that the spectral resolution of DOSSI in the described configuration is approximately 0.95 nm. Further, real-time measurements by DOSSI allowed discrimination between optically identical nanoparticles that were functionalized with two homologous small molecule ligands that bound to the same protein, albeit with different affinity, based purely on their different molecular interaction kinetics— a feat not possible with slower raster-type hyperspectral imaging systems, or other darkfield optical detection systems that solely rely on end-point measurements. Kinetic measurements of plasmon bands by DOSSI can be performed with a relatively simple optical system, thereby opening up the possibility of developing low-cost detectors for arrayed plasmonic diagnostics.

1. Introduction

In the past decade, nanostructured materials of noble metals – typically gold and silver – exhibiting localized surface plasmon resonance (LSPR) have been used for label free optical detection of biomolecular interactions [1–7]. Advantages of LSPR include the fact that it is a label-free detection method that is based on the change in the local refractive index that accompanies binding of a receptor to its ligand – the analyte of interest. This increase in the local refractive index modulates the energy of the plasmon resonance, resulting in a red-shift of the measured LSPR spectrum. Additionally, LSPR spectra are independent of the incident angle of excitation unlike conventional planar SPR. The measured spectra are wavelength-resolved, so that a relatively simple optical detection system with no moving parts can be used for LSPR.

Recently, LSPR has been multiplexed for the detection of multiple, biomolecular interactions on a surface. There are two approaches for multiplexed LSPR detection: (1) *spectrally encoded arrays* in which more than one type of spectroscopically distinct nanoparticles are functionalized with different receptors and the receptor-functionalized nanoparticles are randomly immobilized on a surface or in suspension; detection of a specific

biomolecular interaction then requires imaging of individual NPs and identification of the interaction by the plasmon band of that NP [8]; (2) *spatially encoded arrays* in which a single type of nanoparticle is immobilized on a surface and then different receptors are spatially patterned on the surface; a change in the plasmon band of a spatially demarcated region is then indicative of binding of a specific analyte to the receptor [4–7]. Both spectrally encoded and spatially encoded LSPR arrays require imaging individual nanoparticles over the entire array. Although some of these multiplexed methods exhibit detection sensitivity at comparable levels to current single analyte LSPR sensors [9], spectral multiplexing often involves significant loss of temporal resolution. This is because most spectral imaging techniques collect an entire three-dimensional data cube - two spatial and one spectral. Since photodetectors have at most two dimensions of pixels, collecting a 3D data cube requires scanning the array in time, either physically rastering the stage to collect the missing spatial dimension, or by scanning wavelengths over time to compile the spectral dimension. As a result, the ability of the LSPR sensor to monitor the kinetics of binding events is severely restricted. Achieving the full analytical potential of multiplexed LSPR microarrays hence requires a detection platform capable of simultaneous detection of multiple independent LSPR signals in real-time.

Motivated by the need for a methodology that would allow plasmonic imaging of noble metal nanoparticles on a surface with reasonable spectral, spatial and temporal resolution, we examined the possibility of using compressive detection scheme to achieve this goal. Spectral compression techniques do not rely on collecting the entire three-dimensional data cube, but instead rely on compressing one or more of the data dimensions in such a way that a two dimensional data set can be collected and used to infer information about the entire data cube. For example, compressive detection schemes have been demonstrated which combine two data dimensions (one spatial and one spectral) into one dimension on the detector, and are then reconstructed in post-processing[10]. This technique has been successful in classifying fluorescent beads from snapshot images collected under broadband illumination[11].

In this paper, we describe a new imaging technique that is similar in spirit to the spectral compression. However, in this approach instead of compressing the wavelength dimension, we compress along one spatial dimension taking advantage of the null signal between array spots. Wavelength-resolved spectra are collected simultaneously from an entire field of scattering nanoparticles by imaging the field of view onto a diffraction grating while collecting both the zero-order and first-order diffraction modes with a charge-coupled device (CCD). We term this technique *dual-order snapshot spectral imaging* (DOSSI). The zero-order image provides information regarding physical alignment of the image, thereby allowing the proper deconstruction of spectral information from the first-order diffracted mode.

