

5-2014

Effect of Ca^{2+} on the Steady-State and Time-Resolved Emission Properties of the Genetically Encoded Fluorescent Sensor CatchER

You Zhou
Georgia State University

Kyрил M. Solntsev
Georgia Institute of Technology, solntsev@gatech.edu

Florence Reddish
freddish1@gsu.edu

Shen Tang
Georgia State University, stang3@student.gsu.edu

Jenny J. Yang
Georgia State University, jenny@gsu.edu

Follow this and additional works at: https://scholarworks.gsu.edu/chemistry_facpub

 Part of the [Chemistry Commons](#)

Recommended Citation

EZhuo, Y., Solntsev, K. M., Reddish, F., Tang, S., & Yang, J. J. (2015). Effect of Ca^{2+} on the Steady-State and Time-Resolved Emission Properties of the Genetically Encoded Fluorescent Sensor CatchER. *The Journal of Physical Chemistry B*, 119(6), 2103-2111. DOI: <http://dx.doi.org/10.1021/jp501707n>

This Article is brought to you for free and open access by the Department of Chemistry at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Chemistry Faculty Publications by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.

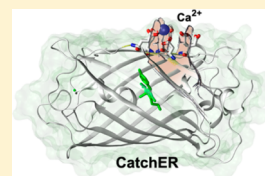
Effect of Ca^{2+} on the Steady-State and Time-Resolved Emission Properties of the Genetically Encoded Fluorescent Sensor CatchER

You Zhuo,[†] Kyril M. Solntsev,^{*,‡} Florence Reddish,[†] Shen Tang,[†] and Jenny J. Yang^{*,†}

[†]Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, United States

[‡]School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0400, United States

ABSTRACT: We previously designed a calcium sensor CatchER (a GFP-based Calcium sensor for detecting high concentrations in the high calcium concentration environment such as ER) with a capability for monitoring calcium ion responses in various types of cells. Calcium binding to CatchER induces the ratiometric changes in the absorption spectra, as well as an increase in fluorescence emission at 510 nm upon excitation at both 395 and 488 nm. Here, we have applied the combination of the steady-state and time-resolved optical methods and Hydrogen/Deuterium isotope exchange to understand the origin of such calcium-induced optical property changes of CatchER. We first demonstrated that calcium binding results in a 44% mean fluorescence lifetime increase of the indirectly excited anionic chromophore. Thus, CatchER is the first protein-based calcium indicator with the single fluorescent moiety to show the direct correlation between the lifetime and calcium binding. Calcium exhibits a strong inhibition on the excited-state proton transfer nonadiabatic geminate recombination in protic (vs deuterio) medium. Analysis of CatchER crystal structures and the MD simulations reveal the proton transfer mechanism in which the disrupted proton migration path in CatchER is rescued by calcium binding. Our finding provides important insights for a strategy to design calcium sensors and suggests that CatchER could be a useful probe for FLIM imaging of calcium in situ.



INTRODUCTION

Calcium transients are essential for intracellular calcium-dependent biological processes.¹ The amplitude, duration, and kinetics of calcium concentration change embody the code to regulate downstream signaling. Such calcium signals are determined by the calcium gradients, the native calcium binding proteins, and the calcium channels as well as pumps. Calcium indicators with a wide range of calcium-binding affinities are needed to probe the spatial-temporal calcium dynamics in different subcellular compartments. The quantitative measurement is critical to understand $[\text{Ca}^{2+}]$ change and the resting calcium level in intracellular calcium stores and subcellular compartments.

For imaging calcium changes in biological systems, much effort has been taken to develop the fluorescent calcium biosensor toolkit.^{2–7} There is a broad range of choices for organic calcium dyes, including the ratiometric indicators, like the Fura and Indo series, and the single wavelength indicators, BAPTA, Fluo, and Rhod series.⁸ These organic dyes have a dissociation constant, K_d , range from 0.2 to 20 μM and are widely applied to monitor the intracellular calcium transients.

Genetically encoded calcium indicators (GECIs), based on fluorescent proteins, benefit the calcium concentration detection in specific cellular compartments, including GCaMP, Cameleon, Pericam, and the Troponin C (TN) series. These GECIs take advantage of the intrinsic calcium-binding properties of calmodulin or troponin C to induce the fluorescence intensity change upon calcium binding.^{4,9–13} These calcium indicators, with K_d in the magnitude from 10^{-7} to 10^{-6} M and slow calcium dissociation rates, are suitable to detect a calcium rise in cytosolic space but not for the

calcium concentration change in the internal calcium stores such as the endoplasmic/sarcoplasmic reticulum (ER/SR). We have previously designed CatchER (Calcium sensor for detecting high concentration in the ER) as a sensitive fluorescence calcium probe with K_d of 0.18 mM.¹⁴ CatchER is created by the addition of a calcium binding site formed by five calcium ligand residues in the beta-barrel in proximity of the phenol group of the chromophore (Cro) from the single-enhanced green fluorescent protein (EGFP) carrying the deprotonated Cro (Figure 1).

However, some major challenges for quantitative monitoring of the calcium cellular responses using the static fluorescence microscopy remain, including uneven dye loading, leakage, and photobleaching. The fluorescence lifetime imaging (FLIM) overcomes the limitations with its advantage of being independent of the concentration of fluorophore as well as the excitation light intensity.^{15,16} In FLIM, lifetime measurement takes place at each pixel, revealing the spatial distribution of the fluorescent molecules, allowing a simultaneous recording of multiple fluorescent labels with reliable discrimination.¹⁷ In addition, it is sensitive to the change of the microenvironment such as pH, the ion strength, hydrophobicity, oxygen concentration, and interaction with other macromolecules and protein–protein/protein–ligand interactions in situ.^{18–21} Thus, it provides insights into the local environment of the

Special Issue: Photoinduced Proton Transfer in Chemistry and Biology Symposium

Received: February 17, 2014

Revised: May 14, 2014

Published: May 16, 2014

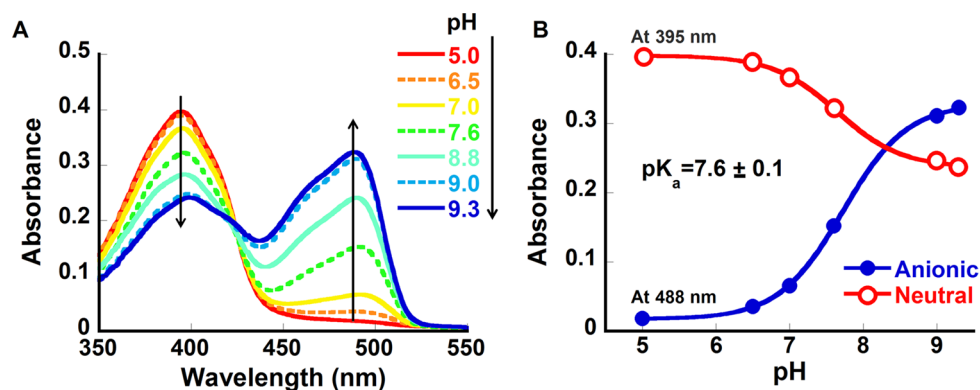


Figure 2. pH dependence of CatchER absorbance. (A) The pH titration from 5 to 9.3; (B) the pK_a fitting by eq 2. The pH titration was carried out using 5 M KOH in a buffer cocktail consisting of 10 mM MES, 10 mM MOPS, and 10 mM CAPS, pH 5.0.

