Published in final edited form as:

Chemphyschem. 2012 March ; 13(4): 1023–1029. doi:10.1002/cphc.201100671.

Optical modulation and selective recovery of Cy5 fluorescence

Chaoyang Fan,

School of Chemistry and Biochemistry and Petit Institute of Bioscience and Bioengineering
Georgia Institute of Technology Atlanta, GA 30332-0400

Jung-Cheng Hsiang, and

School of Chemistry and Biochemistry and Petit Institute of Bioscience and Bioengineering
Georgia Institute of Technology Atlanta, GA 30332-0400

Robert M. Dickson^{*,[a]}

^[a]School of Chemistry and Biochemistry and Petit Institute of Bioscience and Bioengineering
Georgia Institute of Technology Atlanta, GA 30332-0400

Abstract

Fluorescence modulation offers the opportunity to detect low concentration fluorophore signals within high background. Applicable from the single molecule to bulk levels, we demonstrate long-wavelength optical depopulation of dark states that otherwise limit Cy5 fluorescence intensity. By modulated excitation of a long wavelength Cy5 transient absorption, we dynamically modulate Cy5 emission. The frequency dependence enables specification of the dark state timescales enabling optical demodulation-based signal recovery from high background. These dual laser illumination schemes for high sensitivity fluorescence signal recovery easily improve signal to noise ratios by well over an order of magnitude, largely by discrimination against background. Previously limited to very specialized dyes, our utilization of long-lived dark states in Cy5 enables selective detection of this very common single molecule and bulk fluorophore. Although, in principle, the “dark state” can arise from any photoinduced process, we demonstrate that cis-trans photoisomerization, with its unique transient absorption and lifetime enables this sensitivity boosting, long-wavelength modulation to occur in Cy5. Such studies underscore the need for transient absorption studies on common fluorophores to extend the impact of fluorescence modulation for high sensitivity fluorescence imaging in a much wider array of applications.

Keywords

Luminescence; Microscopy; Photochromism; Cyanines; Isomerization

Introduction

Although often considered a zero-background spectroscopic technique, quantitative fluorescence imaging is too-often hampered by competing emission from undesired endogenous fluorophores.^[1-11] Consequently, one needs to identify fluorophores and spectroscopies that combine the sensitivity and selectivity of molecular modulation^[10-16] with fluorescence to uniquely observe signal only from molecules of interest. As absorption spectroscopy needs to overcome both laser noise and signals from similarly absorbing species, sensitivity increases are achieved through selectively shifting only the desired signal to a window with greatly decreased background.^[17-19] The continued development of near

^{*}dickson@chemistry.gatech.edu.

IR emitters^[20, 21] and those with long lifetimes^[6-8] take advantage of (spectrally or temporally) shifting signals away from background emission. Recently, more active molecular modulation approaches have been applied to fluorescence studies, minimizing background contributions by deterministic temporal gating and “locking in” on these specific behaviors, thereby suppressing background signals.^[11, 22] Although largely applied to improve resolution through sequential localization^[23, 24] or point spread function engineering,^[25] such active molecular modulation can also greatly enhance detection sensitivity,^[26] but has required specialized photoswitchable dyes. [11-14, 21-25] Through optical engineering of dark state residence, dual excitation to optically depopulate long-lived states increases overall population in the fluorescence-generating manifold, thereby increasing brightness. If the dark state absorbs at wavelengths longer than those of the collected fluorescence, the extra gain in fluorescence is essentially background-free. Additionally, if the secondary laser intensity is modulated, the modulation waveform is directly encoded on the molecular fluorescence, shifting the signal of interest to a unique, deterministic, shot noise-limited detection frequency.^[12, 13, 27] Attainable modulation frequency is a function of dark state residence, but provides a new dimension for fluorophore discrimination, analogous, but orthogonal to fluorescence lifetime imaging.^[14, 28] Utilizing merocyanine/spiropyran photoswitches, optical lock-in detection (OLID) photobleaches while collecting fluorescence, then photoswitches with UV excitation to regenerate most of the original fluorophore.^[11, 22, 27] The triangular-type waveform is measured through similarly switching, exogenously introduced pure reference dyes for cross-correlation with collected fluorescence to enrich desired signal over unwanted background. By using fluorophores with long-wavelength absorbing metastable dark states, Synchronously Amplified Fluorescence Image Recovery (SAFIRE) modifies OLID effectively by shifting the “recovery” laser from the UV where it generates background (and hence cannot always be on) to the near IR to give a background-free modulation of fluorescence.^[12, 13] In this manner, the modulation waveform applied to the near IR laser is directly encoded on the detected fluorescence, making the use of cross-correlation with pure dye signals unnecessary. [11-13, 27] To date, both OLID and SAFIRE have been somewhat limited in dye choice. OLID requires an emissive photoswitch, SAFIRE gives higher sustained emission (as it does not photoswitch), but requires any fluorescence-limiting dark state to have $>\mu\text{s}$ lifetimes and transient absorption at lower energies than the collected emission. Consequently, to date, only specialized dyes^[12, 13] or advanced photophysical methods (e.g. saturated-FRET^[14] or STED^[15]) in conjunction with common dyes have been utilized.

