Conf-940142--21

LA-UR- 94-695

Title: FLUORESCENCE DETECTION OF SINGLE MOLECULES USING NEAR-FIELD OPTICAL EXCITATION AND TIME CORRELATED PHOTON COUNTING

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Submitted to: Proceedings for the Society of Photonics and Instrumentation Engineers Conference, Los Angeles, CA, Janaury 25-28, 1994, SPIE Proceedings, Vol. 2125, January 1994

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Form No. 836 R5
ST 2629 10/91

Fluorescence detection of single molecules using pulsed near-field optical excitation and time correlated photon counting

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ABSTRACT

Pulsed excitation, time correlated single photon counting and time gated detection are used in near-field optical microscopy to enhance fluorescence images and measure the fluorescence lifetimes of single molecules of Rhodamine 6G on silica surfaces. Time gated detection is used to reject prompt scattered background and to improve the image signal to noise ratio. The excited state lifetime of a single Rhodamine 6G molecule is found to depend on the position of the near-field probe. We attribute the lifetime variations to spontaneous emission rate alterations by the fluorescence reflected from and quenching by the aluminum coated probe.

2. INTRODUCTION

Single molecule detection (SMD) sensitivity was reported recently by two groups using fluorescence near-field scanning optical microscopy (NSOM).[1,2] NSOM is a scanned probe microscopy using a subdiffraction limit optical aperture in close proximity to a surface. SMD spatial resolution has been as high as 50 nm[1] to 90 nm[2]. Single molecule detection was achieved using NSOM under ambient conditions because the small probe area and power of illumination resulted in a low background and associated noise from the probe fiber. The evidence that single molecules were detected in these experiments included several observations: (1) The number of fluorescent objects observed was near the correct order of magnitude for the expected surface coverage.[1,2] (2) The fluorescence signals were within an order of magnitude of estimates based on values for the photophysical parameters in solution.[1,2] (3) Photobleaching times and efficiencies were within the previously observed range for Rhodamine 6G (R6G) on silica.[1] (4) The character of photobleaching[2] and fluorescent images[1] were very different from observations on large numbers of molecules. When a single molecule is photobleached, the fluorescence signal is constant and then abruptly disappears, rather than smoothly decaying as for large numbers of molecules. In addition to the complete loss of fluorescence upon photobleaching, there are also fluctuations in the signal from single R6G molecules on silica that are attributed to small motions of these molecules.[2] Since the physical extent of the probe electric field is ~100 times larger than the molecule or its absorption cross section, a single point-like molecule probes the electric field of the light exiting the tip and the resulting image is a projection of this highly curved field. These tip-field images range from singly peaked to multiple-lobed, the orientation of which depend on the near- and far-field polarization.[1] In the above experiments, CW excitation of molecular fluorescence was used.[1,2]

For SMD in liquids, the background was decreased two or three orders of magnitude, and signal to noise ratio (S/N) was improved when cw excitation[3] was replaced with pulsed excitation, time correlated photon counting (TCPC), and time gated detection.[4] The improvement occurs because the prompt background scatter and fluorescence photons are separated temporally and much of the background and the associated noise can be time-gated out of the counting electronics. In this paper, NSOM is combined with TCPC and time gated detection to improve the SMD S/N ratio. The improved S/N allows more reliable and repeatable observations of image features and physical effects. Also, TCPC is used to measure the fluorescence lifetimes of single R6G molecules on silica. The fluorescence lifetime of a single molecule is found to depend on relative position between the tip and a molecule, but not input power, tip irradiance or tip temperature. Qualitative features of the lifetime dependence on tip position and a skewed distribution of double lobed images are explained by known fluorescence interference and quenching effects near metal surfaces.