This paper reports two sets of experiments that illustrate the utility of DOSSI. First, to ascertain the accuracy of spectral measurements using the DOSSI system, a field of view containing plasmonic gold nanorods was analyzed as a function of the surrounding refractive index. The peak wavelength of the resonant peaks of the nanorods were tracked as they red-shifted due to the increase in the RI of the surrounding medium. Second, to demonstrate its utility for detection of biomolecular interactions, DOSSI was used to track the temporal change in the plasmonic bands of immobilized nanorods that were conjugated with two different receptors that target the same analyte, but with different kinetics.

2. Materials and Methods

2.1 DOSSI System

The foundation of the DOSSI system is a Zeiss Axiovert 200 inverted microscope, configured as shown in Figure 1. Light from an integrated 100W halogen source is incident on the sample via an oil-immersion darkfield condenser (DFC) (NA 1.2). Samples are mounted in a flow cell (Bioptechs FCS3) that is attached to the microscope stage. Scattered light is collected by a 100X Plan-neofluar® (Zeiss) objective iris (OBJ) (NA adjustable 0.7 to 1.3). For real-time observation, an adjustable beam-splitter (BS) can then be introduced into the light path, sending the image to the eyepieces. A final adjustable mirror acts a selector that allows the light to either be directed to a color CCD (Photometrics Coolsnap Color CS) or continue to the grating (150 lines/mm). After being dispersed by the grating, the light is collected by the detection CCD – a Roper Scientific Spec-10 cooled (1340×400 pixels). Both the detection CCD and the grating are constituent components of an Acton SpectraPro 2150i. The inset in Figure 1 shows a representative darkfield image of gold nanorods collected by the color CCD.

The setup is similar to that described by Yeung et al[12, 13] and consists of a conventional slit imaging spectrometer with two important differences. First, a grating is selected that has a relatively coarse line pitch. This reduces the dispersion of the grating, and thereby effectively reduces the spatial extent of the dispersed light when it reaches the CCD detector. For typical spectroscopy applications, optimal resolution is achieved by using a grating with the finest line pitch possible such that the wavelength range of interest of the first diffracted mode of the image covers the full extent of the detector pixels[14]. By using a coarser grating, however, we sacrifice spectral resolution to allow concurrent collection of both the zero-order image and the first order diffracted mode of the image on the same CCD. The simultaneous collection of both diffraction orders allows spatial registration of the image spectral content, which is necessary for accurate reconstruction of LSPR peak shifts.

The second essential characteristic of the DOSSI setup is that the entrance slit is dramatically widened. In conventional slit-imaging spectroscopy, optimum spectral resolution is achieved by matching the size of the entrance slit to the pixel pitch of the detection CCD. This minimizes convolution of the measured spectra which would otherwise be caused by overlapping light being collected by adjacent detector pixels. However, we have shown previously that the width of the entrance slit can be a source of error when measuring spectral density of diffraction limited spots in darkfield due to the spatial distribution of light within the spot[14]. The effect this has on overall spectral resolution is mitigated by virtue of imaging sub-wavelength objects under darkfield illumination. The spatial extent of each spot is essentially self-limiting, because its size is determined by the numeric aperture of the collecting optics. For single particle measurements, we showed that the optimal entrance slit width is $150 \mu\text{m}$. For DOSSI, we opened the entrance slit to its full extent at 2.5 mm. Under 100X magnification, this provides a field of view approximately $25 \mu\text{m} \times 70 \mu\text{m}$ that is spatially and spectrally addressable.

2.3 Gold Nanorod Synthesis

Gold nanorods were chemically synthesized by seed-mediated literature procedures as reported previously [4, 15, 16]. Spherical gold seed particles were first prepared as follows: to a mixture of 7.5 mL of 0.1 M Cetyltrimethylammonium bromide (CTAB) in water and 0.250 mL of 0.01 M Hydrogen tetrachloroaurate trihydrate (HAuCl_4), 0.6 mL of ice cold 0.01 M sodium borohydride (NaBH_4) was added under vigorous stirring. The yellow solution turned brown in color and was then stirred over gentle heat for a few minutes. For preparation of the nanorods, 95 mL of 0.1 M CTAB in water was kept at 29°C (Precision

microprocessor-controlled 280 series water bath), and 4.5 mL of 0.01 M HAuCl_4 , 0.6 mL of silver nitrate, and 0.64 mL of 0.1 M ascorbic acid were added. The mixture was swirled after the addition of each reactant to ensure complete mixing. Finally, 70 μL of gold seed was added, and the mixture was inverted 5 times and incubated overnight, resulting in a blue-colored suspension of gold rods. For comparison to those collected by DOSSI, spectra were collected of the nanorods using the calibrated Spec-10 microspectrophotometer. A representative spectrum is shown in Figure 2. The measured LSPR peak wavelength is 644 ± 8.4 nm and FWHM of 56.1 ± 3.2 nm ($N = 30$).

