

## Fluorescent Probes

# Unraveling Ultrafast Photoinduced Proton Transfer Dynamics in a Fluorescent Protein Biosensor for $\text{Ca}^{2+}$ Imaging

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**Abstract:** Imaging  $\text{Ca}^{2+}$  dynamics in living systems holds great potential to advance neuroscience and cellular biology. G-GECO1.1 is an intensimetric fluorescent protein  $\text{Ca}^{2+}$  biosensor with a Thr-Tyr-Gly chromophore. The protonated chromophore emits green upon photoexcitation via excited-state proton transfer (ESPT). Upon  $\text{Ca}^{2+}$  binding, a significant population of the chromophores becomes deprotonated. It remains elusive how the chromophore structurally evolves prior to and during ESPT, and how it is affected by  $\text{Ca}^{2+}$ . We

use femtosecond stimulated Raman spectroscopy to dissect ESPT in both the  $\text{Ca}^{2+}$ -free and bound states. The protein chromophores exhibit a sub-200 fs vibrational frequency shift due to coherent small-scale proton motions. After wavepackets move out of the Franck–Condon region, ESPT gets faster in the  $\text{Ca}^{2+}$ -bound protein, indicative of the formation of a more hydrophilic environment. These results reveal the governing structure–function relationship of  $\text{Ca}^{2+}$ -sensing protein biosensors.

## Introduction

Intracellular  $\text{Ca}^{2+}$  is a universal second messenger that regulates numerous cellular processes such as neuron firing, muscle contraction, and cancer metastasis.<sup>[1]</sup> Genetically encoded  $\text{Ca}^{2+}$  indicators for optical imaging (GECO), part of the GCaMP family of indicators, consist of a central circularly permuted green fluorescent protein (GFP)<sup>[2]</sup> fused with calmodulin (CaM) and an M13 peptide at the C and N terminus, respectively.<sup>[3]</sup> These biosensors are crucial tools for monitoring complex cellular processes, so substantial efforts are invested in their ongoing development.<sup>[4]</sup> However, the protein engineering efforts so far mostly rely on random mutagenesis, which is time-consuming, labor intensive, and not guided by structural insights. To enable rational design and optimization of the fluorescent protein (FP)-based biosensors from the bottom up, a detailed understanding of fluorescence mechanism is warranted.<sup>[5]</sup>

In our recent report, the green–blue dual-emission CaM–GFP sensor GEM–GECO1 was shown to exhibit different initial structural dynamics from the wild-type (wt)GFP despite having the same serine–tyrosine–glycine (SYG) chromophore. This level of information remains lacking for the threonine–tyrosine–glycine (TYG) chromophore that is a common motif in some of the

most widely used FP variants (e.g., EGFP).<sup>[6]</sup> G-GECO1.1 is such a TYG-based  $\text{Ca}^{2+}$  biosensor derived from GCaMP3;<sup>[4b]</sup> it was engineered to increase the  $\text{Ca}^{2+}$ -dependent fluorescence response by twofold ( $K_d = 618 \text{ nM}$ ).<sup>[4a]</sup> Both the  $\text{Ca}^{2+}$ -free/bound G-GECO1.1 biosensors emit green fluorescence following 400 nm excitation, undergoing excited-state proton transfer (ESPT) to convert the neutral chromophore ( $\text{A}^*$ ) to an intermediate anionic form that is the fluorescent state ( $\text{I}^*$ ). Central to the ESPT reaction coordinate is the phenolic proton located at the end of the tyrosine sidechain, part of the autocyclic three-residue chromophore that is embedded in the protein pocket.

However, it is unclear how  $\text{Ca}^{2+}$  binding in the CaM unit affects the dominant neutral chromophore and ESPT in the adjacent GFP matrix, which plays a crucial role in fluorescence mechanisms of many FP biosensors.<sup>[7]</sup> This represents allostery inside a chimera biosensor at thermal equilibrium because the conformational changes have occurred following  $\text{Ca}^{2+}$  binding to reach the green fluorescent state, which has its distinctive nanosecond lifetime. Although ultrafast techniques were used to study SYG chromophore in wtGFP,<sup>[8]</sup> structural evolution of a TYG chromophore during ESPT remains elusive, particularly for  $\text{Ca}^{2+}$  sensing. A suitable ultrafast spectroscopic tool is thus required to capture molecular snapshots of TYG chromophore *in action*, ideally with high spectral and temporal resolutions as well as a broad spectral coverage to infer its multidimensional potential energy surface (PES) prior to fluorescence.

In this work, we implement femtosecond stimulated Raman spectroscopy (FSRS) to investigate TYG chromophore because FSRS is a technique that can probe photochemical reaction coordinates.<sup>[5,9]</sup> With an actinic pump to induce photochemistry and a Raman picosecond (ps) pump–femtosecond (fs) probe pair, FSRS directly collects time-resolved vibrational spectra in

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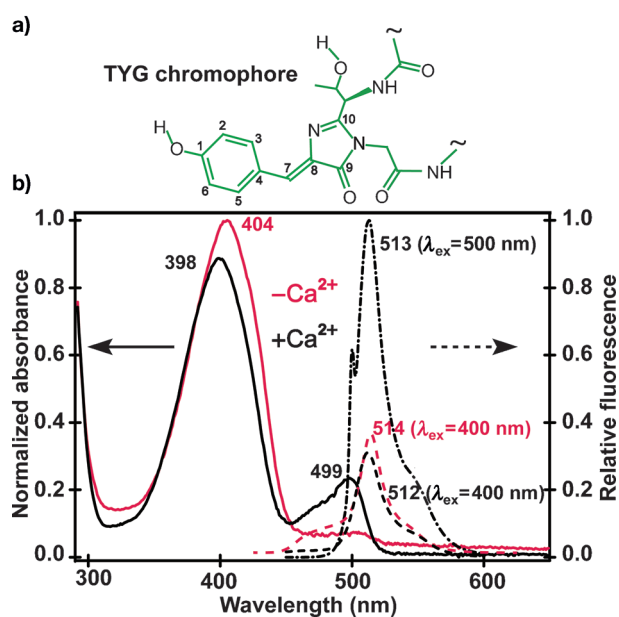
Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/chem.201500491>.

the excited state free from background fluorescence. It can achieve simultaneously high time resolution ( $< 25$  fs) and spectral resolution ( $< 10$   $\text{cm}^{-1}$ ). We exploit FSRS to investigate the  $\text{Ca}^{2+}$ -free and bound G-GECO1.1, aiming to obtain the initial structural evolution of the neutral TYG chromophore following 400 nm excitation and elucidate the effect of  $\text{Ca}^{2+}$  binding on ultrafast ESPT. Transient absorption experiments and molecular dynamics simulations are also performed to corroborate the time constants obtained from FSRS data analysis and to understand the structural basis in the local environment. These dynamics insights will guide the rational design of biosensors with high intensimetric contrast potentially using the 800 nm near-infrared excitation light source (i.e., two-photon absorption).<sup>[4b, 10]</sup>

## Results and Discussion

### Steady-state electronic spectroscopy

In a pH 7.2 aqueous buffer, the ground state ( $S_0$ )  $\text{Ca}^{2+}$ -free G-GECO1.1 biosensor chromophore is mostly protonated and only a very small portion is deprotonated (Figure 1). The UV–



**Figure 1.** The TYG chromophore; a) molecular structure and b) electronic spectroscopy of G-GECO1.1. Normalized absorption (at 276 nm) and relative emission (400 nm excitation) of the  $\text{Ca}^{2+}$ -free/bound biosensors are shown in red/black solid and red/black dashed lines, respectively. The dash-dotted black line displays the fluorescence spectrum of the  $\text{Ca}^{2+}$ -bound biosensor under 500 nm excitation.