3. EXPERIMENTAL DETAILS

Sample preparation,[2] TCPC[4] and NSOM[2] were described previously in detail, and are briefly summarized below for the reader. Samples consisted of fused silica disks spin coated with either $3x10^{-9}$ or 10^{-6} M R6G/methanol solutions so that the average R6G spacing was ~ 1 μm or ~0.07 μm. Fluorescence images of a sample surface were obtained with NSOM. An NSOM probe is a tapered single mode optical fiber that has been coated with aluminum and has a small aperture at the tip (100 to 200 nm diameter for this work). To form images, the probe is scanned in close proximity to a fused silica disk, and optical information is recorded at each position. The tip height is controlled to within a few nanometers using shear force microscopy (SFM).[2] Our microscope is used in illumination mode, where laser light that is coupled into the single mode fiber emerges from the aperture and illuminates the surface. The laser is a mode locked Ar^+ laser operated at 514.5 nm, with an 82 MHz pulse repetition rate and ~150 ps pulse width (FWHM). Fluorescence excited from molecules on the surface is collected and detected using a photon counting Si avalanche photodiode (APD).[2] In this work, the APD photo-pulses were then counted using TCPC techniques, as described elsewhere.[4] In TCPC the time between laser pulses and emitted photons is measured. This timing information is used both to measure fluorescence decay times and to reject prompt scattered light for S/N enhancement. In this experiment, a time-to-amplitude converter (TAC) was used to measure the time between each detected single photon (prompt scatter or delayed fluorescence) and the excitation laser pulse. A histogram of the arrival times of fluorescence photons is a fluorescence decay curve, from which the fluorescence lifetimes of single molecules were obtained. A single channel analyzer (SCA) built into the TAC was used to gate photon counting so that prompt scatter was rejected and delayed photons were counted. For emission rate measurements, two methods were used to count fluorescence photons: (1) At fixed tip positions, a multichannel scaler (MCS) was used to count the time between pairs of detected fluorescence photons,[4] and the average

rate was calculated later. (2) For collecting fluorescence images, the SCA-out signal of the TAC was counted in time intervals 20 to 40 ms long using a conventional photon counter[2] and then recorded by the NSOM scanning electronics.

4. RESULTS AND DISCUSSION

TCPC histograms for an NSOM tip positioned over a single molecule (a), and over a bare silica surface (b) are shown in Fig. 1. The peak in each trace (positioned at zero time) is prompt background scattered light. In (b), a weak tail of delayed background light extends beyond the laser pulse, but is down ~ 3 orders of magnitude from the peak. Since the prompt background is larger than the fraction of the excitation power expected through the long pass spectral filters between the sample and the detector ($< 10^{-8}$),[2] this background is probably Raman scattered light from the ~ 30 cm single mode optical fiber connected to the NSOM tip. In (a), the tail of fluorescence from a single molecule is approximately one order of magnitude larger per bin than the background tail. The integrated prompt background (12,000 photocounts/sec) is comparable to the integrated fluorescence signal in the tail (7,000 photocounts/sec), but the background in the tail region is much less (1,400 photocounts/sec). For rejection of background in emission rate measurements, a time window is set from 0.7 to 10 ns after the peak, and photons arriving in this window are counted (photons arriving at other times are rejected). Assuming shot noise limited operation and using only the noise in the background (signal contributions to the noise at the peak are ignored), the S/N ratio is ~ 32 $(Hz/nW)^{1/2}$ for gated photons in a 0.7 to 10 ns time window, and 10 $(Hz/nW)^{1/2}$ for ungated photons. Time gating decreases the background by an order of magnitude and the noise in the background by a factor of 3.

The reduction in background noise improves images containing single R6G molecules, as shown in Fig. 2. Surface plots (a), (b), and (c) were taken in sequence with the time gate set to (a) reject prompt background, (b) include the background, and then (c) reject the background again. The fewer number of features in (c), as compared with (a), results from photobleaching of some of the molecules during imaging. The background is subtracted in all cases, but is 422 photocounts/ 40 ms higher in (b). Note that the noise in the background between the fluorescent features is considerably larger in (b) compared with (a) and (c), and impedes the reliable identification of features in this image. The RMS background noise reduction by a factor of 3.2 in (a) and (b) allows finer details to be observed in these images.