particle mesh Ewald method. The atomic coordinates were taken from the crystal structures, and proteins were placed in a periodic box of TIP3P water by the AMBER tLEaP module. The system was first treated by 2500 steps of steep descent followed by 2500 steps of conjugate gradient minimization. After minimization, the system was heated to 300 K at 2 fs stepwise for 50 ps using the Langevin thermostat with the collision frequency of 1.0 ps^{-1} . The weak restraint of $1.0 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ was applied. Another 50 ps period was run without restraints to pre-equilibrate the system. Equilibrium runs were made for a 20 ns duration with a 2 fs time step. The temperature was controlled by the Langevin thermostat, and the pressure was maintained by isotropic position scaling with a relaxation time of 2 ps. All hydrogen atoms were constrained using the SHAKE algorithm. The space cut off was 9.0 \AA , and the trajectory file was analyzed by the AMBER ptraj module. The hydrogen bond (H bond) cut off was set as 3.0 \AA for donor–acceptor distance and 120° for a donor–hydrogen–acceptor angle.

RESULTS AND DISCUSSION

pH Effect on the Ground and Excited-State Prototropic Behavior of CatchER. The pH titration of apo-CatchER (calcium-free) monitored by absorption spectroscopy is shown in Figure 2. At pH 5.0, a single peak with the maximum at 395 nm was observed, demonstrating the presence of only neutral Cro at these conditions. As the pH increased, the anionic Cro peak at 488 nm increased with the simultaneous decrease of the neutral absorption peak. At neutral pH, CatchER showed the presence of both the neutral and anionic forms of Cro in the ground (detected by absorption spectroscopy) and in the excited (detected by emission spectroscopy) states. At pH 9.3, the neutral-to-anionic peak ratio was 0.74. At a pH higher than 9.3, the peak of the anionic form began to decrease, and a shoulder around 465 nm was observed (not shown). At the same time, the peak maximum of the neutral Cro was red-shifted, and a shoulder was observed around 425 nm, indicating the degradation of protein at $\text{pH} > 9.3$. The pK_a of CatchER Cro of 7.6 was determined using eq 1. Thus, the anionic form of the Cro was expected to be predominant at pH 10. However, the intensity of the neutral peak absorption was 60% of that at pH 5.0, implying that the portion of neutral Cro was solvent-inaccessible. Similar behavior is known for the wild-type GFP (wt-GFP) that shows very weak pH dependence of its absorption spectra in the pH range of 6–10.^{32,33} Alternatively,

we propose that neutral Cro exists in two environments differing in the arrangement of the proton network around them. One such population can be easily titrated (we name it Cro1), while the other (Cro2) protects the Cro from the pH changes in the outside medium. From known extinction coefficients of the ROH and RO^- in the GFP-type proteins and their synthetic Cros,³⁴ we estimate the relative populations of Cro1 and Cro2 as very close. Such splitting of the ROH population for the number of GFP variants has already been reported.^{35,36}

Figure 3 shows the emission spectra of CatchER at various pH levels. At pH 5.0, the excitation of CatchER led to a two-

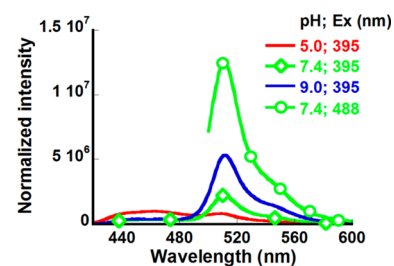


Figure 3. Emission spectra of CatchER in the cocktail buffer at different pH 5.0 (red), 7.4 (green \diamond) and 9.0 (blue) when excited at 395 nm and the emission spectrum of CatchER at pH 7.4 when excited at 488 nm (green \circ). The emission spectra were normalized by the absorbance at the corresponding excitation wavelength.

peak emission spectrum, the high-energy band at 465 nm belonging to the neutral Cro (R^*OH), and the band at 510 nm from the anionic Cro (R^*O^-), formed via ESPT. With pH increase, the relative contribution of the 465 nm emission band decreased together with the relative population of the neutral Cro in the ground state (Figure 2). The emission maxima of the directly and indirectly excited anions (excited at 488 and 395 nm, respectively) are the same within the experimental error.

Fluorescence decay curves of R^*OH and R^*O^- forms of CatchER at various pH are presented in Figure 4. The decay of R^*OH at pH 9.6 belongs only to Cro2, which still exists in the neutral form at these conditions (Figure 2). The nonexponential decay of R^*OH could be caused by the ESPT geminate recombination and nonhomogenous distribution of Cro conformers in the protein.³⁷ Further analysis of the kinetic data is presented below.

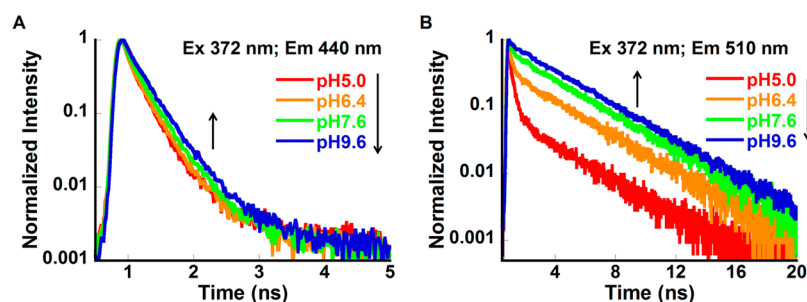


Figure 4. CatchER fluorescence decay at pH 5.0, 7.6, and 9.6. The excitation wavelength was 372 nm, and the emission wavelengths were (A) 440 nm and (B) 510 nm.

Ground and Excited-State Prototropic Behavior of CatchER. Fluorescence decay curves of R^*OH and R^*O^- at pH 7.4 are shown in Figure 5. The decay of R^*OH and the

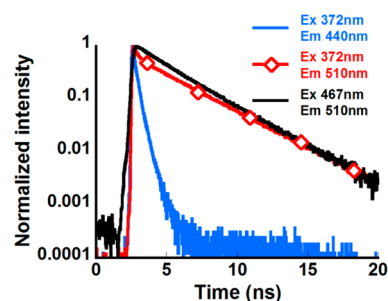


Figure 5. Fluorescence decay curves of CatchER at pH 7.4 excited and monitored at various wavelengths.

directly excited (at 467 nm) R^*O^- form could be fitted by the double-exponential expressions with the average lifetimes of 0.19 and 2.61 ns, respectively (Table 1). For the indirectly excited (at 372 nm) R^*O^- form, a fast quenching component within the first 2 ns was detected, which was followed by the long time asymptotic decay closely approaching the lifetime of the directly excited R^*O^- (Table 1). To make sure that our observation is not an experimental artifact, we have measured the fluorescence kinetics of the wt-GFP, and its several variants, and reproduced the published data. None of the known fluorescence proteins exhibited such fluorescence kinetic

behavior of the indirectly excited R^*O^- , showing the characteristic biphasic decay.

