Published in final edited form as:

J Phys Chem Lett. 2017 February 16; 8(4): 733–736. doi:10.1021/acs.jpclett.6b02816.

Improved Fluorescent Protein Contrast and Discrimination by Optically Controlling Dark State Lifetimes

Yen-Cheng Chen and Robert M. Dickson*

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

Abstract

Modulation and optical control of photoswitchable fluorescent protein (PS-FP) dark state lifetimes drastically improves sensitivity and selectivity in fluorescence imaging. The dark state population of PS-FPs generates an out-of-phase fluorescence component relative to the sinusoidally modulated 488nm laser excitation. Because this apparent phase advanced emission results from slow recovery to the fluorescent manifold, we hasten recovery and, therefore, modulation frequency by varying co-illumination intensity at 405nm. As 405nm illumination regenerates the fluorescent ground state more rapidly than via thermal recovery, we experimentally demonstrate that secondary illumination can control PS-FPs dark state lifetime to act as an additional dimension for discriminating spatially and spectrally overlapping emitters. This experimental combination of out of phase imaging after optical modulation (OPIOM) and synchronously amplified fluorescence image recovery (SAFIRE) optically controls the fluorescent protein dark state lifetimes for improved time resolution, with the resulting modulation-based selective signal recovery being quantitatively modeled. The combined experimental results and quantitative numerical simulations further demonstrate the potential of SAFIRE-OPIOM for wide-field biological imaging with improved speed, sensitivity, and optical resolution over other modulation-based fluorescence microscopies.

Graphical abstract

* dickson@chemistry.gatech.edu.

Supporting Information

Experimental methods, three-state model, simulation parameters. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Fluorescent microscopy; optical modulation; photoswitchable proteins; dark state lifetime; phase advance; SAFIRE; OPIOM

Fluorescence labeling enables direct imaging of subcellular positions and dynamics. When the bright and dark states of fluorescent labels can be optically controlled, however, both resolution¹⁻⁵ and contrast/selectivity can be further enhanced.⁶⁻¹⁵ Instead of waiting for ground state recovery from photoinduced dark states, optically induced dark state depopulation as in SAFIRE (Synchronously Amplified Image Recovery)⁷ more rapidly repopulates the bright manifold of states than through natural decay. This approach has been effective in selectively modulating the fluorescence of certain dyes for drastically improved imaging sensitivity and fluorophore discrimination. Such long-wavelength co-illumination depopulates the dark state with a rate proportional to the secondary laser intensity, thereby providing an additional dimension for discriminating fluorophores in complex biological environments.⁸⁻¹⁰

Complementary to SAFIRE's use of frequency-dependent modulation depth, out-of-phase imaging after optical modulation (OPIOM)¹⁵ utilizes an optically created bath of dark state molecules to continuously repopulate the emissive manifold of photoswitchable fluorescent proteins (PS-FPs).¹⁶⁻²¹ When the modulation period is comparable to the natural dark state relaxation time, an apparent phase advance²² is observed relative to the modulation waveform. This "advance" arises from the continuous, slow flow of population from the dark to the bright manifold of states at a rate that is slower than the modulation frequency. This nonlinear response to sinusoidal excitation of the ground state distorts the fluorescence response from the modulation waveform, with the rise in fluorescence being more rapid than the fall.²²⁻²⁴ As the entire fluorescence response is directly modulated with the primary excitation laser modulation waveform, the out of phase component is very small unless an extremely high dark state fraction is achievable. Thus, only long-lived photoswitches have been used for imaging contrast, with the amplitude of out-of-phase signals being related to the dark state lifetime.¹⁵ While SAFIRE can also be applied to modulate fluorophores with short-lived dark states, OPIOM, OLID (optical lock-in detection),^{6, 25} and SAFIRE⁷⁻¹³ can all utilize PS-FP photophysics to discriminate PS-FP signals from similarly emitting non-photoswitchable fluorophores.^{6, 10, 15}

Using only a single excitation source, OPIOM relies on producing large steady-state dark state populations that slowly relax to the ground state.¹⁵ Under these circumstances, modulated Dronpa-2 (Dronpa-M159T) fluorescence shows a phase shift of fluorescence relative to the modulated excitation waveform, but extremely slow, 4.93 mHz, modulation frequencies must be used.¹⁵ SAFIRE offers the opportunity to improve on this promising scheme by also employing a second laser to hasten ground state recovery and increase detected fluorescence intensity and imaging rates ~1000-fold over the original OPIOM implementation. Thus, the combination of SAFIRE with OPIOM offers the opportunity to control the dark state lifetime for improved sensitivity and dynamics.