visible spectrum of the  $\text{Ca}^{2+}$ -free state has the absorption maximum at 404 nm that corresponds to the neutral form of the chromophore, with a small shoulder at approximately 505 nm attributed to the anionic form.<sup>[6, 11]</sup> Upon  $\text{Ca}^{2+}$  binding, the shoulder peak intensity increases significantly, indicative of more chromophore deprotonation in  $S_0$ , matching ground-state FSRS data (Figure S1 in the Supporting Information). This result is consistent with the measured  $\text{pK}_a$  values of about

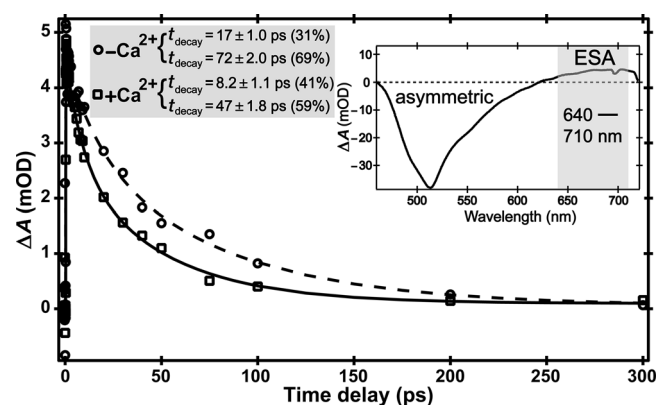
10.2/7.5 for the  $\text{Ca}^{2+}$ -free/bound biosensors, respectively.<sup>[4a]</sup> The green fluorescence of  $\text{Ca}^{2+}$ -bound state with 500 nm excitation is greatly enhanced mainly due to direct emission from photoexcited deprotonated chromophore, which has a larger quantum yield ( $\text{QY} = 0.46$ ) with  $\text{Ca}^{2+}$  than that without  $\text{Ca}^{2+}$  (0.20, likely due to solvent quenching effect).<sup>[3c, 4a, 12]</sup>

### Ground-state FSRS measurements and computations

The ground-state FSRS results of G-GECO1.1 are shown in Figures S1 and S2 in the Supporting Information, as well as Figure 3 (bottom; see below). The  $S_0$  Raman mode assignments (Table S1 in the Supporting Information) are determined by comparing the experimental spectra with DFT calculations at the RB3LYP/6-31G + (d,p) level in Gaussian 09.<sup>[13]</sup> Most of the  $S_0$  Raman frequencies are insensitive to  $\text{Ca}^{2+}$ ; however, a few peak changes are notable. Upon  $\text{Ca}^{2+}$  binding, the 1547  $\text{cm}^{-1}$  mode (mainly C=C and C=N stretching) redshifts to 1543  $\text{cm}^{-1}$  with a significantly increased intensity, the 1040  $\text{cm}^{-1}$  mode (mainly imidazolinone ring deformation and C–H rocking) redshifts to 1037  $\text{cm}^{-1}$  with a marked intensity drop, while the 1335  $\text{cm}^{-1}$  mode (mostly C<sub>1</sub>OH rocking, phenol ring-H rocking, and bridge C<sub>7</sub>–H rocking) blueshifts to 1344  $\text{cm}^{-1}$  and strengthens. These collateral spectral changes reflect the modification of H-bonding network and the chromophore protonation state upon  $\text{Ca}^{2+}$  binding.

### Femtosecond transient absorption spectroscopy

Transient absorption spectroscopy<sup>[14]</sup> on G-GECO1.1 reveals a broad positive peak attributed to the excited-state absorption (ESA) of the  $A^*$  state and a negative peak mainly due to the stimulated emission from  $I^*$  (Figure 2). The femtosecond 400 nm pump pulse induces electronic state changes that are probed by a weaker supercontinuum white light in the visible to near-infrared range (see the Experimental Section). The transient absorption spectrum at 8 ps time delay (Figure 2, insert)



**Figure 2.** Femtosecond transient absorption spectroscopy on the  $\text{Ca}^{2+}$ -free (circles) and bound (squares) G-GECO1.1 protein biosensor. The double-exponential fits are shown in dashed and solid curves, respectively, with the decay time constants and relative weights noted above. A typical transient absorption spectrum at 8 ps time delay is displayed in the insert and the excited-state absorption (ESA) region is highlighted by the light shading.

has an asymmetric line-shape of the broad negative signal and a positive feature above 630 nm (see the Supporting Information). The integrated signal intensity between 640–710 nm is plotted against the time delay between the pump and probe pulses to compare the response of  $\text{Ca}^{2+}$ -free/bound biosensors, which both exhibit bi-exponential decay. The least-squares fitting results convoluted with the 140 fs instrument cross-correlation time reveal that the ESA feature attributed to  $\text{A}^*$  decays faster upon  $\text{Ca}^{2+}$  binding. Because transient absorption directly reports on  $\text{S}_1$  population dynamics, this result is useful in correlating with the time-dependent vibrational spectra from FSRs measurements (see below) to provide a vivid picture and comprehensive understanding of chromophore structural dynamics from  $\text{A}^*$  to  $\text{I}^*$  via ESPT.