Examples of the finer details observable with improved signal to noise ratio are shown in Fig. 3. Two images are shown of the same region of a silica disk with a submonolayer coverage of R6G molecules. These two images were obtained with orthogonal excitation polarization, as indicated in the caption. Note that the images contain many double lobed features with an orientation aligned with the field polarization. This is a demonstration that the absorption by these fluorescent objects is polarization dependent, i.e., they have the linear transition dipole character of single

molecules.[1] An interpretation of the various image shapes is that the dipoles have different fixed orientations and the emission rate is proportional to the square of the projection of the highly curved electric field onto the dipole axis.[1] The strengths of the lobes are not equally distributed top to bottom (a) and left and right (b), as one might expect from a statistical distribution of orientations, but rather are more peaked on the bottom in (a) and to the right in (b). The asymmetry in the lobe heights is consistent with other effects observed below.

TCPC and time gated detection were used also to measure the lifetimes of individual molecules. In our first experiments, the tip was positioned over the brightest part of an image, and TCPC was performed until the molecule photobleached (observed as an abrupt decrease of the emission intensity to the background level[2]). Fluorescence decay curves for individual R6G molecules on silica are shown in Fig. 4. To obtain these curves, a background histogram is generated also on a bare portion of the sample, and the background is subtracted from the single molecule data before taking the logarithm. As seen in Fig. 4, the fluorescence is a single exponential when the background is subtracted. In these experiments, there appear to be many different lifetimes for single R6G molecules on silica ranging from ~ 1 to 5 ns. For a tip pulled back ~ 1 μm from a higher coverage surface (estimated to have 5.3×10^{10} R6G/cm^2 = 5.3×10^2 R6G/μm^2), the decay curve is a single exponential with a lifetime of $\tau = 3.7 \pm 0.3$ ns. This value is in agreement with the value of 3.5 ns obtained previously for conventional far-field excitation of R6G on silica,[5] and will be referred to below as the bulk lifetime. This implies that all molecules on the surface have the same intrinsic lifetime rather than the 1 to 5 ns distribution of lifetimes displayed in Fig. 4. With the tip engaged with the high coverage surface, the fluorescence decay then becomes non-exponential (the tip is within ~ 10 nm of the surface and there are ~ 25 molecules at different positions within the near-field region).

To examine why the single exponential decay curve became nonexponential when the tip was engaged with the surface with many R6G molecules near the tip, fluorescence lifetimes were measured as a function of tip position across spatially well separated single molecules on a lower coverage surface. Figure 5 shows the selection of such a molecule and the tip path for position dependent lifetime measurements. First, an image of a region was obtained as shown in Fig. 5 (a). The boxed area in (a) is shown as a surface plot in (b). In the upper left part of the boxed area there is a terminated feature reminiscent of a moon on the horizon. We interpret this as a molecule that bleached during one of the line scans in the image (seen as an abrupt loss of signal to the background during a scan in Fig. 5 (b)). Other molecules have intensity fluctuations such as the feature in the lower right corner of (a) that has a dark line through the lower lobe and the feature in the upper left corner of (a) that has a noisy appearance. These intensity fluctuations are probably due to small motions of the molecules that change their orientation and the strength of their absorption and emission during successive scans.[2] The feature in the lower right corner of the box is stable and was selected for fluorescence lifetime dependence on tip position. In contrast to other molecules, this molecule did not photobleach before the completion

of the lifetime measurements described below.

Figures 6 and 7 show the emission rate and lifetime measurements taken at fixed positions along the arrow in the plane of Fig. 5 (the tip is engaged with the surface throughout these measurements). The measurements were performed at two excitation power levels differing by a factor of 9. In Fig. 6, the emission rate as a function of position shows the doublet profile taken along the arrow in Fig. 5. The emission rates approximately scale with the far-field power, indicating that the irradiance is well below the saturation value. At each position of the tip, a decay curve is generated, the background from a bare region is subtracted, and the data are fitted to a single exponential function. Single exponential decays are found at each position, but the extracted lifetimes are strongly dependent on the position of the tip as shown in Fig. 7. The lifetimes start at values well below the bulk lifetime (3.7 ns), pass through and then exceed this value, and begin to fall again on the trailing edge. (Similar traces are observed in moving from left to right across one of the lobes instead of both lobes.) The lifetimes do not depend on the power levels used to make these measurements, and therefore are not a function of the tip irradiance or tip temperature. The most reasonable conclusion is that the fluorescence lifetime depends on the position of a single molecule near the tip.

