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Resolving Single Fluorophores Within Dense Ensembles: Contrast Limits of Tip-Enhanced Fluorescence Microscopy

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Most biological samples that could benefit from study with apertureless varieties of near-field microscopy (e.g. membranes) are composed of a relatively high-density ensemble of various proteins, lipids, or other biomolecules. To study these samples, a near-field microscope should be sensitive to individual molecules within the ensemble while maintaining nanoscale resolution. This is challenging because multiple molecules within the laser focus elevate the background signal, thus lowering the signal-to-noise ratio and reducing the sensitivity. Here, we investigated the limits of one-photon fluorescence as a contrast mechanism in nanoscale-resolution tip-enhanced optical microscopy, a technique we call tip-enhanced fluorescence microscopy (TEFM). Specifically, we examined the magnitude of tip-induced signal enhancement needed to resolve individual fluorophores within densely-packed ensembles [1].

To resolve an individual molecule using TEFM, the near-field signal from that molecule must be approximately the same as the background signal originating from all the molecules within the diffraction limited (far-field) laser focus, $C = S_{NF}/S_{BG} > 1$, where C is the image contrast, and S_{NF} and S_{BG} are the near-field and background signals, respectively. We developed a model for our oscillating-tip TEFM, which was then used to predict the amount of signal enhancement needed to achieve $C > 1$ for single fluorophores within high density samples. For this calculation, we assumed a fluorophore density of 10,000 μm^{-2}, which corresponds to one fluorophore for every 10×10 nm^2 corresponding roughly to the lateral extent of the tip-enhanced zone. For contact-mode imaging using a focused linearly-polarized Gaussian laser beam, a signal enhancement factor of $f_{CM} \sim 2{,}500$ is required in this high density limit [1].

When operating the TEFM in tapping mode, the near field fluorescence signal is modulated with the tip height. Through the use of a lock-in amplifier, the near-field signal can be demodulated, resulting in much lower constraints on the signal enhancement factor. We mathematically modeled the behavior of the lock-in amplifier in tapping mode; to achieve $C > 1$ in this case, the required signal enhancement factor, f_{TM}, follows the relationship:

$$f_{TM} > \frac{1}{\alpha\gamma}\sqrt{\frac{3\pi\beta N_{FA}}{4k}}$$

where k is the efficiency of the optical system, β is a parameter characterizing the filtering quality of the lock-in amplifier, N_{FA} is the number of fluorophores in the diffraction-limited focal area, and α

and γ depend on the tip-oscillation amplitude and are related to the lock-in amplifier and the effective time the tip spends near the sample, respectively [1]. The model has been validated in part by its agreement with experimental results (Fig. 1) using values for the above variables extracted from experimental measurements (no free parameters). We found that tapping mode imaging coupled with lock-in demodulation increased image contrast by nearly two orders of magnitude. This reduces the requisite signal enhancement factor to $f_{TM} \sim 18$ for radially polarized incident light. Signal enhancement factors as large as ~20 have been demonstrated for quantum dots using commercially available Si tips [2]. It should be possible to achieve even larger signal enhancement factors using metal tips in combination with fluorophores with low intrinsic quantum yield [3, 4].

In summary, our analysis indicates that it should be possible to resolve individual molecules within dense ensembles where the fluorophores are separated by only ~10 nm, which may ultimately lead to ultra-high resolution structural studies of intact biomolecular networks.

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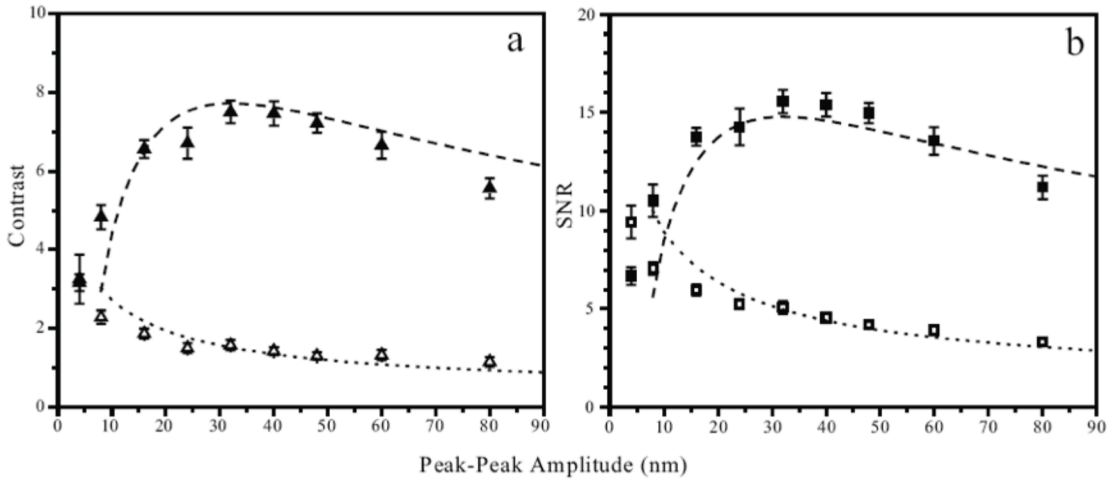

FIG. 1. TEFM image contrast, panel (a), and signal-to-noise ratio, panel (b), for isolated quantum dots as a function of the tip oscillation amplitude. Data were obtained using commercial silicon tips. Data points correspond to the average value of ~15 measurements for the lock-in demodulation signal (closed symbols) and the scalar photon sum (open symbols). Dashed and dotted lines are the corresponding theoretical predictions.

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