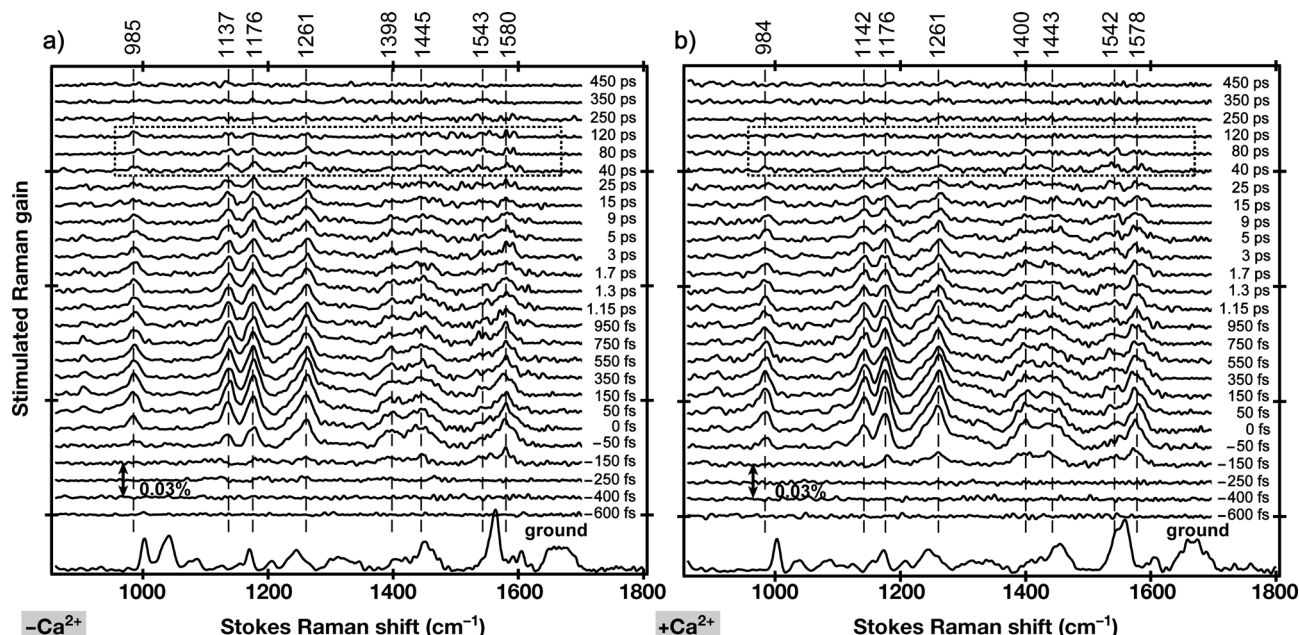
### Excited-state time-resolved FSRs

To elucidate the ESPT reaction coordinate from time zero of photoexcitation, we use a 400 nm actinic pump to specifically excite the neutral chromophore (Figure 1) where the anionic form has negligible absorption. Figure 3 presents time-resolved excited-state ( $\text{S}_1$ ) FSRs data for the  $\text{Ca}^{2+}$ -free and bound G-GECO1.1 up to 450 ps. The  $\text{S}_1$  Raman peaks vanish after approximately 120 ps, showing that ESPT occurs in both cases.<sup>[5,8b]</sup> The weakened product feature (e.g., at  $\sim 1305\text{ cm}^{-1}$  for partially deprotonated chromophore in  $\text{S}_1$ ,  $\text{I}^*$  state) may indicate an altered excited-state PES from the SYG chromophore,<sup>[5,8b]</sup> resulting in reduced polarizability hence decreased Raman intensity of the nascent  $\text{I}^*$  modes. Tunable FSRs with a different Raman pump wavelength can enhance  $\text{I}^*$  features (see below). Peak assignments for  $\text{S}_1$  vibrational modes

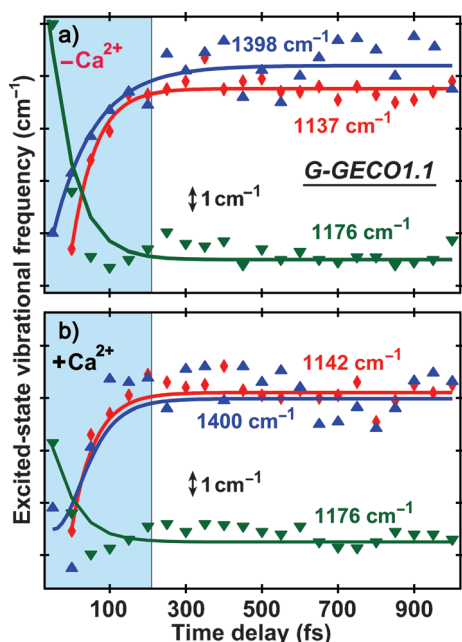
(Table S2 in the Supporting Information) are facilitated by comparison with TD-DFT calculation results at the RB3LYP/6-31G + (d,p) level.<sup>[13]</sup>

Notably, the  $\text{S}_1$  spectra of the TYG chromophore largely resemble the SYG chromophore particularly for modes above  $1000\text{ cm}^{-1}$ . This is because the two chromophores only differ by a methyl group at imidazolinone-ring end while high-frequency modes are largely localized vibrations. Marked exceptions are two peaks at about  $1543$  and  $1580\text{ cm}^{-1}$ , unique to TYG because only a single peak at approximately  $1565\text{ cm}^{-1}$  is observed for SYG, for example, in wtGFP and GEM-GECO1.<sup>[5,8b]</sup> Below  $1000\text{ cm}^{-1}$ , a strong  $985\text{ cm}^{-1}$  peak in TYG (G-GECO1.1) has a  $957\text{ cm}^{-1}$  shoulder, which likely represents a redshift from the corresponding  $967\text{ cm}^{-1}$  peak in SYG (GEM-GECO1).<sup>[5]</sup> The latter two modes both involve strong phenol-ring deformation, and the additional methyl group in TYG decreases the mode frequency by a modest amount of approximately  $10\text{ cm}^{-1}$ . The  $985\text{ cm}^{-1}$  mode mainly involves the bridge  $\text{C}_7\text{--H}$  rocking and imidazolinone-ring motions (Table S2 in the Supporting Information), and the extra methyl group in TYG may contribute to the increased polarizability on the imidazolinone ring that leads to a larger Raman gain.<sup>[15]</sup>

To infer the transient structural evolution of the photoexcited biosensor chromophore, we perform multi-Gaussian least-squares fitting of excited-state Raman peaks and plot the peak positions and integrated intensities versus time. Figure 4 presents the vibrational mode frequency of the  $\text{Ca}^{2+}$ -free/bound biosensors for delay time up to 1 ps. Several modes exhibit frequency shifts within the first approximately 200 fs following photoexcitation. Results based on single-exponential fitting show that the blueshift time constant is about 100/70 fs



**Figure 3.** Time-resolved excited-state FSRs spectra of: a)  $\text{Ca}^{2+}$ -free, and b)  $\text{Ca}^{2+}$ -bound G-GECO1.1 following 400 nm photoexcitation. The aqueous-buffer subtracted  $\text{S}_0$  spectrum is plotted at the bottom to compare with the  $\text{S}_0$ -subtracted  $\text{S}_1$  Raman spectra. All the spectral baselines drawn are shown in the Supporting Information (Figure S2). The dotted rectangles highlight the difference of mode intensities between the  $\text{Ca}^{2+}$ -free and bound biosensors after 40 ps. The double-headed line shows the Raman gain magnitude of 0.03%. The excited-state vibrational modes are indicated by dashed lines and labeled above.



**Figure 4.** Ultrafast vibrational frequency shifts of the 1137/1142 (red), 1176/1176 (green), and 1398/1400  $\text{cm}^{-1}$  (blue) modes in the  $\text{Ca}^{2+}$ -free/bound G-GECO1.1. The double-headed line represents a frequency magnitude of 1  $\text{cm}^{-1}$ . The 1176  $\text{cm}^{-1}$  mode is vertically offset for comparison. The cyan shading highlights the mode shifts within 250 fs following photoexcitation.

( $\sim 1400 \text{ cm}^{-1}$  mode), 70/60 fs ( $\sim 1140 \text{ cm}^{-1}$  mode), while the redshift time constant is 60/60 fs (1176  $\text{cm}^{-1}$  mode) for the  $\text{Ca}^{2+}$ -free/bound biosensors, respectively. These ultrafast vibrational frequency changes suggest that the chromophore undergoes a characteristic sub-200 fs transition from a locally excited (LE) state to an adjacent charge-transfer (CT) state, which is typical for a photoacidic chromophore with a phenolic moiety.<sup>[8d,16]</sup> Since heavy atoms on the molecular skeleton cannot move on such a short timescale, we tentatively attribute the structural origin to a transient, small-scale proton motion along the pre-existing H-bonding chain. Notably, this distinct phase of frequency shift occurs in  $S_1$  as the photoexcited wavepacket starts to move out of the Franck–Condon (FC) region. This shows a larger time gap between initial electron motions and proton motions than the proton-coupled electron transfer case,<sup>[17]</sup> which would otherwise suggest an instantaneous change of the mode frequency.

