Supporting Information

Optically Modulated Fluorophores for Selective Fluorescence Signal Recovery

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S1. Sample Preparation

Samples for ensemble experiments were prepared by spin casting 50 nM Ag nanodot solutions in saturated PVA on a coverslip and imaged through a 15 μM solution of Cy5. To demonstrate single molecule signal recovery, separate samples of Ag nanodots (~500 pM, or 100-fold lower concentration than that used for ensemble experiments) in saturated PVA solution and Cy5 (200 nM) in saturated PVA solution were separately spin cast on to coverslips. Cell samples were prepared such that NIH 3T3 cells were washed with PBS with 2 mM $MgCl_2$ once and then loaded with NHS-LC-Biotin (Pierce) (1 mg/mL in PBS with 2 mM Mg^{2+}) and incubated at 4º C for 15 min, then fixed with 4% formaldehyde at r.t for 15 min. After 3 washes, cells were incubated with avidin (Pierce) (1 mg/mL) for half an hour and then incubated with biotinylated ssDNA-Ag nanodots for 30 minutes and washed three times with phosphate buffer (pH7) and mounted onto thick glass slides (Erie Scientific, Portsmouth, NH).

S2. Signal Extraction for Whole Image Demodulation

Modulation of Ag nanodot fluorescent signals was achieved by simultaneous excitation with the primary excitation laser (633 nm) and with an intensity modulated secondary laser (805 nm) in an epifluorescence setup. The secondary laser results in selective fluorescence increase solely resulting from photobrightened Ag nanodots. This occurs precisely at the secondary laser modulation frequency. Imaging of ensemble Ag nanodot signals was performed by imaging (60x, 1.2NA, Andor iXon EMCCD, 16μm pixels) with constant defocused 633 nm-excitation (3 kW/cm^2) and optically chopping a focused 805 nm-excitation (2 kW/cm^2) at 10Hz with a camera frame rate of 100 Hz for a total of 10 seconds. Cellular imaging was performed by imaging (60x, 1.45NA, Andor iXon EMCCD, 16μm pixels) with constant 633 nm excitation (1.5 kW/cm^2) and 805 nm laser (23 kW/cm^2) modulated at 1Hz with a camera frame rate of 10Hz. Single molecule signal extraction was performed by imaging, (1.4NA, 150x total magnification, Andor iXon (front illuminated) 7.8 μm pixels) a sample of Cy 5 (200 nM) and 710-nm-emitting Ag nanodots (~500 pM) in a PVA film under constant 633 nm excitation

(800 W/cm^2), and simultaneous 805 nm excitation (6 kW/cm^2) optically chopped at 4 Hz with a frame rate of 40 Hz. Fourier transforms of intensity versus time for each individual pixel, within the entire image stack, were calculated. Each pixel's amplitude at the modulation frequency was extracted, with these values directly yielding the demodulated image.

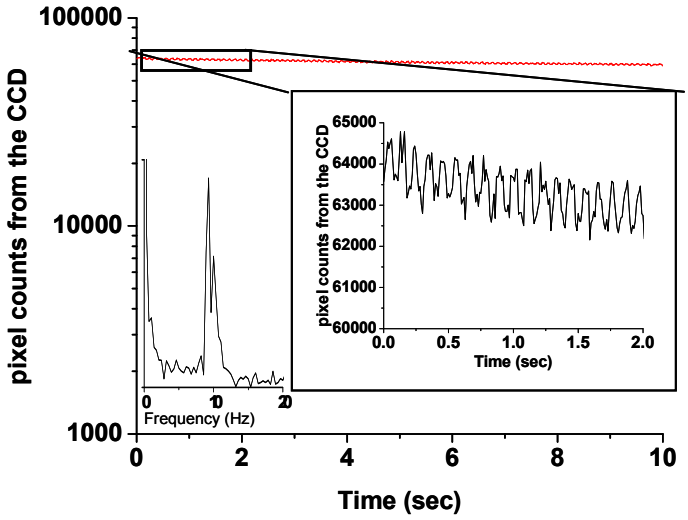

Figure 1S. Ensemble Pixel Time Trace and Fourier Transform

Time trace of an individual pixel for 10 sec with **insets** of (right) a zoomed in region of the time trace revealing the modulated intensity (from Ag nanodots) over the high Cy5 background and (left) the Fourier transform of the same time trace showing the modulation frequency component.

