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Plasmon-Enhanced Fluorescence

Thousand-fold Enhancement of Single-Molecule Fluorescence Near a Single Gold Nanorod**

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Optical detection of single molecules mostly relies on their fluorescence because of the high contrast of this technique against the background. Since their invention in the early 1990s, single-molecule fluorescence microscopy and spectroscopy have spread to many fields in chemistry, physics, and biology, and have provided unique access to nanometer scales.[1] New developments have yielded insight into a wide range of phenomena, including the dynamics of enzymes[2] and the subwavelength arrangement of cellular components.[3] A primary requirement of this technique is that the emission rate of the molecules under study must be as high as possible. However, an overwhelming majority of the strongly absorbing molecules (called chromophores) fluoresce only weakly and hence are not detectable by conventional single-molecule fluorescence techniques. Prominent examples are metal complexes and many biological chromophores, such as hemes. Herein, we will show how fluorescence enhancement by a plasmonic nanoparticle may be harnessed to overcome this limitation and extend single-molecule studies to weakly emitting species.

The enhancement of single-molecule fluorescence near a plasmonic nanoparticle may arise from two factors. 1) In contrast to dielectrics, metals support a collective response of conduction electrons, which can concentrate the optical field in the vicinity of a particle. Excitation can be enhanced by this high local field, particularly close to tips or protrusions.[4–6] This lightning rod effect is further amplified by resonance if the excitation frequency coincides with a surface plasmon resonance (SPR) of the particle. 2) The rate of emission by a fluorophore can also be enhanced by a similar antenna effect.[7] This Purcell effect can be seen as an enhanced density of optical states accessible for decay for a dipole at the position of the molecule, or, equivalently, as enhancement of the transition dipole moment by electric currents in the nanoparticle antenna.[8] The Purcell effect may not only change the intensity of the emission, but also its spectral shape, fluorescence lifetime, and quantum yield. As metals also enhance nonradiative decay rates, they may quench fluorescence. The balance between enhancement and quenching depends on the exact position and orientation of the fluorophore with respect to the nanoparticle.

By the late 1990s, a number of near-field optics experiments had shown the existence of distinct regimes of enhancement and quenching in the interaction of a single molecule with a metal tip.[9] At distances less than 5 nm, molecular excitations are efficiently dissipated in the metal by a mechanism similar to Förster transfer. At intermediate distances (10–50 nm), however, currents in the metal can actually enhance the radiating dipole and boost emission by the optical antenna effect. Later measurements on single gold nanospheres by the research groups of Novotny and Sandoghdar have confirmed these two regimes,[10] although field enhancement by a single sphere is rather modest (only a factor 3 or so). More recently, researchers have put molecules in interaction with lithographically fabricated nanostructures designed for strong field enhancement. Prominent examples are the directional Yagi-Uda antenna by the research group of Van Hulst[11] and the bowtie nano-antenna by the research groups of Hecht[12] and Moerner.[13] In the latter work, a fluorescence intensity enhancement of up to 1340 was demonstrated for a molecule with a low quantum yield.[13]

Enhancing the fluorescence by a lithographically made metal nanostructure has a number of drawbacks. 1) The structures are often difficult and expensive to fabricate. 2) As the gold films produced by standard evaporation or sputtering techniques are polycrystalline, their plasmon resonances are significantly broadened and weakened. In contrast to those structures, chemically synthesized gold nanorods are easy to make. Their single-crystalline structure generates narrow and intense plasmon resonances.[6,14] Theory predicts fluorescence enhancements of up to several thousand times at the tips of gold nanorods.[15,16] Experimental realizations, so far, have lagged far behind this prediction, with a maximum single-molecule fluorescence enhancement of only 40-fold, as achieved by Fu et al.[17] The main hurdle is the accurate positioning of a single fluorophore in the region of the highest field enhancement.

Here we report large enhancements of single-molecule fluorescence, up to 1100 times, by chemically synthesized gold nanorods. The reported enhancement is one to two orders of magnitude higher than in previous reports.[17,18] We have achieved such high enhancements by 1) selecting a dye with significant overlap with the surface plasmon of nanorods and 2) allowing *single* dye molecules to diffuse slowly through the

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near-field of a *single* nanorod. As individual fluorophores explore the field profile near the particle, a molecule will occasionally diffuse through the most favorable position where the enhancement is maximum. Monitoring the fluorescence with a high enough time resolution, we determine the maximum enhancement by analyzing bursts in fluorescence time traces.

