Modulated Fluorescence Correlation Spectroscopy Enables Quantitative Ag Nanodot Signal Recovery

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Supplemental Information

S1. Data collection

Home built software written in C controlled a National Instruments PCI-6602 counting card which recorded incoming photon arrival pulses from an avalanche photodiode (APD), marking the absolute arrival times – the "macrotime". An electro optical modulator (EOM) which modulates the secondary laser excitation intensity is controlled via a function generator, is synchronized to a second function generator whose output also synchronizes the photon arrival times to the secondary laser modulation frequency (Fig. S1). Photon time stamps were then plotted as a histogram by subtracting integer modulation periods such that all photon arrival times were within a single modulation period, revealing the clear high/low structure, as in Fig. 3. Equal duration portions of the dual-laser (higher counts) and one-laser (lower counts) regions of the modulation cycle were manually selected and correlations performed according to Eq. 2.

S2. Modulation Depth

Optical enhancement due to secondary excitation results from the optically induced depopulation of nonemissive states. Relative enhancement is defined as (D-S)/S, where D is the fluorescence intensity from dual laser excitation and S is the fluorescence intensity from single laser excitation. The relative enhancement vs. modulation frequency is plotted in Fig. S4. Enhancement decreases as the modulation frequency approaches the sum of photophysical rates into and out of the nonemissive state. At these high frequencies, modulation becomes faster than the time needed to fully establish steady-state populations of ground and dark states that result in modulation.

S3. Derivation of the correlation subtraction function

Taking signal as S[t], background as B[t], and enhancement with secondary laser excitation being c*S[t], where c is a constant that is greater or equal to 1, we can recover the true background-free signal autocorrelation through correlation subtraction. Starting with the addition and subtraction of single (S[t]+B[t]) and dual (cS[t]+B[t]) laser portions of the modulation cycle, the following correlation manipulation is simplified to yield Eq. 2 of the manuscript:

$$\int_{-\infty}^{\infty}(cS[t]+B[t])(cS[t+\tau]+B[t+\tau])\,dt+\int_{-\infty}^{\infty}(S[t]+B[t])(S[t+\tau]+B[t+\tau])\,dt$$
$$-\int_{-\infty}^{\infty}(cS[t]+B[t])(S[t+\tau]+B[t+\tau])\,dt-\int_{-\infty}^{\infty}(S[t]+B[t])(cS[t+\tau]+B[t+\tau])\,dt$$

Expand the first term:

$$\int_{-\infty}^{\infty}(cS[t]+B[t])(cS[t+\tau]+B[t+\tau])\,dt$$

$$=\int_{-\infty}^{\infty}c^2S[t]S[t+\tau]\,dt+\int_{-\infty}^{\infty}B[t]B[t+\tau]\,dt+\int_{-\infty}^{\infty}cB[t]S[t+\tau]\,dt+\int_{-\infty}^{\infty}cS[t]B[t+\tau]\,dt$$

$$=c^2\int_{-\infty}^{\infty}S[t]S[t+\tau]\,dt+\int_{-\infty}^{\infty}B[t]B[t+\tau]\,dt+c\int_{-\infty}^{\infty}B[t]S[t+\tau]\,dt+c\int_{-\infty}^{\infty}S[t]B[t+\tau]\,dt$$

Expand the second term (autocorrelation of second half of the cycle (no secondary laser)),

$$\int_{-\infty}^{\infty}(S[t]+B[t])(S[t+\tau]+B[t+\tau])\,dt$$

$$=\int_{-\infty}^{\infty}(S[t]S[t+\tau]+B[t]B[t+\tau]+B[t]S[t+\tau]+S[t]B[t+\tau])\,dt$$

$$=\int_{-\infty}^{\infty}S[t]S[t+\tau]\,dt+\int_{-\infty}^{\infty}B[t]B[t+\tau]\,dt+\int_{-\infty}^{\infty}B[t]S[t+\tau]\,dt+\int_{-\infty}^{\infty}S[t]B[t+\tau]\,dt$$