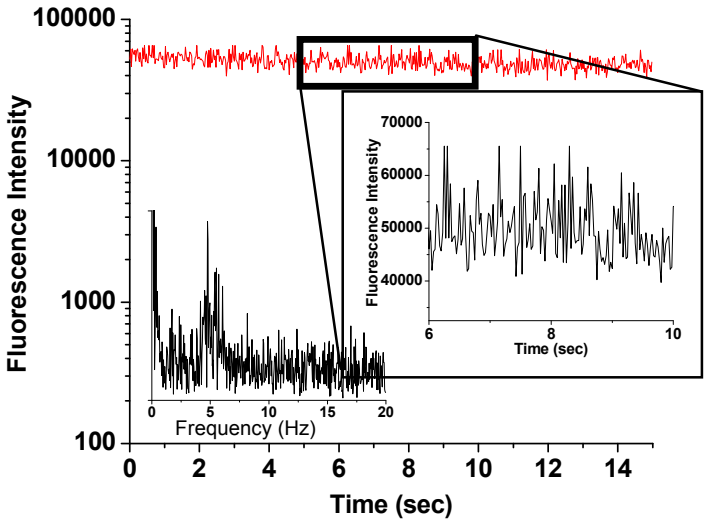

Figure 2S. Single Molecule Dipole Pixel Time Trace and Fourier Transform

Time trace of an individual pixel showing the fluorescence intensity for 15 sec with insets of an expanded region of the time trace revealing the modulated intensity (from Ag nanodots) above the Cy5 background and the Fourier transform of the same time trace showing the frequency component matching the original modulation frequency. As a very low frequency chopper was used for modulation, the modulation frequency is broadened over a few Hz.

S3. Single Molecule Photophysical Characterization

Correlation analysis of individual Ag nanocluster molecules was used to verify the origin of the enhancement realized upon simultaneously exciting with a low energy secondary laser. The microsecond blinking dynamics of Ag nanoclusters exhibited both in polymer matrices and in aqueous environments (ref 22) is readily observed through correlation analysis. In this way the effect of the secondary laser on the dark state dynamics of individual molecules isolated in PVA films can be directly monitored. Under typical excitation conditions (633 nm 1.8 kW/cm^2) the autocorrelation of a single molecule time trace showed a dark state decay time of 6 μs (fig 3s A). When exposed to low intensity secondary laser (805 nm 700W/cm^2) the correlation time shifts to 4.4 μs (fig 3s B) and the contrast of the correlation significantly decreases confirming the depopulation of the dark state.[1] Increasing the intensity of the 805 nm laser completely eliminates the microsecond component in the correlation (fig 3s C). Thus the observed fluorescence enhancement results directly from the depopulation of the dark state. While for the most part blinking is limited to the microsecond regime (ref 22) some infrequent longer time scale blinking of individual molecules has been observed particularly at higher excitation intensities.(ref 22) Whole image demodulation using Ag nanocluster labeled cells revealed some similar blinking behavior suggesting single molecule labeling. This blinking has no effect on the over all demodulation process as the modulation frequency is still applied to the fluorescent signal.

Interestingly, this blinking can be ameliorated by increasing the spacer length between the biotin and the oligonucleotide scaffold.

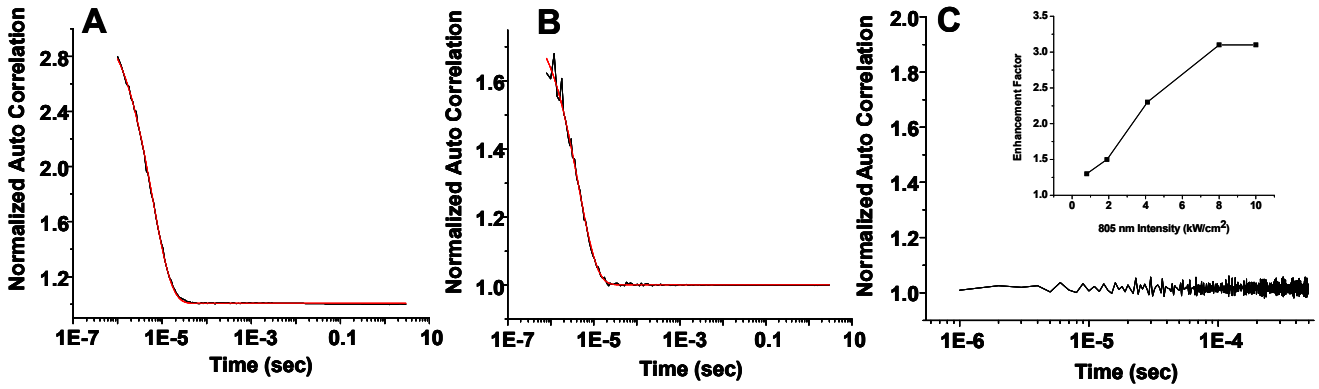

Figure 3S. Autocorrelation Analysis of Single Laser vs Dual Laser Excitation of Single Molecules