Interestingly, the frequency gap between the two phenol ring-H rocking with some bridge  $\text{C}_7\text{--H}$  rocking modes ( $\sim 1154$  and  $1170 \text{ cm}^{-1}$  in  $S_0$ , Table S1 in the Supporting Information) of  $16/18 \text{ cm}^{-1}$  increases to  $56/44 \text{ cm}^{-1}$  at time zero in  $S_1$ , but reduces to  $39/34 \text{ cm}^{-1}$  after approximately 200 fs for the  $\text{Ca}^{2+}$ -free/bound G-GECO1.1 chromophore, respectively. The corresponding vibrational frequency gap is  $19/17 \text{ cm}^{-1}$  (in  $S_0$ ) and  $42/33 \text{ cm}^{-1}$  (in  $S_1$ , after  $\sim 200$  fs) for the  $\text{Ca}^{2+}$ -free/bound GEM-GECO1, respectively.<sup>[5]</sup> This slightly larger gap between the two modes, particularly for the  $\text{Ca}^{2+}$ -free state, is consistent with a more protonated state in  $S_0$  and larger photoexcited changes of the SYG versus TYG chromophore. The dynamic plot of the  $1409 \text{ cm}^{-1}$  mode (mainly phenol ring  $\text{C}=\text{C}$  stretching and  $\text{C}_1\text{OH}$

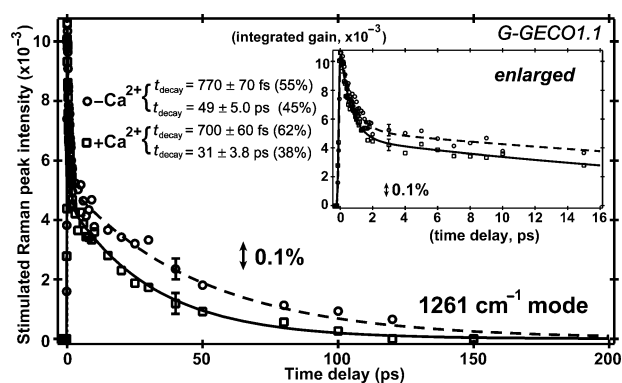
rocking) shows similar frequency evolution to reach  $1398/1400 \text{ cm}^{-1}$ . Moreover, the frequency gap variation shows that the photoinduced electronic redistribution is far from equilibrium and precedes the onset of coherent proton motions. To dissipate the sudden influx of incident photon energy, the chromophore slightly lengthens its phenolic O–H bond and the charge on the oxygen atom promptly redistributes. After this initial stage, the chromophore reaches a relatively more stable excited state as skeletal motions start to evolve.<sup>[8b]</sup> A similar phenomenon was observed in another GECO biosensor with SYG chromophore; Figure S3 in the Supporting Information shows the early-time (e.g.,  $< 1$  ps) frequency evolution of several  $A^*$  marker bands in the  $\text{Ca}^{2+}$ -free GEM-GECO1 biosensor.<sup>[18]</sup> An ultrafast LE-CT transition may be ubiquitous in FP biosensors with pre-existing H-bonds so the proton can efficiently move in response to the chromophore photoacidity, and compensates for the “overshoot” of initial charge separation in the LE state. In subsequent stages after the coherent motions diminish, we consider that the proton-transfer pathway mainly depends on the H-bond length: when it is short, a single-well PES forms and a deprotonated state is reached via initial proton motions; when it is long, a transition to a nearby ESPT-prone state occurs and initial proton motions play more of a preparatory role.<sup>[19]</sup>

To delineate the  $\text{Ca}^{2+}$ -binding effect on ESPT, we analyze the time-resolved excited-state FSRS spectra and find that after approximately 40 ps, the  $A^*$  peak intensity of the  $\text{Ca}^{2+}$ -bound biosensor is much weaker than the  $\text{Ca}^{2+}$ -free case (highlighted by two dotted boxes in Figure 3). ESPT seems to become faster upon  $\text{Ca}^{2+}$  binding while the observed peak frequencies remain largely unchanged. Notably, the emergence of  $I^*$  features is not obvious in the time-resolved spectra on the picosecond timescale while  $A^*$  features decay away. Given that the typical fluorescence lifetime is nanosecond, the photoexcited TYG chromophore remains in  $S_1$  as ESPT progresses. The observed peak evolution pattern may be largely due to the different resonance conditions induced by 800 nm Raman pump that specifically enhances  $A^*$  features.

It is important to note that although the observed Raman modes of the TYG chromophore between about  $900\text{--}1800 \text{ cm}^{-1}$  typically involve multiple atoms and are not highly localized, they act as sensitive probes to the multidimensional PES and reaction coordinate that govern both vibrational relaxation and the ESPT barrier crossing.<sup>[8b,20]</sup> The mode-specific kinetic analysis and detailed comparison between the time constants and the associated vibrational motions (aided by computations) are thus key to understanding the “in between” states and their transient dynamics that either accompany or facilitate the photophysical and photochemical reaction pathways.

Figure 5 presents the kinetic peak intensity plot of the  $1261 \text{ cm}^{-1}$  mode (mainly phenolic  $\text{C}_1\text{--O}$  stretching and ring-H rocking, see Table S2 in the Supporting Information). Double-exponential fitting yields similar first decay time constants of approximately 770/700 fs, but different second decay time constants of about 50/30 ps for the  $\text{Ca}^{2+}$ -free/bound biosensors, respectively (Table S2). The initial FC dynamics on the



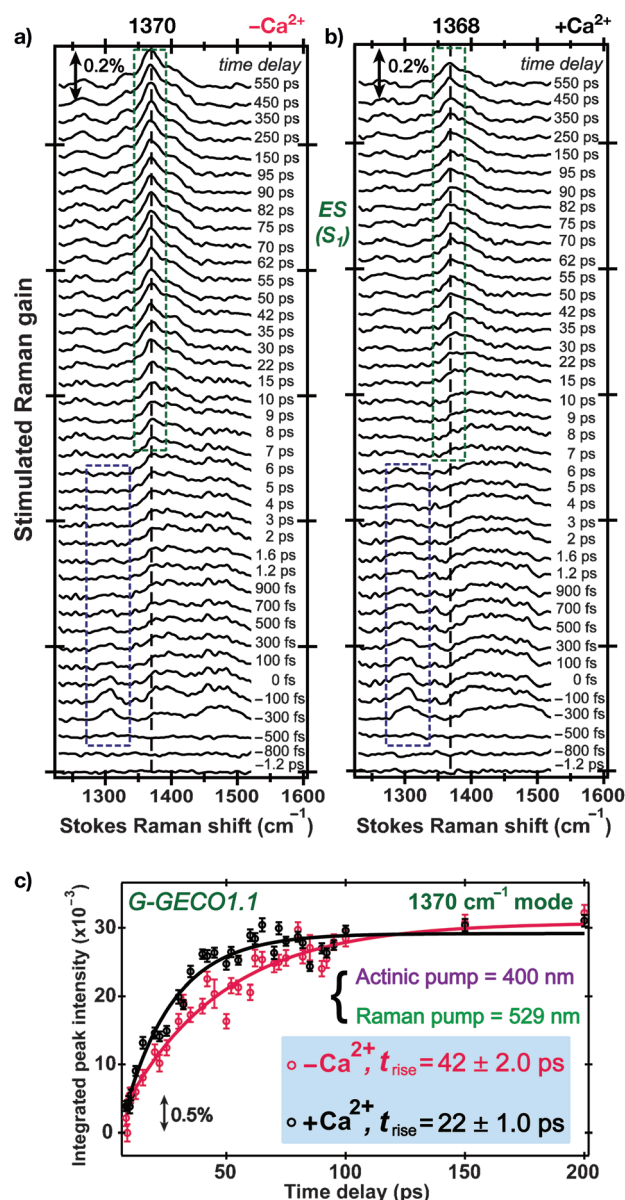


**Figure 5.** Time-resolved intensity of the  $1261\text{ cm}^{-1}$  mode in  $Ca^{2+}$ -free (circles) and bound (squares) G-GECO1.1 within 200 ps. The double-exponential fitting results are listed. The insert shows an expanded plot up to 16 ps. The double-headed line represents a stimulated Raman gain of 0.1%. The  $-Ca^{2+}$  peak is normalized to the stronger  $+Ca^{2+}$  peak with a scaling factor of 1.15. The typical error bar (1 s.d.) is depicted on one time delay point in each data trace.

sub-ps timescale commonly involve collective skeletal motions of the chromophore<sup>[5,8b]</sup> that may also be affected by local solvation dynamics in its immediate vicinity. We surmise that the vibronic wavepacket dynamics are strongly dependent on the PES that involves nearby reorienting water molecules. However, the faster second decay of  $A^*$  modes in the  $Ca^{2+}$ -bound G-GECO1.1 biosensor reveals that ESPT accelerates (rate increases by  $\sim 1.6$  times) in a presumably more efficient H-bonding network.