Usually a source of blinking or photoswitching, photo-isomerisation is common in a variety of organic dyes.^[22-24, 29-35] The photoisomerization process tends to cause non-radiative relaxation and leads to an isomer with at least slightly different absorption and emission characteristics.^[30-37] Such isomerization-based optical switches have found utility in super-resolution fluorescence imaging^[24, 25, 38] and imaging of intracellular dynamics.^[11, 22] Unfortunately, the vast majority of these optical switches have thermally stable dark states that must be recovered through high-energy co-illumination, thereby introducing additional background into the detection channel. In principle, the photoisomerisation process can also be used for SAFIRE if the dark state is metastable (enabling fast recovery and continual emission) and the isomerisation can be reversed with low energy secondary co-illumination. Here we demonstrate that the very commonly used Cy5 is an outstanding dye for SAFIRE-based signal recovery, likely resulting from its propensity for cistrans isomerization and corresponding red-shifted absorption.^[32] The ability to selectively optically modulate the fluorescence of bright emitters enables demodulation-based selective signal recovery with excellent signal to noise improvements. Identification of bright fluorophores with optically depopulatable dark states in different spectral regions will enable a new dimension for sensitivity and labeling optimization in high background environments.

Results and Discussion

Cyanine dyes have extremely strong oscillator strengths and very narrow absorptions.^[39] Their ability to be synthetically modified for a given purpose, and their excellent photophysics make them excellent fluorescent probes in biology and materials science.^[40-42] Recent applications in reactive oxygen sensing,^[41] medical imaging,^[42, 43] and super-resolution imaging^[23, 24] highlight the importance of cyanines, and Cy5 in particular. The photophysics of these “simple” particle-in-a-box-like molecules,^[40, 42] however, is surprisingly rich. Although maintaining high fluorescence and low photobleaching quantum yields, Cy5 also readily isomerizes upon photoexcitation.^[31-33, 40] This photoisomer plays a dominant role in the photoswitching and dynamics of Cy5 fluorescence. Cy5 excited-state photophysics have been unraveled by both fluorescence correlation^[31] and transient absorption experiments.^[32, 33] In order to avoid obfuscation by the diffusive component, the former are limited to measuring contributions to the intensity fluctuations of states with lifetimes roughly an order of magnitude shorter than the $\sim 100\ \mu\text{s}$ diffusion time through the laser focus.^[31] Transient absorption has no such time resolution issues, if the sensitivity and timing are sufficient to probe long-lived states. Consequently although photoisomerization will lead to an additional off time in the FCS studies, its contribution can be clouded by diffusion and the faster triplet dynamics.^[31] Transient absorption, however, clearly discriminates between the $\sim 10\text{-}\mu\text{s}$ triplet states and the $\sim 150\ \mu\text{s}$ -lived cis-Cy5 isomer, the latter not being affected by heavy atom presence.^[32] Unlike the shorter-lived ($\sim 10\ \mu\text{s}$) and blue-shifted triplet state of the trans isomer, the cis-Cy5 singlet-singlet transition has been shown by transient absorption to be $\sim 45\ \text{nm}$ red-shifted from the trans-Cy5 singlet-singlet absorption.^[32, 33] While the trans-Cy5 provides a strongly emitting “on” state, the cis-Cy5 is often regarded as a “dim” state, as it is less efficiently excited at the trans-Cy5 excitation wavelength and likely has lower yield and further red-shifted emission.^[32] Importantly, transient absorption clearly shows that the longest-lived, $150\text{-}\mu\text{s}$ transient arises from cis-Cy5 and absorbs maximally at $690\ \text{nm}$.^[32, 33] By exciting into this transient absorption, we expect to be able to switch the cis-Cy5 back to trans-Cy5 faster than it naturally decays using low energy laser co-illumination, thereby enabling its use in SAFIRE-based signal recovery.

