Quantitative determination of the full switching cycle of photochromic fluorescent proteins
Supporting Information

Anaïs C. Bourges, Benjamien Moeyaert, Thi Yen Hang Bui,
Franziska Bierbuesse, Wim Vandenberg, Peter Dedecker

Department of Chemistry, KU Leuven, Belgium

peter.dedecker@kuleuven.be

Supplementary note 1: derivation of the theoretical model

The model shown in main text Figure 1C can be described using conceptually straightforward analytical expressions. We begin by introducing c_c and c_t, the total concentrations of proteins in the cis and trans state:

$$c_c = [CH] + [C^-] \tag{S.1}$$
$$c_t = [TH] + [T^-] \tag{S.2}$$

The assumption of fast pH equilibria allows us to reduce the four-state system to a much simpler one consisting of only two species:

$$c_c \underset{k_{tc}}{\overset{k_{ct}}{\rightleftharpoons}} c_t \tag{S.3}$$

where the rate constants are given by

$$k_{ct} = k_{ct}^{H} \cdot (1 - f_c^-) + k_{ct}^{\text{-H}} \cdot f_c^- \tag{S.4}$$
$$k_{tc} = \left(k_{tc}^{H} + k_{tc}^{H,therm}\right) \cdot (1 - f_t^-) + \left(k_{tc}^{\text{-H}} + k_{tc}^{\text{-H,therm}}\right) \cdot f_t^- \tag{S.5}$$

Here f_c^- is the fraction of the cis-state molecules that are in the deprotonated state, and f_t^- is the fraction of the trans-state molecules that are in the deprotonated state:

$$f_c^- = \frac{K_c}{[H^+] + K_c} \tag{S.6}$$
$$f_t^- = \frac{K_t}{[H^+] + K_t} \tag{S.7}$$

These parameters also make it straightforward to determine the concentrations of all four chromophore species from the simplified two-state model:

$$[C^-] = f_c^- \cdot c_c \qquad [CH] = (1 - f_c^-) \cdot c_c \tag{S.8}$$
$$[T^-] = f_t^- \cdot c_t \qquad [TH] = (1 - f_t^-) \cdot c_t \tag{S.9}$$

Using this pH dependence, we can rewrite equations (S.4) and (S.5) into the following form:

$$k_{ct} = \frac{k_{ct}^{H}}{10^{pH-pKac} + 1} + \frac{k_{ct}^{\text{-H}}}{10^{pKac-pH} + 1} \tag{S.10}$$
$$k_{tc} = \frac{k_{tc}^{H} + k_{tc}^{H,therm}}{10^{pH-pKat} + 1} + \frac{k_{tc}^{\text{-H}} + k_{tc}^{\text{-H,therm}}}{10^{pKat-pH} + 1} \tag{S.11}$$

Our concern is to understand how the chromophore species evolve in time when exposed to irradiation with light. The rate equations for the system in equation (S.3) are given by

$$\frac{\mathrm{d}c_\mathrm{c}}{\mathrm{d}t} = -k_\mathrm{ct}c_\mathrm{c} + k_\mathrm{tc}c_\mathrm{t} \tag{S.12}$$

$$\frac{\mathrm{d}c_\mathrm{t}}{\mathrm{d}t} = \quad k_\mathrm{ct}c_\mathrm{c} - k_\mathrm{tc}c_\mathrm{t} \tag{S.13}$$

which is a simple two-component equilibrium giving rise to well-known solutions:

$$c_\mathrm{c}(t) = \frac{c_{\mathrm{c},0}\left(k_\mathrm{ct}e^{-(k_\mathrm{ct}+k_\mathrm{tc})t} + k_\mathrm{tc}\right) + c_{\mathrm{t},0}k_\mathrm{tc}\left(1 - e^{-(k_\mathrm{ct}+k_\mathrm{tc})t}\right)}{k_\mathrm{ct} + k_\mathrm{tc}} \tag{S.14}$$

$$c_\mathrm{t}(t) = \frac{c_{\mathrm{t},0}\left(k_\mathrm{tc}e^{-(k_\mathrm{ct}+k_\mathrm{tc})t} + k_\mathrm{ct}\right) + c_{\mathrm{c},0}k_\mathrm{ct}\left(1 - e^{-(k_\mathrm{ct}+k_\mathrm{tc})t}\right)}{k_\mathrm{ct} + k_\mathrm{tc}} \tag{S.15}$$

where $c_{\mathrm{c},0}$ and $c_{\mathrm{t},0}$ are the concentrations of proteins with cis and trans chromophores at $t = 0$. Experimentally, we can measure the different species via their absorption spectra, though in practice the component spectra display considerable overlap and also see slight changes as a function of pH, making it difficult to distinguish their contributions. The fluorescence emission, in contrast, is assumed to arise only from the C^- state and therefore appears to offer prospects for a more simple and robust measurement. Accordingly, we focus on this state and express equation (S.14) in terms of the fraction of proteins that is in the cis state f_c:

$$f_\mathrm{c}(t) = \frac{c_\mathrm{c}}{c_\mathrm{c} + c_\mathrm{t}} \tag{S.16}$$

$$= \frac{(f_{\mathrm{c},0}k_\mathrm{ct} - (1 - f_{\mathrm{c},0})k_\mathrm{tc})e^{-(k_\mathrm{ct}+k_\mathrm{tc})t} + k_\mathrm{tc}}{k_\mathrm{ct} + k_\mathrm{tc}} \tag{S.17}$$

with $f_{\mathrm{c},0}$ the fraction of proteins in the cis state at $t = 0$. The resulting expression is an exponential function of the well-known form $A\exp(-t/\tau) + y_0$, where

$$A = f_{\mathrm{c},0} - y_0 \tag{S.18}$$

$$\tau = \frac{1}{k_\mathrm{ct} + k_\mathrm{tc}} \tag{S.19}$$

$$y_0 = \frac{k_\mathrm{tc}}{k_\mathrm{ct} + k_\mathrm{tc}} \tag{S.20}$$

The rate constants governing the reduced system can therefore be determined by fitting a measured $f_\mathrm{c}(t)$ trace with such an exponential function, recovering these using

$$k_\mathrm{ct} = \frac{1 - y_0}{\tau} \tag{S.21}$$

$$k_\mathrm{tc} = \frac{y_0}{\tau} \tag{S.22}$$

The resulting k_ct and k_tc values then provide access to the underlying molecular rate constants by repeating such measurements for multiple pH values and fitting these to equations (S.10) and (S.11).