Upon calcium addition, the fluorescence decay of the directly excited R^*OH and R^*O^- did not differ significantly (Figure 6). These results are in agreement with the previous observation that the quantum yield of CatchER was not affected by calcium when excited at 488 nm.¹⁴

Meanwhile, the amplitude of the initial fast decay component observed in the indirectly excited R^*O^- was dramatically reduced, and the lifetime for the long-time asymptotic decay increased in the presence of calcium (Figure 6). The average lifetime of the indirectly excited anionic form was increased by 44% from 1.51 to 2.18 ns when calcium was bound to CatchER.

At pH 7.4, both the neutral and anionic Cro were present in the absorption spectra, with the peak height ratio A_{RO^-}/A_{ROH} of 0.43 (as shown in Figure 7). The anionic emission maxima was observed at 510 nm when excited at either 395 or 488 nm, and the intensity of the indirectly excited R^*O^- was 45% of the directly excited species. With the addition of calcium, the population of the neutral and the anionic Cro was changed, leading to the ratio A_{RO^-}/A_{ROH} increase to 0.58. The steady-state fluorescence spectra showed that the R^*O^- intensity was increased by 50% and 30% upon calcium binding when excited at 395 and 488 nm, respectively.

At the same time, in the normalized emission spectra (Figure 8), the peak height ratio $I_{R^*OH}/I_{R^*O^-}$ was higher for apo- than for the holo-CatchER. It is known that $I_{R^*OH}/I_{R^*O^-} \sim 1/(k_d\tau')$, where k_d is the proton dissociation rate during ESPT and τ' is average lifetime of the indirectly excited R^*O^- .³⁸ The higher

Table 1. Values of the Time Constants (τ_i) and Normalized (to 1) Pre-Exponential Factors (A_i) of the Multi-Exponential Function Fitting the ps-Emission Transients of CatchER in Various Solvents^a

in H ₂ O		τ_1 (ns)	A_1	τ_2 (ns)	A_2	τ_3 (ns)	A_3	τ_{ave} (ns)
Ex372; Em440	Apo	0.15 ± 0.01	0.81 ± 0.01	0.37 ± 0.01	0.19 ± 0.01	NA	NA	0.19 ± 0.01
	Holo	0.17 ± 0.01	0.81 ± 0.01	0.41 ± 0.01	0.19 ± 0.01	NA	NA	0.22 ± 0.01
Ex372; Em510	Apo	0.16 ± 0.03	0.47 ± 0.03	0.73 ± 0.24	0.09 ± 0.04	3.14 ± 0.03	0.44 ± 0.02	1.51 ± 0.10
	Holo	0.21 ± 0.01	0.37 ± 0.02	3.34 ± 0.03	0.63 ± 0.02	NA	NA	2.18 ± 0.06
Ex467; Em510	Apo	2.01 ± 0.14	0.75 ± 0.11	4.47 ± 0.87	0.25 ± 0.11	NA	NA	2.61 ± 0.06
	Holo	2.09 ± 0.03	0.77 ± 0.01	4.73 ± 0.10	0.23 ± 0.01	NA	NA	2.54 ± 0.19
in D ₂ O		τ_1 (ns)	A_1	τ_2 (ns)	A_2	τ_3 (ns)	A_3	τ_{ave} (ns)
Ex372; Em440	Apo	0.12 ± 0.01	0.79 ± 0.08	0.31 ± 0.06	0.21 ± 0.06	NA	NA	0.16 ± 0.01
	Holo	0.14 ± 0.01	0.87 ± 0.01	0.40 ± 0.01	0.13 ± 0.01	NA	NA	0.17 ± 0.01
Ex372; Em510	Apo	0.10 ± 0.02	0.67 ± 0.07	0.39 ± 0.10	0.17 ± 0.07	3.06 ± 0.04	0.15 ± 0.01	0.61 ± 0.03
	Holo	0.13 ± 0.01	0.60 ± 0.04	0.41 ± 0.10	0.15 ± 0.05	3.42 ± 0.01	0.25 ± 0.01	0.99 ± 0.05
Ex467; Em510	Apo	2.18 ± 0.24	0.81 ± 0.01	5.24 ± 0.10	0.19 ± 0.02	NA	NA	2.55 ± 0.16
	Holo	1.81 ± 0.03	0.56 ± 0.16	3.62 ± 0.46	0.44 ± 0.16	NA	NA	2.60 ± 0.07

^aThe amplitude-weighted average lifetimes of τ_{ave} were calculated as $[\tau_{ave} = \sum_{i=1}^n (A_i\tau_i / \sum_{i=1}^n A_i)]$.

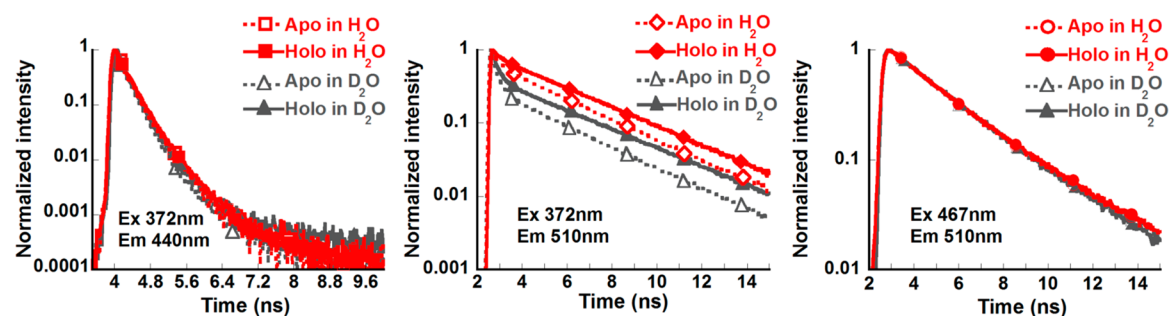


Figure 6. Fluorescence decay traces of Ca^{2+} -free CatchER (Apo, open symbol, dash line) and CatchER supplemented with 10 mM Ca^{2+} (Holo, closed symbol, solid line) in H_2O (red) and D_2O (gray). Protein samples were prepared in 10 mM Tris, pH 7.4.