To actively control dark state lifetime, we excite PS-FP fluorescence from rsFastLime¹⁸⁻¹⁹ immobilized within fixed cells with a sinusoidally modulated primary laser (488nm, Figure 1) as in OPIOM, but also co-illuminate the dark state with cw secondary excitation at 405nm. Without co-illumination at 405nm, even modest ~1W/cm² 488nm illumination leads to nearly all rsFastLime molecules becoming rapidly trapped in the long-lived (~480 seconds)¹⁸ dark state, decreasing total fluorescence. The 405nm co-illumination more rapidly regenerates the fluorophore ground state than possible through natural decay. Changing 405-nm secondary intensity thereby tunes the dark state lifetime, providing a mechanism by which one can shift the maximum modulation frequency for faster imaging or improved molecular discrimination.¹⁰

Longer-lived dark states increase steady-state dark state populations and increase dark state hysteresis to result in a larger out-of-phase signal component. This limits imaging speed as very low modulation frequencies must be used for contrast enhancement using OPIOM.¹⁵ To maximize out-of-phase signals, the modulation frequency must be comparable to the dark-state lifetime. If the modulation is too slow, the dark state decays before the modulation cycle completes; if it is too fast, the molecules cannot reestablish steady-state dark and bright populations within each modulation period. Thus, modulating too slowly minimizes the out-of-phase component, while modulating too rapidly decreases modulation depth. Consequently, the long-lived (>1 minute¹⁸) dark states of PS-FPs make live cell imaging with OPIOM somewhat impractical. Co-illumination of sinusoidally 488nm-excited rsFastLime with adjustable 405nm intensity, however, offers the ability to tune the dark-state lifetime to any timescale from minutes to milliseconds, while also controlling the magnitude of out-of-phase fluorescence (Figure 2A).

Because the introduction of a second laser to OPIOM controls dark state lifetime, this scheme offers the potential for greatly improved signal discrimination and increased imaging rates. Since the maximum out-of-phase signal is determined by the 405nm laser intensity, illumination of rsFastLime samples with non-uniform 405nm intensity profiles encodes different out-of-phase responses on each CCD pixel (Figure 2B). This provides an additional dimension to discriminate fluorophores based on either natural^{8, 10, 15} or 405nm intensity dependent dark state lifetimes. Because rsFastLime¹⁸⁻¹⁹ is such an efficient photoswitch, low 488 and 405nm intensities can be used. This approach minimizes photobleaching and phototoxicity effects, while being able to record out-of-phase signals with CCD acquisition on the sub-second time scale. Similar to SAFIRE and OLID, secondary laser-enhanced

OPIOM (SAFIRE-OPIOM) enables discrimination of spectrally overlapped fluorophores on the basis of their photophysical dark state dynamics.

Utilizing our three-state model for rsFastLime, numerical simulations using published photophysical parameters¹⁹⁻²¹ can be used to accurately model the observed phase advance (Figure 3, with details given in the Supplementary Information). Both experimental data and numerical simulation produce similar modulation frequency-responses (Figure 3), offering predictive and modeling ability for recording the out-of-phase PS-FP fluorescence.