In actual experiment, however, we measure the observed fluorescence in time $F(t)$, rather than $f_\mathrm{c}(t)$. This induces a discrepancy since $F(t)$ is proportional to the C^- population while $f_\mathrm{c}(t)$ includes both the CH and the C^- states. Our goal is therefore to convert $F(t)$ into $f_\mathrm{c}(t)$. We typically do not know the value of $f_{\mathrm{c},0}$ for an unirradiated sample, since it is difficult to reliably estimate this parameter from the absorption spectra, especially over a broad pH range. Accordingly, in this study we assume that the cis state is considerably more thermally stable than the trans state, as described for most FPs [1, 2], and hence that $f_{\mathrm{c},0} = 1$. We can then obtain the desired f_c trace simply by dividing the observed fluorescence by the fluorescence observed before the sample was irradiated. Expressed quantitatively:

$$f_\mathrm{c}(t) = \frac{F(t)}{F(t = 0)} \tag{S.23}$$

This equation may appear surprising at first sight. However, at any given pH the ratio between the amount of C^- and CH is constant, and therefore the concentration of C^-, and hence the overall fluorescence, is always proportional to f_c. Furthermore, the value of this proportionality constant or any other proportionality constants (i.e. the total concentration of the fluorophore) need not be known since they are automatically taken into account in the normalization.

Methods

Protein purification and handling

All His-tagged proteins were cloned in a pRSETb vectors and expressed and purified as described previously [3]. The obtained proteins were stored in the dark in a TN 100/300 buffer (Tris 100 mM/NaCl 300 mM at pH 7.4) at 4°C. All experiments were done at 23 °C. Care was taken to ensure that the samples were not exposed to light prior to the experiments. Purified proteins were diluted in 1.5 mL of universal buffer (50 mM citric acid, 50 mM glycine, 50 mM KH_2PO4, 50 mM Tris and 100 mM NaCl adjusted from pH 4 to 12) into a cuvette equipped with a magnetic stirrer (at 725 rpm, STERICO) that was used to homogenize the solution over the full duration of each measurement.

pKa and extinction coefficient determination

The chromophore pKa's were determined on samples in which the fluorophores were assumed to be entirely in the cis or in the trans state. To determine the pKa of the cis state, this titration and analysis were performed on unirradiated samples. To determine the pKa of the trans state, the protein solution at pH 7.4 was irradiated with light at 488 nm until extinction of the fluorescence. Then, part of the sample was quickly diluted in one of the buffered solutions while absorption spectra were continuously acquired with a time resolution of 0.5 s. We then used the first absorption spectrum that did not appear perturbed by the addition itself for the quantitative analysis.

We determined extinction coefficients for both the protonated and deprotonated species, each time at the absorption maximum. Since the exact peak wavelengths differ between the different proteins, we here denote measurements performed at the peak wavelength of the protonated species with the sub- or superscript '390', and at the peak wavelength of the depronotated species with the sub- or superscript '490'.

The extinction coefficients for TH and C^- were obtained using the alkaline denaturation method. Briefly, the proteins were denatured at a solution pH of approximately 12.5, where we assume that the denatured GFP chromophore has a peak extinction coefficient of 44,000 M^{-1} cm^{-1} at 447 nm) [4]. We used this approach to determine the absolute protein concentration. Comparison with an unirradiated solution at pH 11 then allowed us to determine $\epsilon^{\text{C-}}_{490}$, while for the trans species we diluted a solution irradiated with 488 nm light into pH 6.5, resulting in a value for $\epsilon^{\text{TH}}_{390}$.

Determining the extinction coefficients of CH and T^- is less straightforward due to the low pKa of the cis and the high pKa of the trans state. Accordingly, we determined these from pH titration data, since plotting A_{490} and A_{390} against pH shows clear interconversion of the protonated and deprotonated species (Supplementary Figure S7). For the cis species, and entirely analogous for the trans species, it must be the case that

$$\frac{d[\text{C}^-]}{d\,\text{pH}} = -\frac{d[\text{CH}]}{d\,\text{pH}} \tag{S.24}$$

Combination with Lambert-Beer's law then leads to

$$\frac{dA^{490}}{d\,\text{pH}} = s_{490} = \epsilon^{\text{C-}}_{490} l \frac{d[\text{C}^-]}{d\,\text{pH}} + \epsilon^{\text{CH}}_{490} l \frac{d[\text{CH}]}{d\,\text{pH}} \tag{S.25}$$

$$\frac{dA^{390}}{d\,\text{pH}} = s_{390} = \epsilon^{\text{C-}}_{390} l \frac{d[\text{C}^-]}{d\,\text{pH}} + \epsilon^{\text{CH}}_{390} l \frac{d[\text{CH}]}{d\,\text{pH}} \tag{S.26}$$

where we denote the slope of the absorbance as s and l is the length of the optical path. Combining these equations shows that

$$\frac{s_{490}}{s_{390}} = \frac{\epsilon^{\text{C-}}_{490} - \epsilon^{\text{CH}}_{490}}{\epsilon^{\text{C-}}_{390} - \epsilon^{\text{CH}}_{390}} \tag{S.27}$$

and an entirely analogous expression for TH and T^-.