2.4 Substrate Preparation

Glass coverslips, used as substrates for immobilization of gold nanorods were cleaned with RBS detergent at 80 °C, sonicated for 10 min, and rinsed with water. The slides were further cleaned by sonication in a 1:1 mixture of methanol/hydrochloric acid for 30 min, then rinsed thoroughly with water and ethanol, and dried overnight in an oven at 60 °C. After cooling, the slides were incubated in a 10% solution of 3-Aminopropyltriethoxysilane (APTES) in ethanol for 15 min, resulting in the formation of an amine-terminated silane self-assembled monolayer (SAM) on the glass surface. After thorough rinsing and sonicating in ethanol (5×1 min each), the slides were dried in an oven for 3 h at 120 °C[3].

2.5 Bulk Refractive Index Detection

Eight DOSSI images were collected of the same nanorod substrate under each of five surrounding refractive index solutions containing 0, 20, 40, 60, and 80 percent glycerol (v/v) in water using the flow cell mounted on the imaging system. The refractive index of these solutions is 1.333, 1.355, 1.392, 1.419, and 1.450 respectively. Image acquisition times varied from 0.2 to 0.4 seconds before the detector CCD was saturated. Between acquisition of each image, the microscope focus was purposely misaligned and then refocused to avoid potential influences of focus drift.

2.6 Time-Resolved Binding Measurements

To demonstrate the advantages of the snapshot capabilities of DOSSI data collection, a second experiment was conducted with a field of view of two populations of gold nanorods – some of the nanorods were decorated with biotin, while the remainder of the nanorods were decorated with iminobiotin. Both biotin and iminobiotin bind specifically to streptavidin, though the kinetics of binding and affinities greatly differ. The goal of these experiments was to determine if the DOSSI system is capable of distinguishing members these populations based on their binding kinetics as measured by LSPR of individual nanorods – a feat that is impossible for slower, scanning hyperspectral imaging techniques. We term members of these populations biotin-nanorods (bNRs) and iminobiotin nanorods (ibNRs). Samples containing both types of nanorods were prepared by first binding bare gold nanorods to the glass substrate, and then conjugating iminobiotin to their gold surfaces. Images were collected of the sample in this state for later identification of the ibNRs. Then, a suspension of gold nanorods that had been previously conjugated with biotin were bound to the surface, thereby introducing the bNR population. Figure 3 shows a schematic cartoon of the fabrication of the employed nanorod substrates.

The ibNRs were created by dropping 20 μL of a dilute (1:15 in water) nanorod solution on to the APTES modified glass surface, allowed to incubate for 10s, and then rinsed with water. This incubation method has empirically been determined to yield nanorod surface coverages amenable to darkfield imaging and microspectroscopy [4]. In order to conjugate the NHS-terminated iminobiotin to the nanorod surface, the gold rods were first incubated in a 1:1 solution of (1-Mercaptoundec-11-yl)hexa(ethylene glycol) ($\text{HS-EG}_6\text{-NH}_2$) and (1-Mercaptoundec-11-yl)hexa(ethylene glycol) ($\text{HS-EG}_6\text{-COOH}$) (1 mM total thiol) in ethanol

for 1 h resulting in an amine-presenting monolayer on the nanorod surfaces, spaced by oligoethylene glycol moieties. Then, the surface was incubated with a 1 mM solution of NHS-iminobiotin in 50 mM sodium borate buffer at pH 8.0 for 2 h, which conjugated the iminobiotin to the gold nanorods.