Secondary laser-induced fluorescence enhancement results from faster than natural, laser-induced depopulation of the fluorophore dark (or dim) state. If the blinking timescales are appropriately fast, fluorescence correlation spectroscopy (FCS) is a powerful tool for studying transitions to and from such dark states, and is readily analyzable in terms of a 3-state photophysical model.^[44] Duty-cycle modulation of the primary excitation laser or introduction of a secondary laser enables more complex photophysical models to be evaluated.^[45] To elucidate some of the interactions, we performed single ($594\ \text{nm}$ cw)- and dual-laser ($594\ \text{nm}$ cw and $710\ \text{nm}$ cw) FCS of aqueous Cy5 (Fig. 1), collecting fluorescence from $660\text{--}694\ \text{nm}$. Secondary laser illumination simultaneously increases brightness/molecule and should decrease contrast in the photoinduced dark state component by reducing fluctuations associated with dark state residence. Interestingly, it is the slow component timescale ($>100\ \mu\text{s}$) that appears to change most significantly upon secondary illumination, appearing to shift to longer time with increased contrast. The fast component contrast also appears to reduce slightly. This shift of the slower component suggests that 1) the slower component corresponds to both diffusion and a photophysical state with a secondary laser-induced faster decay, 2) this slow component is convolved with and therefore is masked by the diffusive timescale with single laser illumination, 3) this long-lived dark state builds up significant steady state population. Any secondary laser-induced shortening of a timescale otherwise convolved with the diffusive timescale would result in the bifurcation of the single decay into an apparently new, faster decay, and a *longer*, better approximation of the true diffusion correlation time, as the two processes become

temporally separated.^[16, 46] To demonstrate this we utilized Monte Carlo simulations of FCS with a 70 μ s on time, 170 μ s diffusion time, and a ~90 μ s off time under primary laser illumination that decreases to and 5 μ s with secondary laser co-illumination (Fig. 1, inset). Such dark state transient absorption shifts the off time away from the diffusive timescale, reproducing the shift to longer diffusion times while also shortening faster photophysical timescale.

The timescale changes observed in FCS suggest that one can modulate the secondary laser intensity to dynamically shift population between the fluorescence-generating and dark state manifolds, thereby modulating detected fluorescence.^[47, 48] At frequencies below the inverse dark state lifetime, the secondary laser modulation waveform is directly encoded on the collected fluorescence and Fourier transformation readily and selectively recovers the modulated fluorescence signal. Using this long-lived dark state and the published transient absorption spectra, aqueous Cy5 fluorescence was generated with simultaneous primary (594 nm) and secondary (710 nm) illumination through a 60 $\times$ water objective. The secondary laser was square-wave modulated at 700 Hz (Figure 2A). Fluorescence in 100- μ s bins does not clearly show modulation, but the Fourier transform yields a clear peak at the modulation frequency (Figure 2B).

Brightness/molecule increases by ~40% with dual illumination, but the somewhat small energy difference between collected fluorescence and secondary illumination wavelength gives rise to a small portion of the increased fluorescence (<15% of the additional signal, or 6% of the total) arising from secondary illumination alone. Not reduced through additional secondary laser filtering, such weak emission could arise from concentration effects,^[49] two-photon Cy5 fluorescence, or natural thermal population of excited vibrational or cis-Cy5 states,^[50, 51] the latter possibly giving emission if cis- and trans-Cy5 share a common excited state. In contrast to STED-based SAFIRE,^[15] such weak emission can increase the apparent enhancement due to secondary illumination, improving the SAFIRE signal. We choose to discriminate against this minor component, however, through dual modulation.^[15] As the desired signal is excited by the primary laser and increased by the secondary, the desired signal is linearly proportional to both laser intensities. Consequently, the pure, modulated signal arises at the sum and difference of the different primary and secondary modulation frequencies,^[15] effectively discriminating against all primary only and secondary only induced backgrounds (Figure 2C).

