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Optically Activated Delayed Fluorescence

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Abstract

We harness the photophysics of few-atom silver nanoclusters to create the first fluorophores capable of Optically Activated Delayed Fluorescence (OADF). In analogy with thermally activated delayed fluorescence, often resulting from oxygen- or collision-activated reverse intersystem crossing from triplet levels, this optically controllable/reactivated visible emission occurs with the same 2.2 ns fluorescence lifetime as produced with primary excitation alone, but is excited with near infrared light from either of two distinct, long-lived photopopulated dark states. In addition to faster ground state recovery under long-wavelength co-illumination, this “repumped” visible fluorescence occurs many microseconds after visible excitation, and only when gated by secondary near IR excitation of ~1–100 microsecond lived dark excited states. By deciphering the Ag nanocluster photophysics, we demonstrate that OADF improves upon previous optical modulation schemes for near complete background rejection in fluorescence detection. Likely extensible to other fluorophores with photopopulatable excited dark states, OADF holds potential for drastically improving fluorescence signal recovery from high backgrounds.

Graphical abstract

Fluorescence in complex biological environments depends on both signal strength and background interference. Inherent contrast is often low, demanding that bright fluorophores are attached to identify target species. Often obscured by both endogenous and exogenous cellular emitters,^{1–2} visualization of low concentration species, weak binding interactions, and spectrally overlapping emitters are limited by available dyes, available spectral real

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Supporting Information: Supporting information is provided that contains fluorescence excitation/emission spectra, fluorescence lifetime data, photophysical modeling details and additional experimental details for background-free detection using OADF.

estate, and obscuring background. Because organic fluorophores commonly exhibit near unity oscillator strengths, improving signal brightness typically demands the creation of larger cross-section, nanoparticle-based emitters with many excitable electrons per particle.³⁻⁴ In fact, a wide array of quantum-confined or molecular scale inorganic and carbon-based emitters have been demonstrated to exhibit superior brightness in many spectral regions.^{3, 5-10} Unfortunately, the increased size, propensity to aggregate, potential toxicity concerns, and multiple points of attachment of such materials can significantly perturb biological processes.^{11,12}

We consider an alternate approach that avoids nearly all background interference. Signal visibility dramatically improves through both passive and active background reduction. Common passive background reduction schemes utilize large nonlinear cross sections,¹³⁻¹⁴ red-shifted emitters,¹⁵⁻¹⁶ or FRET/quenched pairs¹⁷⁻¹⁸ to minimize spectral overlap with endogenous biological fluorophores, or time gating to temporally collect emission from long-lived emissive states.¹⁹⁻²⁰ While effective for background reduction, limitations in dye excitation and emission characteristics still enable only a limited number of strong emitters to be simultaneously visualized through spectral or temporal resolution.

Exhibiting good chemical stability in a variety of biological media, Ag nanoclusters were introduced to circumvent many of the challenges in fluorescence detection within high background.²¹⁻²³ Ag nanoclusters demonstrated improved photostability and brightness relative to organic dyes in all spectral regions, while maintaining relatively small size. These properties continue to motivate studies to further strengthen the DNA – Ag cluster interactions needed for long-term chemical stability in live cell imaging.²⁴ Recently, we demonstrated Ag nanoclusters to be the first class of fluorophores whose emission can be dynamically brightened by optically shuttling molecules between dark and bright states more rapidly than would occur without long-wavelength co-excitation.^{22, 25} Modulating the intensity of the long-wavelength secondary illumination alters the ground state population and encodes the secondary laser modulation frequency on the collected fluorescence.²² Fluorescence demodulation results in active background reduction, as unmodulatable background does not contribute to signals recovered at the narrow-bandwidth modulation frequency. Because each modulatable fluorophore's natural dark state lifetime determines its own characteristic modulation frequency response, the dark state lifetime becomes an additional dimension on which spectrally overlapping fluorophores can be distinguished.²⁶ We have since expanded this concept to more traditional fluorophores.^{22, 27-31} Importantly, because dark state excitation in SAFIRE (synchronously amplified fluorescence intensity recovery)³¹ occurs with wavelengths longer those of the molecular emission, modulation adds no additional background to the detection channel, and the total number of collected photons either increases or stays the same as without modulation.³² As endogenous emitters are not modulatable, only the signal of interest is recovered at the modulation frequency to simultaneously improve signal sensitivity and discrimination.^{26, 32}