A suspension of bioinylated gold nanorods (bNR) was created by first chemisorbing the gold nanorods on APTES functionalized cover glass by overnight incubation. The same procedure that was used to conjugate iminobiotin to the glass was then used, to bind biotin to the gold surface of the rods. First, the gold nanorod-modified coverslip was incubated in a 1:1 solution of HS-EG₆-COOH / HS-EG₃-OH in ethanol (1 mM total thiol) for 1 hour, then rinsed in ethanol then water. The coverslip was then incubated in 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ 0.05 M *N*-hydroxysuccinimide (NHS) for 20 min, then rinsed with water. The coverslip was immediately incubated in 1 mM biotin-amine for 1 hour, rinsed with water, then incubated in 1 M ethanolamine pH 8.5 for 10 min to quench any unreacted sites. Finally, in order to get a suspension of nanorods, the biotin-functionalized nanorods were removed from the coverslip by sonication[17]. Specifically, the coverslip was incubated in a 1:1 solution of HS-EG₆-COOH / HS-EG₃-OH in water (1 mM total thiol) and sonicated for 1 h. The thiol solution containing the nanorods was then centrifuged and resuspended in water to remove excess thiol. This bNR suspension was then diluted in water 1:15 (v/v) and drop coated onto a coverglass already containing ibNRs. The droplet was rinsed away after a 10 s incubation and was then stored in water until use.

DOSSI images were collected continuously as a 1 μ M streptavidin solution in 50 mM ammonium carbonate, pH 9.5 was flowed over the glass surface with both ibNRs and bNRs. After exposure to this solution for 20 min, the sample was rinsed with 10 mM sodium acetate, pH 5.0. The incubation pH and rinsing pH were selected to exploit the pH-sensitivity of iminobiotin and maximize the difference in streptavidin binding kinetics between the ibNRs and bNRs. Specifically, the equilibrium dissociation constant (k_d) of the iminobiotin-streptavidin interaction has been shown to vary linearly with pH - from 10^{-6} M at pH 5 to 10^{-10} M at pH 9 [18], whereas the k_d of biotin-streptavidin is 10^{-14} M [18]. For acquisition of these images, a 50/50 beamsplitter was used to send half of the light to the microscope eyepiece, so that sample focus could be continuously maintained manually. As a result of this split, image acquisition times were increased to 1–2 seconds.

3. Results and Discussion

3.1 DOSSI Images

Figure 4 shows a typical raw DOSSI micrograph of gold nanorods under darkfield illumination. The zero-order mode is visible in the left-hand portion of the image, with bright spots corresponding to nanorod scattering. The right-hand portion of the image is the first-order diffraction mode of the image and contains corresponding nanorod spots that are spread out along the x-dimension. These bright smears in the right-hand portion correspond to plasmon bands of individual gold particles.

The first steps of DOSSI data interpretation are identical to that of typical microspectroscopy. First, contributions due to dark current were removed by subtracting an image collected for the same integration time, but with no light exposure. Source correction was next performed by collecting a single source spectrum from each DOSSI image by averaging together lines from the first-order portion of the image that did not exhibit nanorod spots. Each row of the first-order image was then divided by that acquired source spectrum. Figure 5 shows a source-corrected DOSSI image.

The x-position of the spectral peaks in the first-order image is determined by two factors. First, the spectral peak position is determined by the starting position of the spot in the zero-order image. The final position of the peak is determined by the distribution of its spectral content. The dispersion of the grating will shift longer wavelength components further to the right in the first-order image. LSPR red-shifts are measured as small perturbations in the positions of these peaks along the positive x-direction in first-order image. In order to observe small shifts in these peak locations, it is necessary to decouple contributions from these two potential sources, else mechanical drift caused by physical movement of the microscope stage relative to the detector could be misconstrued as an LSPR peak shift.

For the purposes of the experiments described herein, we restricted DOSSI to measurement of relative shifts in resonance peaks, and did not attempt to measure the absolute wavelength of the peaks. However, we note that this is not a fundamental limitation of the system, as the dispersion of the grating is constant across the Y-dimension in the field of view, so that the 'center' blaze wavelength of each first-order diffracted peak will be the same distance away from its zero-order spot on the CCD. Even if small angular deviations in the ray trace of these spots exist, the system could easily be calibrated to take this effect into account by post-processing.