Modulation frequency provides an additional dimension in signal visibility, analogous to fluorescence lifetimes. As with frequency domain fluorescence lifetime determinations, the modulation depth will be frequency dependent, decreasing as the characteristic dark state lifetime is approached.^[28] At frequencies faster than the characteristic dark state decay, the molecular system cannot fully re-establish full steady-state dark and fluorescent manifold populations that are attainable at lower frequencies. In SAFIRE, modulation depends on shifting of populations between bright and dark manifolds through laser excitation. The on and off times, therefore, provide limits on how fast such populations can be redistributed. Consequently, the modulation frequency dependence should be consistent with the natural lifetime of the dark state giving rise to observed enhancements. Modulation frequencies exceeding the inverse of the dark state lifetime provide insufficient time for steady state distributions to be established, thereby decreasing modulation depth due to sluggish molecular response. While providing temporal discrimination among modulatable fluorophores, higher frequency modulation can not only allow faster imaging, but also offers larger shifts from typically low-frequency background noise, thereby improving sensitivity. The modulation frequency dependence of Cy5 is shown in Figure 3 for various primary wavelengths and intensities, further demonstrating the role of a relatively long-lived Cy5 dark state in the modulation, suggesting formation of cis-Cy5. Enhancement is nearly

constant from DC to ~2 kHz, but rapidly decreases with higher modulation frequencies, limited by molecular response. At 100 kHz, the enhancement factor is close to 0, indicating the modulation period of 10 μ s is too short to buildup the dark state. This timescale and that observed in our FRET-based modulation experiments^[14] are indicative of the ~150 μ s cis-Cy5 component giving the dynamic signal enhancement. Characteristic times, τ , were extracted from fits of enhancement dependence to that for normalized frequency dependent lifetime modulation depth, m :

$$m = \frac{A}{\sqrt{1 + (2\pi\nu)^2 \tau^2}}.$$

The frequency dependent modulation depth yields a time, τ , of ~35 μ s for moderate primary laser intensities that shortens slightly at high primary intensities. Unlike frequency domain fluorescence lifetimes resulting from changing primary laser modulation frequency and detecting the direct fluorescence response, we are modulating the secondary laser that depopulates the dark state to regenerate fluorescence. Importantly, the characteristic time scale here is a convolution of on, natural off, and secondary laser-induced off times. The frequency dependence of the enhancement is characteristic of Cy5 photophysics, reflecting the time necessary for buildup and decay of the modulatable dark state. Using a three-state model, we simulated the modulated frequency dependence, to discover that the characteristic off time should be on the order of 100 microseconds to match the observed frequency response (Fig. 3). Increased primary excitation, however, shifts the characteristic frequency to higher values, thereby indicating that primary illumination artificially shortens the observed off times. These frequency response curves nicely complement the FCS data, again indicating that the longer lived (~150 μ s) cis-Cy5 state is the likely species giving rise to Cy5 fluorescence modulation.

Having previously used Cy5 as unenhanceable background with 805nm secondary illumination,^[13] enhancement is highly dependent on secondary laser wavelength and intensity as the secondary wavelength alters the steady-state population of cis-Cy5. The wavelength dependences on these factors are shown in Figure 4. In these measurements, the secondary laser was modulated at 1 kHz and fluorescence binned in 10 μ s bins for frequency analysis.

Generally, enhancement (or modulation depth) increases with increasing primary laser intensity, resulting from faster population of the dark state (Fig. 4A). Secondary laser illumination more rapidly depopulates the dark state, decreasing the off time and enhancing emission. Enhancement is initially linearly dependent on secondary laser intensity, but saturates at sufficiently high intensity to depopulate the dark state faster than its creation (Fig. 4B). Consistent with the solution absorption spectrum (690 nm max) of cis-Cy5,^[32] enhancement is quite sensitive to the secondary laser wavelength, dropping significantly from 710 nm to 740 nm (Fig. 4C). At high intensity, the primary laser may also excite the dark state, leading to a lack of independence of these excitations (Fig. 4A). Although intensities exceeding 5.1 kW cm⁻² further increase fluorescence, they begin to decrease enhancement depth, likely due to primary laser-induced back isomerization. The primary laser can not only shorten the on time for Cy5 from trans to cis states, but also inefficiently shorten the off time for Cy5 back isomerization from cis to trans. Therefore, at high primary intensity, the off time is also shortened, thereby decreasing the enhancement depth. 633 nm primary excitation produces similar results, but with a lower intensity necessary to reach maximal enhancement, consistent with stronger trans-Cy5 absorption at 633 nm (Fig. 4D).