To render the analysis solvable, we now assume that $\epsilon^{\text{CH}}_{490}$ and $\epsilon^{\text{TH}}_{490}$ are negligible (Supplementary Figure S4). The sought-after extinction coefficients can then be determined via

$$\epsilon^{\text{CH}}_{390} = \epsilon^{\text{C-}}_{390} - \frac{s_{390}}{s_{490}} \epsilon^{\text{C-}}_{490} \tag{S.28}$$

$$\epsilon^{\text{T-}}_{490} = \frac{s_{490}}{s_{390}} \left(\epsilon^{\text{T-}}_{390} - \epsilon^{\text{TH}}_{390} \right) \tag{S.29}$$

To determine the slopes of the pH titration measurement, we fit the 390 and 490 nm absorbances with a sigmoidal fit function of the form

$$A = A_0 + \frac{A_\mathrm{m}}{1 + \exp\left(\frac{\mathrm{pKa-pH}}{rate}\right)} \tag{S.30}$$

with A_0 and A_m the absorbance at the base and the max, respectively. Taking the derivative of this equation shows that the slope s at the inflection point (pH = pKa) is given by

$$s = \frac{A_m}{4rate} \tag{S.31}$$

The sigmoidal fit can be used to further expand equation (S.28). At high pH, the protein is exclusively in the deprotonated state, and therefore

$$\frac{A_0^{390} + A_m^{390}}{\epsilon_{390}^{\text{C-}}} = \frac{A_0^{490} + A_m^{490}}{\epsilon_{490}^{\text{C-}}} \tag{S.32}$$

$$\epsilon_{390}^{\text{C-}} = \frac{A_0^{390} + A_m^{390}}{A_0^{490} + A_m^{490}} \epsilon_{490}^{\text{C-}} \tag{S.33}$$

since $A_0 + A_m$ is the absorbance in the limit of high pH (but in the absence of denaturation). Substitution into equation (S.28) shows that

$$\epsilon_{390}^{\text{CH}} = \left(\frac{A_0^{390} + A_m^{390}}{A_0^{490} + A_m^{490}} - \frac{s_{390}}{s_{490}}\right) \epsilon_{490}^{\text{C-}} \tag{S.34}$$

which is the equation that we used to determine this extinction coefficient.

Our derivation does not make any assumptions about the nature of C^- and T^-, and therefore an entirely analogous relationship to equation (S.33) also exists for T^-, by simply replacing C^- with T^-. Substitution of this equation into (S.29) and rearrangement leads to

$$\epsilon_{490}^{\text{T-}} = \left(\frac{A_0^{390} + A_m^{390}}{A_0^{490} + A_m^{490}} - \frac{s_{390}}{s_{490}}\right)^{-1} \epsilon_{390}^{\text{TH}} \tag{S.35}$$

Equation (S.35) is the inverse of equation (S.34), as is to be expected since there are no assumptions specific to the cis or trans state in this derivation. Given knowledge of the extinction coefficients at the peak absorption wavelengths, we then determined the extinction coefficients at the laser illumination wavelengths (405 and 488 nm) by scaling these to the observed absorption spectra of the species involved.

Photochromism measurements

The photochromism measurements were performed in cuvette using a previously described setup [3]. Briefly, the solutions were irradiated with a 488 nm laser light at 15, 81 or 145 mW (OxLasers 488 nm 200 mW) at the top of the cuvette and emission and/or absorption spectra were measured every few seconds. 405 nm irradiation was performed similarly using light at 0.8, 3.5 or 7 mW (Coherent Cube 404 nm 100mW). Off-switching was achieved with several irradiation steps of 2 s (except 1.5 s for rsGreenF and 5 s for SkylanS) with a 488 nm laser at 145 mW and the on-switching with 5 s irradiation steps (except 3 s for rsGreen1 and rsGreenF) of the 405 nm laser. Fluorescence timetraces were generated by integrating the observed emission spectra, smoothing the traces in time using binomial smoothing, and normalizing these to 1 at $t = 0$. The raw acquired data and procedures used to process these are available on Zenodo at `https://doi.org/10.5281/zenodo.8041934`.

Analysis of the time-resolved fluorescence traces

The obtained photochromism traces (Figure 3 and Figure S6) were then fitted to the analytical model. Briefly, we fitted each off- and on-switching step as well as the thermal relaxation with single-exponential functions, providing a value for the decay time τ and plateau y_0. For on- and off-switching, these values were averaged over the repeated switching events in each sample (three off-switching events with 488 nm and two on-switching events with 405 nm). We then used these values to obtain k_ct and k_tc using equations (S.21) and

(S.22). To compensate for thermal relaxation, the value of k_{tc}^{therm} obtained via single-exponential fitting of the dark recovery was subtracted from k_{tc}^{405} and k_{tc}^{488} for each measured pH value. We then fitted the k_{ct} and k_{tc} values measured at all pH values using equations (S.10) and (S.11) (setting $k_{tc}^{H,therm}$ to zero since thermal relaxation was already compensated for in the previous step). In performing this analysis, we noted that at high pH the k_{tc} values were close to zero, reflecting that the 488 nm induced switching was too slow to efficiently compete with thermal relaxation (Figure 4 and Figure S6). Accordingly, we repeated these measurements with higher intensities of the 488 nm irradiation.