LSPR shifts were measured by calculating relative peak locations between the same nanorod spectrum in the two first-order regions of the corresponding DOSSI images. First, physical misalignment of a sample was removed with an image alignment algorithm [19] that was applied to the zero-order region of the DOSSI images. Alignment was performed by maximizing the cross correlation of the images. Alignment is calculated to a specified fraction of a pixel by up-sampling the cross-correlation matrix by the inverse of the defined fraction. For the studies described herein, an up-sampling factor of 100 was specified, which registered images to within 0.01 pixels. The alignment parameters determined for the zero-order images were then applied to the first-order images.

Next, a peak finding algorithm was applied to each line of the aligned first-order images [20]. The algorithm operates by smoothing the data with a running average, and then locates peaks by thresholding the derivative of the line scan above a specified level. A least-squares fit to a parabola was applied to the spectral region around each potential peak. The peaks were then screened by height and width to exclude spectral features not expected to arise from nanorod scattering. The peak location was considered the vertex of the fit parabola and has units of pixels but is not restricted to integer values as it is extracted from a least-squares fit. Because most LSPR peaks exist on more than one row of the detection CCD, the spectra are averaged together before peak fitting is applied. LSPR shifts are calculated in units of pixels as the difference in peak location in a reference image and the test image.

3.2 Bulk Refractive Index Detection

Figure 6A shows an x-axis line scan from a DOSSI image collected of bare nanorods immobilized on glass in water. The first 200 pixels are from the zero-order mode image, and two peaks corresponding to nanorod scattering spots can be observed. The difference in SNR from the two regions of the image is due to the source correction applied to the first-order region. The remainder of the line scan is the source corrected first-order diffraction mode of the image. Two peaks are observed, one corresponding to the LSPR of each particle. The two particles are physically separated by approximately 50 pixels in the zero-order mode. The two peaks in the first-order more are also separated by approximately 50 pixels, indicating that the two nanorods have LSPR peaks at a similar peak wavelength. Figure 6B shows a plot that includes line scans from five images collected from the same sample in different mixtures of water and glycerol. Both peaks were observed to red-shift approximately 30 pixels (towards the right) due to the increase in local RI.

Forty DOSSI images –eight images in each of five water/glycerol solutions– were analyzed to quantitatively assess the system. Each first-order image contained approximately 150 nanorod spots. The relationship between peak pixel position and peak wavelength was calculated using the dispersion of the grating to be 1.49 pixels/nm. This value allows conversion of pixels to nm. The variability in the measurement of each peak was determined by calculating the standard deviation of the location of a peak over the eight images collected under identical refractive index. The average standard deviation of individual peak locations was 1.42 pixels. Applying the dispersion relationship, this uncertainty translates to 0.95 nm. We term this value the peak measurement uncertainty and it is a measure of how accurately the system can measure the spectral peak location of any individual spot within the DOSSI image. The FWHM of the peaks was 93.1 ± 7.8 pixels, which corresponds to 62.5 ± 5.2 nm, in reasonable agreement with the mean FWHM of 56.1 nm measured by conventional microspectroscopy. The slight increase in FWHM measured by DOSSI is attributed to lower dispersion grating which increases the relative size of the diffraction-limited spot, and thus its convolution in the wavelength dimension.

To determine the overall RI shift, the measured spectral shift of each of the approximately 150 particle spots was considered. Figure 7 shows a plot of the mean peak pixel shift as a function of refractive index. For this measurement, each peak shift was measured relative to DOSSI images in water. A linear fit to this plot yields a slope that gives an approximate sensitivity of 204 pixels/RIU, which translates to 137 nm/RIU by the dispersion relation of the grating used for these measurements. This agrees well with the theoretically predicted value of 141 nm/RIU calculated for gold nanorods that resonate at 644 nm in water[21, 22]. The error bars in the plot are the standard deviations of the mean peak shift (from all nanorod peaks) across the eight images collected at each RI. We term this value the image uncertainty, as it is based on the average shifts from every spot measured in the DOSSI image. The mean image uncertainty for the experiment described is 0.57 pixels, or 0.38 nm. This value describes how accurately the entire image can be used as a predictor for average LSPR shift of the sample as a whole. The increased accuracy relative to the single peak measurement uncertainty of 0.98 nm is gained from the statistical advantage of using many spots to measure one value, but is slightly offset by the particle-to-particle variance in RI sensitivity.