Expand the third term (the cross correlations between the first and second halves of the cycle):

$$\int_{-\infty}^{\infty}(cS[t]+B[t])(S[t+\tau]+B[t+\tau])\,dt$$

$$= \int_{-\infty}^{\infty} (cS[t]S[t+\tau] + B[t]B[t+\tau] + B[t]S[t+\tau] + cS[t]B[t+\tau])\, dt$$

$$= c\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt + \int_{-\infty}^{\infty} B[t]B[t+\tau]\, dt + \int_{-\infty}^{\infty} B[t]S[t+\tau]\, dt + c\int_{-\infty}^{\infty} S[t]B[t+\tau]\, dt$$

Expand the fourth term (the other cross correlation between the first and second halves of the cycle):

$$\int_{-\infty}^{\infty} (S[t] + B[t])(cS[t+\tau] + B[t+\tau])\, dt$$

$$= \int_{-\infty}^{\infty} (cS[t]S[t+\tau] + B[t]B[t+\tau] + cB[t]S[t+\tau] + S[t]B[t+\tau])\, dt$$

$$= c\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt + \int_{-\infty}^{\infty} B[t]B[t+\tau]\, dt + c\int_{-\infty}^{\infty} B[t]S[t+\tau]\, dt + \int_{-\infty}^{\infty} S[t]B[t+\tau]\, dt$$

Combine the expanded forms with the correct signs:

$$\begin{aligned} c^2\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt + \int_{-\infty}^{\infty} B[t]B[t+\tau]\, dt + c\int_{-\infty}^{\infty} B[t]S[t+\tau]\, dt + c\int_{-\infty}^{\infty} S[t]B[t+\tau]\, dt \\ + \int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt + \int_{-\infty}^{\infty} B[t]B[t+\tau]\, dt + \int_{-\infty}^{\infty} B[t]S[t+\tau]\, dt + \int_{-\infty}^{\infty} S[t]B[t+\tau]\, dt \\ - c\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt - \int_{-\infty}^{\infty} B[t]B[t+\tau]\, dt - \int_{-\infty}^{\infty} B[t]S[t+\tau]\, dt - c\int_{-\infty}^{\infty} S[t]B[t+\tau]\, dt \\ - c\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt - \int_{-\infty}^{\infty} B[t]B[t+\tau]\, dt - c\int_{-\infty}^{\infty} B[t]S[t+\tau]\, dt - \int_{-\infty}^{\infty} S[t]B[t+\tau]\, dt \end{aligned}$$

Collect like terms (that cancel), then simplify to become:

$$= c^2\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt - 2c\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt + \int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt$$