Metal surfaces in close proximity to radiating dipoles are known to influence the fluorescence decay time strongly.[6] The results of applying an approximate theory[7] to calculate the lifetime of an R6G-like molecule as a function of the distance from a planar aluminum surface are shown in Fig. 8. Two distinct physical phenomena occur. As the molecule approaches from infinity, it is influenced more and more strongly by the reflected and retarded field from the metal. Spontaneous emission is suppressed or enhanced depending on the phase of the reflected field. In the far zone, the lifetime can have values both shorter and longer than without the metal. As the molecule approaches to within ~50 nm of the metal surface, direct energy transfer from the molecule to the metal begins to dominate the excited state decay, and the lifetime approaches zero as $\sim d^3$. For other metallic structures such as thin films[8] and spheroids[9], the two processes, interference in the far zone and quenching in the near zone, are still present, though they may be altered by an order of magnitude or more. The simple model in Fig. 8 is sufficient for illustrating that very close to the metal fluorescence is quenched, and at intermediate distances the spontaneous emission may be suppressed so that the lifetime exceeds the bulk value. The data in Fig. 7 and Fig. 3 are in agreement with the qualitative features of Fig. 8.

Since the lifetime is shorter than the bulk lifetime on the left side of Fig. 7, the metal quenching effect is stronger there (perhaps there is an angle between the tip face and the surface, or a bulge in the metal coating, and the metal is closer to the surface on this side of the image). In the middle of Fig. 7, the lifetime exceeds the bulk lifetime, and reflected wave interference effects must dominate. The half width of the images in this work are ~ 100 nm, which is approximately the physical aperture radius. The phase change for a reflected and retarded wave may be on the order of 180 degrees for a molecule in the middle of such a metal structure, and it is reasonable to

suppose that spontaneous emission suppression and longer lifetimes could result. In examining Fig. 3 (a), the distribution of doublets was found to be peaked more strongly on the lower lobe. This may be a result of the strong quenching by the metal on the upper side, which reduces the quantum efficiency for emission and the signal size. (The dipole orientation leading to the roughly equal strengths of the lobes in Fig. 6 would probably have resulted in a stronger emission on the upper lobe in the absence of quenching.) In Fig. 4, the tip was positioned over the brightest part of a single molecule image. Since this position depends on the orientation of the molecule, the measurements were made on molecules in different positions relative to the tip, which resulted in the different lifetimes found in these initial experiments.

5. CONCLUSIONS

We have demonstrated that pulsed excitation and time gated detection are useful for improving the S/N in NSOM since the background is dominated by prompt Raman scattering in the probe optical fiber. Improved S/N led to clearer images of the tip electric field, as probed by single R6G point dipoles. The fluorescence lifetimes of single R6G molecules on silica are found to vary with the lateral displacement of a molecule relative to the center of the tip. A likely explanation is that interference and quenching effects by the metal coating on the tip alters the emission rate and lifetime.

6. REFERENCES

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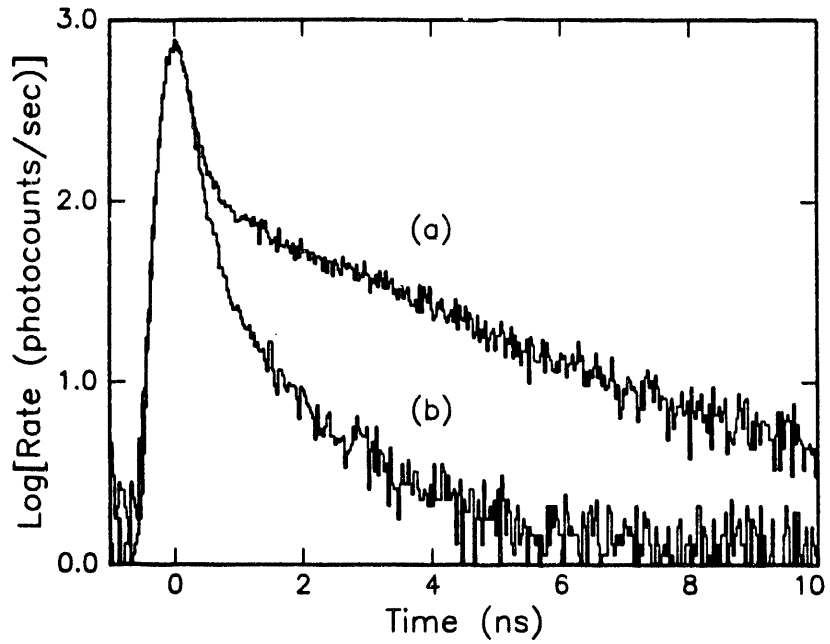