3.3 Temporally-Resolved Protein-Ligand Binding

Both biotin and iminobiotin exhibit reversible and specific binding of streptavidin. Incubation in a streptavidin-containing solution of the nanorod-modified glass surface with immobilized biotin and iminobiotin causes streptavidin molecules to bind to both types of nanorods. Binding increases the local RI, which results in a red-shift of the LSPR spectrum of both types of nanorods. Similarly, upon dissociation of the streptavidin molecules by rinsing in buffer, the nanorod LSPR peaks blue-shift back to their native original wavelengths. Figure 8 shows the LSPR peak locations of an ibNR and a bNR from the same DOSSI image as the surface is incubated in a solution of streptavidin, and subsequently rinsed in buffer. For each nanorod, the streptavidin-biotin and streptavidin-iminobiotin dissociation rate constant (k_d) was determined by applying a least-squares fit to the rinse portion of the curve. As there is no analyte during rinsing, the kinetics follow first order dissociation described by the following rate equation:

$$X = X_0 + X_{MAX} e^{-k_d \cdot t} \quad (1)$$

where X is the LSPR peak location at time t , X_0 is the initial peak position, and X_{MAX} is the maximum peak shift observed before rinsing. Once k_d is determined, a second fit is applied

to the binding (association) region of the kinetics to determine the association rate constant (k_a) from the following rate equation:

$$X = X_0 + X_{MAX} \left(1 - e^{-(Ck_a + k_d)t} \right) \quad (2)$$

where C is the streptavidin concentration at time t , and the other parameters are as described for equation 1. Figure 8 shows the kinetics of an ibNR and a bNR as measured by DOSSI and fits to the association and dissociation portion of the kinetics, as described above.

Table 1 displays the k_d and k_a of the two nanorod populations measured by DOSSI ($N_{bNR} = 24$, $N_{ibNR} = 21$) and their respective values from the literature [18, 23, 24]. The final column lists the mean and standard deviation of the corresponding LSPR shifts of the nanorod populations at saturation with streptavidin before rinsing. The final row labeled Bulk RI lists an 'effective' k_a of the fluid handling system. These value were obtained a by applying the same binding fit to time-resolved nanorod LSPR shifts as the bulk medium is replaced as described above (data not shown). This value describes the effective rate at which the fluid handling system can effectively replace the medium at the sample surface. In an ideal system, this value would be infinitely high signifying immediate replacement, but the laminar flow conditions at the sample surface and short sensing distance (tens of nanometers) of the nanorods results in diffusion-limited rates for sample introduction at a rate described by this value.

The values for the net LSPR shift of each population are summarized in the final column in Table 1. These values describe the final, steady-state red-shift that results from the change in local RI due to binding of streptavidin to the bNR or ibNR before rinsing, and represent an end-point measurement. The large standard deviations of these measurements result from two sources. As mentioned above, measurement error of the DOSSI system introduces ~0.95 nm of uncertainty to the measured values. The remainder of this variation is a result of the size and shape dispersity of the nanorods, which modulates the RI sensitivity of each nanorod [25]. Because of these variations within each nanorod population, such end-point measurements are statistically unable to clearly differentiate between bNRs and ibNRs (ANOVA, $p = 0.093$).

The values acquired by the DOSSI system for the association rate constants (k_a) of both interactions are clearly below that to the values reported in the literature [23, 24]. However, we note that the measured values are close to the effective k_a of the transport-limited bulk RI exchange experiments. This value is between four and ten orders of magnitude lower than the values in the literature, indicating much slower association rates in our experimental set-up. We therefore conclude that both the bNR-streptavidin and ibNR-streptavidin interactions are dominated by diffusion-limited association kinetics. Because of this, the measured k_a are unable to discriminate between the two interactions (ANOVA, $p = 0.92$).

The measured dissociation rate constants (k_d) for biotin-streptavidin and iminobiotin-streptavidin interactions are also listed in Table 1. We note that both dissociation rate constants measured by DOSSI are significantly lower than the effective diffusion-limited binding rate of -3.62 (assuming the same diffusion-limited dissociation). This indicates that the true dissociation kinetics are dominated by the –relatively slower– molecular dissociation of streptavidin from either ligand, and not by diffusion. This is confirmed by the good agreement between the dissociation rate constants measured by DOSSI with those previously reported in the literature [18, 23]. Furthermore, the two populations of nanorods exhibit significantly different means (ANOVA, $p = 9.1 \times 10^{-3}$) illustrating that their k_d clearly differentiates ibNRs from bNRs.