Fluorophores signals that can be modulated with long-wavelength secondary excitation are readily recovered from high background via selective demodulation. By overlapping primary and modulated secondary lasers within the sample Cy5 fluorescence can be extracted in the presence of obscuring fluorescent background (Fig. 5). To demonstrate removal of obscuring dye signals, Cy5 was added to a Texas red solution with ~10-fold higher emission than that of Cy5. An identical sample of Texas red diluted in water (instead of Cy5 solution) was also imaged with secondary laser modulation (Fig. 5A), serving as a control. The microscope was focused ~30 μm into solution and the primary laser was defocused. A CCD camera placed at a primary image plane collects both defocused emission at the sample plane and out of focus emission above and below the sample focus, appearing as a broad emissive area. Demodulation at the 3 Hz secondary laser modulation frequency, however, only recovers emission from the dual-illuminated spot (Fig. 5D), effectively eliminating out of focus background and all Texas red emission, while also providing optical sectioning resulting from signal originating from only where the two beams overlap. The Texas red control (no Cy5) gives no signal at the modulation frequency (Fig. 5B).

Conclusion

The rich photophysics of Cy5 in solution can be harnessed for modulation-based signal recovery utilizing a long-lived transient that is largely obscured by diffusion-limited FCS studies. Presumably resulting from optically induced forward and reverse trans-cis isomerization, the spectral shifts associated with this change give rise to optically modulatable dark states at energies lower than those of collected fluorescence. The modulation timescale and further evidence for the cis-Cy5 state being responsible for modulation is supported by the measured modulation frequency dependence that tails off near 10 kHz. Using this long-lived state, Cy5 fluorescence is readily recovered from high background in solution. The broad range of applications of Cy5 fluorescence^[24, 34, 40-42, 52] offers the opportunity for greatly improved sensitivity in high background detection and imaging. This is the first commonly available, routinely used, and very bright dye identified that is capable of optically modulated fluorescence. Our characterization of its frequency response further provides an additional dimension for ultrahigh sensitivity contrast imaging.

Experimental Section

Cy5 NHS ester (GE Biosciences) was used as received. Cy5 solutions were prepared by dissolution in deionized water (18 M Ω) followed by dilution to experimental concentrations. All dual laser experiments were performed on an inverted microscope (Olympus IX71) using a 60 $\times$ water immersion objective (Olympus 1.2 NA). All solution data was taken by focusing 30 microns into solution. Signal was collected in a confocal arrangement with a 50- μm multimode fiber serving as the pinhole and directing the emission to a photon-counting avalanche photodiode (APD, Perkin Elmer). Intensity trajectories were recorded using a Becker-Hickl SPC-630 single photon counting board to timestamp individual photon arrival times. Primary excitation was performed at either 594 nm or 633 nm (Melles Griot, He-Ne lasers), and secondary co-illumination was with a Ti:sapphire laser (Coherent Mira 900) with wavelength ranging from 700 nm to 740 nm. Primary and secondary excitation beams were spatially overlapped in the microscope after combining on a dichroic mirror. Appropriate band pass filters were used to efficiently block both the primary excitation and the lower energy secondary laser, with an additional short-pass filter being used to fully block the secondary beam. Mechanical choppers and an electro-optic modulator (Conoptics) were used to modulate the incident laser beams, with recorded time traces being binned no less than 10-fold faster than the modulation frequency. When CCD collection was used, frame rates were synchronized to the modulation frequency, but were also collected at 10

times higher frequency in an epifluorescence geometry with a larger (defocused) primary laser excitation area and a smaller, focused secondary laser spot. Images were processed by Fourier transformation of the time trace of individual pixel intensities to reveal the frequency components. The corresponding amplitude at the modulation frequency of each pixel was used to recover the demodulated image.

Acknowledgments

The authors gratefully acknowledge financial support from NIH R01GM086195.

((This paper is contributed in honor and admiration of Professor Bräuchle's outstanding scientific contributions.))