Figure 1. Time correlated single photon counting histograms taken (a) over a single Rhodamine 6G molecule, (b) over bare substrate. The excitation power emerging into the far-field was 33 nW.

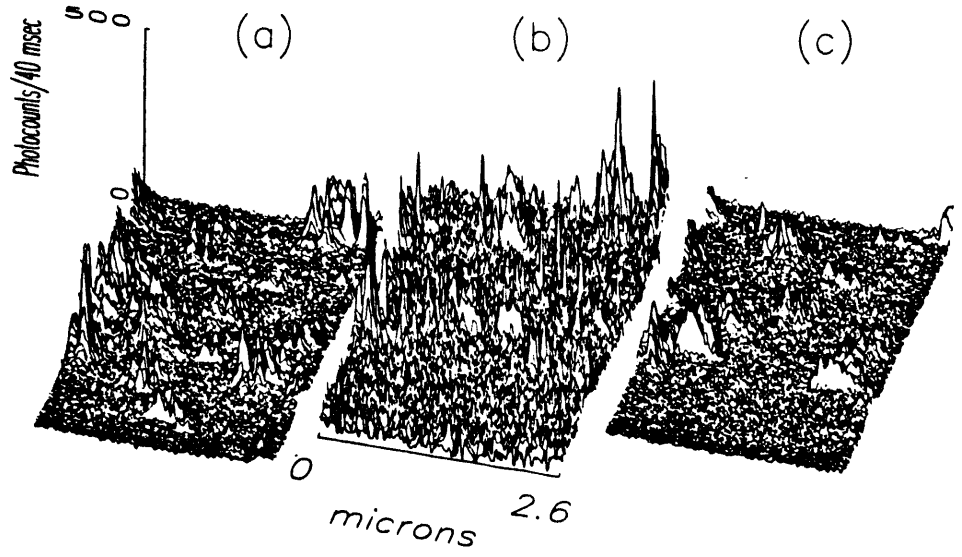

Figure 2. Noise reduction in fluorescence NSOM images using pulsed excitation and time gated detection. A sequence of fluorescence images were collected in the same region of the sample by counting photons [(a) and (c)] in a gated time window of 0.7 to 10 ns after the peak in Fig. 1, or (b) with ungated counting (prompt background photons included). An increased average background level of 422 photocounts/40 msec has been subtracted from (b). The excitation power measured in the far-field was 15.6 nW.

