Single-Molecule Surface Plasmon-Coupled Emission with Plasmonic Gratings

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Supporting Information

ABSTRACT: The ability to image single molecules (SM) has been the dream of scientists for centuries, and because of the substantial recent advances in microscopy, individual fluorescent molecules can now be observed on a regular basis. However, the development of such imaging systems was not without dilemmas, such as the detection and separation of individual fluorescence emissions. One method to solve this problem utilized surface plasmon resonance (SPR) to enhance the emission intensity of SMs. Although enhancing the SM emission intensity has yielded promising results, this method does not fully utilize the unique plasmonic properties that could vastly improve the SM imaging capabilities. Here, we use SPR excitation as well as surface plasmon-coupled emission from a high-definition digital versatile disc grating structure to image and identify different fluorophores using the angular emission of individual molecules. Our results have important implications for research in multiplexed SM spectroscopy and SM fluorescence imaging.

1. INTRODUCTION

Surface plasmon resonance (SPR) is a unique phenomenon wherein a resonant charge oscillation forms at the surface of a metallic grating structure as a result of light impingement on the surface at a specific angle of incidence. This charge oscillation and resulting high-intensity electromagnetic (EM) field can interact with and excite nearby dipoles, such as fluorescent molecules, and enhance the fluorescence intensity. In this case, we fabricated nanoscale gratings ($\Lambda = 400$ nm, $H = 55$ nm) using a high-definition digital versatile disc (HDDVD) as a master mold in a soft-lithography process (see Figure S1a). This process yields very similar results to those of other grating fabrication methods, such as E-beam lithography, but is much less expensive and is less time consuming. After depositing a thin, 100 nm silver coating, the plasmonic gratings are capable of enhancing the fluorescence emission of rhodamine 6G (R6G) and cyanine 5 (Cy5) dye films by as much as 100–200× compared to that from glass substrates using a relatively simple epifluorescence microscope for excitation and imaging.1–3 Additionally, these gratings can couple wavelengths of light over a much wider angle range than prism-based SPR platforms. The wider coupling capability of plasmonic gratings makes these platforms ideally suited for the wide-angle excitation provided by microscope objectives, as opposed to narrow dispersion, laser-based excitation.1,2 We have previously demonstrated that we are capable of imaging a wide range of fluorophore concentrations using these plasmonic gratings and an epifluorescence microscope.2

However, an intriguing observation was made when examining single-molecule (SM) fluorescence on silver gratings with different polarization filters and at different focal heights (Figure S1b). The emission intensity of a large SM population was found to exhibit an angular emission profile that was similar to the anticipated emission angle range of surface plasmon-coupled emission (SPCE), as illustrated in Figure 1a. SPCE is a phenomenon wherein a dipole, such as a fluorescent molecule, can nonradiatively transfer energy to a plasmonic substrate, such as a metallic grating. The transferred energy is converted into either a radiative plasmon or lossy surface plasmon. Radiative plasmons are subsequently emitted from plasmonic substrates as photons over a specific emission angle range, whereas surface plasmons decay into heat.4 To date, SPCE has been primarily studied on the macroscale using the projected emission pattern from flat metal films and hemispherical lenses in a modified Kretschmann or Otto configuration.4–6 Consequently, very little to no information is available on SPCE imaging on the SM size scale.7 As SPCE and SPR are, in a sense, opposites of each other, wherein light is coupled at a specific angle to form SPR and radiative surface plasmons are...
emitted at a specific angle in SPCE, we can use the SPR dispersion (Figure S2) to predict the angular emission of a specific fluorophore (Figure 1b). Additional information on fitting of the angular emission and determination of theoretical SPCE emission ranges has been provided in the Methods section. By studying the SM emission from SPCE, it is possible to not only obtain the emission angle range but also to obtain substantially more information about the molecular position in the EM field and dipole orientation. As the emission angle range is also unique to the fluorophore emission spectrum, it can be used to identify different fluorescent molecules on, for example, a multiplexed fluorescent sample.