In contrast to the largely symmetric illumination and dark state lifetimes in Figure 2B, co-illumination with asymmetric 405nm intensities provides an opportunity to utilize rsFastLime dark state lifetime as a probe of spatial position. The accuracy of our numerical simulations compared to our experimental results (Figures 2&3, and SI) enables us to simulate microscopy images of rsFastLime fluorescence employing a linear spatial intensity gradient at 405nm. rsFastLime photophysics and positions are simulated at slightly different spatial positions, and final images are convolved with a point spread function using BlurLab3D.²⁶

If a sufficiently large anisotropic spatial gradient (~6-fold intensity change over 50nm) is used, two rsFastLime molecules separated by 50nm become readily resolved based on their different frequency-dependent out of phase modulated fluorescence responses (Figure 4). As long as the separation between labeled features is less than the minimum resolvable frequency difference, sub-diffraction spatial information from the 405nm intensity gradient is recoverable from observed dark state lifetimes. Thus, optical control of dark state lifetime with 405nm intensity, offers a new dimension to resolve otherwise identical emitters, as each molecule experiences different 405nm intensities. In contrast to localization or point-spread function engineering methods, utilizing the out-of-phase signal resulting from controlling dark-state lifetimes in this SAFIRE-OPIOM approach suggests that one can simultaneously exclude auto-fluorescent background and non-modulatable emitters, while also potentially distinguishing emitters separated by less than the diffraction limit, all at moderate excitation intensities.

Under dual illumination, with a spatially anisotropic secondary illumination pattern, each PS-FP exhibits modulated emission with a unique out-of-phase modulation response curve. Thus, spectral unmixing of the modulation responses improves signal discrimination as in OPIOM by looking only at the out of phase response, while simultaneously enabling faster imaging and optical control of emitters through SAFIRE. Our accurate numerical simulations of optical response also suggest that molecules separated by less than the diffraction limit can be directly resolved by application of a spatially anisotropic 405nm intensity gradient, coupled with spectral unmixing of modulation responses. Thus, SAFIRE-OPIOM should speed imaging rates and enable dark state lifetime to be used as a new dimension for improving spatial resolution, while retaining the original signal discrimination abilities of both OPIOM and SAFIRE.

Photophysical control of PS-FP dark state lifetime can be utilized to simultaneously improve imaging time resolution, discriminate fluorophores based on dark state lifetime, and offers

the potential for super-resolution microscopy. Accelerating dark state decay with low intensity co-illumination at 405nm also drastically increases imaging speed of OPIOM, potentially allowing live cell imaging and dynamics to be probed. Quantitative numerical models have been developed to provide predictive ability and utilization of out of phase signals in biological imaging. This combined SAFIRE-OPIOM approach is likely to find wide application in high sensitivity biological imaging, and can now be more readily applied to studying biological interactions and dynamics with improved speed and sensitivity.

Experimental methods

see supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors gratefully acknowledge cell samples from C.J. Fahrni and D. Bourassa and support from NIH R21EB020371 and the Vasser Woolley Foundation.

References

1. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*. 2006; 313(5793):1642–1645. [PubMed: 16902090]
2. Dertinger T, Colyer R, Iyer G, Weiss S, Enderlein J. Fast, background-free, 3D super-resolution optical fluctuation imaging (SOFI). *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106(52):22287–22292. [PubMed: 20018714]
3. Dedecker P, Mo GC, Dertinger T, Zhang J. Widely accessible method for superresolution fluorescence imaging of living systems. *Proc. Natl. Acad. Sci. U.S.A.* 2012; 109(27):10909–10914. [PubMed: 22711840]
4. Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods*. 2006; 3(10):793–795. [PubMed: 16896339]
5. Danzl JG, Sidenstein SC, Gregor C, Urban NT, Ilgen P, Jakobs S, Hell SW. Coordinate-targeted fluorescence nanoscopy with multiple off states. *Nat. Photon.* 2016; 10(2):122–128.
6. Marriott G, Mao S, Sakata T, Ran J, Jackson DK, Petchprayoon C, Gomez TJ, Warp E, Tulyathan O, Aaron HL, Isacoff EY, Yan Y. Optical lock-in detection imaging microscopy for contrast-enhanced imaging in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105(46):17789–17794. [PubMed: 19004775]
7. Richards CI, Hsiang JC, Dickson RM. Synchronously Amplified Fluorescence Image Recovery (SAFIRE). *J. Phys. Chem. B*. 2010; 114(1):660–665. [PubMed: 19902923]
8. Jablonski AE, Hsiang JC, Bagchi P, Hull N, Richards CI, Fahrni CJ, Dickson RM. Signal Discrimination Between Fluorescent Proteins in Live Cells by Long-wavelength Optical Modulation. *J. Phys. Chem. Lett.* 2012; 3(23):3585–3591. [PubMed: 23419973]
9. Jablonski AE, Vegh RB, Hsiang JC, Bommarius B, Chen YC, Solntsev KM, Bommarius AS, Tolbert LM, Dickson RM. Optically modulatable blue fluorescent proteins. *J. Am. Chem. Soc.* 2013; 135(44):16410–16417. [PubMed: 24099419]
10. Chen YC, Jablonski AE, Issaeva I, Bourassa D, Hsiang JC, Fahrni CJ, Dickson RM. Optically Modulated Photoswitchable Fluorescent Proteins Yield Improved Biological Imaging Sensitivity. *J. Am. Chem. Soc.* 2015; 137(40):12764–12767. [PubMed: 26402244]