References

- [1]. Weiss S. *Science*. 1999; 283:1676–1683. [PubMed: 10073925]
- [2]. Orrit M, Bernard J. *Phys. Rev. Lett.* 1990; 65:2716–2719. [PubMed: 10042674]
- [3]. Lin B, Urayama S, Saroufeem RMG, Matthews DL, Demos SG. *Opt. Express*. 2010; 18:21074–21082. [PubMed: 20941003]
- [4]. Chang WT, Yang YC, Lu HH, Li I-L, Liao I. *J. Am. Chem. Soc.* 2010; 132:1744–1745. [PubMed: 20102188]
- [5]. Bierwagen J, Testa I, Folling J, Wenzel D, Jakobs S, Eggeling C, Hell SW. *Nano. Lett.* 2010; 10:4249–4252. [PubMed: 20831171]
- [6]. Elgass K, Caesar K, Wanke D, Harter K, Meixner AJ, Schleifenbaum F. *Anal. Bioanal. Chem.* 2010; 398:1919–1925. [PubMed: 20811880]
- [7]. Sun Y, Phipps J, Elson DS, Stoy H, Tinling S, Meier J, Poirier B, Chuang FS, Farwell DG, Marcu L. *Opt. Lett.* 2009; 34:2081–2083. [PubMed: 19572006]
- [8]. Ramanujan VK, Zhang JH, Biener E, Herman B. *J. Biomed. Opt.* 2005; 10:0514071–0514011.
- [9]. Hess ST, Gould TJ, Gudheti MV, Maas SA, Mills KD, Zimmerberg J. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:17370–17375. [PubMed: 17959773]
- [10]. Aubin JE. *J. Histochem. Cytochem.* 1979; 27:36–43. [PubMed: 220325]
- [11]. Marriott G, Mao S, Sakata T, Ran J, Jackson D. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:17789–17794. [PubMed: 19004775]
- [12]. Richards CI, Hsiang J-C, Dickson RM. *J. Phys. Chem. B.* 2010; 114:660–665. [PubMed: 19902923]
- [13]. Richards CI, Hsiang J-C, Senapati D, Patel S, Yu J, Vosch T, Dickson RM. *J. Am. Chem. Soc.* 2009; 131:4619–4621. [PubMed: 19284790]
- [14]. Richards CI, Hsiang J-C, Khalil AM, Hull NP, Dickson RM. *J. Am. Chem. Soc.* 2010; 132:6318–6323. [PubMed: 20397664]
- [15]. Fan C, Hsiang J-C, Jablonski AE, Dickson RM. *Chem. Sci.* 2011; 2:1080–1085. [PubMed: 22262992]
- [16]. Jung G, Wiehler J, Steipe B, Bräuchle C, Zumbusch A. *ChemPhysChem*. 2001; 2:392–396.
- [17]. Cordonnier M, Uy D, Dickson RM, Kerr KE, Zhang Y, Oka T. *J. Chem. Phys.* 2000; 113:3181–3193.
- [18]. Moerner WE, Kador L. *Phys. Rev. Lett.* 1989; 62:2535–2538. [PubMed: 10040013]
- [19]. Bawendi MG, Rehfuss BD, Oka T. *J. Chem. Phys.* 1990; 93:6200–6209.
- [20]. Petty JT, Fan CY, Story SP, Sengupta B, Iyer AS, Prudowsky Z, Dickson RM. *J. Phys. Chem. Lett.* 2010; 1:2524–2529. [PubMed: 21116486]
- [21]. Petty JT, Fan CY, Story SP, Sengupta B, Sartin M, Hsiang JC, Perry JW, Dickson RM. *J. Phys. Chem. B.* 2011; 115:7996–8003. [PubMed: 21568292]
- [22]. Yan YL, Marriott ME, Petchprayoon C, Marriott G. *Biochem. J.* 2011; 433:411–422. [PubMed: 21235524]
- [23]. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF. *Science*. 2006; 313:1642–1645. [PubMed: 16902090]