Gold nanorods with average dimensions of 25 nm × 60 nm (Figure 1 a) were synthesized by the seed-mediated growth method.[19] The longitudinal plasmon resonance of these nanorods is approximately at 650 nm in glycerol. A calculated

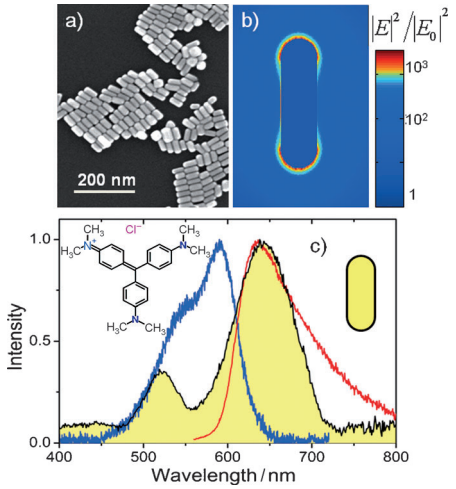

Figure 1. a) SEM image of a drop of gold nanorod suspension dried on a silicon chip. On average, the nanorods are (25 ± 4) nm wide and (58 ± 7) nm long. b) Map of the near-field optical intensity, calculated using the discrete dipole approximation for a gold nanorod with 9 nm width and 31 nm length. c) Extinction spectra of gold nanorods dispersed in glycerol (shaded area), absorption (blue), and fluorescence (red) spectra of CV in glycerol. Inset: chemical structure of CV (also shown in Figure S1).

map of the local light intensity around a gold nanorod excited at the longitudinal SPR resonance is displayed in Figure 1 b and shows two regions of high enhancement in the vicinity of the tips. Figure 1 c shows a bulk extinction spectrum of these nanorods dispersed in glycerol. For our single particle studies, the nanorods, coated with cetyl-trimethylammonium bromide (CTAB), were isolated on a glass coverslip by spin coating from a water suspension. After spin coating, the residual CTAB was removed by washing 2–3 times with water and by subsequent UV/ozone treatment for 30 minutes.

We selected crystal violet (CV) molecules for the enhancement study. CV has its absorption maximum at 596 nm and its emission maximum at 640 nm, ensuring a significant overlap with the SPR of the nanorods (Figure 1 c). CV has a low fluorescence yield (QY) of 0.019.[20] It has been previously reported that fluorescence enhancement depends on the QY of the dye and higher enhancements can be achieved for lower QY.[13,15,16,21] Low QYs also offer better contrast against the fluorescence background of unenhanced molecules.

Confocal microscopy was performed on our home-built microscope, assembled on an inverted optical microscope. The details of the setup are described elsewhere.[22,23] Briefly, a circularly polarized helium–neon laser (633 nm) or an Ar-ion laser (514 nm) was used as excitation source. An oil immersion objective with a numerical aperture (NA) of 1.25 focused the excitation laser to a diffraction-limited spot (about 300 nm in diameter). The red-shifted fluorescence from the sample was collected by the same objective and was separated from the excitation laser by a notch filter (removing 514 or 633 nm, according to the excitation wavelength). Fluorescence was detected by an avalanche photodiode (Figure 2 a) or a liquid-nitrogen-cooled CCD. Images were acquired by scanning the sample across the focus with a piezo-scanning stage.

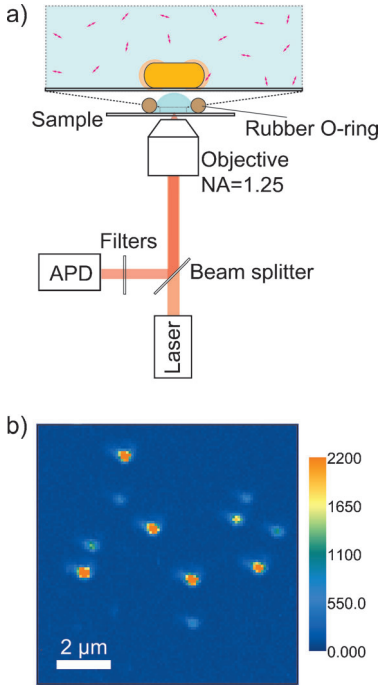

Figure 2. a) A simplified scheme of the experimental setup (NA = numerical aperture, APD = avalanche photodiode. The small double-sided arrows represent single CV molecules diffusing in solution and the yellow body represents a nanorod. b) A photoluminescence image of the nanorods isolated on a glass coverslip and immersed in glycerol doped with 100 nM CV. A circularly polarized 633 nm laser was used as the excitation source. The excitation intensity was 5 kW cm^{-2}. The image consists of 120 × 120 pixels with an integration time of 10 ms per pixel.