11. Hsiang JC, Jablonski AE, Dickson RM. Optically modulated fluorescence bioimaging: visualizing obscured fluorophores in high background. *Acc. Chem. Res.* 2014; 47(5):1545–1554. [PubMed: 24725021]
12. Petty JT, Fan C, Story SP, Sengupta B, Sartin M, Hsiang JC, Perry JW, Dickson RM. Optically enhanced, near-IR, silver cluster emission altered by single base changes in the DNA template. *J. Phys. Chem. B.* 2011; 115(24):7996–8003. [PubMed: 21568292]
13. Fan C, Hsiang JC, Dickson RM. Optical modulation and selective recovery of Cy5 fluorescence. *Chemphyschem : a European journal of chemical physics and physical chemistry.* 2012; 13(4): 1023–1029. [PubMed: 22086764]
14. Li AD, Zhan C, Hu D, Wan W, Yao J. Photoswitchable nanoprobe offer unlimited brightness in frequency-domain imaging. *J. Am. Chem. Soc.* 2011; 133(20):7628–7631. [PubMed: 21539363]
15. Querard J, Markus TZ, Plamont MA, Gauron C, Wang P, Espagne A, Volovitch M, Vriz S, Croquette V, Gautier A, Le Saux T, Jullien L. Photoswitching kinetics and phase-sensitive detection add discriminative dimensions for selective fluorescence imaging. *Angew. Chem. Int. Ed.* 2015; 54(9):2633–2637.
16. Ando R, Mizuno H, Miyawaki A. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science.* 2004; 306(5700):1370–1373. [PubMed: 15550670]
17. Habuchi S, Ando R, Dedecker P, Verheijen W, Mizuno H, Miyawaki A, Hofkens J. Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102(27):9511–9516. [PubMed: 15972810]
18. Stiel AC, Trowitzsch S, Weber G, Andresen M, Eggeling C, Hell SW, Jakobs S, Wahl MC. 1.8 Å bright-state structure of the reversibly switchable fluorescent protein Dronpa guides the generation of fast switching variants. *Biochem. J.* 2007; 402(1):35–42. [PubMed: 17117927]
19. Andresen M, Stiel AC, Folling J, Wenzel D, Schonle A, Egner A, Eggeling C, Hell SW, Jakobs S. Photoswitchable fluorescent proteins enable monochromatic multilabel imaging and dual color fluorescence nanoscopy. *Nat. Biotechnol.* 2008; 26(9):1035–1040. [PubMed: 18724362]
20. Kao YT, Zhu X, Min W. Protein-flexibility mediated coupling between photoswitching kinetics and surrounding viscosity of a photochromic fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 2012; 109(9):3220–3225. [PubMed: 22328153]
21. Yadav D, Lacombe F, Dozova N, Rappaport F, Plaza P, Espagne A. Real-time monitoring of chromophore isomerization and deprotonation during the photoactivation of the fluorescent protein Dronpa. *J. Phys. Chem. B.* 2015; 119(6):2404–2414. [PubMed: 25325882]
22. Gatzogiannis E, Zhu X, Kao Y-T, Min W. Observation of Frequency-Domain Fluorescence Anomalous Phase Advance Due to Dark-State Hysteresis. *J. Phys. Chem. Lett.* 2011; 2(5):461–466.
23. Yonemaru Y, Yamanaka M, Smith NI, Kawata S, Fujita K. Saturated excitation microscopy with optimized excitation modulation. *Chemphyschem : a European journal of chemical physics and physical chemistry.* 2014; 15(4):743–749. [PubMed: 24488765]
24. Manna P, Jimenez R. Time and frequency-domain measurement of ground-state recovery times in red fluorescent proteins. *J. Phys. Chem. B.* 2015; 119(15):4944–4954. [PubMed: 25781915]
25. Du, G., Marriott, G., Yan, Y. An improved optical lock-in detection method for contrast-enhanced imaging in living cells. *Bioinform. Biomed. Eng; (iCBBE), 2010 4th International Conference;* 2010. p. 1-5.
26. Ursell, T., Huang, KC. BlurLab -- 3D Microscopy Simulation Package v0.9. <https://simtk.org/projects/blurlab>
27. Dougherty, B. Diffraction PSF 3D (ImageJ). http://imagej.net/Diffraction_PSF_3D