- [24]. Bates M, Huang B, Dempsey GT, Zhuang X. *Science*. 2007; 317:1749–1753. [PubMed: 17702910]
- [25]. Hofmann M, Eggeling C, Jakobs S, Hell SW. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:17565–17569. [PubMed: 16314572]
- [26]. Min W, Lu S, Chong S, Roy R, Holtom GR, Xie XS. *Nature*. 2009; 461:1105–1109. [PubMed: 19847261]
- [27]. Li ADQ, Zhan C, Hu D, Wan W, Yao J. J. *Am. Chem. Soc.* 2011; 133:7628–7631. [PubMed: 21539363]
- [28]. Lakowicz, JR. *Principles of Fluorescence Spectroscopy*. Third Edition. Lakowicz, JR., editor. Springer; New York: 2006.
- [29]. Habuchi S, Ando R, Dedecker P, Verheijen W, Mizuno H, Miyawaki A, Hofkens J. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:9511–9516. [PubMed: 15972810]
- [30]. Widengren J, Mets U, Rigler R. *Chem. Phys.* 1999; 250:171–186.
- [31]. Widengren J, Schwille P. J. *Phys. Chem. A*. 2000; 104:6416–6428.
- [32]. Huang ZX, Ji DM, Wang SF, Xia AD, Koberling F, Patting M, Erdmann R. J. *Phys. Chem. A*. 2006; 110:45–50. [PubMed: 16392838]
- [33]. Huang ZX, Ji DM, Xia AD, Koberling F, Patting M, Erdmann R. J. *Am. Chem. Soc.* 2005; 127:8064–8066. [PubMed: 15926831]
- [34]. Dempsey GT, Bates M, Kowtoniuk WE, Liu DR, Tsien RY, Zhuang X. J. *Am. Chem. Soc.* 2009; 131:18192–18193. [PubMed: 19961226]
- [35]. Jia K, Wan Y, Xia AD, Li SY, Gong FB, Yang GQ. J. *Phys. Chem. A*. 2007; 111:1593–1597. [PubMed: 17295461]
- [36]. Chmyrov A, Sanden T, Widengren J. *Anal. Chem.* 2010; 82:9998–10005. [PubMed: 21105696]
- [37]. Hunt PA, Robb MA. J. *Am. Chem. Soc.* 2005; 127:5720–5726. [PubMed: 15826214]
- [38]. Schwenker MA, Bock H, Hofmann M, Jakobs S, Bewersdorf J, Eggeling C, Hell SW. *Microsc. Res. Tech.* 2007; 70:269–280. [PubMed: 17262791]
- [39]. Emerson ES, Conlin MA, Rosenoff AE, Norland KS, Rodriguez H, Chin D, Bird GR. J. *Phys. Chem.* 1967; 71:2396–2403.
- [40]. Mishra A, Behera RK, Behera PK, Mishra BK, Behera GB. *Chem. Rev.* 2000; 100:1973–2012. [PubMed: 11749281]
- [41]. Oushiki D, Kojima H, Terai T, Arita M, Hanaoka K, Urano Y, Nagano T. J. *Am. Chem. Soc.* 2010; 132:2795–2801. [PubMed: 20136129]
- [42]. Levitus M, Ranjit S. Q. *Rev. Biophys.* 2011; 44:123–151. [PubMed: 21108866]
- [43]. Intes X, Ripoll J, Chen Y, Nioka S, Yodh AG, Chance B. *Med. Phys.* 2003; 30:1039–1047. [PubMed: 12852527]
- [44]. Haustein E, Schwille P. *Annu. Rev. Biophys. and Biomol. Struct.* 2007; 36:151–169. [PubMed: 17477838]
- [45]. English DS, Harbron EJ, Barbara PF. J. *Phys. Chem. A*. 2000; 104:9057–9061.
- [46]. Daglio SC, Banterle N, D'Alfonso L, Collini M, Chirico G. J. *Phys. Chem. B*. 2011; 115:10311–10321. [PubMed: 21736327]
- [47]. Eggeling C, Volkmer A, Seidel CAM. *ChemPhysChem*. 2005; 6:791–804. [PubMed: 15884061]
- [48]. Ringemann C, Schonle A, Giske A, von Middendorff C, Hell SW, Eggeling C. *ChemPhysChem*. 2008; 9:612–624. [PubMed: 18324718]
- [49]. Monguzzi A, Mezyk J, Scotognella F, Tubino R, Meinardi F. *Phys. Rev. B*. 2008:78.
- [50]. Clark JL, Rumbles G. *Phys. Rev. Lett.* 1996; 76:2037–2040. [PubMed: 10060590]
- [51]. Blau W, Dankesreiter W, Penzkofer A. *Chem. Phys.* 1984; 85:473–479.
- [52]. Heilemann M, Margeat E, Kasper R, Sauer M, Tinnefeld P. J. *Am. Chem. Soc.* 2005; 127:3801–3806. [PubMed: 15771514]

Figure 1.