The obtained values of k_{ct} could be reliably fitted and also recovered the pKa of the cis state expected from the pH titration described previously (Figure 4 and Supplementary Figure S5). The fitting of the k_{tc} values was more difficult due to the (very) high pKa of the trans states (Figure S5). We also observed different behaviors in the values of k_{tc} resulting from 405 nm irradiation. rsGreen1, rsFastLime, ffDronpaF, and SkylanS show a higher value at lower pH that transitions to a lower plateau level with an apparent pKa close to that of the cis state. In GMarsQ, in contrast, the transition is shifted to higher pH and the plateau is located at a higher level. Finally, rsGreenF does display the expected behavior with k_{tc}^{405} being at a plateau and then showing a transition to a lower value at a pH close to the pKa of the trans chromophore. Our data does not allow us to unambiguously explain these different behaviours, which may arise if only a pH-dependent subset of the TH state is reactivated, if residual absorption of the 405 nm light by the C^- state occurs, or if the protonation of other residues in the protein affects the on-switching. Accordingly, the value of the low-pH plateau was used to determine the value of k_{tc}^{H}.

We then used the obtained rate constants (k_{ct}^{H}, k_{ct}^{-H}, k_{tc}^{H}, and k_{tc}^{-H}) to estimate the quantum efficiencies of the four light-induced transitions, as is described in the materials and methods. The resulting values are shown in Table 1. Of the 6 proteins studied, an incomplete set of quantum efficiencies have previously been published only for rsGreen1 (0.14 for TH to CH and 0.0042 for C^- to T^-) and rsGreenF (0.18 and 0.0087) [5], which is close to the values determined here. To our knowledge, this is the first time that the photoisomerization quantum efficiencies of all four chromophore states have been determined, though only for two of the proteins. The values for the other proteins could not be fully determined since the pKa trans values do not permit significant proton abstraction before pH-induced denaturation occurs.

Determination of the isomerization quantum yields

The rate constant k for an isomerization process can be expressed as

$$k = k_{exc}\phi \tag{S.36}$$

with k_{exc} the excitation rate of the fluorophore and ϕ the quantum efficiency of the process. Likewise we can write that

$$k_{exc} = \sigma I \tag{S.37}$$

with σ the molecular cross section of the fluorophore and I the intensity of the irradiation in units of photons per area per unit time. This expression is valid as long as there is no saturation of the excited state, which is not the case under the conditions used here. σ is given by

$$\sigma = \frac{\ln(10) \cdot \epsilon \cdot 1000}{N_A} \tag{S.38}$$

with ϵ the extinction coefficient at 405 or 488 nm and N_A Avegadro's number. The calculation of the light intensity is in principle straightforwardly done, but is rendered more complex because the laser does not illuminate the cuvette with a flat profile. However, the cuvette is stirred with a mixing time that we estimate to be considerably less than a second in duration. Accordingly, we approximate the apparent irradiation intensity as

$$I = \frac{P\lambda A}{hc} \tag{S.39}$$

with λ the wavelength of the light, c the speed of light, h Planck's constant, and A the surface area of the solution in the cuvette perpendicular to the direction of the laser illumination (in cm^2) and P the power of the laser (in W).

Given these considerations, the isomerization quantum yields were then obtained by inserting the measured rate of isomeration k and the k_{exc} calculated for the relevant extinction coefficient and excitation intensity into equation (S.36), solving for ϕ.

Data availability

The raw data and analysis code have been deposited on Zenodo at `https://doi.org/10.5281/zenodo.8041934`.