$$= (c-1)^2\int_{-\infty}^{\infty} S[t]S[t+\tau]\, dt$$

$$= (c-1)^2 \text{Autocorrelation}[S[t], S[t]]$$

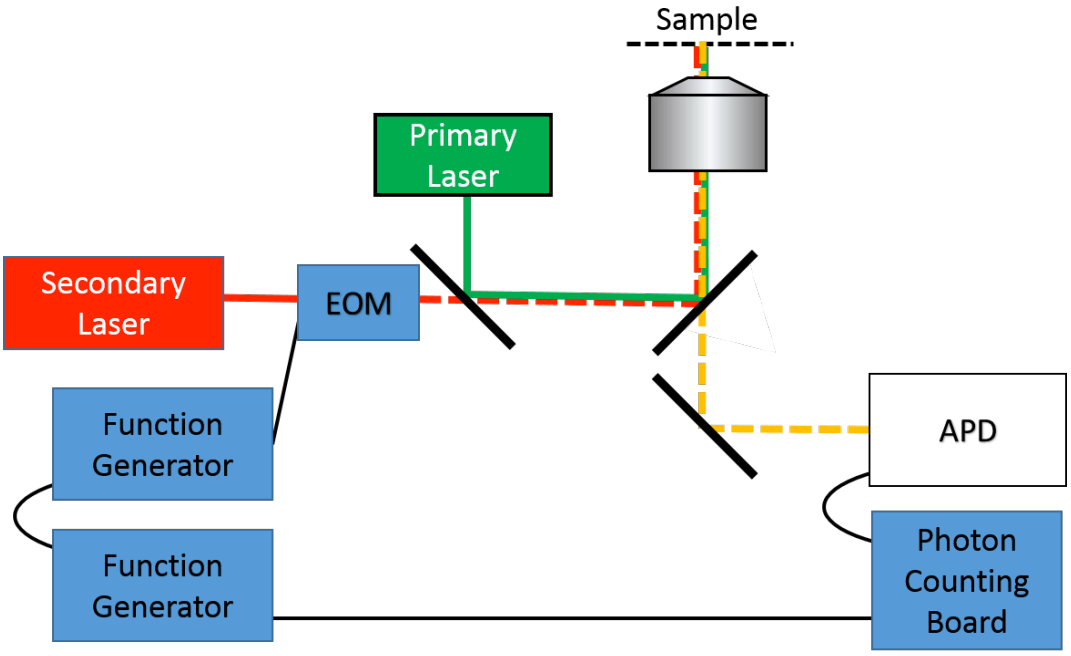

Figure S1. Experimental setup for modulated correlation spectroscopy using either 1 Hz or 100 kHz square-wave secondary co-illumination, as shown in Fig. 3.