2. RESULTS AND DISCUSSION

To determine whether the angular emission was due to SPCE and not dipole-related phenomena, SMs of R6G embedded in a 33 nm poly(methylsilsequioxane) (PMSSQ) thin film were imaged using an excitation polarizer and a rotating polarized emission analyzer. Given the much longer exposure time required to acquire SM images (∼10 s, see Methods), the resulting images were assumed to contain the full angular emission range for each SM. During imaging, we observed two populations of SM emissions (Figure S3): the first population exhibited the response seen in Figure 2a, whereas the second population exhibited the response seen in Figure 2b. In Figure 2a, two lobes or “split emission” per molecule were observed wherein the emission was primarily P-polarized. The absence of S-polarized light in the SM emission is in agreement with the SPCE theory, as P-polarization is the only polarization that is capable of being emitted by a plasmonic grating from a radiative surface plasmon.4 If the emission polarization was due to the dipole orientation alone, we would expect the orientation of the split emission to rotate with the polarized analyzer, which was only observed from SMs that were part of the second population.9

Additionally, the SPCE mechanism is highly dependent on the transfer of energy from the dipole back to the grating to form a radiative plasmon, which only occurs at short distances (10–250 nm) from the grating surface.4 The time necessary to nonradiatively transfer energy from the dipole to the grating (∼10−9 s) is on the same time scale as that for fluorescence resonance energy transfer and surface energy transfer (SET). This time scale is similar to the fluorescence emission time scale (∼10−9–10−7 s) from the dipole itself. The variation in the proximity of the dipoles in the PMSSQ film enables the observation of both SPCE (Figure 2a) and SPR-excited dipole fluorescence emissions (Figure 2b). Roughly half of the fluorophores observed in each fluorescent image exhibited SPCE-based split-emission behavior, as seen in Figure S3. The remaining half of the observed molecules exhibit SPR-excited fluorescent emissions from SM or multiple molecule behavior. A difference in emission shapes between the two populations can also be observed by increasing the height of the objective’s image plane relative to that of the sample, as seen in Figure 2b. The split-emission SM seen in Figure 2c spreads outward from the molecule at a definable emission angle, whereas the second population SM (Figure 2d) displayed an inverted, conical emission similar to that observed by Böhmer with defocused images.10 Two additional fluorophores were imaged, FITC and Cy5, at concentrations within the SM behavior range (1 μM−1 pM), to further test whether the split emission observed in the first population is the result of SPCE. On the basis of the SPR dispersion and excitation/emission ranges of the two dyes, FITC is expected to have a wider and Cy5, a narrower emission angle range. As anticipated, split-emission patterns were also observed with FITC and Cy5, which had different angular...
emission ranges (Figure 3). The angular emission profiles were obtained using the intensity profiles at known focal heights. Furthermore, points of interest, such as the peaks, full widths at half-maximum (FWHMs), and valleys of the profiles, at different heights were fitted using linear models for each fluorophore. The resulting angles for each linear model were then applied to the in-focus intensity profile to obtain an emission angle vs. intensity relation for FITC (Figure 3a), R6G (Figure 3b), and Cy5 (Figure 3c).

The obtained emission profiles for each dye correspond well with the predicted SPCE emission range (grey lines), with some variations in intensity between the two lobes of the split emissions. In theory, an excited dye molecule located in the middle of a grating groove has an equal probability of nonradiatively transferring its energy to either grating ridge paralleling the groove. However, closer proximity to either ridge will result in a higher probability of energy transfer according to the SET model.11 As the placement of the dye molecule is random within the pits of the grating structure, the molecule may be located closer to one side of the groove than the other. To further illustrate the difference in lobe-to-lobe intensity, a histogram comparing the relative intensities of the two lobes for several R6G molecules has been provided in Figure S4. It was found that most of the observed SMs (~75%) had less than 5% variation in the intensity emitted from the same molecule between lobes, and the maximum variation in intensity observed between the lobes was ~30%. It may be possible to locate the precise XYZ location of a molecule within the grating groove on the basis of the relative intensity of the two lobes and focal plane location, but this requires a secondary localization precision method to confirm the location of the molecules, which is beyond the scope of our measurement capabilities.

The intensity profiles of two representative SMs for each population were also compared to determine the image resolution in Figure 4. The FWHM was found to be ~327 nm on the basis of the intensity profile of the diffraction-limited population (Figure 4a). Given that the wavelengths of most of the photons collected in these images are between 542 and 600 nm and that an airy disc pattern with distinct zero- and first-order diffraction modes can be seen, this population is certainly diffraction-limited. However, the lobes of the split emission (Figure 4b) have a much better resolution, with an FWHM of ~233 and ~217 nm for each lobe. Additionally, the spacing between the two lobes was shorter at ~164 nm. The diffraction limit can be roughly estimated to be between 230 and 260 nm on the basis of the Rayleigh criterion, which is larger than the FWHM and a much larger spacing of the split-emission pattern. This increase in image resolution can be expected if the split emission is due to SPCE, as subdiffraction limit information would be transmitted into the far field.