A. Autocorrelation of the time trace from an individual Ag nanocluster excited with a 633 nm laser showing a clear microsecond blinking component. **B**. Autocorrelation of the same molecule now simultaneously excited with low intensity 805 nm laser (700W/cm^2). The correlation shows a reduction in the correlation contrast and slight shift to a faster time scale. **C.** Autocorrelation of the same molecule now simultaneously excited with a higher intensity 805 nm laser (5 kW/cm^2)showing the complete elimination of the microsecond blinking component verifying that excitation with the second laser rapidly depopulates the dark state. **Inset** showing the typical linear dependence of the enhancement factor on the intensity of the secondary laser, and enhancement saturation at higher intensities.

In order to definitively verify single molecule signal extraction, a spin coated PVA film containing Cy 5 (200 nM) and 710-nm-emitting Ag nanodots (~500 pM), which results in widely spaced molecules embedded in the film, was imaged with constant 633 nm excitation and an 805 nm laser modulated at 4 Hz. Subsequent demodulation resulted in images of individual molecules of Ag nanoclusters while the signal from the high concentration Cy 5 was completely eliminated (fig 4s).

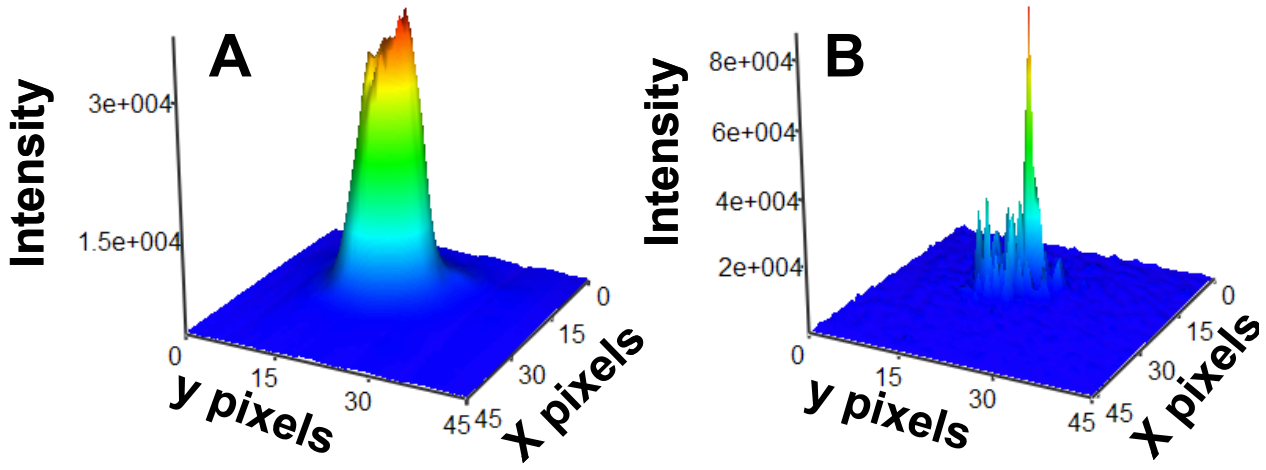

Figure 4S. Demodulated Image of Individual Ag NC's and high Concentration Cy 5 in a PVA Film

Cy 5 (200 nM) and 710-nm-emitting Ag nanodots (~500 pM) in a PVA film imaged (1.4NA, 150x total magnification, Andor iXon (front illuminated), 7.8mM pixels, 40 Hz) under constant 633 nm excitation (800 W/cm^2) and simultaneous 805 nm excitation (6 kW/cm^2) optically chopped at 4 Hz. **A**. 3-D plot of a typical 25 ms-exposure ccd frame of the film showing the high Cy 5 fluorescent background. **B**. 3-D plot of the demodulated signal obtained from A with a 10 second time constant, showing the recovered emission from an otherwise obscured (presumably) individual Ag nanodot.

(1) Yip, W. T.; Hu, D. H.; Yu, J.; Vanden Bout, D. A.; Barbara, P. F. *J. Phys. Chem. A* **1998**, *102*, 7564-7575.