### Tunable excited-state FSRS confirming $I^*$ features

To confirm the occurrence of ESPT and generation of  $I^*$ , we have developed tunable FSRS that achieves resonance Raman enhancement for transient species.<sup>[9c]</sup> The time-resolved FSRS data in Figure 6a and b using an approximately 530 nm Raman pump (see the Experimental Section) show the emergence of  $I^*$  features on the 22–42 ps timescale (see Figure 6c for the integrated peak intensity of the about  $1370\text{ cm}^{-1}$  modes with and without  $Ca^{2+}$ ) that matches the  $A^*$  decay dynamics (Figure 3 and Figure 5). Notably, we performed TD-DFT calculations with RB3LYP 6-31G+(d,p) basis sets on the deprotonated TYG chromophore in vacuo and found a prominent vibrational normal mode at approximately  $1372\text{ cm}^{-1}$  after multiplication of a frequency scaling factor of 0.96.<sup>[21]</sup> The major components of this mode consist of the phenolic C=O stretching, phenol ring-H rocking, and bridge  $C_7$ -H rocking motions. This vibrational mode is absent in the same level of calculation result of a protonated TYG chromophore, which explains why we did not observe such a mode in the conventional FSRS data with an 800 nm Raman pump prior to ESPT (Figure 3). The approximately  $1370\text{ cm}^{-1}$   $I^*$  modes represent a blueshift from the about  $1300\text{ cm}^{-1}$   $A^*$  modes in Figure 6a and b, which may act as a sensitive spectral probe involving the bridge  $C_7$ -H rocking with phenolic CO stretching to track the ESPT progress. Besides a similar trend, additional discussion on the small difference between fitted time constants in Figure 5 and Figure 6c can be found in the Supporting Information.



**Figure 6.** Time-resolved tunable FSRS spectra of: a)  $Ca^{2+}$ -free, and b)  $Ca^{2+}$ -bound G-GECO1.1 biosensor with 400 nm actinic pump and 529 nm Raman pump. The blue (green) dashed boxes highlight the vibrational modes that show decay (rise) dynamics before (after) approximately 6 ps, attributed to  $A^*$  ( $I^*$ ) modes, respectively. The double-headed corner shows the Raman gain magnitude of 0.2%. c) Kinetic plot of the  $1370$  ( $1368$ )  $\text{cm}^{-1}$  mode in the  $Ca^{2+}$ -free (bound) samples is shown in red (black), respectively. The single exponential fits (solid lines) are overlaid with data points (hollow circles). The intensity trace of the  $Ca^{2+}$ -free biosensor is vertically offset to match the  $Ca^{2+}$ -bound data point at approximately 10 ps time delay to compensate for the difference in the Raman signal strength. The magnitude of the signal rise within 200 ps is comparable for both samples. The fitted time constants are noted in the Figure.

The tunable FSRS results clearly correlate the consumption of reactant and generation of product state, confirming that the main photochemical pathway is ESPT, which becomes faster by approximately 2 times in the G-GECO1.1 biosensor upon  $Ca^{2+}$  binding. The observed  $S_1$  Raman frequencies change from the  $1261$  and  $1300\text{ cm}^{-1}$  modes in  $A^*$  (Figure 3

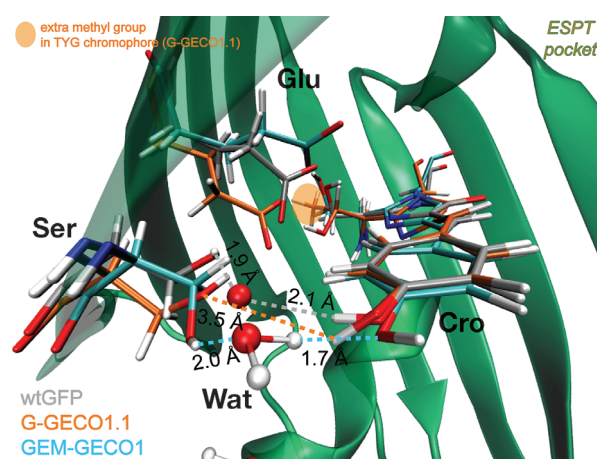
and Table S2 in the Supporting Information) to the  $1370\text{ cm}^{-1}$  mode in  $I^*$  (Figure 6), which is consistent with the increase of double-bond character of phenolic  $C_1\text{--O(H)}$  bond,<sup>[5,8b]</sup> hence deprotonation of the protein chromophore on the ps time-scale. Detailed analysis on tunable FSRs data yields richer information about the multidimensional ESPT reaction barrier crossing to  $I^*$  formation, and will be reported in a future study.

### Molecular dynamics (MD) simulation

Because the crystal structure of G-GECO1.1 is unavailable, we performed a 15 ns MD simulation (see Experimental Section and Supporting Information) to find the  $S_0$  equilibrium structure of the biosensor with all the point mutations from the parent GCaMP3 crystal structure (PDB ID: 3SG3).<sup>[4b]</sup> Figure 7 shows the simulated relative geometry between the chromophore phenolic hydroxyl and the adjacent serine residue along the ESPT chain, which turns out to be in between wtGFP<sup>[8b]</sup> and GEM-GECO1.<sup>[5]</sup> In wtGFP (PDB ID: 2WUR),<sup>[22]</sup> the conserved  $H_2O$  molecule is above and to one side of the SYG chromophore phenolic hydroxyl group. In GEM-GECO1, 10 ns MD simulations<sup>[5]</sup> found two  $H_2O$  molecules within close proximity of the SYG chromophore phenolic hydroxyl and the Ser118 side-chain hydroxyl. In G-GECO1.1, the equilibrated structure after 12 ns from MD simulations shows a chromophore–Ser118 geometry in between wtGFP and GEM-GECO1, and the distance between the phenolic proton and the Ser118 hydroxyl oxygen is approximately  $3.5\text{ Å}$ . Consequently, a bridging  $H_2O$  molecule is expected to facilitate the initial ESPT in the G-GECO1.1 chromophore pocket with access to labile solvent molecules through the  $\beta$ -barrel opening.