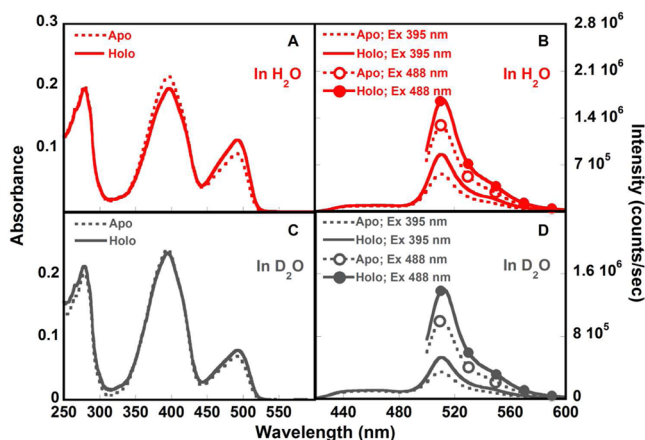


Figure 7. Absorption and fluorescence spectra of CatchER in H_2O (red, A and B) and D_2O (gray, C and D), in the absence (Apo, dash line) and presence of 10 mM Ca^{2+} (Holo, solid line). Protein samples were prepared in 10 mM Tris, pH 7.4.

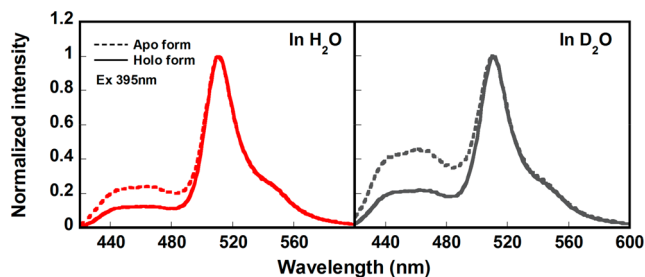


Figure 8. Normalized fluorescence emission spectra ($\lambda_{\text{ex}} 395 \text{ nm}$) of Ca^{2+} -free CatchER (Apo, dash line) and CatchER in the presence of 10 mM Ca^{2+} (Holo, solid line) in H_2O (red) and D_2O (gray).

ratio $I_{\text{R}^*\text{OH}}/I_{\text{R}^*\text{O}^-}$ in apo-CatchER than holo-CatchER indicated the larger population of R^*O^- remained in the excited state with the additional calcium. Therefore, the calcium binding shifts the acid–base equilibrium of the CatchER Cro to the ground state toward the anion. On the other hand, the calcium binding reduces the fast deactivation component in the decay of R^*O^- .

Kinetic Isotope Effect. The fluorescence decay of apo- and holo-CatchER was measured in H_2O and D_2O -based buffers to obtain the H/D kinetic isotope effect (KIE). The latter may reveal the rate-limiting steps in the photoinduced proton-transfer cascade. No kinetic H/D isotope effect was found in the fluorescence decay of the directly excited neutral and anionic forms at room temperature, meaning that rates of proton dissociation as well as the decay of the R^*OH and

R^*O^- are nonsensitive to H/D exchange. However, a substantial H/D isotopic effect was observed for the indirectly excited R^*O^- (Figure 6), for which the average lifetime was 1.5 ns in H_2O and 610 ps in D_2O . Interestingly, the amplitude of the short-time quenching component was larger in D_2O than in H_2O (Table 1). Altogether, the H/D KIE and the Ca^{2+} effects on the nonadiabatic geminate recombination were similar.

Analysis of CatchER Hydrogen-Bond Network by Molecular Dynamics Simulation. To provide the structural basis for the observed spectral and prototropic behavior of CatchER, we have performed the MD simulation in the static state using our recently determined X-ray structures of calcium free (PDB ID: 4L13) and calcium loaded (PDB ID: 4L11) CatchER.³¹ Table 2 shows the probability of hydrogen bonds

Table 2. Hydrogen Bond Analysis in the Cro Environment by MD Simulation

donor ^a	acceptor ^a	occupancy	
		apo	holo
CRO-OG1	E222-OE2	88.5	91.5
WAT1	CRO-OH	15.5	N/A
CRO-OH	WAT1	N/A	92.0
CRO-OH	H148-ND1	41.5	N/A
WAT1	S205-OG	N/A	6.5
S205-OG	E222-OE2	85	98.5
H148-N	T203-O	44.5	54.5
WAT2	E222-OE1	16.5	NA
WAT2	T203-OG1	10.5	31
WAT3	E222-OE1	43	40
WAT2	WAT3	24.5	35.5
Q69-NE2	WAT2	40.5	68.5

^aThe residue and atoms names of H-bond donors and acceptors were given based on the pdb files.

(H bonds) formed in the Cro environment, including Cro, H148, T203, S205, E222, and bridging water molecules labeled as in Figure 9. In holo-CatchER, the hydrogen-bond network extends from the Cro tyrosyl (Cro–OH), WAT1, S205-OG, and finally to E222-OE2. The Cro tyrosyl served as an H-bond donor to the bridging water molecule WAT1 connecting S205 with an occupancy of 92%. The probability to form the H bond between the S205 side chain (S205-OG) and WAT1 was 6.5%, while the formation between S205 and E222 was 98.5%. In contrast, WAT1 was the H bond donor for the Cro tyrosyl with the occurrence of 15.5% in apo-CatchER, and the H-bond between the S205 hydroxyl and the bridging water (WAT1) was missing. Instead, the Cro tyrosyl served as the H-bond donor for the H148-ND1 with occupancy of 41.5%.

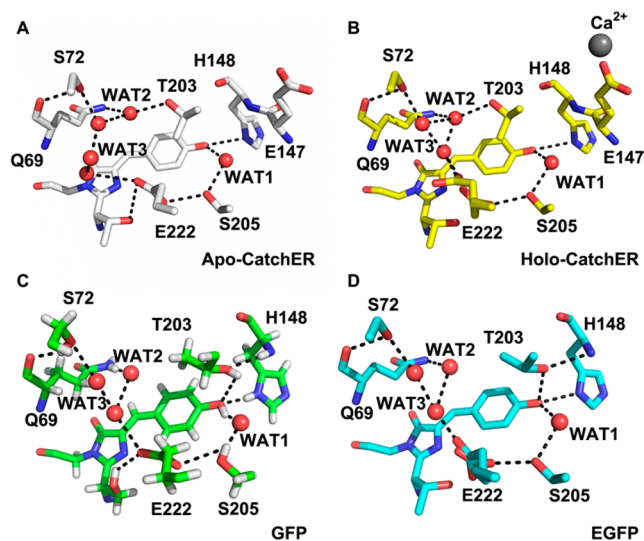


Figure 9. Comparison of the crystal structures of (A) apo-CatchER (PDB ID: 4L13), (B) Ca^{2+} -CatchER (PDB ID: 4L11), (C) GFP (PDB ID: 2WUR), and (D) EGFP (PDB ID: 4EUL). The H bonds are shown in the black dash line with the cutoff of 3.5 Å. Only side chains were shown in S72, T203, S205, and E222.

On the other side from the side chain of Q69, a proton can migrate to E222. Here, water molecules WAT2 and WAT3 connect the side chains of E222, Q69, and T203. In both apo and Ca^{2+} -loaded CatchER, the water molecule WAT2 serves as the proton donor for both T203 side chain and WAT3. Although the probability of WAT2-WAT3-E222 was higher in Ca^{2+} -CatchER, it was found that WAT2 can directly form the H bond to the E222 side chain with occupancy of 16.5%. Ca^{2+} effect on the proton wire to E222 is not significant based on the 20 ns MD simulation.