The readily accessible dark states, coupled with strong emission make Ag nanoclusters the ideal emitters to understand the photophysical interactions that lead to improved optical modulation,^{21, 23, 33-36} paving the way for applications with more traditional emitters. Excitation of the few Ag atom chromophore produces both strong visible fluorescence and

high populations of transient, photoinduced dark states that are optically depopulated with near infrared co-illumination.^{22, 31, 37} Utilizing the advantages of molecular modulation, these and related modulatable fluorophores have resulted in new high-sensitivity, fluorophore-selective detection and imaging schemes in high background biological environments that improve sensitivity both in bulk and on the single molecule scale.^{22, 25–26, 30, 32} Unlike modulatable organic dyes and fluorescent proteins reported to date which often undergo non-radiative cis-trans isomerization to switch between bright and dark ground state manifolds,^{38–39} Ag-DNA nanodot modulation repetitively builds up and depopulates a large fractional electronic excited dark state population that lives for tens of microseconds.³⁷ Although typically modeled as 3 state systems, consisting of a ground state, an emissive excited state, and a single, ~10 μ s-lived electronically excited dark state, we demonstrate OADF and new photophysics from a particularly promising 630 nm-emitting Ag nanocluster that exhibits >100% modulation depths from two distinct dark states. Using CW excitation with correlation-based background subtraction, this emitter enabled accurate recovery of nanocluster concentrations from high obscuring backgrounds,²⁵ without need for time-gated detection of very long-lived fluorescence.⁴⁰

We utilize pulsed primary excitation to not only significantly improve understanding of the states leading to this 630 nm Ag nanocluster modulation, but also develop the first fluorophores exhibiting optically activated delayed fluorescence (OADF). Under pulsed-CW excitation, these nanoclusters clearly exhibit two long-lived (electronically excited) dark states, and generate additional visible fluorescence photons solely resulting from near-IR (CW or pulsed) secondary illumination of the optically prepared Ag nanocluster dark states. More analogous to thermally activated delayed fluorescence (TADF)^{41–43} than to fluorescence upconversion from weakly coupled states of lanthanide ions,⁴⁴ such repumped fluorescence exhibits the same emission and lifetime as primary-only induced emission, but can be regenerated from near-IR depopulation of the microsecond-lived photoinduced dark states. This sequential two-photon process leads to modulation not only by increasing dark state population for the primary laser to excite, but also by near-IR repumping of the emissive excited state to yield visible, ns-lived fluorescence, many microseconds after the initial visible primary excitation pulse. This OADF is a fundamentally new paradigm in fluorescence detection and can be utilized to recover truly background-free fluorescence.

Synthesis of Ag-DNA

The single-stranded (ss)-oligonucleotide CCCCAACTCC was obtained from Integrated DNA Technologies (IDT) and used to prepare Ag-DNA clusters according to previously reported methods.^{25, 34} Briefly, lyophilized and desalted oligonucleotides (Integrated DNA Technologies) were hydrated, and concentrations were measured by the absorbances at 260 nm and extinction coefficients from the nearest-neighbor approximation. 40 nmoles of the DNA were combined with 22.4 nmoles of Ag⁺ (11 Ag⁺:DNA) and water to give a total volume of 40 μ L. Next, 12.6 nmoles of BH₄⁻ was added within 30s of combining NaBH₄ and water (6:11 BH₄:Ag), vortexed for 1 minute and incubated at room temperature for >5 hrs. The mixture is then refrigerated (~4 °C) for long-term storage.²⁵ Fluorescence excitation and emission spectra and data showing the 2.2ns fluorescence lifetime are reported in the Supporting Information (Figs S1 & S2).