Supplementary table and figures

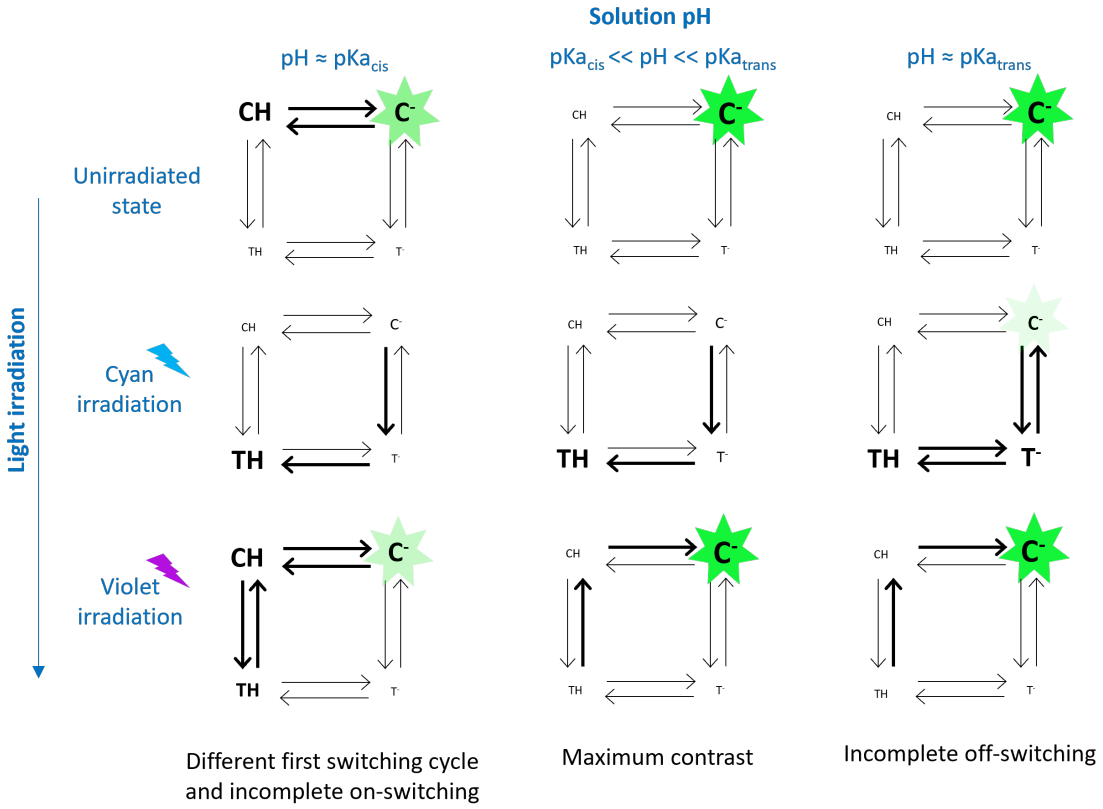


Figure S 1: Graphical explanation of the different initial switching cycles and incomplete photochromism according to our four-state model, starting from unirradiated proteins (the 'initial state'). The font size indicates the occupancy of each state, while the green star indicates the observed fluorescence brightness. When pKa cis ≈ pKa trans, the violet recovery illumination can do no better than to set up an equilibrium between CH and TH, thus preventing full recovery of the fluorescence. When pKa cis ≪ pH ≪ pKa trans, a maximum fluorescence is expected with a complete extinction of the fluorescence with 488 nm irradiation and complete recovery with 405 nm irradiation. However at pH ≈pKa cis, the 488 nm irradiation can do no better than to set up an equilibrium between C^- and T^-, leading to a reduced switching contrast.

References

1. Andresen, M. *et al.* Structural basis for reversible photoswitching in Dronpa. *Proc. Natl. Acad. Sci. USA* **104,** 13005–13009 (32 2007). ppublish.
2. Christou, N. E. *et al.* Disentangling Chromophore States in a Reversibly Switchable Green Fluorescent Protein: Mechanistic Insights from NMR Spectroscopy. *J. Am. Chem. Soc.* **143,** 7521–7530 (2021). ppublish.
3. Moeyaert, B. *et al.* Green-to-Red Photoconvertible Dronpa Mutant for Multimodal Super-resolution Fluorescence Microscopy. *ACS Nano* **8,** 1664–1673 (2014).
4. Bokman, S. H. & Ward, W. W. Renaturation of Aequorea green-fluorescent protein. *Biochem. Biophys. Res. Commun.* **101,** 1372–1380 (1981).
5. Duwé, S. *et al.* Expression-Enhanced Fluorescent Proteins Based on Enhanced Green Fluorescent Protein for Super-resolution Microscopy. *ACS Nano* **9,** 9528–9541 (2015).