4. Conclusion

We have shown that DOSSI can be effectively used to collect spectrally-resolved LSPR images of a full field of view of sparsely located gold nanorods. Measurement uncertainty analysis of gold nanorod LSPR peaks indicates that the spectral uncertainty of DOSSI is approximately 0.95 nm. In addition, real-time measurements were made which allowed discrimination between two otherwise-identical populations of gold nanorods based purely on their molecular interaction kinetics – a feat that is not possible with slower raster-type hyperspectral imaging systems, or other systems relying solely on end-point measurements.

Because of these capabilities, DOSSI offers the possibility of spatially resolved real-time plasmonic spectroscopy of immobilized noble metal nanorods. DOSSI is applicable to both spectrally encoded or spatially encoded plasmonic arrays, as long as individual nanorods in the field of view are separated from their closest neighbor in the spatial dimension to be dispersed spectrally by a distance that is sufficient to prevent overlap of their spectra in the first order diffracted image. DOSSI has other ancillary advantages: it is cheap, and it is simple to assemble and calibrate, as the only components required beyond those of a typical optical microscope are a grating and an entrance slit. For these reasons, we believe DOSSI will prove to be a useful technique for the real-time interrogation of LSPR arrays and other optical microarrays.

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Fig. 1.
Basic schematic of the DOSSI system and a representative image of gold nanorods as collected by the color CCD (inset). Scale bar indicates 5 μm .

Fig. 2. Scattering spectrum of a single gold nanorod as measured by conventional microspectroscopy.

Fig. 3.

Schematic cartoon of sample substrate preparation. 1) COOH-terminated SAM applied to bNRs. 2) biotin conjugated to bNR. 3) bNRs sonicated into suspension. 4) COOH-terminated SAM applied ibNR. 5) iminobiotin conjugated to ibNRs. 6) introduction of bNRs to ibNR sample.

Fig. 4.

Raw DOSSI micrograph of gold nanorods under darkfield illumination. Collected by a single CCD, the left-hand portion shows the zero-order mode of the nanorod field, and the right hand portion shows the first-order diffraction mode of the same field of view. The vertical axis of the DOSSI image corresponds to the vertical axis of the sample, and the horizontal axis of the DOSSI image corresponds to the convoluted spatial/spectral axis of the sample. The intensity map is false colored for visual clarity.

Fig. 5. Zero-order mode (left) and first-order diffracted mode (right) of a single source- corrected DOSSI image.

Fig. 6.

A) Line scan of a DOSSI image. The first 200 pixels are the zero-order mode and the remaining image is the source corrected first-order mode. B) DOSSI line scans of two nanorods on the same pixel row incubated in five glycerol/water mixtures with increasing refractive index.

Fig. 7. Mean peak shift recorded for DOSSI imaging of gold nanorods as a function of refractive index. Error bars indicate standard deviations from eight images at each RI.

Fig. 8. Data points (black dots) and fits to the binding (blue lines) and rinsing (red lines) of representative biotin-conjugated (top) and iminobiotin-conjugated (bottom) gold nanorods. The dotted line indicates the time point at which the rinse was initiated.

Table 1

Binding constants and endpoint LSPR shifts

	$\text{Log}_{10}(k_d) \text{ (Ms}^{-1}\text{)}$		$\text{Log}_{10}(k_a) \text{ (s}^{-1}\text{)}$		$\Delta\lambda_{\text{LSPR}} \text{ (nm)}$
	DOSSI	Literature	DOSSI	Literature	
Iminobiotin- rods	-5.14 ± 0.36	-6 [18]	3.64 ± 0.27	7 - 9 [23]	6.88 ± 1.93
Biotin-rods	-13.65 ± 0.26	-14 [18]	3.66 ± 0.26	13 [24]	5.48 ± 1.23
Bulk RI	-	-	3.62 ± 0.096	-	-

± indicates 95 percent confidence interval