Bulk nanocluster emission was studied both in aqueous solution and after immobilization in poly(vinyl alcohol) (PVA, (Sigma-Aldrich)) films. Samples immobilized in PVA were prepared by diluting the stock solutions of the clusters 1:1 in a saturated solution of PVA (9 – 10 kDa MW, dissolved in water at a concentration of 15% (w/v)) and evaporated at room temperature onto glass coverslips. Fluorescence intensity trajectories were obtained using spatially overlapped pulsed primary excitation and either continuous wave (CW) or ps-pulsed secondary laser excitation. Primary excitation of the 630 nm emitters was accomplished with either a 532 nm pulsed laser (Uniphase, 10kHz, 800 ps) or a 560-nm pulsed diode (PicoQuant, LDH-D-TA-560). CW secondary illumination was accomplished at ~803 nm with either a fiber-coupled diode laser (Thorlabs, LP808-SA40) or a Ti:sapphire laser operating in continuous wave mode. Pulsed secondary excitation was performed with ~6 ps pulses from a mode-locked Ti:sapphire laser (Coherent Mira 900). Fluorescence was collected in a confocal geometry on an inverted microscope (Olympus IX-71) with a 60× 1.2 NA water objective. A 100-μm multimode fiber was used as a used as a pinhole to route emission to an avalanche photodiode (APD). Photon arrival times were recorded with a time correlated single photon counting board (Becker-Hickl, SPC-630) in conjunction with a multichannel router (Becker-Hickl, HRT-41). Optical band-pass filters centered near the emission wavelength of the specific dye were used to efficiently block both the primary excitation and the lower energy secondary laser excitation while allowing fluorescence through. Data analysis was performed in MATLAB (Mathworks, r2015a).

Highly fluorescent Ag nanoclusters produce significant dark state populations at moderate excitation intensities (~kW/cm²).²³ Depopulation of these dark states is enhanced with coincident near IR excitation to brighten overall visible emission by more rapidly repopulating the original ground state.^{22–23} The dark state decay rate, k_{off} , is the sum of the natural decay rate, k_{off}^0 , and the secondary laser induced depopulation, $I_{\text{sec}} \sigma_D \Phi_{\text{rev1}} \lambda_{\text{sec}} h^{-1} c^{-1}$, with parameters as defined in Equation (1). Changing the intensity of this near IR secondary laser then modulates Ag nanocluster fluorescence by altering ground vs. dark state populations. Steady-state studies of Ag nanocluster photophysical dynamics are typically well fit by including a single dark state that regenerates the ground state of the fluorescent manifold upon secondary excitation. The strong modulation of the 630 nm emitter, however, leads to the new and general concept of OADF.

To demonstrate and reveal the crucial features of fluorophores capable of optically activated delayed fluorescence, we probed Ag 630 nm emitters with pulsed primary/secondary CW and pulsed primary/pulsed secondary excitations. Using low repetition rate pulsed primary excitation (532nm, 10 kHz) allowed investigation of photopopulated dark state dynamics between primary laser pulses (Figure 1). Primary excitation alone produces 630 nm fluorescence with a 2.2 ns lifetime (Fig. S2). Although secondary illumination at 803 nm is completely blocked by bandpass filters, and no nanocluster fluorescence is observed with secondary excitation alone, near IR co-illumination in combination with 532 nm primary excitation significantly increases detected 630 nm fluorescence, both within and *between* the primary pulses.

The secondary laser-excited fluorescence decays biexponentially between successive primary pulses (Fig. 1, Fig. S3, & 2A,B), indicating that two optically reversible dark states result from primary excitation. Decay rates from both dark states increase linearly with secondary laser intensity but exhibit ~4-fold different slopes (Fig. 2). Extrapolation of the measured decay rates to zero secondary intensity yields the natural dark state lifetimes (Table 1). Thus, the near IR secondary excitation repumps population to the emissive excited state from each of the two dark states to generate OADF up to 100 μ s after primary excitation. The intensity of near IR secondary laser-excited 630 nm emission is proportional to the dark state populations, which are significant due to a ~5% dark state quantum yield that generates <100 μ s-lived dark states. This optically activated delayed fluorescence results from dark states that are too energetically different from the emissive state to yield TADF, but near IR secondary excitation can regenerate the emissive excited states long after primary laser-induced dark state populations are established.