This finding may explain the absence of a dominant low-frequency mode that projects onto the ESPT reaction coordinate, and the subsequently lengthened ESPT ( $\sim 50\text{ ps}$ , Table S2 in the Supporting Information) versus GEM-GECO1 ( $\sim 30\text{ ps}$ )<sup>[5]</sup> in the  $Ca^{2+}$ -free state. Furthermore, ESPT takes more time in G-GECO1.1 than wtGFP mainly due to labile bridging  $H_2O$  molecules, which may better solvate the  $A^*$  state in the first approximately 1 ps and hence increase the ESPT barrier after the photoexcited wavepacket slides down the PES toward the  $A^*$  well. In contrast, the conserved  $H_2O$  molecule above and to one side of the chromophore in wtGFP has less flexibility and cannot stabilize or solvate the photoexcited chromophore effectively. The wavepacket thus stays relatively high on the  $A^*$  PES which makes the ESPT barrier crossing easier and faster ( $\sim 5\text{ ps}$ ).<sup>[8b]</sup>

Due to the extra sidechain methyl group in the TYG chromophore of G-GECO1.1 (Figure 7, orange), the Thr223 hydroxyl can rotate to better H bond with the nearby Glu135. Therefore, if the aforementioned initial small-scale coherent proton motions occur concertedly<sup>[18,23]</sup> at the imidazolinone end that may lead to higher electronic polarizability,<sup>[15]</sup> it can explain the increased  $985\text{ cm}^{-1}$  mode intensity (Figure 3 and Figure S2 in the Supporting Information) in comparison to our previous results<sup>[5,8b]</sup> for wtGFP and GEM-GECO1. This specific G-GECO1.1 configuration may also be responsible for a faster sub-ps decay of the  $985\text{ cm}^{-1}$  mode intensity (Table S2 in the Support-



**Figure 7.** Illustration of different ESPT geometries in the protein chromophore pockets (enlarged) of three related GFPs: wtGFP (silver backbone), G-GECO1.1 (orange), and GEM-GECO1 (cyan). The extra methyl group of the TYG chromophore in G-GECO1.1 is highlighted by the light orange ellipse shadow. To better illustrate the chromophore location and small geometrical differences, only the  $\beta$ -barrel in G-GECO1.1 is shown (green strands on the background). The dashed lines (colored accordingly with various proteins) represent the H bonds between the chromophore phenolic hydroxyl group and the nearby serine (Ser118 for GEM-GECO1 and G-GECO1.1, and Ser205 for wtGFP) via a bridging water molecule. The H-bonding lengths [ $\text{Å}$ ] involving the spatially closer  $H_2O$  molecule are labeled in black. Nitrogen, oxygen, and hydrogen atoms are depicted in blue, red, and white, respectively.

ing Information) because a rearranged H-bonding network can be quickly established after the vibronic wavepacket moves out of the initial FC region following 400 nm photoexcitation.

### Interplay between photoacidity and local environment

Can the observed ESPT difference be correlated with  $pK_a$  change of the chromophore? Based on thermodynamics,<sup>[24]</sup> the excited-state  $pK_a$  can be estimated using the Förster equation:

$$pK_a^* = pK_a - h(\nu_1 - \nu_2)/2.3 k_B T \quad (1)$$

where  $\nu_1$  and  $\nu_2$  are absorption and emission peak frequencies in  $s^{-1}$  of the neutral and anionic species, respectively. Given the measured  $pK_a$  values of 10.2/7.5,  $pK_a^*$  is  $-1.03/-4.36$  for  $Ca^{2+}$ -free/bound biosensor chromophore, respectively. This indicates that the  $Ca^{2+}$ -bound biosensor has a greater tendency to lose the proton. These results agree with Brønsted correlation that higher  $pK_a$  leads to slower acid catalysis reaction rate.<sup>[25]</sup> Though this correlation has been verified in small photoacids,<sup>[26]</sup> it is notable that the large protein matrix complies with the same principle. In G-GECO1.1 upon  $Ca^{2+}$  binding, the CaM domain wraps around the M13 peptide, enabling residues at the newly formed FP–CaM interface to modify the local environment of the TYG chromophore (Figure 7). In particular, the positively charged Arg377 of the CaM domain that swings into the interfacial region can interact with the phenolic hydroxyl of the chromophore via an intervening water molecule

and extend the H-bonding network in the  $\text{Ca}^{2+}$ -bound protein pocket (see the section below for discussion). This results in a more favorable environment to facilitate ESPT with faster proton transfer while stabilizing the deprotonated TYG chromophore.<sup>[3c,12]</sup>

In contrast to our earlier work on FPs and FP biosensors,<sup>[5,8b]</sup> no dominant low-frequency modulation modes were found in G-GECO1.1 to exhibit quantum beats. Possible reasons include: 1) TYG chromophore may experience less coherent skeletal motions due to a bulkier sidechain; 2) the anharmonic coupling matrix is intricate, so skeletal motions do not strongly couple to other Raman modes; 3) the relative geometry between the TYG chromophore and Ser118 favors neither the phenolic ring wagging nor rocking, as illustrated in Figure 7; and 4) the chromophore environment promotes direct proton transfer via pre-existing H bonds on the sub-ps to ps timescale, followed by large-scale proton transfer on the tens of ps timescale that depends on proton acceptor (e.g., surrounding  $\text{H}_2\text{O}$  molecules, and protein residues along the ESPT chain) properties including orientations, mobility, and flexibility.<sup>[9b,16a]</sup>

### Functional relevance of the interfacial residue Arg377

Based on the  $\text{Ca}^{2+}$ -bound GCaMP2 structure (PDB ID: 3EVR)<sup>[3c]</sup> and GCaMP3 structure (PDB ID: 3SG3),<sup>[4b]</sup> the chromophore deprotonation is expected to occur upon  $\text{Ca}^{2+}$  binding because the interfacial Arg377 residue occupies a functionally similar position as His148 in wtGFP to participate in ESPT chain involving the phenolic hydroxyl of the chromophore.<sup>[3c,6,12,27]</sup> Though  $\text{Ca}^{2+}$  does not directly interact with the chromophore, the structural reorganization induced by the allosteric  $\text{Ca}^{2+}$  binding changes the chromophore dynamics. In GEM-GECO1, Arg377 is replaced by Pro377 that cannot H-bond to proton donor or acceptor,<sup>[4a]</sup> besides its conformational rigidity to disrupt secondary or tertiary structure of the protein chimera particularly at the interfacial region of the  $\text{Ca}^{2+}$ -bound state. As shown in the absorption and emission spectra of GEM-GECO1 and its single-residue mutants, the green–blue dual-emission capability of the ratiometric biosensor is diminished with P377R mutation,<sup>[5]</sup> corroborating the functional relevance of this strategic interfacial residue. In other words, an adjacent Pro377 can help trap a photoexcited neutral chromophore so blue fluorescence dominates, whereas Arg377 facilitates ESPT leading to green fluorescence. In this current work on G-GECO1.1, the increase of ESPT rate in the  $\text{Ca}^{2+}$ -bound protein biosensor is consistent with our interpretation that Arg377 participates in the modified extensive H-bonding network involving the phenolic hydroxyl group of the chromophore. As a result, green fluorescence dominates in both the  $\text{Ca}^{2+}$ -free and bound (equilibrium) states of the intensimetric biosensor.

Furthermore, the  $\text{Ca}^{2+}$ -free G-GECO1.1 biosensor shows weak blue fluorescence as evinced by the 466 nm shoulder to the blue side of its dominant green emission at 514 nm (Figure 1). This suggests that the absence of interfacial Arg377 (due to extended conformation of the CaM domain in the  $\text{Ca}^{2+}$ -free state, also confirmed by MD simulations) contributes to the increased hydrophobicity of the chromophore pocket that

favors an increased ESPT barrier and a lengthened ESPT time constant, as well as trapping of the photoexcited protonated chromophore to some extent.<sup>[5]</sup> Nevertheless, it is notable that both the  $\text{Ca}^{2+}$ -free and bound G-GECO1.1 resembles wtGFP<sup>[8b,11,22]</sup> more than EGFP,<sup>[6,28]</sup> as Figure 1 exhibits a dominant protonated chromophore in both states (aqueous buffer solution pH 7.2) with green fluorescence upon 400 nm excitation.