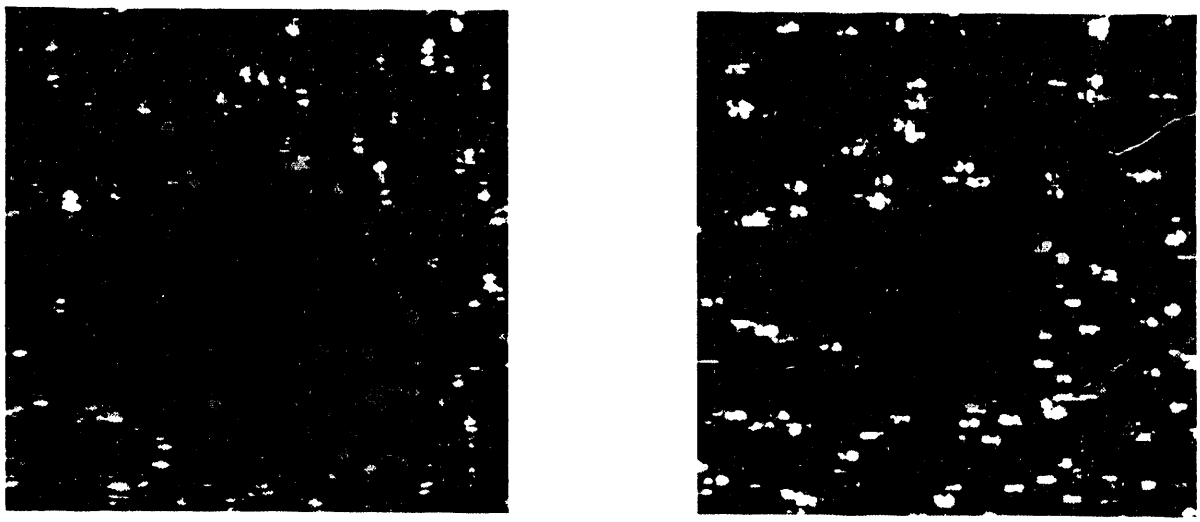

Figure 3. Field polarization dependence in images of single Rhodamine 6G molecules on silica. The polarization of the excitation light emitted into the far-field is predominantly polarized (a) vertically and (b) horizontally. The images are 7.7 μm wide and 8.2 μm high.

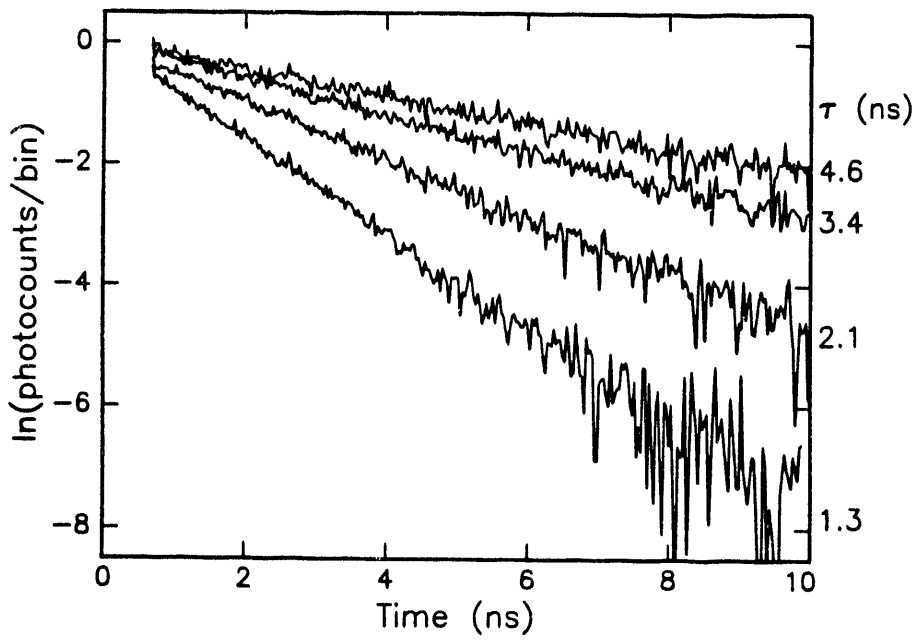

Figure 4. Fluorescence decay curves for probe tips fixed in position over different R6G molecules. The lifetime obtained from a linear fit to each curve is indicated on the left side of the figure.