Pulsed primary/pulsed secondary excitation yields additional photophysical insight. The fluorescence intensity between primary pulses is proportional to the product of the dark state population, dark state cross section, and reverse quantum yield. Utilizing pulsed secondary excitation removes any CW secondary intensity-induced decay of the dark state, as only a single excitation cycle is possible within the ps-pulse width. Directly probing the OADF-generating process, 630 nm emitters were irradiated with pulsed 560 nm excitation and time-delayed, 6-ps secondary laser pulses at 803 nm (Fig. 3). Sequential two-photon, 630 nm fluorescence (OADF, Fig. 3, inset) is readily detected as a second pulse following the primary-induced fluorescence with the same 2.2 ns emissive lifetime. As with pulsed-CW experiments, no emission was observed with secondary illumination alone. The primary fluorescence:OADF intensity ratio of ~50, directly reports on the relative populations of dark and ground states resulting from primary excitation.

The photophysics leading to OADF are modeled by the coupled rate equations in Equation 1. To initially estimate the parameters, the secondary intensity dependence of the pulsed-CW decays yields the product of dark state fraction, dark state absorption cross section, and reverse quantum yield. The ratio of the pulsed secondary- to pulsed primary-induced fluorescence cannot exceed the forward dark state quantum yields. For example, if one defines the number of molecules excited by the primary pulse as 1, the initial dark state fraction equals the sum of forward dark state quantum yields. If secondary excitation saturates the dark state absorptions, this secondary:primary fluorescence ratio should approach $(\Phi_{D1} \Phi_{revD1S1} + \Phi_{D2} \Phi_{revD2S1})$. Using the

$$\begin{aligned}
 \frac{d}{dt} \begin{pmatrix} S_0 \\ S_1 \\ D_1 \\ D_2 \end{pmatrix} = & \begin{pmatrix} -I_{\text{Pri}}[t]\sigma_{S_0}\frac{\lambda}{hc} & k_F & k_{D_1S_0} & k_{D_2S_0} \\ -I_{\text{Pri}}[t]\sigma_{S_0}\frac{\lambda}{hc} & -k_{S_1D_1} - k_{S_1D_2} - k_F & I_{\text{Sec}}[t]\sigma_{D_1}\frac{\lambda}{hc}\Phi_{\text{rev}D_1S_1} & I_{\text{Sec}}[t]\sigma_{D_2}\frac{\lambda}{hc}\Phi_{\text{rev}D_2S_1} \\ 0 & k_{S_1D_1} & -k_{D_1S_0} - I_{\text{Sec}}[t]\sigma_{D_1}\frac{\lambda}{hc}\Phi_{\text{rev}D_1S_1} & 0 \\ 0 & k_{S_1D_2} & 0 & -k_{D_2S_0} - I_{\text{Sec}}[t]\sigma_{D_2}\frac{\lambda}{hc}\Phi_{\text{rev}D_2S_1} \end{pmatrix} \cdot \begin{pmatrix} S_0 \\ S_1 \\ D_1 \\ D_2 \end{pmatrix} \\
 & \quad \quad \quad (1)
 \end{aligned}$$

experimental secondary:primary ratio of peak intensities of 1:50 and the assumption that $\Phi_{D1} = \Phi_{D2} = 0.05$ and $\Phi_{\text{rev}D1S1} = \Phi_{\text{rev}D2S1}$, one estimates reverse yields $\Phi_{\text{rev}D1S1} = \Phi_{\text{rev}D2S1}$ of 0.2.