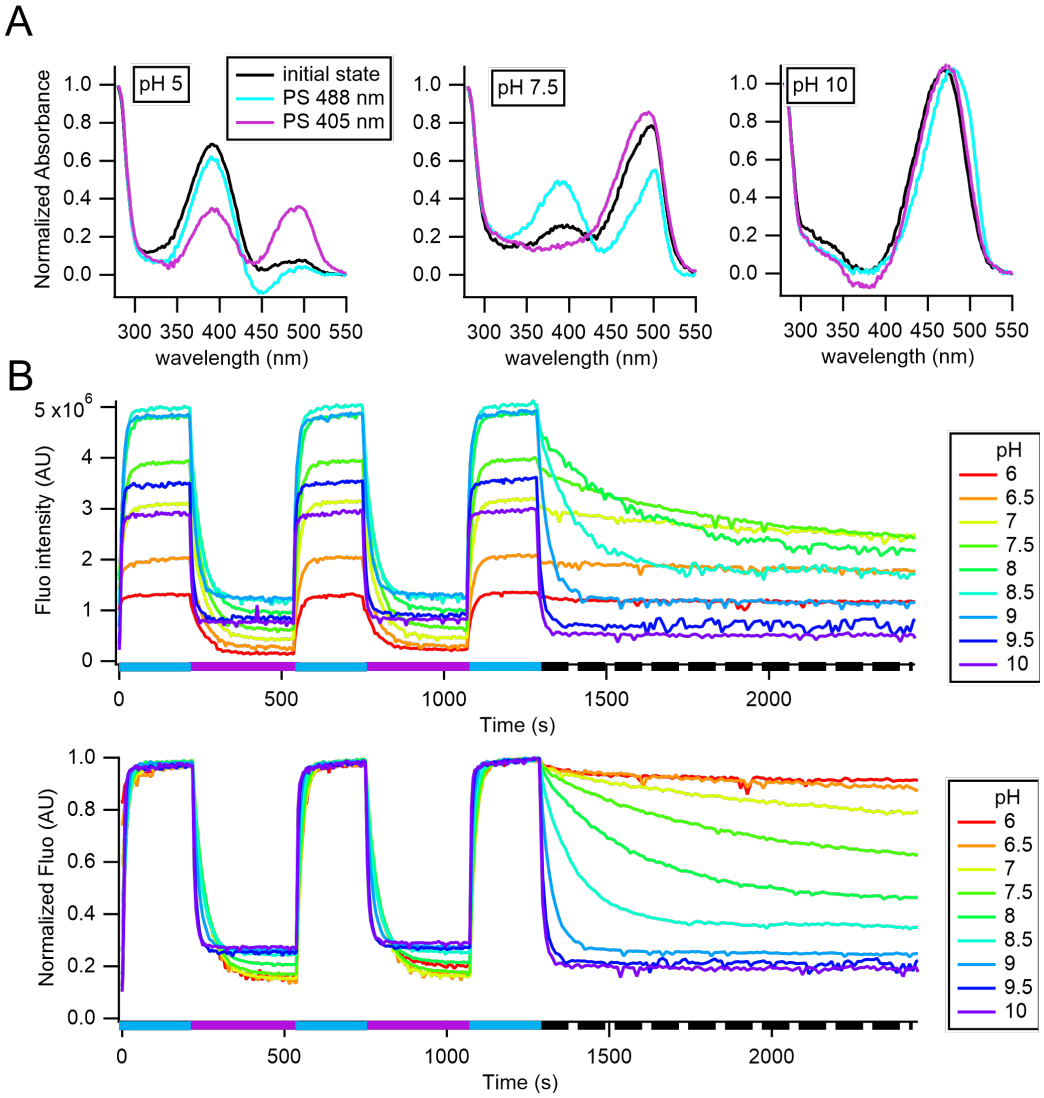


Figure S 2: Photochromism cycles recorded on a green positive switcher, a mutant of Padron (Countdown23). (A) Absorbance spectra of the initial state and of the photostationary states induced irradiation with 488 and 405 nm light at 3 different pHs. (B) Raw and normalized fluorescence intensity traces from multiple photo switching cycles (cyan and violet lines) and thermal recovery (black dashed line) at different pH. The on-switching was achieved with several irradiation steps of 5 s with a 488 nm laser at 145 mW and the off-switching with 5 s irradiation steps of the 405 nm laser at 3.5 mW. TAs expected for positive switchers, the cis pKas of this Padron mutant is smaller than the trans pKa (7 and 5 respectively). At pH 5, proteins are mainly protonated in the cis state after irradiation with the 488 nm laser (absorbance at pH 5, cyan curve) while in the trans state about half of proteins are deprotonated (violet curve). At pH 10, the protein is mostly deprotonated in the cis state and fully deprotonated in the trans state. The two absorption peaks around 490 nm are probably associated to the deprotonated cis and trans states, with the deprotonated cis peak at 500 nm (cyan curve). The superposition of the initial state and PS 405 nm reinforces our suspicion that the proteins are at least partially in a trans state at steady state and not completely in the cis state as assumed for the negative switchers. We thank Maria Ingaramo (Calico Life Sciences LLC, USA) for donating the plasmid for the Padron mutant (countdown23).

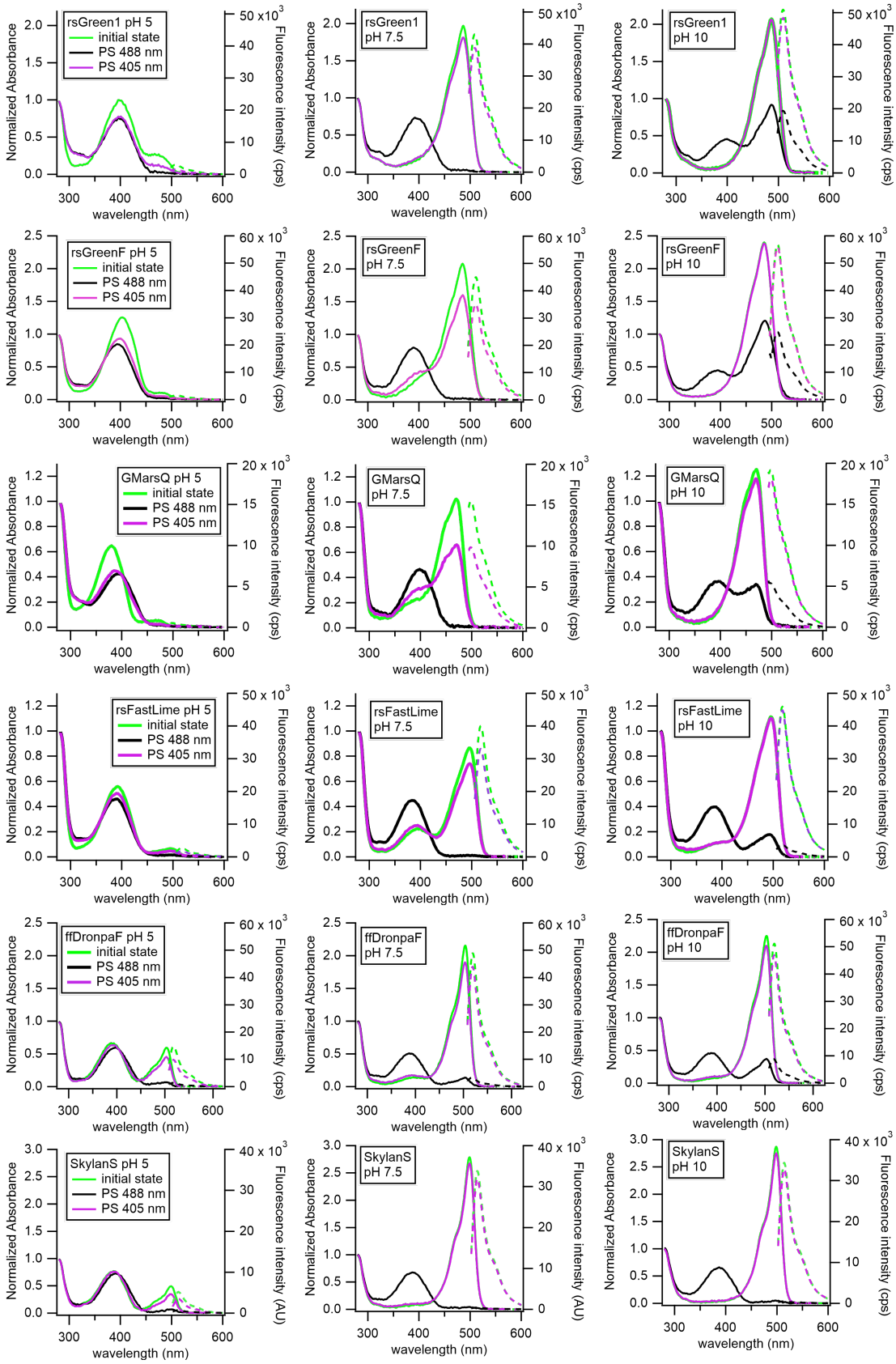


Figure S 3: Absorbance and fluorescence emission (dashed lines) spectra of unirradiated proteins and of the 2 photostationary states induced by laser irradiations at 488 and 405 nm (PS 488 nm and PS 405 nm, respectively), at 3 different pHs.