The ability to obtain subdiffraction limit resolution using a plasmonic grating, as well as obtaining angle-based spectroscopic information from individual molecules via simple defocus microscopy techniques, has many advantages over conventional super resolution microscopy. First, defocus
microscopy is much less expensive than conventional super resolution as a spectrometer and highly sensitive camera are no longer required. Another advantage is the ability to identify fluorescent molecules on the basis of their emission spectrums without the need for an emission filter to screen out other types of fluorescent molecules, which has many applications in filterless, multiplexed imaging.

3. CONCLUSIONS

In summary, we have demonstrated that it is possible to image SM SPCE from plasmonic gratings using a simple epifluorescence microscope. From these images, we can extract the angular emission profiles for individual dye molecules that can be used to identify the type of fluorophore and improve localization precision. Plasmonic gratings can also be used to improve the image resolution to below the diffraction limit. This imaging technique can be easily applied to other SM research applications by substituting glass or quartz substrates for plasmonic gratings to greatly improve fluorophore identification and emission intensity image resolution.

4. METHODS

4.1. Grating Fabrication. PMSSQ gratings were fabricated using a soft-lithography process, with cleaned HDDVD (Memorex) halves as master molds to cast poly(dimethylsiloxane) (PDMS, Sylgard-184; Gelest) stamps. The grating profile was transferred to silicon wafers by spin casting (3000 rpm, 30 s) a 3 wt % solution of PMSSQ (Techniglass) dissolved in pure ethanol onto the PDMS stamp and placing the stamping in contact with the wafer for ~5 s before removal. PMSSQ gratings were cured at 60 °C for 3 h on a hotplate. The gratings were coated with a 5 nm thin titanium film followed by a 100 nm thin silver film using RF sputtering. Silver was protected from corrosion using a 10 nm thin Al2O3 film deposited via atomic layer deposition.1

4.2. Sample Preparation and Analysis. FITC, R6G, and Cy5 were diluted to 10−15 M in 200 proof ethanol with 1 wt % PMSSQ and spin-casted onto the finished gratings to yield a 33 nm PMSSQ thin film, which was cured at 60 °C for 10 min. The fluorophore density of the film was measured to be ~4 molecules per 10×10 μm² area. The molecular emissions used for emission angle analysis were confirmed to be emitted from individual molecules on the basis of the observation of a single-step photobleaching event in a time trace analysis for each molecule (Figure S5).

Reflectivity peak position and FWHM were used to obtain the air-incident SPR dispersion (θSPCE,air), which was converted to oil-incident (n = 1.518) SPR dispersion. The normalized emission spectra (Iλ fluorophore) for FITC, R6G, and Cy5 were multiplied by the respective bandpass emission filter transmission spectra (Tλ em filter), the microscope objective’s transmission spectrum (Tobjective), and the spectral sensitivity of the camera (qCMOS). The resulting spectra were multiplied by the oil-incident SPR dispersion to convert the intensity related to wavelength into angular emission

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I_{\text{emission}}(\theta) = I_{\lambda \text{ fluorophore}}(\lambda) \cdot T_{\text{em filter}}(\lambda) \cdot T_{\text{objective}}(\lambda) \cdot q_{\text{CMOS}}(\lambda) \cdot \theta_{\text{SPCE, oil}}(\lambda)
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ImageJ software was used to obtain the intensity profiles of individual fluorophores at known focal plane heights. The intensity profile points were fitted across all of the image planes to determine the angular spread of the intensity profile as the SM was defocused.

4.3. Experimental Setup. Grating reflectivity was captured using a variable angle spectroscopic ellipsometer. Samples were imaged using an Olympus BX51WI epifluorescence microscope equipped with a Lambda XL light source; excitation polarizer; fluorescence filter cubes for FITC, R6G, and Cy5; rotatable, polarized analyzer; UAPON 100×OI TIRF objective (1.49 NA); and an ORCA-Flash 2.8 CMOS camera. Fluorescence videos were captured with an exposure time of 10 s. A diagram of the experimental setup has been included in the Supporting Information document (Figure S1).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00104. Atomic force microscopy and schematic of the imaging setup; SPR dispersion plots for HDDVD gratings overlayed with the excitation and emission spectra of FITC, R6G, and CY5 dyes; brightfield and fluorescence images of SMs; time trace of a molecule exhibiting a split-emission pattern; and histogram of the split-emission lobe-to- lobe intensity variation (PDF)

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Notes
The authors declare no competing financial interest.

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