For given primary and secondary intensities, the rate constants and estimated forward and reverse quantum yields can be combined with the state connectivities (Fig. 4) to model the time-dependent state populations (Eqn. 1). Each dark state, D_n , is depopulated at the sum of a natural decay rate, k_{DnS0} , and the product of secondary intensity, I_{Sec} , dark state absorption cross section, $\sigma_{Dn\in\{1,2\}}$, and the reverse quantum yield to the excited emissive state, $\Phi_{\text{rev}DnS1}$, with the factor $\lambda \text{ h}^{-1}\text{c}^{-1}$ to convert to Hz. Natural rate constants for fluorescence

(k_F), and between each pair of dark ($D_{n \in \{1,2\}}$) or bright ($S_{m \in \{0,1\}}$) states are indicated with subscripts indicating the initial state on the left and the final state on the right. These coupled rate equations (Eqn. 1) are readily integrated for constant rate coefficients to yield state populations at any time, t .

Pulsed excitation is simulated by integrating equation 1 using experimental parameters, but the time varying excitations require piecewise integration to calculate time-dependent state populations. Pulsed primary/CW secondary excitation is modeled for the first 1 ns (primary excitation pulse width) with both primary and secondary intensities being non-zero. For the rest of the excitation cycle, the primary intensity is set to zero. For both portions of this cycle, the secondary intensity is constant. The matrix for each portion of the cycle is exponentiated over the relevant illumination timescale, and the resulting state populations generate the starting values for the next illumination conditions/cycle. Fluorescence trajectories are given by the S_1 excited state fractional population vs. time.

Pulsed-pulsed excitation is similarly simulated, but with four separate periods/cycle: (1) $I_{Pri}=0$, $I_{Sec}=0$ (2) $I_{Pri}=0$, $I_{Sec}=0$, (3) $I_{Pri}=0$, $I_{Sec}=0$, (4) $I_{Pri}=0$, $I_{Sec}=0$, with an adjustable delay between the two pulses, and experimental peak intensities for both primary and secondary illumination being used. Simulations of pulsed-CW and pulsed-pulsed excited fluorescence then yield kinetic parameters that reproduce experimental excited state dynamics (Table 1, Fig. 5). Photophysical parameters in each environment were optimized with global fits to best reproduce experimental data (details in supporting information).

Optical modulation typically occurs through accelerated dark state depopulation to increase primary laser-excited emission. Obscured in CW-CW modulation experiments, OADF also increases the modulation signal, but can be selectively recovered without background if pulsed primary excitation is used. Waiting until after all primary-excited background emission has decayed, optically activated delayed photons from pulsed/CW-excited 630 nm emitting nanoclusters were used to recover background-free signal from aqueous Ag-DNA nanodots in a high, spectrally overlapping sulforhodamine 101 (SR101, 4.5ns lifetime,⁴⁵ excitation/emission spectra shown in Fig. S4) background. The SR101/630 nm nanocluster solution was excited with pulsed 560 nm primary excitation at 10 MHz and CW 805 nm secondary excitation. Pure 630 nm emitting Ag nanodot samples of known concentration were compared to standardize and confirm concentration recovery (Fig 6B). Within the mixed samples (~17nM Ag nanodots and ~85nM SR101), combined emission from both Ag nanodots and SR101 is collected upon primary excitation. Only Ag nanodots yield delayed OADF, however, enabling collection >50ns after primary excitation to recover Ag nanodot emission, devoid of SR101 signals (Figure 6 C,D). Thus, by post-acquisition subsampling the fluorescence such that photons arriving within 50ns of primary excitation are temporally excluded from the analysis, only Ag nanocluster OADF was collected and correlated. Background-free fluorescence intensity correlation functions were calculated from the OADF photon arrival times and fitted to a standard diffusion model.²⁵ The true Ag nanodot concentration of ~17nM was quantitatively recovered through post-processing-based time-gating, confirming complete exclusion of obscuring SR101 emission (Figure 6B,D). The Ag nanocluster OADF emission originates from the primary laser-prepared dark states, with emission only being observed for transitions to the emissive excited level that produce

fluorescence. Since OADF starts from the dark manifold of states, on-off blinking dynamics are not seen in the repumped correlation functions (Fig 6 C,D). Therefore, only the diffusional dynamics of the Ag nanodots are recovered, without any overlaying photophysical dynamics or background signals.