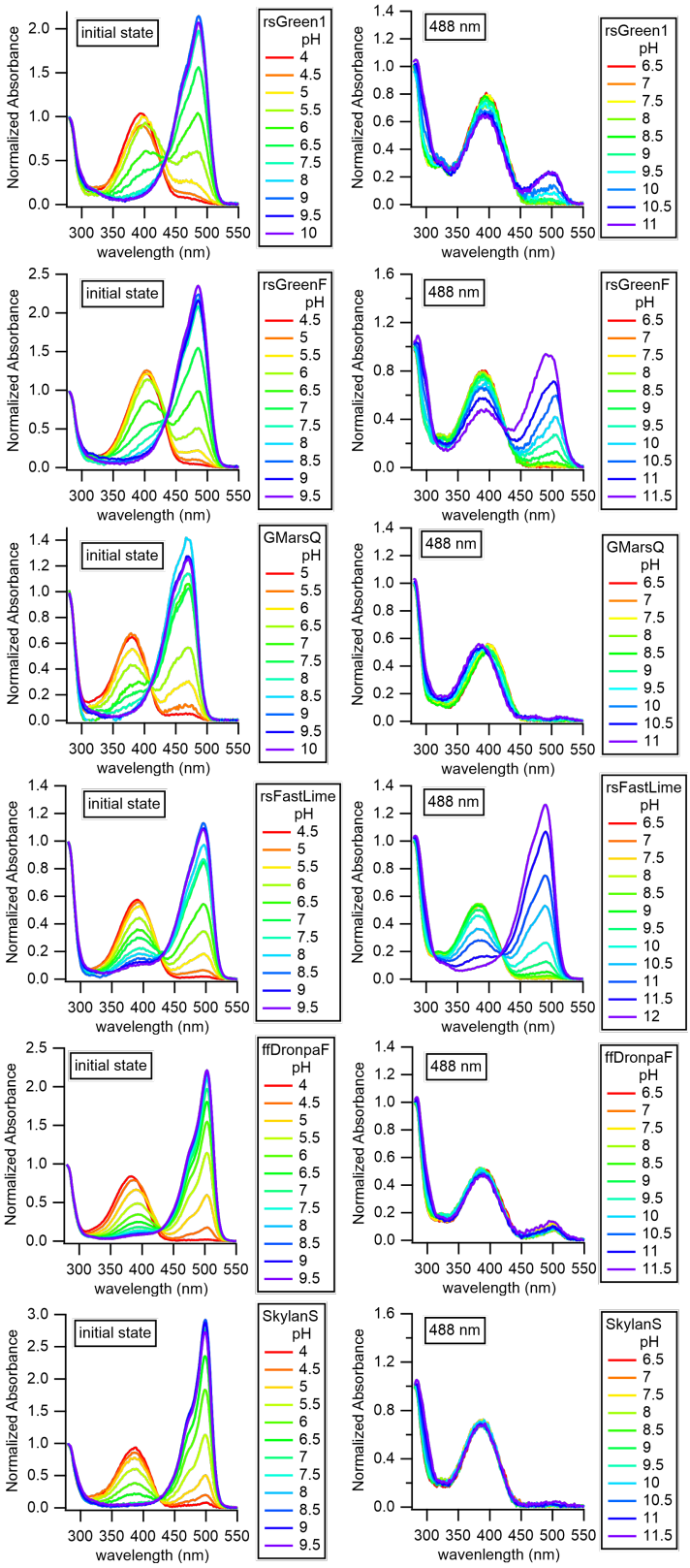


Figure S 4: pKa titration of purified proteins. 'Initial state' refers to unirradiated proteins, '488 nm' means that the sample was irradiated with 488 nm light at pH 7.5 and then diluted in the appropriate buffer. The absorbance spectra are normalized to 280 nm. Traces of the absorbance evolution are shown in Supplementary Figure S7.