Figure 2 b shows a typical photoluminescence image of the nanorods isolated on a glass coverslip and immersed in glycerol doped with 100 nM CV. The excitation wavelength was 633 nm. Each bright spot in Figure 2 b arises from photoluminescence of a single nanorod. This is confirmed by measuring a photoluminescence spectrum of each bright spot. We used 514 nm laser excitation to record the luminescence spectra of the nanorods. The 514 nm excitation was preferred because it is energetically far from the longitudinal SPR of the nanorods (Figure 1 c) and thus enabled us to

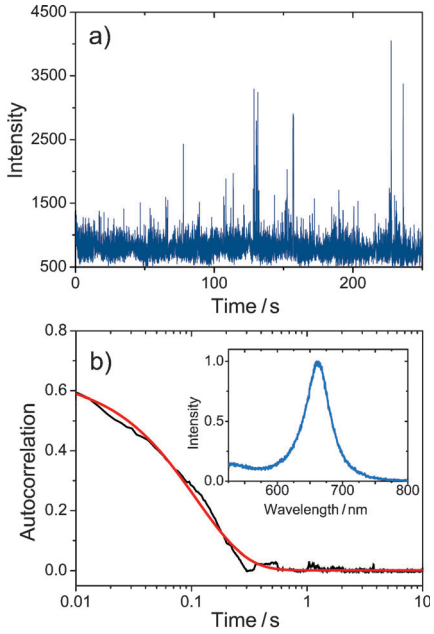

Figure 3. a) A fluorescence intensity time trace taken on a single gold nanorod immersed in glycerol doped with 100 nM CV molecules. b) The luminescence spectrum of this nanorod is shown in the inset. The Lorentzian lineshape of the plasmon resonance confirms the presence of a single nanorod. Autocorrelation curve (black) of the fluorescence bursts shown in (a). A single exponential fit (red) yields a correlation time of 120 ms.

record the full nanorod emission spectrum. We found approximately 90% (35 out of 40 spots) of the bright spots to stem from single nanorods, evidenced by their narrow spectral width and Lorentzian line shape. An example spectrum is shown in Figure 3 (we note that scattering spectra are more commonly used in the literature to check for single nanorods, but, as we have recently shown that scattering spectra closely resemble luminescence spectra, the latter can be used as well[23]). Figure 2b, recorded with excitation at 633 nm, shows a large variation of the photoluminescence intensity among the bright spots. This intensity variation is due to volume differences from nanorod to nanorod and most importantly to the different positions of their surface plasmon resonance with respect to the excitation.

Fluorescence time traces taken on individual nanorods show fluorescence bursts. Figure 3a shows a typical fluorescence intensity trace taken on a single nanorod, the spectrum of which is shown in the inset of Figure 3b. The large background signal of the fluorescence trace, about 700 counts in 10 ms, comes from all the CV molecules present in the focal volume of the excitation laser as well as from some intrinsic luminescence of the nanorod. The contribution of CV molecules can be deduced from a fluorescence trace taken under the same experimental conditions but on an area without a nanorod. Figure S2 shows such a trace with an average count of about 170 per 10 ms bin time. At the given CV concentration of 100 nM, we expect approximately 60 molecules in the focal volume at any given time (considering a focal volume of 1 fL). From this, we estimate on average of about 2–3 counts/molecule/10 ms. It is interesting to compare this signal to the intrinsic photoluminescence of the nanorod. This nanorod appears to be about two orders of magnitude brighter (about 500 counts per 10 ms) than an individual CV molecule under our experimental conditions, even though the nanorods are known to have a much lower luminescence QY (10^{-5} or less)[23,24] than CV ($\approx 10^{-2}$). This high brightness is explained by the very high absorption cross-section of the nanorod at resonance (about 10^3 nm^2) compared to a CV molecule (about 10^{-2} nm^2).

To verify that the observed fluorescence bursts are coming from CV molecules, we performed blank experiments where we measured fluorescence time traces of single gold nanorods under identical conditions but immersed in pure glycerol. We did not observe any fluorescence burst, as shown in the example trace in the Supporting Information (Figure S3). Moreover, we recorded fluorescence time traces on single gold nanorods immersed in glycerol solutions with different concentrations (0, 10, and 100 nM) of CV. Figure S3 shows an occurrence of fluorescence bursts approximately an order of magnitude larger (about 30 bursts to about 200 bursts in a 200 s time window) in the solution with higher CV concentration.

The previous experiments show that the fluorescence bursts must be due to enhanced fluorescence of CV molecules. Such fluorescence bursts are also observed in standard fluorescence correlation spectroscopy (FCS) where single fluorophores diffuse through the focal volume of the excitation laser. Those experiments, however, are performed at very low concentrations (typically tens of pM to 1 nM[25]) so that only one molecule (or very few ones) is present in the focal volume at any given time. In this experiment, we used a CV concentration of 100 nM, corresponding to approximately 60 molecules in the focal volume. We therefore do not expect any significant fluctuation of fluorescence in a confocal fluorescence time trace. This is confirmed by the absence of fluctuations in a time trace recorded from a 100 nM CV solution in glycerol in the absence of gold nanorods (Figure S2). We also rule out the possibility of aggregates of CV, as these molecules carry a net positive charge and form very stable solutions in polar solvents. We did not observe any significant shift of the absorption maximum or change in the shape of the absorption spectra of CV dissolved in glycerol as a function of the concentration (up to 30 μM) and time (6 h) as shown in Figure S4. Moreover, the fluorescence intensity bursts were predominantly polarized along the long axis of the nanorod (Figure S5). These results confirm that the fluorescence enhancement is caused by the presence of a nanorod.