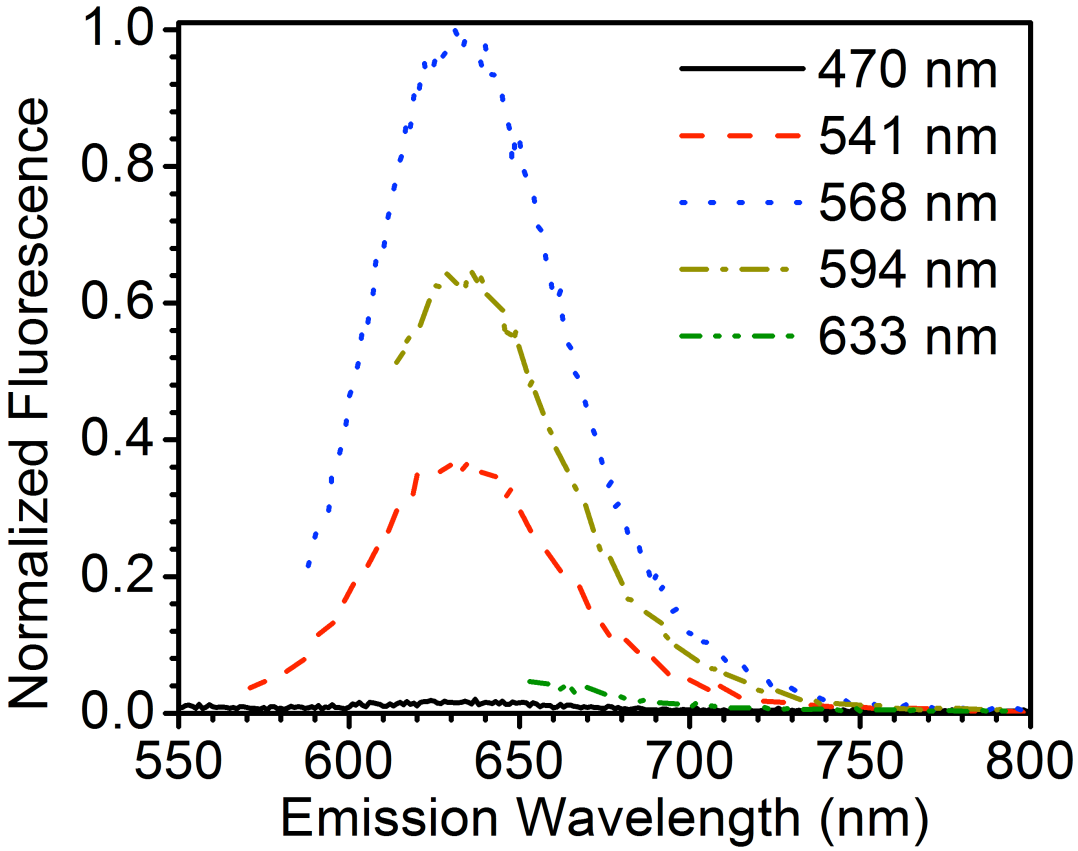

Figure S2. Independence of fluorescence spectrum on excitation wavelength. The fluorescence emission spectra of 630nm emitting Ag nanodot are unchanged, even when collected at multiple excitation wavelengths.

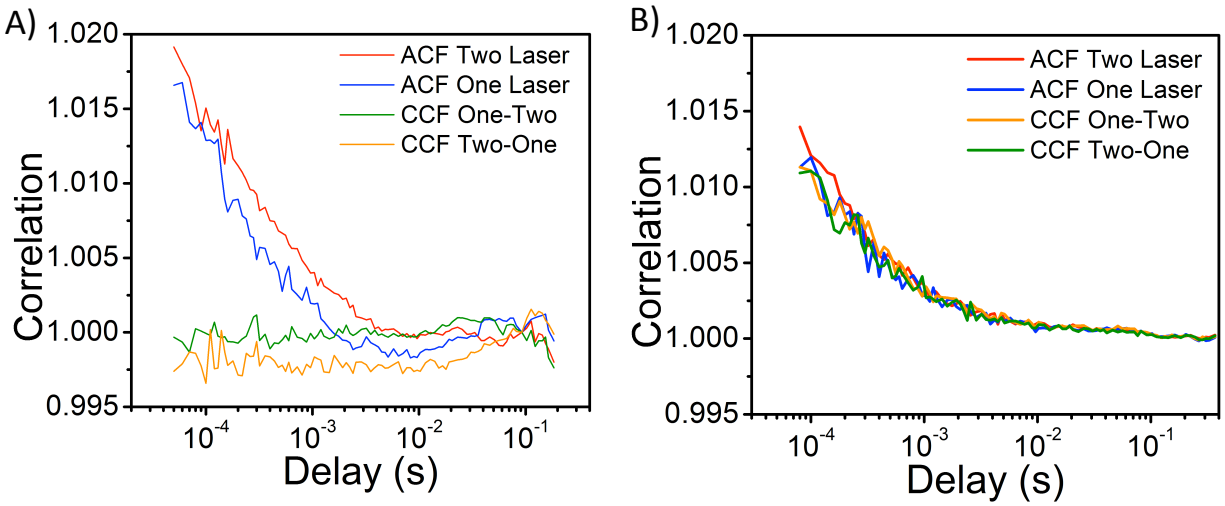

Figure S3. Expanded plots of various correlation subtraction functions from Figure 3 of main manuscript. In A, the 1-Hz cross-correlation amplitudes are extremely low, as there are no correlations between single and dual laser excitation halves of the period. As a result, the subtraction leads to a large amplitude and subsequently fewer extracted number of molecules. In contrast, 100-kHz cross correlations in B are nearly as high in amplitude as the autocorrelations, leading to more complete background subtraction and improved recovery of molecules. Extracted excitation dimensions for both data sets are w_{xy} = 0.5 μm and w_z = 1.5μm. Recovered diffusion coefficients from correlation subtraction fits for 100 kHz and 1 Hz are $1.8 \pm 0.6 \times 10^{-10}$ m^2s^{-1} and $2.0 \pm 0.6 \times 10^{-10} m^2s^{-1}$, respectively.