Structural Effects in CatchER on the Ground State Spectra. The unique optical properties of GFP has attracted extensive research interest for decades.³⁹ In 1996, Chattoraj and co-workers first proposed there were different forms (the protonated neutral Cro as A form, an intermediate I form, and the deprotonated anionic Cro as B form) in GFP, which can convert to each other by proton migration.⁴⁰ In the same year, the crystal structures of wt-GFP and its S65T mutant, known as EGFP, were published.^{41,42} The spectral property was altered due to the mutation S65T in EGFP, where the anionic deprotonated Cro with the absorption maximum wavelength at 488 nm dominates, while wt-GFP has a larger population of neutral protonated Cro at pH 7.0. With five mutations on the β -barrel surface of EGFP, the spectral property of CatchER is closer to wt-GFP than EGFP, where the protonated neutral Cro is dominated at pH 7.0.

The crystal structure of CatchER, recently determined by us,³¹ demonstrates that the side chain of T203 is in the opposite position of what it shows in both GFP and EGFP (as shown in Figure 9). Fewer H-bond donors around the CatchER Cro, compared to wt-GFP⁴³ and EGFP,⁴⁴ lead to the tyrosyl group being prone to be protonated, which is also observed in cpEGFP 149–144.^{11,45} The pH profile of apo- and holo-CatchER absorption spectra suggests that the pK_a of holo-CatchER is a little lower than the apo form, implying that the Cro is more deprotonated upon calcium binding.¹⁴ Since calcium binding does not alter the conformation of T203, perhaps such acid–base equilibrium shifts originated from

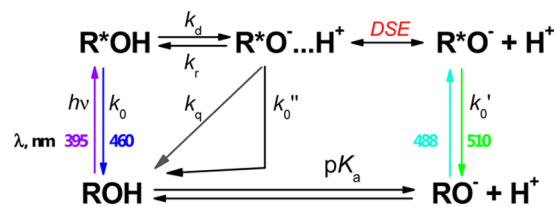
other residues in the Cro H-bond network. It is observed that the distance between H148-ND1 and the tyrosyl of Cro is 3.3 and 3.2 Å, a reasonable range for a H-bond formation in both apo and holo forms of CatchER, respectively. Upon calcium binding, the electrostatic neutralization of one ligand, E147, results in the redistribution of electron density through bonds by inductive effect, which in turn may increase the pK_a of the H148 side chain. Thus, the protonated side chain of H148 can help to stabilize the deprotonated Cro tyrosyl. This assumption is in agreement with the MD simulation results that the deprotonated H148-ND1 is the H-bond acceptor in the pair (Cro-tyrosyl)-(H148-ND1) in apo-CatchER but not in the holo form.

Calcium Effect on CatchER Fluorescence Lifetimes and ESPT. Previously, we have discovered that the optical properties of CatchER resemble those of several GFP mutants. Excitation of both the neutral and the anion forms resulted in an emission band with a maximum at 510 nm (Figure 3). Therefore, the simplest kinetic scheme describing the prototropic behavior of CatchER may be first depicted by Scheme 1 that is different from the wt-GFP with intermediate anionic states.⁴⁰

The ground-state population of CatchER is represented by Cro1 and Cro2, differing in their pH-susceptibility and not existing in the dynamic equilibrium (otherwise both populations would be deprotonated at pH 9.3). These Cros are spectrally indistinguishable in both absorption and emission spectra, therefore, the observed ROH, RO^- , R^*OH , and R^*O^- spectral bands are the superposition of Cro1 and Cro2 spectra. The excited-state R^*OH decay lifetimes of pure Cro2 at pH 9.6 and the 1/1 Cro1–Cro2 mixture at pH 5.0 (Figure 4A) do not differ by more than a factor of 2, demonstrating similar ESPT dissociation reactivity for both forms. Although the Cro1 and Cro2 cannot be differentiated in both apo-CatchER crystal structure,³¹ the side chain of E222 in Ca^{2+} -CatchER shows a double conformer in a 1:1 occupancy, probably responsible for Cro1 and Cro2 populations.

The kinetic Scheme 1, however, does not explain the deviation from mono- and biexponential fluorescence decays of R^*OH and R^*O^- often observed experimentally for strong photoacids.^{46,47} To explain this phenomenon, Agmon and co-workers^{48–51} expanded Scheme 1 into two reaction steps (Scheme 2). The photoinduced protolytic dissociation of

Scheme 2. Two-Step Diffusion-Influenced ESPT Kinetic Scheme



R^*OH , with an intrinsic rate constant k_d , leads to formation of the contact ion pair (CIP) $\text{R}^*\text{O}^- \cdots \text{H}^+$, whereas adiabatic recombination, with rate constant k_r , may reform the excited acid. Additionally, back protonation may proceed also by a nonadiabatic pathway, involving proton quenching (non-adiabatic geminate recombination) with a rate constant k_q . Separation of a CIP from the contact radius to infinity is described by the transient numerical solution of the Debye–

Smoluchowski equation (DSE). As in Scheme 1, all excited species decay to the ground state, but the decay rate k_0'' for the CIP is usually much slower than all chemical and diffusion processes and can be ignored. Since no analytical solution exists for the system of coupled DSEs describing the time evolution of R^*OH and R^*O^- , the solution can be obtained numerically by the publically available SSDP package.⁵² One of the characteristic features of the R^*O^- decay in the presence of proton quenching is the unique biphasic decay of this transient observed in a number of “super” photoacids. It is important to stress, that for complicated proteinous systems such as CatchER, several input parameters required by the SSDP include a number of variables almost impossible to estimate realistically (diffusion coefficient of proton, the radius of reaction sphere, outer boundary conditions, the profile of the electrostatic potential inside the protein, etc.). Therefore, we will not attempt to fit the full profile of the R^*OH and R^*O^- fluorescence decay curves in CatchER. Instead, we will analyze the overall characteristic decay profiles of these species, especially the indirectly excited R^*O^- . As mentioned earlier, the characteristic shape of its decay is a fingerprint of the nonadiabatic proton geminate recombination. Therefore, our work is the first observation of this unique kinetic behavior in fluorescent proteins.

The ESPT dissociation rate, k_d , as well as both k_0 and k_0' were calcium-independent. The average fluorescence lifetime of the directly excited anionic Cro was consistent with the reported lifetime of EGFP (2.54 ns)⁵³ in an acceptable error range. However, the nonadiabatic geminate recombination rate, k_q , was inhibited by Ca^{2+} , as can be judged by the magnitude of the fast-decaying component in R^*O^- , since for $k_q = 0$, the R^*O^- would decay monoexponentially. As a result, a larger population of R^*O^- remained in the excited state, resulting in a higher ratio $I_{R^*O^-} / I_{R^*OH}$, which was in agreement with the observation shown in Figure 8. Calcium binding also leads to the increase of the long-time asymptotic decay, τ_0' , when the anionic form R^*O^- was indirectly excited.