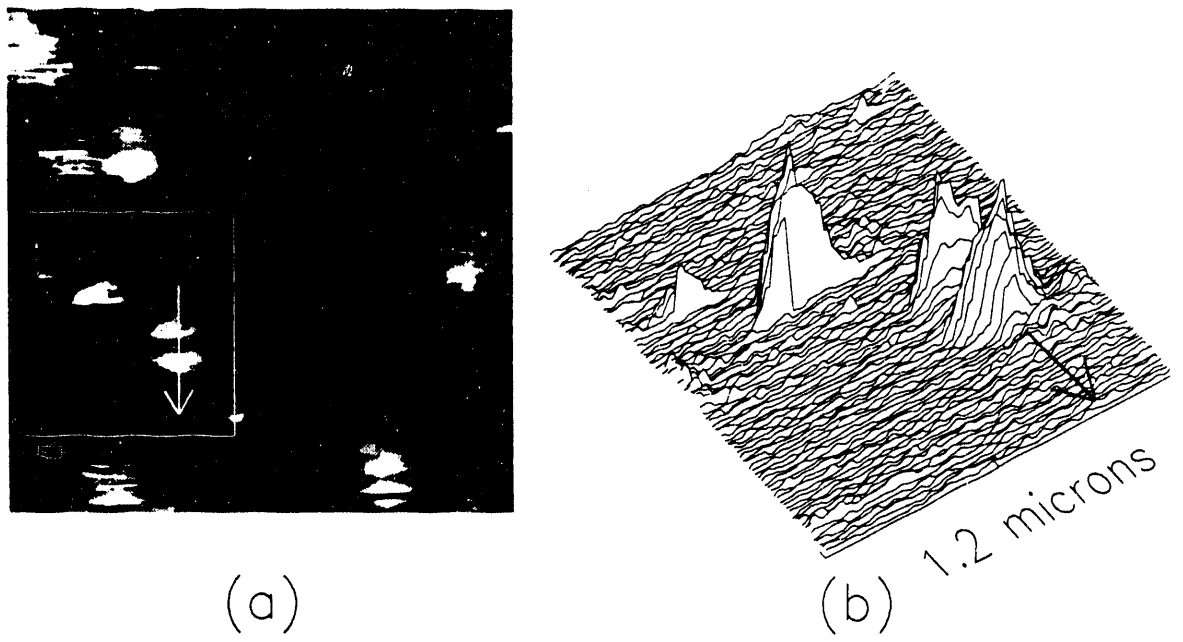

Figure 5. Selection of a single molecule for lifetime dependence on tip position. (a) is a 2.6 X 2.7 μm fluorescence image. The box in (a) is shown as a surface plot in (b). The arrows show the path across the doublet image of a single R6G molecule used for lifetime measurements.

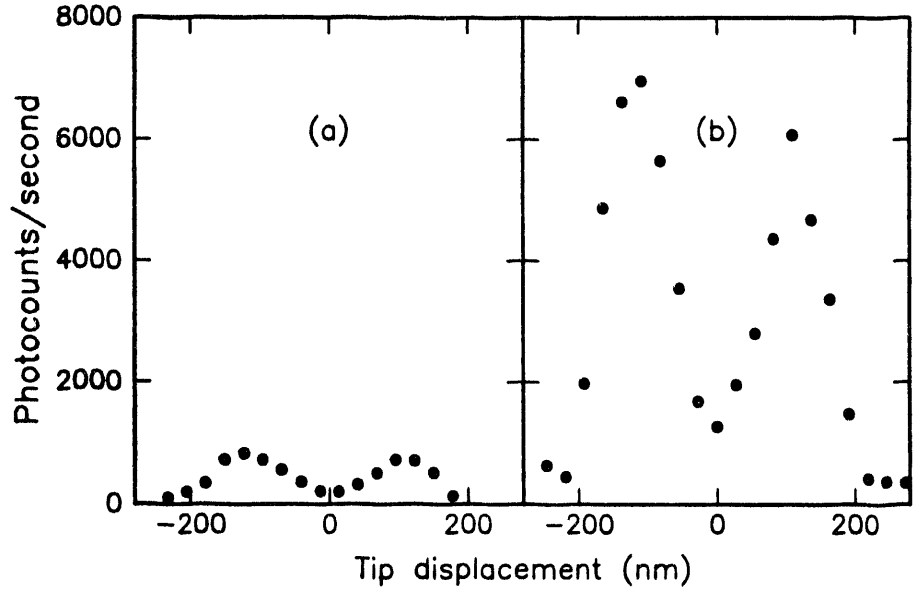

Figure 6. Emission profiles across the single molecule doublet in Fig. 5 taken at two power levels. (a) The far-field power emerging from the tip was 2.5 nW, and (b) was increased 9 times to 22 nW.