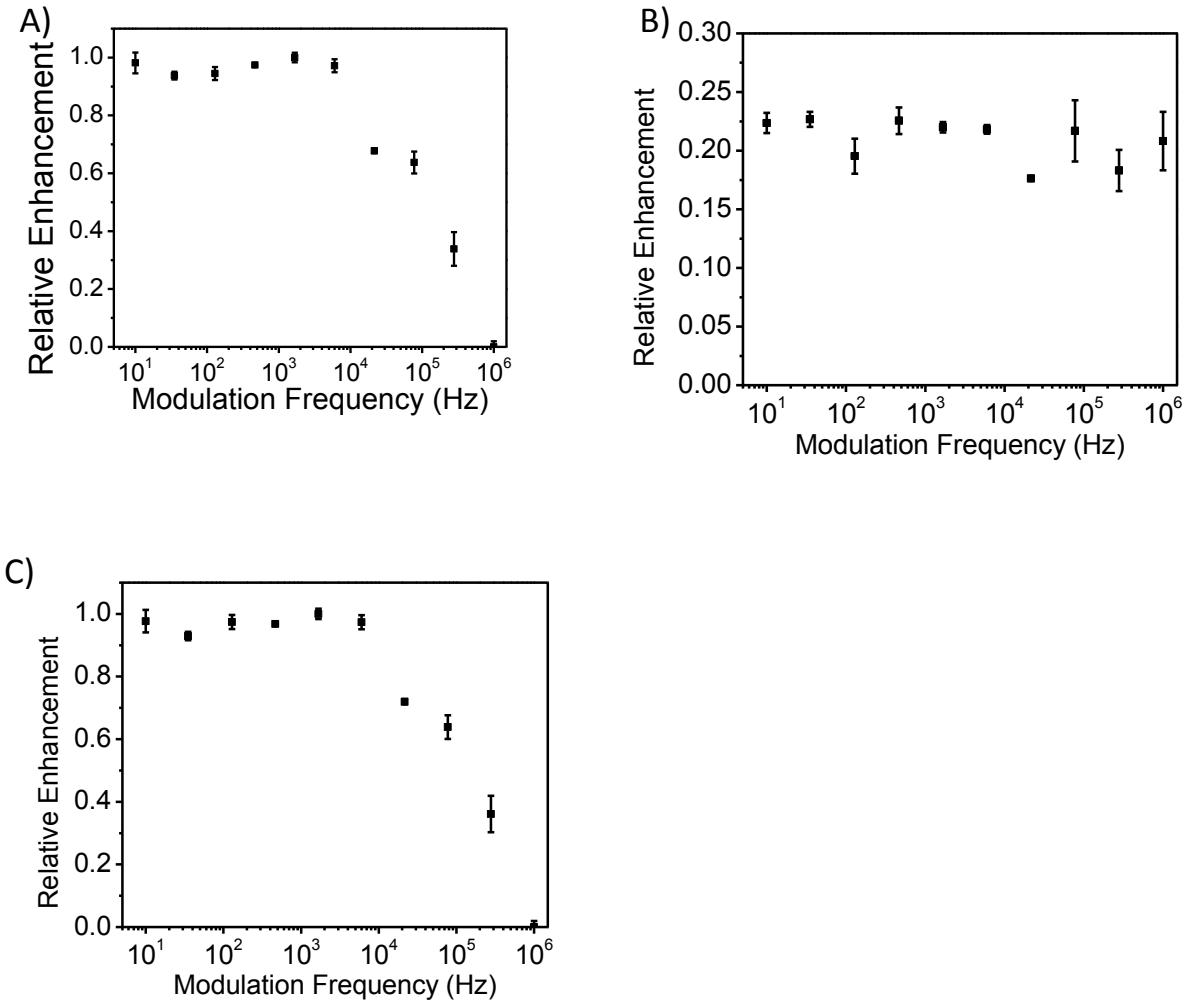

Figure S4. A) Normalized 630nm Ag cluster fluorescence enhancement as a function of modulation frequency. B) Secondary laser scattered light from coverslip vs. modulation frequency as the frequency-dependent control of detector and electronics response. C) Modulation frequency-dependent fluorescence enhancement normalized to secondary laser scattered light.