Possible both in bulk and using correlation methods, our active approach using OADF collects and can utilize all photons from all emitters, and can map out dark state dynamics. Correlation functions within high background have been recovered using both CW-CW modulated correlation subtraction²⁵ and long-lived (~20ns) fluorophores with passive time gating in TCSPC⁴⁰ to preferentially collect the long-lived fluorescence after most background emission has decayed. While successful, this passive approach cannot multiplex to discriminate multiple emitters with different dark state lifetimes as has been demonstrated with modulatable fluorophores,²⁶ nor is the background removal as complete, as few-ns-lived emitters can still contribute significant emission at 20ns. In Figure 6, both the SR101:Ag nanocluster concentrations and total number of SR101:Ag nanocluster photons collected are in a ratio of ~5:1. Using the measured count rates, the Ag nanocluster signal/ (background of Ag nanocluster and SR101 emission) is (48,000 photons/sec)/(281,000 photons/sec), or 0.17. Because the pure Ag nanocluster signal cannot be identified within the overwhelming, 5-fold higher SR101 signals, the Ag nanocluster signal cannot be distinguished. By using only the OADF photons beyond 50ns, the Ag nanocluster count rates (5500 photons/sec) are recovered without SR101 background. The only background in OADF results from detector dark counts (~300 Hz) and counting noise from the Ag nanocluster emission., giving a S/N of $5500/\sqrt{5500+300} = 72$ for the repumped photons in a 1-second interval. Thus, the Ag nanocluster signal visibility effectively increases >400-fold from 0.17 to 72 in a 1-second interval using only the repumped photons. Although passive time-gating with long-lived fluorophores also can give good discrimination, the improvement in signal visibility resulting from OADF enables even higher signal discrimination of emitters as emission is observed at very long times – well after all other emitters have decayed. Further, species with similar fluorescence lifetimes can be discriminated as it is the dark state residence that extends the timescale for active OADF signal recovery.

While standard correlation and single molecule experiments using CW excitation of pure Ag nanodot emitters have suggested that a 3-state model (ground state, excited state and one dark state) is sufficient to explain Ag nanocluster photophysical dynamics,^{23, 31, 37, 46} CW-excited photophysical correlations preferentially build up the longer-lived dark state, minimizing the contribution from the shorter-lived dark state. Thus, correlation contrast arising from dark state shelving yields a much larger amplitude corresponding to the sum of rates in $(I_{\text{Pri}}[t]\sigma_{S_0}^{\lambda}\Phi_{D_1})$ and out $(k_{D_1S_0})$ of the longer-lived dark state. Thus, only through pulsed-CW and pulsed-pulsed experiments can the additional dark state be resolved, allowing a better model of the photophysical dynamics of Ag nanoclusters to be constructed and OADF to be used for improved signal recovery.

These detailed studies of Ag nanocluster photophysics enable the observed OADF to measure only the repumped visible fluorescence resulting from near IR secondary excitation

of the long-lived dark states. As the dark state lifetimes are tens of μ s, delayed fluorescence at higher energy is readily observed only from Ag nanoclusters. Because background emitters do not produce such repumped emission, Ag-DNA signals are uniquely recovered from the background-free OADF signals. Such optically activated delayed fluorescence is a new concept in fluorescence detection. Not limited to Ag nanocluster emitters, OADF should be observable from organic dyes with appropriate long-wavelength excitable dark states and is likely to find many uses in high sensitivity imaging and detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

630 nm emitter with an average power 6 W/cm² 532 nm pulsed and 9 kW/cm² 803 nm CW excitation immobilized in PVA. **Inset:** (Expanded region after primary pulse) Increased secondary intensity increases the dark state decay and initial brightness of the same sample. Single exponentials did not fit sufficiently, especially as the secondary intensity is increased. A semi-log plot is shown as Fig. S3 in the Supporting Information.