In 1997 and 2000, Palm and Remington reported the crystal structures of several mutants and summarized the spectral relevance to the three forms (A, B, and I).^{54,55} The ESPT pathway was first revealed as the tyrosyl of the Cro, the bridging water molecule (WAT1), S205 and E222. Agmon and co-workers extended the view of the proton conduction in the protein matrix along 1D proton wires and described the proton pathways, including the proton exiting the β -barrel by rotation of T203 and the slow re-entry (μs) from the opposite side of GFP.^{56–58} In this theory, T203 hydroxyl in the anionic B-state of GFP accepts the proton of Cro–OH and donates the H-bond to the main chain of H148, so-called the “out state” of T203, which is observed in the Ca^{2+} -GCaMP2 (PDB ID: 3EVR) and EGFP (PDB ID: 4EUL),^{44,45} as well as in the wt-GFP crystal structure of higher resolution (PDB ID: 2WUR). In the acid A-state of GFP, T203 adopts the “in” conformer, in which the side chain of T203 rotates 120° from the “out” state and T203-OH is not able to form the H bond to Cro–OH. The wt-GFP (PDB ID: 1GFL) and circularly permuted EGFP (PDB ID: 3EVP) show the conformation of T203 as the “in” state.

As shown in Figure 9, we observed only the “in” state of the T203 side chain in both apo- and Ca^{2+} -CatchER crystal structures. On the basis of the MD simulation, the water molecule WAT2 serves as the H-bond donor for both T203 hydroxyl and indirectly for E222 via the other water molecules.

The H bond between Q69-WAT2-T203 can stabilize the side-chain position and make the rotation less possible. The absence of the “threonine switch”, proposed by Agmon,⁵⁶ would prevent the proton from escaping the beta-barrel and increase its probability to recombine with the parent R^*O^- during ESPT.

In the 20 ns MD simulation, it was observed that the proton wire, consisting of the Cro tyrosyl, the bridging water (WAT1), S205, and E222, was disrupted by the introduction of the negatively charged Ca^{2+} binding site, which was known to be pivotal for ESPT. Ca^{2+} binding rescued this proton transfer pathway. Such a disturbed proton wire (Cro-WAT1-S205-E222) in apo-CatchER can explain the larger contribution of the nonadiabatic geminate recombination phenomenon due to the lack of an efficient proton migration path.

The broken proton migration pathway also explains the inverse H/D kinetic isotope effect. It is known that the diffusion constant of the deuterium ion is 1.4-fold smaller than that of the proton.^{59,60} The deuteron could be restricted in the Cro environment, facilitating the reprotonation of the latter. We hypothesize that in H_2O , the probability of the proton to escape from the Cro vicinity is higher, causing less geminate recombination. Therefore, in the static fluorescence spectra, the higher ratio $I_{R^*OH} / I_{R^*O^-}$ of CatchER in D_2O (Figure 8) does not mean slower proton dissociation, which leads to an H/D KIE of about 5 in wt-GFP for the lifetime of the indirectly excited R^*O^- .⁴⁰ On the contrary, the slow diffusion of deuterium makes it easier to reprotonate the deprotonated excited Cro (both adiabatically and nonadiabatically), especially in the situation where the deuterium has little chance to travel to the H-bond acceptor, leading to the shorter average lifetime of indirectly excited R^*O^- . As a result, larger geminate proton quenching of R^*O^- was observed in D_2O , which resulted in the decreased average fluorescent lifetime.

CONCLUSIONS

In summary, binding of Ca^{2+} to CatchER results in the increase of the dominating green emission from this protein. Such fluorescence increase is caused by the combination of thermodynamic (change of the ground-state acid–base equilibrium) and kinetic factors. The latter is mostly based on the unusual dependence of the nonadiabatic proton geminate recombination on Ca^{2+} binding and H/D isotope exchange. The longer average lifetime of indirectly excited anionic Cro (caused by retarded proton geminate recombination) measured in the presence of calcium versus the apo-CatchER is proposed to arise from the different arrangement of the proton wire with and without calcium binding.

Our results demonstrate that CatchER is the first example of proton nonadiabatic geminate recombination in fluorescent proteins. Such unusual utilization of this photoinduced process in metalloproteomics opens new horizons in this rapidly expanding area. Our finding here reveals a new strategy to tune the fluorescence properties of the metalloproteins by metal binding that alters the intraprotein photoinduced proton transfer. CatchER is also the first GECI of the single wavelength calcium detection that has the feature of calcium-dependent lifetime change. The results about the calcium-induced lifetime changes of R-CaMP are yet to be reported.⁶¹ For the FRET pair based calcium indicator TN-XXL, the donor lifetime decrease was observed upon calcium binding. However, the small dynamic range of the donor lifetime change (10–20%) and the complexity of the multiexponential decay were

not optimal for the FLIM.^{62,63} Hence, such an increase (>40%) of the average lifetime (caused by the change of the amplitude ratio for two decaying components) of indirect excitation of R*O⁻ enables CatchER to quantitatively monitor calcium dynamics by lifetime imaging in the future.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: solntsev@gatech.edu. Address: School of Chemistry and Biochemistry, Georgia Institute of Technology, 901 Atlantic Dr., Atlanta, GA, 30332-0400, United States. Tel: 404-385-3117. Fax: 404-894-7452.

*E-mail: jenny@gsu.edu. Address: Department of Chemistry, Georgia State University, University Plaza, Atlanta, GA 30303, United States. Tel: 404-413-5520. Fax: 404-413-5551.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Noam Agmon, Irene Weber, and Ying Zhang for helpful discussions. We also thank Jingjuan Qiao, Cassie Miller, and Kathy Meenach for their critical review and assistance. This work is supported, in part, by NIH Grants GM081749 and EB007268 and a Brain & Behavior (BB) seed grant to J.J.Y.; a BB fellowship to Y.Z., and K.M.S. acknowledges generous support from the National Science Foundation (CHE-1213047).