The maximum intensity of the fluorescence burst shown in Figure 3a is 4050 counts/10 ms, corresponding to an increase of 3350 counts/10 ms over the background signal of 700 counts/10 ms (fluorescence from background CV and nanorod). This increase is due to the enhanced fluorescence from one single CV molecule. Based on the fact that each CV molecule (when not enhanced) produces about 3 counts per 10 ms, this burst leads to a calculated fluorescence enhancement factor of about 1120. We can rule out the possibility that this enhancement is caused by more than one CV molecule

because at the given concentration of 100 nM, less than 0.001 CV molecules are present in the near-field of a nanorod (about 10^4 nm^3). We repeated the fluorescence enhancement study on 21 nanorods and found large variations in the maximum fluorescence enhancement among them. The lowest maximum enhancement we found was 165 and the highest one 1150 (Figure S6).

The large fluorescence enhancements also allow us to perform a fluorescence correlation study at a CV concentration of 100 nM. Figure 3b shows the autocorrelation curve (black) of the fluorescence bursts from the time trace shown in Figure 3a. A single exponential fit (red) to the autocorrelation curve yields a correlation time of 120 ms. We repeated this procedure for 21 other nanorods and found that most of them show a single exponential decay with an average correlation time of (100 ± 40) ms. For a few nanorods, we also see an additional slower component with a correlation time of up to several seconds (Figure S7). Using the bulk viscosity of glycerol, we can estimate a single CV molecule to spend a few milliseconds in the near-field of the nanorod (see the Supporting Information). This time is almost two orders of magnitude shorter than the average correlation time recovered from the autocorrelation curves. This discrepancy might indicate significant sticking of CV to the nanorod or to the substrate, followed by bleaching, as found in a previous report.[21] More work is needed to clarify this point.

Fluorescence enhancement results from both excitation enhancement and emission enhancement by the nanorod. At resonance, the field enhancement factor in the near-field of the rod, resulting from a combination of lightning rod effect and of plasmonic resonance, can exceed 30.[4,15] This factor can lead to intensity enhancements of about 1000 for the excitation rate, and/or to a similar factor for the spontaneous emission rate, depending on the respective spectral overlaps of the excitation line, of the fluorescence band, and of the plasmon resonance. Careful adjustment of the overlap between the surface plasmon resonance of a gold nanorod and the molecular absorption and emission spectra, has been predicted from theory to yield fluorescence enhancements of up to several thousand.[15] In our experiment, as fluorescence traces were recorded with a 633 nm laser which is very close to the surface plasmon resonance of the nanorods, we expect a large excitation enhancement. We note that our excitation intensity is rather low (each CV molecule absorbs about 10^6 photons per second) and even with a maximum excitation enhancement of 1000, the molecule is well below its saturation limit (total decay rate of 10^{11} s^{-1}, see the Supporting Information) and thus can take full advantage of the excitation enhancement. Moreover, the emission rate can also be enhanced as the plasmon resonance of the nanorods is close to the fluorescence maximum of the CV molecules. Thus the high enhancement that we observe here is most likely a combination of excitation and emission enhancements. The exact contribution of each of these factors will depend on the plasmon resonance of each particle and hence the overall enhancement would vary from nanorod to nanorod. Indeed, we observe a broad distribution of enhancement among the nanorods (Figure S6) we have studied. A quantitative estimation, however, requires more experimental and theoretical work and will be communicated in a separate article.

In conclusion, we report large fluorescence enhancements of up to one-thousand-fold by chemically synthesized gold nanorods. This large enhancement is achieved by selecting a dye with its absorption and emission very close to the surface plasmon resonance of the nanorods and by measuring the maximum enhancement corresponding to the optimum position of the fluorophore with respect to the nanorod. We demonstrate an application of this large enhancement to perform correlation spectroscopy at a concentration of fluorophores two orders of magnitude higher (100 nM) than commonly used (10 pM to 1 nM). This promises a wider application of fluorescence correlation spectroscopy to many systems, notably in biology, where the analyte concentrations can be very high and cannot be arbitrarily reduced. We note that the enhancement factors reported here are still well below the theoretically predicted values. Further improvements including the use of dyes with a better spectral overlap with the nanorods and a still lower intrinsic quantum yield will be explored in the near future.

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