Figure 2.

Secondary laser-induced dark state decays in (A) water and (B) PVA. Dark state decays of the 630 nm emitter are plotted as a function of varying CW 805 nm secondary intensity with constant pulsed 532 nm primary intensity (6 kW/cm² avg. intensity). Ag nanoclusters in both solution and PVA films exhibit two different dark state decay rates. Both long- and short-lived decays yield linear fits. The slopes yield the product of dark state (fractional) population, dark state absorption cross section, and reverse quantum yield. Photophysical parameters with 1 σ uncertainties from linear fits in A and B are presented in Table 1.

Figure 3.

Experimental fluorescence signal resulting from pulsed 560 nm (1.5 kW/cm^2 , avg. intensity) and pulsed 803-nm (880 W/cm^2 , avg. intensity) excitation of aqueous 630 nm emitters.

Inset: Magnification of the 630 nm fluorescence resulting from the 803 nm pulsed excitation after dark state preparation by pulsed primary excitation at 560 nm (10 MHz repetition rate). Both primary- and secondary-excited fluorescence exhibit 2.2 ns fluorescence lifetimes.

Figure 4. Schematic of DNA-silver cluster photophysics. Initial fluorescence is a result of primary, higher energy wavelength excitation, where transitions into a dark state ($I_{Pri}\sigma_{S0} \Phi_D$) occur. The application of a second, longer wavelength laser causes repumping of dark states back into the emissive state, providing a direct means for optically activated delayed fluorescence.

Figure 5.

Simulated S₁ excited state fraction in a single 100 μs primary and secondary illumination period for (A) pulsed-CW and (B) pulsed-pulsed excitation. The emissive S₁ population is proportional to fluorescence signal and is shown as the continuous black line. Simulated 1 ns primary pulses are shown as green vertical lines in both (A) and (B). Secondary excitation is represented by the red lines – a continuous excitation is represented by the horizontal red line in the (A) pulsed-CW simulation and as a 1 ns wide vertical line in the (B) pulsed-pulsed simulation.

Figure 6.

Photon arrival histograms of an aqueous 630 nm Ag-DNA clusters, depicting the relatively rapid fluorescence decay and relatively longer lived decay. (A) Fluorescence histogram of pure Ag-DNA clusters (positive control), showing all photons (black) and only those recorded outside the fluorescent lifetime (red, >50 ns). (B) Corresponding correlation decays as a result of the full fluorescence trace (black) and subsampled fluorescence (red), providing a control for the concentration recovery. (C) Plot of fluorescence signal from a mixture of SR101 and 630 nm emitters, and D shows the corresponding correlation decays for the full (blue) and subsampled photons (red). The black plot in (D) is the FCS curve from an Ag-DNA cluster only sample (B), and is repeated in (D) for comparison.

Table 1

Dark state photophysical parameters for 630 nm Ag nanocluster emitters.

Ag-DNA Environment	τ_1 (μ S)	τ_2 (μ s)	$\sigma_{D_1} \Phi_{\text{rev}D_1S_1}$ ($\text{cm}^2 10^{-18}$)	$\sigma_{D_2} \Phi_{\text{rev}D_2S_1}$ ($\text{cm}^2 10^{-18}$)
PVA	8.7 ± 0.4	42.6 ± 1.3	1.54 ± 0.09	0.18 ± 0.01
Aqueous solution	5.8 ± 0.3	12.8 ± 0.2	1.03 ± 0.05	0.18 ± 0.01

$\sigma_{D_1}, \sigma_{D_2}$: absorption cross sections for states D_1 and D_2 , respectively

$\Phi_{\text{rev}D_1S_1}, \Phi_{\text{rev}D_2S_1}$: Reverse quantum yields to the excited emissive state from D_1 and D_2

Errors are standard deviations determined from the fits.