REFERENCES

- (1) Clapham, D. E. Calcium signaling. *Cell* **2007**, *131*, 1047–1058.
- (2) Rizzuto, R.; Simpson, A. W.; Brini, M.; Pozzan, T. Rapid changes of mitochondrial Ca²⁺ revealed by specifically targeted recombinant aequorin. *Nature* **1992**, *358*, 325–327.
- (3) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **1985**, *260*, 3440–3450.
- (4) Miyawaki, A.; Llopis, J.; Heim, R.; McCaffery, J. M.; Adams, J. A.; Ikura, M.; Tsien, R. Y. Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature* **1997**, *388*, 882–887.
- (5) Griesbeck, O.; Baird, G. S.; Campbell, R. E.; Zacharias, D. A.; Tsien, R. Y. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J. Biol. Chem.* **2001**, *276*, 29188–29194.
- (6) Chen, T. W.; Wardill, T. J.; Sun, Y.; Pulver, S. R.; Renninger, S. L.; Baohan, A.; Schreiter, E. R.; Kerr, R. A.; Orger, M. B.; Jayaraman, V.; et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* **2013**, *499*, 295–300.
- (7) Zhao, Y.; Araki, S.; Wu, J.; Teramoto, T.; Chang, Y. F.; Nakano, M.; Abdelfattah, A. S.; Fujiwara, M.; Ishihara, T.; Nagai, T.; et al. An expanded palette of genetically encoded Ca(2) indicators. *Science* **2011**, *333*, 1888–1891.
- (8) Paredes, R. M.; Etzler, J. C.; Watts, L. T.; Zheng, W.; Lechleiter, J. D. Chemical calcium indicators. *Methods* **2008**, *46*, 143–151.
- (9) Heim, N.; Griesbeck, O. Genetically encoded indicators of cellular calcium dynamics based on troponin C and green fluorescent protein. *J. Biol. Chem.* **2004**, *279*, 14280–14286.
- (10) Whitaker, M. Genetically encoded probes for measurement of intracellular calcium. *Methods Cell Biol.* **2010**, *99*, 153–182.
- (11) Nakai, J.; Ohkura, M.; Imoto, K. A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nat. Biotechnol.* **2001**, *19*, 137–141.
- (12) Nagai, T.; Yamada, S.; Tominaga, T.; Ichikawa, M.; Miyawaki, A. Expanded dynamic range of fluorescent indicators for Ca(2+) by circularly permuted yellow fluorescent proteins. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10554–10559.
- (13) Griesbeck, O.; Baird, G. S.; Campbell, R. E.; Zacharias, D. A.; Tsien, R. Y. Reducing the environmental sensitivity of yellow fluorescent protein: Mechanism and applications. *J. Biol. Chem.* **2001**, *276*, 29188–29194.
- (14) Tang, S.; Wong, H. C.; Wang, Z. M.; Huang, Y.; Zou, J.; Zhuo, Y.; Pennati, A.; Gadda, G.; Delbono, O.; Yang, J. J. Design and application of a class of sensors to monitor Ca²⁺ dynamics in high Ca²⁺ concentration cellular compartments. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 16265–16270.
- (15) Lakowicz, J. R.; Geddes, C. D. *Topics in Fluorescence Spectroscopy: Lifetime-Based Sensing*; Plenum Press: New York, 1991.
- (16) Lakowicz, J. R.; Szmajdzinski, H.; Nowaczyk, K.; Berndt, K. W.; Johnson, M. Fluorescence lifetime imaging. *Anal. Biochem.* **1992**, *202*, 316–330.
- (17) Kumar, A. T. N.; Raymond, S. B.; Bacskai, B. J.; Boas, D. A. Comparison of frequency-domain and time-domain fluorescence lifetime tomography. *Opt. Lett.* **2008**, *33*, 470–472.
- (18) Gannot, I.; Ron, I.; Hekmat, F.; Chernomordik, V.; Gandjbakhche, A. Functional optical detection based on pH dependent fluorescence lifetime. *Lasers Surg. Med.* **2004**, *35*, 342–348.
- (19) Harvey, C. D.; Yasuda, R.; Zhong, H.; Svoboda, K. The spread of Ras activity triggered by activation of a single dendritic spine. *Science* **2008**, *321*, 136–140.
- (20) Yasuda, R.; Harvey, C. D.; Zhong, H.; Sobczyk, A.; van Aelst, L.; Svoboda, K. Supersensitive Ras activation in dendrites and spines revealed by two-photon fluorescence lifetime imaging. *Nat. Neurosci.* **2006**, *9*, 283–291.
- (21) Huang, P. C.; Chiu, T. Y.; Wang, L. C.; Teng, H. C.; Kao, F. J.; Yang, D. M. Visualization of the Orail homodimer and the functional coupling of Orail-STIM1 by live-cell fluorescence lifetime imaging. *Microsc. Microanal.* **2010**, *16*, 313–326.
- (22) Bloch, S.; Lesage, F.; McIntosh, L.; Gandjbakhche, A.; Liang, K.; Achilefu, S. Whole-body fluorescence lifetime imaging of a tumor-targeted near-infrared molecular probe in mice. *J. Biomed. Opt.* **2005**, *10*, 054003.
- (23) Godavarty, A.; Sevick-Muraca, E. M.; Eppstein, M. J. Three-dimensional fluorescence lifetime tomography. *Med. Phys.* **2005**, *32*, 992–1000.
- (24) Ardeshirpour, Y.; Chernomordik, V.; Zielinski, R.; Capala, J.; Griffiths, G.; Vasalatiy, O.; Smirnov, A. V.; Knutson, J. R.; Lyakhov, I.; Achilefu, S.; et al. In vivo fluorescence lifetime imaging monitors binding of specific probes to cancer biomarkers. *PLoS One* **2012**, *7*, e31881.
- (25) Lakowicz, J. R.; Szmajdzinski, H.; Nowaczyk, K.; Johnson, M. L. Fluorescence lifetime imaging of calcium using Quin-2. *Cell Calcium* **1992**, *13*, 131–147.
- (26) Lakowicz, J. R.; Szmajdzinski, H.; Nowaczyk, K.; Lederer, W. J.; Kirby, M. S.; Johnson, M. L. Fluorescence lifetime imaging of intracellular calcium in COS cells using Quin-2. *Cell Calcium* **1994**, *15*, 7–27.
- (27) Agronskaia, A. V.; Tertoolen, L.; Gerritsen, H. C. Fast fluorescence lifetime imaging of calcium in living cells. *J. Biomed. Opt.* **2004**, *9*, 1230–1237.
- (28) Meech, S. R. Primary Photophysical Processes in Chromoproteins. In *Fluorescent Proteins I*; Jung, G., Ed.; Springer: New York, 2012.
- (29) Chirico, G.; Collini, M.; D'Alfonso, L.; Caccia, M.; Daglio, S. C.; Campanini, B. Green Fluorescent Protein Photodynamics As a Tool for Fluorescence Correlative Studies and Applications. In *Fluorescent Proteins II*, Jung, G., Ed.; Springer: New York, 2012.
- (30) Helms, V.; Gu, W. Proton Travel in Green Fluorescent Protein. In *Fluorescent Proteins I*; Jung, G., Ed.; Springer: New York, 2012.
- (31) Zhang, Y.; Reddish, F.; Tang, S.; Zhuo, Y.; Wang, Y.; Yang, J. J.; Weber, I. T. Structural basis for a hand-like site of the calcium sensor CatchER with fast kinetics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2013**, *69*, 2309–2319.
- (32) Ward, W. W. Properties of the Coelenterate Green Fluorescent Proteins. In *Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical Applications*; DeLuca, M. A.; McElroy, W. D., Eds.; Academic Press: New York, 1981; pp 225–234.