Figure 1.

Modulated PS-FP, rsFastLime, fluorescence time traces from sinusoidally intensity modulated 488-nm excitation. Normalized curves are plotted relative to modulated fluorescein emission collected under identical conditions. Samples were co-illuminated at 405nm with intensities of (A) 0.25W/cm² and (B) 3W/cm². Fluorescein emission tracks the excitation waveform, while rsFastLime fluorescence appears advanced in phase. The phase advance at a given modulation frequency changes with 405nm laser intensity.

Figure 2.

Experimental out-of-phase signals (FL_{out}) upon varied 405nm laser intensity. (A) Out-of-phase (cosine) rsFastLime fluorescence amplitude resulting from sinusoidally modulated, spatially homogeneous 488nm (8W/cm² average intensity) and continuous, homogeneous 405nm laser excitation for several 405nm intensities. At increasing 405nm intensities, the out of phase maxima shift from low to high frequency. (B) (Left) Using spatially heterogeneous, weakly focused 405nm co-illumination within a much larger, spatially homogeneous wide field 488nm illumination, the out-of-phase rsFastLime fluorescence maximum (FL_{out}) increases in frequency at spatial positions exhibiting higher 405nm secondary intensities. The raw fluorescence and out of phase maxima at each pixel are shown in the two images, with the color indicating the maximum of the out of phase signal at each pixel, while outside the 405nm illumination spot, the fluorophores are unobservable (UnObs, black), as they are switched into the dark state under 488nm illumination alone. (Right) The plot shows the out of phase amplitude vs. 488nm modulation frequency for rsFastLime at low (square), medium (circle) and high (triangle) 405nm intensity. Scale bar: 1 μ m. 488nm intensity, 40W/cm², is spatially invariant over the images.

Figure 3.

Comparison between experimental data (red) and numerical simulation (black) using a three-state model of rsFastLime emission and photoswitching. rsFastLime is co-illuminated with 8W/cm² at 488nm and (Left) 0.25W/cm², (Center) 1W/cm², and (Right) 3W/cm² at 405nm.

Figure 4.

Numerically simulated resolution of two diffraction-limited molecular emission patterns, separated by 50nm, and illuminated at 6-fold different 405nm intensities. (A) With the recovered FL_{out} intensities at two 405nm intensities, we utilize spatially homogeneous 0.5Hz, 1Hz, 5Hz, and 10Hz modulated 488nm excitation. (B) After point spread function convolution (MATLAB BlurLab v0.9²⁶ and Diffraction PSF 3D)²⁷ and FL_{out} analysis, spectral unmixing of the modulation spectra enables recovery of the correct molecular positions, with a recovered distance of 49.2nm. Scale bar: 250 nm.