The correlation between the strategic importance of Arg377 and the increased ESPT rate in the  $\text{Ca}^{2+}$ -bound G-GECO1.1 suggests a potential route to apply our current findings to rationally design FP-based biosensors with better fluorescence contrast upon 400 nm excitation, and potentially using fs 800 nm excitation that takes advantage of the two-photon absorption cross-section of FPs.<sup>[4b,10]</sup> Deeper penetration depth can be achieved with near-infrared light irradiation in comparison to the visible 500 nm excitation. Furthermore, this work reveals the interplay between *chromophore photoacidity* and protein pocket *local environment* on the intrinsic *molecular timescale*, laying the foundation to systematically perform site-specific mutagenesis around the circularly permuted (cp)GFP chromophore<sup>[4a,5]</sup> and benchmark the  $\text{Ca}^{2+}$ -dependent intensimetric emission signal contrast with the fundamental output (i.e., 800 nm) of commercially available Ti:sapphire laser sources. Key functional residues will be identified to efficiently improve the FP-based biosensors aided by de novo design of proteins.<sup>[29]</sup>

### Conclusion

In summary, we have used time-resolved FSRS to dissect the multidimensional excited-state structural evolution of a new intensimetric FP biosensor for  $\text{Ca}^{2+}$  imaging. In particular, the  $\text{I}^*$  features are selectively enhanced using a tunable FSRS setup with 529 nm Raman pump. A sub-200 fs transition from an LE to CT state is evinced by ultrafast frequency shift of several vibrational marker bands of TYG chromophore in the electronic excited state. This feature arises from chromophore photoacidity of the GFP-derived biosensors, following initial electronic redistribution upon photoexcitation, regardless of  $\text{Ca}^{2+}$  binding or point mutations. The functional role of initial coherent small-scale proton motions may trigger a subsequent solvent rearrangement phase that occurs on the approximately 700 fs timescale, corroborated by the first decay time constant of most of the  $\text{S}_1$  modes (Table S2 in the Supporting Information). On the longer ps timescale, the effect of the protein pocket environment becomes more pronounced. Upon  $\text{Ca}^{2+}$  binding in G-GECO1.1, a further drop of the chromophore  $\text{pK}_a^*$  is accompanied by a faster ESPT process, corroborated by emergence of deprotonated species on the 42 (22) ps timescale for the  $\text{Ca}^{2+}$ -free (bound) biosensor, respectively. This unique series of spectroscopic results aided by MD simulations of the chromophore pocket substantiate the strategic importance of interfacial residues between CaM and GFP in modulating the biosensor fluorescence to achieve better  $\text{Ca}^{2+}$ -sensing capability. On the methodology side, FSRS proves to be a platform



technology to elucidate molecular fluorescence mechanisms and dynamically enable bioimaging and biomedical advances.

## Experimental Section

### Fluorescent protein biosensor preparation

The sample preparation of G-GECO1.1 protein solution for in vitro spectroscopic characterization was performed according to previously reported protocol.<sup>[5]</sup> Briefly, *E. coli* DH10B cells were transformed with the pTorPE plasmid harboring His<sub>6</sub>-G-GECO1.1.<sup>[4a]</sup> The transformed *E. coli* formed colonies on the nutrient agar plate supplemented with ampicillin, which were then picked and inoculated in 1 L of a modified terrific broth containing 20 g lysogeny broth mix, 7 g yeast extract, 14 g tryptone, 2.2 g KH<sub>2</sub>PO<sub>4</sub>, 9.2 g K<sub>2</sub>HPO<sub>4</sub>, and 8 mL glycerol (pH 7.20, sterilized) at 30 °C for two days. The cells from the culture were obtained by centrifugation, resuspended in standard Tris-buffered saline (TBS) at pH 7.4, and then lysed by French press. After another round of centrifugation, G-GECO1.1 protein in the clarified solution was purified using Ni-NTA affinity chromatography, followed by buffer exchange in MOPS buffer with either 10 mM EGTA (Ca<sup>2+</sup>-free sample) or 10 mM Ca-EGTA (Ca<sup>2+</sup>-bound sample). The concentrated G-GECO1.1 protein solution (OD > 10 cm at 400 nm) was used for in vitro characterization by steady-state electronic spectroscopy (e.g., UV/Vis and fluorescence, Figure 1) and time-resolved FSRS measurements (e.g., Figure 3 and Figure 6). Notably, the absorbance ratio of the 404 nm peak (neutral form) to the 505 nm peak (anionic form) is approximately 19 for the Ca<sup>2+</sup>-free G-GECO1.1 in Figure 1. Given that the latter form has about twice the extinction coefficient,<sup>[2,6]</sup> the molar ratio of neutral to anionic chromophores in the system is about 38, which confirms that the concentration of the anionic form in the sample system is negligible. This ensures high selectivity of using the 400 nm photoexcitation pulse to specifically track the structural dynamics of the neutral chromophore inside the biosensor as discussed in the main text.

### Femtosecond transient absorption

In brief, the fundamental laser source at approximately 35 fs, 800 nm, 2 mJ per pulse with 1 kHz repetition rate is provided by an fs mode-locked Ti:sapphire oscillator (Mantis-5) and regenerative amplifier (Legend Elite-USP-1K-HE, Coherent). The transient absorption setup uses a portion (~200 mW) of the fundamental output, which is separated into two arms by a 30%R:70%T beamsplitter. The transmitted arm goes through a  $\beta$ -barium borate (BBO) crystal to generate an fs pump pulse centered at 400 nm via second harmonic generation. The reflected beam is focused on a 2 mm thick sapphire plate to generate a supercontinuum white light as probe pulse (ca. 470–720 nm).<sup>[30]</sup> The pump pulse is then attenuated by neutral density filter to approximately 0.5 mW and the probe power is about 50  $\mu$ W. Both pulses are focused by the same concave mirror onto a 1 mm thick quartz sample cell. The pump pulse traverses a motorized delay stage (NRT150, Thorlabs) to control the time delay to the subsequent probe pulse up to 1 ns. The aqueous protein sample concentration is prepared to have its OD = 0.5 per mm at approximately 400 nm (i.e., absorption peak of the ground-state protonated chromophore, see Figure 1). The main absorption peak of the deprotonated chromophore shifts from 505 to 499 nm upon Ca<sup>2+</sup> binding. To minimize thermal effect and photodegradation of proteins, sample solution is constantly stirred by a miniature magnetized stir bar below the laser focal point during spectroscopic data collection.

### FSRS instrumentation

The experimental FSRS setup has been described previously.<sup>[9a,b,31]</sup> The laser output undergoes a three-way split and is used to generate a Raman pump–probe pair and a preceding actinic pump for photoexcitation. An approximately 40 fs, 500  $\mu$ W actinic pump pulse centered at 400 nm is generated from second harmonic generation in a 0.3 mm thick BBO crystal (Type I, phase-matching angle  $\theta = 29.2^\circ$ ) and prism (Suprasil-1, CVI Melles Griot) compression. A Raman pump pulse with 800 nm center wavelength, approximately 6 mW power, and 3.5 ps pulse duration is achieved by using a home-built grating-slit-based spectral filter. A 100 nJ per pulse white light supercontinuum at 840–940 nm is generated to act as the Raman probe by focusing a small portion of the fundamental pulse in a 2 mm thick Z-cut sapphire crystal plate, followed by fused silica prism compression to roughly 35 fs. All three pulses are collimated and incident on the sample in a noncollinear geometry with an approximately 7° crossing angle between the Raman pump and actinic pump on either side of the centered Raman probe beam. A pinhole after the sample cell selects the probe beam carrying the FSRS signal to go into the spectrograph and CCD camera.