- (33) Campbell, T. N.; Choy, F. Y. M. The Effect of pH on Green Fluorescent Protein: A Brief Review. *Mol. Biol. Today* **2001**, *2*, 1–4.
- (34) Dong, J.; Solntsev, K. M.; Tolbert, L. M. Solvatochromism of the green fluorescence protein chromophore and its derivatives. *J. Am. Chem. Soc.* **2006**, *128*, 12038–12039.
- (35) Leiderman, P.; Genosar, L.; Huppert, D.; Shu, X.; Remington, S. J.; Solntsev, K. M.; Tolbert, L. M. Ultrafast excited-state dynamics in the green fluorescent protein variant S65T/H148D. 3. Short- and long-time dynamics of the excited-state proton transfer. *Biochemistry* **2007**, *46*, 12026–12036.
- (36) Fron, E.; Van der Auweraer, M.; Moeyaert, B.; Michiels, J.; Mizuno, H.; Hofkens, J.; Adam, V. Revealing the excited-state dynamics of the fluorescent protein Dendra2. *J. Phys. Chem. B* **2013**, *117*, 2300–2313.
- (37) Gepshtein, R.; Leiderman, P.; Huppert, D. Origin of the nonexponential dynamics of excited-state proton transfer in wt-green fluorescent protein. *J. Phys. Chem. B* **2008**, *112*, 7203–7210.
- (38) Solntsev, K. M.; Al-Ainain, S. A.; Il'ichev, Y. V.; Kuzmin, M. G. Effects of long-chain alkyl substituents on the protolytic reactions of naphthols. *J. Photochem. Photobiol., A: Chemistry* **2005**, *175*, 178–191.
- (39) Dedecker, P.; De Schryver, F. C.; Hofkens, J. Fluorescent proteins: Shine on, you crazy diamond. *J. Am. Chem. Soc.* **2013**, *135*, 2387–2402.
- (40) Chatteraj, M.; King, B. A.; Bublitz, G. U.; Boxer, S. G. Ultra-fast excited state dynamics in green fluorescent protein: Multiple states and proton transfer. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8362–8367.
- (41) Yang, F.; Moss, L. G.; Phillips, G. N., Jr. The molecular structure of green fluorescent protein. *Nat. Biotechnol.* **1996**, *14*, 1246–1251.
- (42) Ormo, M.; Cubitt, A. B.; Kallio, K.; Gross, L. A.; Tsien, R. Y.; Remington, S. J. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* **1996**, *273*, 1392–1395.
- (43) Shinobu, A.; Palm, G. J.; Schierbeek, A. J.; Agmon, N. Visualizing proton antenna in a high-resolution green fluorescent protein structure. *J. Am. Chem. Soc.* **2010**, *132*, 11093–11102.
- (44) Arpino, J. A.; Rizkallah, P. J.; Jones, D. D. Crystal structure of enhanced green fluorescent protein to 1.35 Å resolution reveals alternative conformations for Glu222. *PLoS One* **2012**, *7*, e47132.
- (45) Wang, Q.; Shui, B.; Kotlikoff, M. I.; Sondermann, H. Structural basis for calcium sensing by GCaMP2. *Structure* **2008**, *16*, 1817–1827.
- (46) Solntsev, K. M.; Agmon, N. Dual asymptotic behavior in geminate diffusion-influenced reaction. *Chem. Phys. Lett.* **2000**, *320*, 262–268.
- (47) Solntsev, K. M.; Sullivan, E. N.; Tolbert, L. M.; Ashkenazi, S.; Leiderman, P.; Huppert, D. Excited-state proton transfer reactions of 10-hydroxycamptothecin. *J. Am. Chem. Soc.* **2004**, *126*, 12701–12708.
- (48) Pines, E.; Huppert, D.; Agmon, N. Geminate recombination in excited-state proton-transfer reactions: Numerical solution of the Debye–Smoluchowski equation with backreaction and comparison with experimental results. *J. Chem. Phys.* **1988**, *88*, 5620–5630.
- (49) Agmon, N.; Pines, E.; Huppert, D. Geminate recombination in proton-transfer reactions. II. Comparison of diffusional and kinetic schemes. *J. Chem. Phys.* **1988**, *88*, 5631–5638.
- (50) Gopich, I. V.; Solntsev, K. M.; Agmon, N. Excited-state reversible geminate reaction. I. Two different lifetimes. *J. Chem. Phys.* **1999**, *110*, 2164–2174.
- (51) Agmon, N. Excited-state reversible geminate reaction. II. Contact geminate quenching. *J. Chem. Phys.* **1999**, *110*, 2175–2180.
- (52) Krissinel, E. B.; Agmon, N. Spherical symmetric diffusion problem. *J. Comput. Chem.* **1996**, *17*, 1085–1098.
- (53) van Thor, J. J. Photoreactions and dynamics of the green fluorescent protein. *Chem. Soc. Rev.* **2009**, *38*, 2935–2950.
- (54) Palm, G. J.; Zdanov, A.; Gaitanaris, G. A.; Stauber, R.; Pavlakis, G. N.; Wlodawer, A. The structural basis for spectral variations in green fluorescent protein. *Nat. Struct. Biol.* **1997**, *4*, 361–365.
- (55) Remington, S. J. Structural basis for understanding spectral variations in green fluorescent protein. *Methods Enzymol.* **2000**, *305*, 196–211.
- (56) Agmon, N. Proton pathways in green fluorescence protein. *Biophys. J.* **2005**, *88*, 2452–2461.
- (57) Leiderman, P.; Huppert, D.; Agmon, N. Transition in the temperature-dependence of GFP fluorescence: From proton wires to proton exit. *Biophys. J.* **2006**, *90*, 1009–1018.
- (58) Agmon, N. Kinetics of switchable proton escape from a proton-wire within green fluorescence protein. *J. Phys. Chem. B* **2007**, *111*, 7870–7878.
- (59) Roberts, N. K.; Northey, H. L. Proton and deuteron mobility in normal and heavy-water solutions of electrolytes. *J. Chem. Soc., Faraday Trans. 1* **1974**, *70*, 253–262.
- (60) Lewis, G. N.; Doody, T. C. The mobility of ions in (HHO)-H-2-O-2. *J. Am. Chem. Soc.* **1933**, *55*, 3504–3506.
- (61) Akerboom, J.; Carreras Calderon, N.; Tian, L.; Wabnig, S.; Prigge, M.; Tolo, J.; Gordus, A.; Orger, M. B.; Severi, K. E.; Macklin, J. J.; et al. Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. *Front. Mol. Neurosci.* **2013**, *6*, 2.
- (62) Geiger, A.; Russo, L.; Gensch, T.; Thestrup, T.; Becker, S.; Hopfner, K. P.; Griesinger, C.; Witte, G.; Griesbeck, O. Correlating calcium binding, Förster resonance energy transfer, and conformational change in the biosensor TN-XXL. *Biophys. J.* **2012**, *102*, 2401–2410.
- (63) Mank, M.; Santos, A. F.; Drenth, S.; Mrsic-Flogel, T. D.; Hofer, S. B.; Stein, V.; Hendel, T.; Reiff, D. F.; Levelt, C.; Borst, A.; et al. A genetically encoded calcium indicator for chronic in vivo two-photon imaging. *Nat. Methods* **2008**, *5*, 805–811.