Single- and dual-laser illuminated FCS of aqueous Cy5 solutions. Solid square data, red line fit: Primary laser alone (594 nm, $\sim 1.4 \text{ kW cm}^{-2}$); Open circle data, blue line fit: secondary laser (710 nm, $\sim 12 \text{ kW cm}^{-2}$) co-illumination. Inset: Monte Carlo simulated FCS curves. The solid red line was simulated with primary laser alone (594 nm), in which the $\tau_{\text{on}} \sim 70 \mu\text{s}$, $\tau_{\text{off}} \sim 90 \mu\text{s}$, and $\tau_{\text{diffusion}} \sim 170 \mu\text{s}$. The simulation in blue used the same parameters as the red curve, but with secondary laser co-illumination. Dark state absorption at the secondary laser wavelength reduces τ_{off} to $\sim 5 \mu\text{s}$, without changing other timescales. In both data and simulation, the long timescale shifts to longer times and increases in contrast. The opposite effects are observed in the shorter timescale.

Figure 2.

Modulated Cy5 fluorescence under dual illumination with primary (594 nm) and secondary (710 nm) laser intensities of 1.7 kW cm^{-2} and 12 kW cm^{-2} , respectively. (A) A portion of an aqueous Cy5 fluorescence trajectory, modulated at 700 Hz. (B) Fourier transform of the time trace in A. (C) Fourier transform of Cy5 fluorescence in solution under dual modulation (primary laser chopped at 550 Hz and the secondary laser chopped at 700 Hz). In addition to primary only and secondary only modulation peaks, sum (1250 Hz) and difference (150 Hz) peaks are observed in addition to much weaker combination and overtones at 950 Hz ($2\nu_{\text{primary}} - \nu_{\text{secondary}}$) and 1100 Hz. Note that at 100% of the fluorescence is modulated at the primary laser modulation frequency, so it is normalized to an amplitude of 1 in panel C to show the sum and difference peaks

Figure 3.

Experimental (solid black squares) and simulated (open red circles) modulation frequency dependent fluorescence enhancement of aqueous Cy5. Simulations are based on a three energy level model, consisting of ground, bright, and dark states. Primary excitation is varied in A-D as follows: A. 633 nm and 1.8 kW cm⁻², B. 633 nm and 7.7 kW cm⁻², C. 594 nm and 1.7 kW cm⁻², D. 594 nm and 4.2 kW cm⁻². For all panels, secondary excitation is at 710 nm and 12 kW cm⁻². For each frequency, the modulation depth was normalized by the DC component after subtracting any secondary laser-only induced background. This normalized Fourier amplitude is converted to the enhancement factor (y-axis) for ideal square wave modulation. Characteristic modulation frequencies (where the modulation amplitude reached half its initial value) for each excitation condition were extracted and compared with frequency dependent simulations.

Figure 4.

Intensity and spectral dependence of Cy5 fluorescence enhancement using a secondary laser modulation frequency of 1000 Hz. A. Enhancement vs. 594 nm primary laser intensity with constant secondary laser (710 nm) intensity at 12 kW cm⁻². Enhancement dependence on secondary laser intensity using primary laser intensity of 2.3 kW cm⁻² at 594 nm. C. Enhancement dependence on the secondary laser wavelength with primary (594 nm) and secondary intensities of 2.3 kW cm⁻² and 12 kW cm⁻², respectively. D. 633 nm primary laser intensity dependence using secondary laser (710 nm) intensity of 12 kW cm⁻². enhancement was calculated from the normalized Fourier amplitude at modulated frequency, assuming the ideal square wave of the fluorescence emission.

Figure 5.

Cy5 fluorescence image recovery upon demodulation with high background. A. Average image for the Texas red only solution. B. The recovered image from A upon demodulation showing no signal at the modulation frequency (3 Hz). C. Average image for the Texas Red + Cy5 solution. D. The recovered image from C when demodulated at 3 Hz, removing the Texas red background and recovering only the Cy5 emission.