For the time-resolved excited-state FSRS data collection, the three laser pulses are focused on the protein sample solution (OD  $\approx$  1 per mm at 400 nm, close to the absorption peak of the neutral chromophore, see Figure 1), which continuously flows to avoid thermal effects.<sup>[8b]</sup> The Raman pump and probe pulses are temporally overlapped to maximize the ground state ( $S_0$ ) spectral intensity of a solvent standard, with the arrival of the actinic pump tuned by a stepper-motor-driven linear translation stage. The stimulated Raman signal that is collinear with the probe pulse enters a spectrograph with a 600 grooves mm<sup>-1</sup>, 1  $\mu$ m blaze ruled reflective grating, and is dispersed and imaged onto a 1340  $\times$  100 CCD array (PIXIS 100F, Princeton Instruments) that is binned vertically. A 500 Hz optical chopper in the Raman pump beampath enables the acquisition of one FSRS spectrum in 2 ms. Twelve sets of excited state ( $S_1$ ) FSRS data are collected at each time delay from –5 to 650 ps with randomized time points to avoid heat effect or backlash. The  $S_0$  Raman signal is periodically collected throughout the data collection to monitor sample and laser conditions. A separate  $S_0$  FSRS measurement is performed with only the Raman pump and probe pair. Sixty sets of the  $S_0$  Raman spectra are obtained and averaged (Figure S1 in the Supporting Information), followed by least-squares fitting with multiple Gaussian peaks. A small percentage (~10%) of this fitted  $S_0$  spectrum is then added back to fill the small dips in the ground-state-subtracted FSRS spectra to obtain the pure positive  $S_1$  vibrational features.<sup>[5]</sup> Broad, smooth, and featureless spline baselines are then drawn (Figure S2 in the Supporting Information) and subsequently subtracted from the time-resolved FSRS data traces to yield the  $S_1$  vibrational (Raman) spectra presented in Figure 3. It is apparent that the transient Raman peaks have *high enough* signal-to-noise ratio so the small uncertainty of baseline drawing becomes insignificant and negligible to the subsequent vibrational peak kinetic analysis and mechanistic understanding of the photophysical and photochemical reaction pathways.

The sample integrity is verified by UV/Vis spectroscopy (Figure 1) before and after the time-resolved FSRS data collection that typically takes 1–2 h depending upon the number of data sets being recorded.<sup>[5,9b]</sup> The approximately 500  $\mu$ W femtosecond actinic pump and 6 mW picosecond Raman pump pulses are within a comfortable range of performing such experiments, because up to 1 mW actinic pump and 8 mW Raman pump pulses were used without detectable photodegradation to the sample. The focusing



diameter of both pulses on the sample cell is about 300  $\mu\text{m}$ , larger than the focusing diameter of the Raman probe pulse at about 200  $\mu\text{m}$ . The quartz flow cell with a suitable sample solution flow rate controlled by a peristaltic pump helps to dissipate the local heat effect or any thermally induced damage to the protein biosensors in aqueous buffer solution. Moreover, during the time-resolved FSRS data collection, the reproducible ground-state spectra periodically recorded throughout the excited-state scan confirms that the protein sample maintains its integrity under the pulsed laser irradiation conditions employed in our FSRS experiments.<sup>[5,8b,18]</sup>

To retrieve the dynamic information of these transient  $S_1$  Raman modes, we perform the least-squares fitting of each time-resolved spectrum across the detection window. Gaussian profiles work well for the protein chromophore modes largely due to inhomogeneous broadening in solution.<sup>[15,32]</sup> The center frequency and integrated intensity of individual vibrational mode are then plotted against delay time, followed by multi-exponential fitting to obtain the characteristic time constants and infer the conformational dynamics of the photoexcited chromophore.

In the tunable FSRS setup that has been recently developed in our laboratory,<sup>[9c]</sup> we collect time-resolved Stokes FSRS data with an approximately 530 nm Raman pump pulse and a 540–585 nm Raman probe pulse (supercontinuum white light to the red side of the Raman pump). The actinic pump pulse remains at 400 nm to selectively excite the neutral chromophore of the biosensor samples with and without  $\text{Ca}^{2+}$ . We use the 1200 grooves  $\text{mm}^{-1}$  ruled reflective grating with 500 nm blaze wavelength in the imaging spectrograph (Princeton Instruments SP2356) to disperse and record the signal onto the  $1340 \times 100$  CCD array (PIXIS 100F, Princeton Instruments) that is synchronized with the laser system at 1 kHz repetition rate.

### Molecular dynamics simulation methods

The starting coordinates for our molecular dynamics (MD) simulations are taken from the crystal structure of a parent GFP–calmodulin (CaM) protein complex G-CaMP3 in the  $\text{Ca}^{2+}$ -bound state (PDB ID: 3SG3).<sup>[4b]</sup> To mimic the  $\text{Ca}^{2+}$ -free environment, the CaM part of 3SG3 is replaced by an independent  $\text{Ca}^{2+}$ -free CaM crystal structure (PDB ID: 1CFD)<sup>[33]</sup> with all the calcium ions deleted. The crystallized water molecules in both structures were kept. The missing residues (residue number 144–157) are added and all the point mutations specific to G-GECO1.1<sup>[4a]</sup> are made using the SwissPDB Viewer.<sup>[34]</sup> Before the equilibrium MD simulations are performed, we use the AmberTools13 package<sup>[35]</sup> to add hydrogen (H) atoms to the protein, and the counter ions are supplied to neutralize the entire system. We solvate the protein in a box of 27774 TIP3P water molecules with periodic boundary within a 15 Å radius of the protein. To obtain an ensemble equilibrium structure to correlate the key mutations with functional changes of the chromophore local environment (in the absence of a crystal structure), we perform 15 ns MD simulations of the  $\text{Ca}^{2+}$ -free G-GECO1.1 protein complex using Amber12 program. The Langevin thermostat is used to maintain the protein system temperature at 300 K with a  $2 \text{ ps}^{-1}$  collision frequency. The SHAKE method that enacts bond-length constraints is employed to maintain the rigidity of all bonds involving the H atoms. The time step is 2 fs and the output atomic coordinates are recorded every 1000 steps.

### Acknowledgements

We thank Liangdong Zhu for transient absorption measurements and Dr. Fangyuan Han for Igor programming. This work is supported by the Oregon State University (OSU) Faculty Research Start-up Grant and General Research Fund (to C.F.), Natural Sciences and Engineering Research Council of Canada and Canadian Institutes of Health Research (to R.E.C.), and a University of Alberta fellowship and an Alberta Innovates scholarship (to Y.Z.). We appreciate the 2014 OSU chemistry departmental summer research fellowships including the David P. Shoemaker Memorial Research Project (to B.G.O.) and the Dorothy and Ramon Barnes Graduate Fellowship (to Y.W.). We also acknowledge the recent NSF CAREER Award (CHE-1455353) to C.F.

**Keywords:** calcium ion sensing • excited state proton transport • femtochemistry • fluorescent probes • vibrational spectroscopy

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Received: February 6, 2015

Published online on March 11, 2015