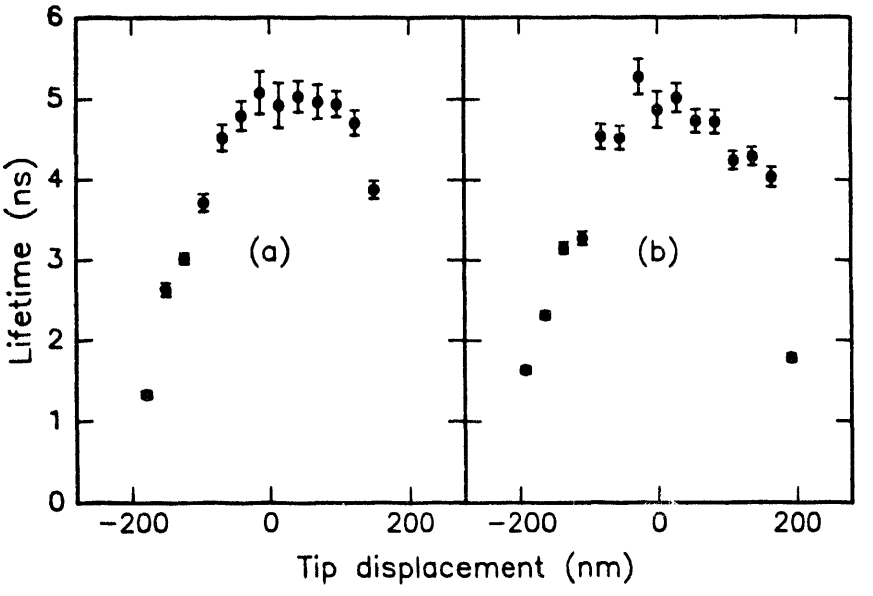

Figure 7. Single molecule fluorescence lifetime dependence on tip position. The lifetimes in (a) and (b) were computed from TAC histograms obtained concurrently with the emission rate data in Fig. 6. The lifetimes depend on tip position, but do not vary with power emerging at the probe tip.

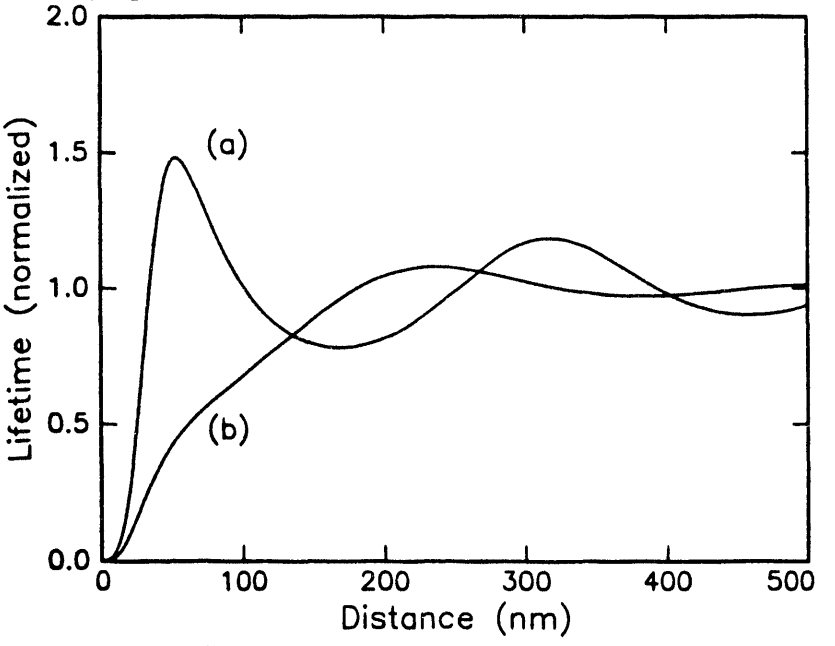

Figure 8. Fluorescence lifetime dependence on distance from an infinite half space of aluminum. Two physically distinct phenomena occur when a radiating dipole approaches a metal surface: (1) the reflected light interacts with the radiator and results in lifetimes shorter and longer than without the metal, and (2) in the near zone, the lifetime is dramatically decreased by rapid energy transfer to the metal. The radiating dipole is (a) parallel and (b) perpendicular to the surface.

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