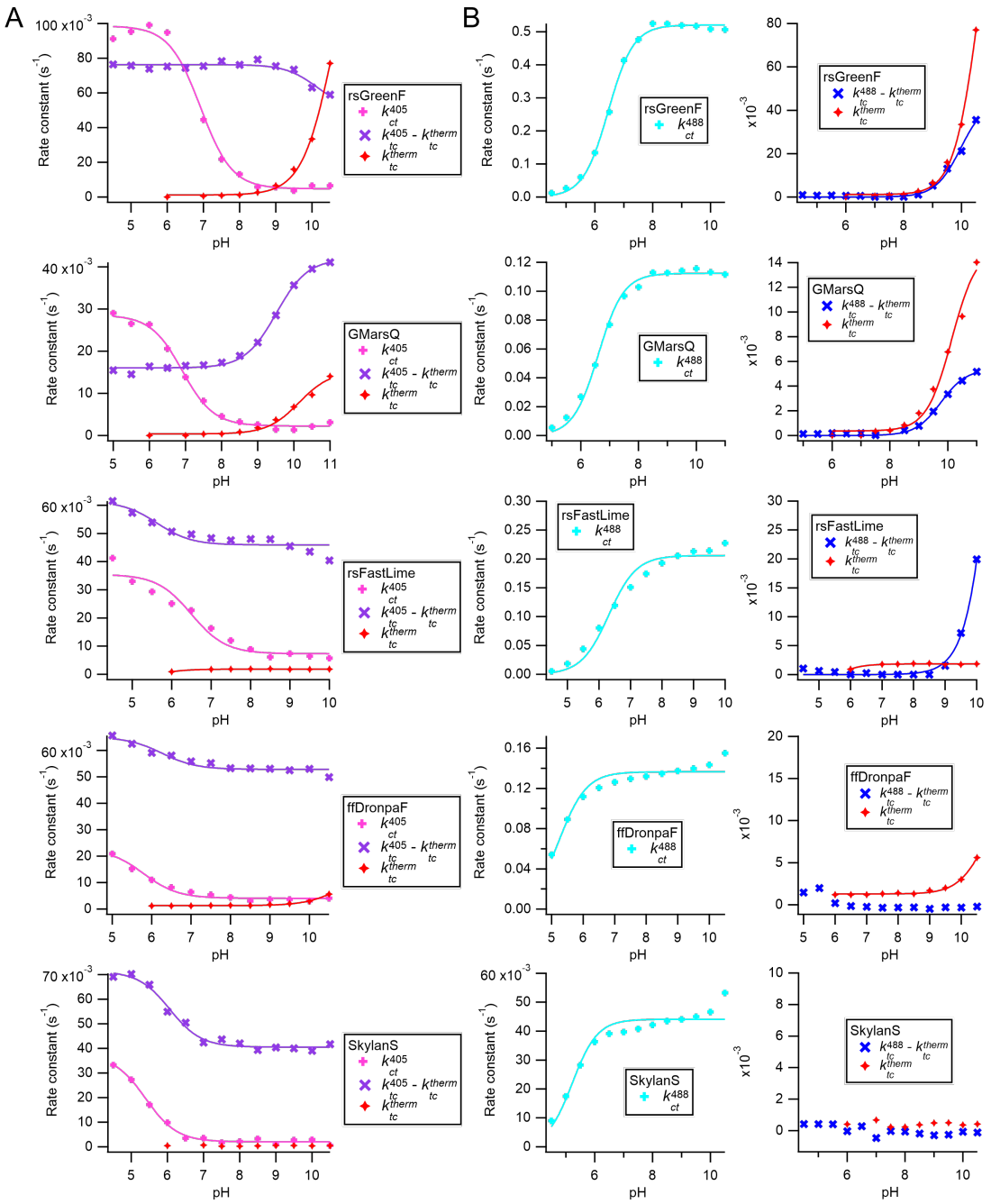
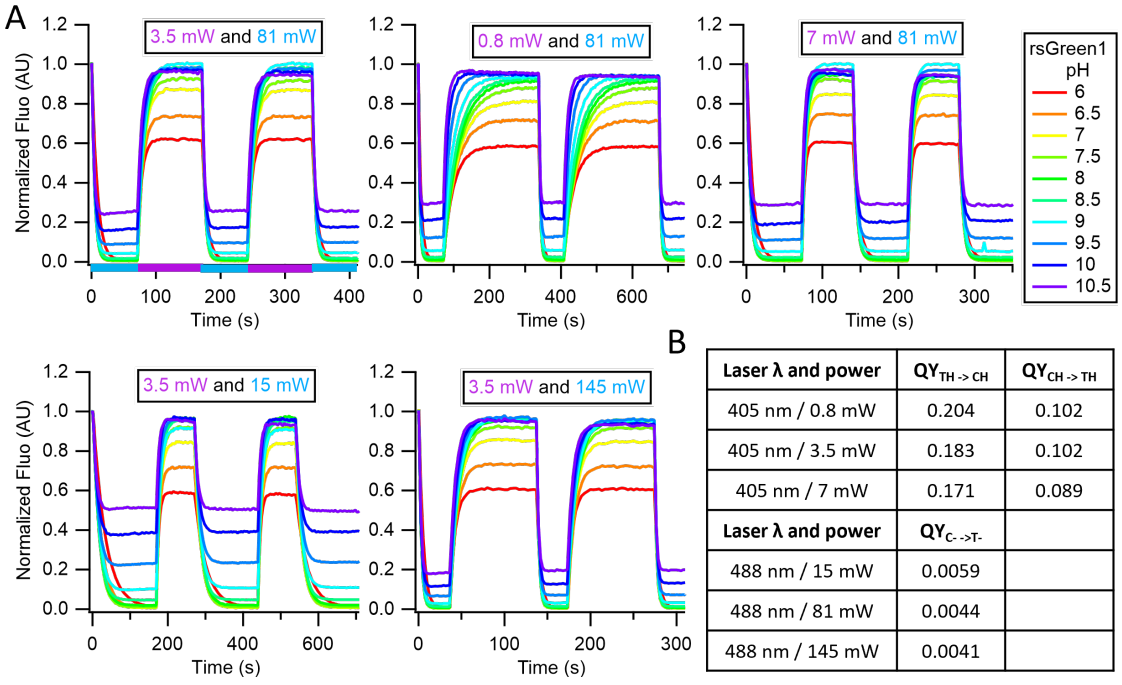


Figure S 5: k_{ct} and k_{tc} values determined for photochromism measurements on the indicated proteins and pH values, measured during (A) on-switching and (B) off-switching. Off-switching was achieved with several irradiation steps of 2 s (except 1.5 s for rsGreenF and 5 s for SkylanS) with a 488 nm laser at 145 mW and the on-switching with 5 s irradiation steps (except 3 s for rsGreen1) of the 405 nm laser at 3.5 mW. Solid lines indicate fits to the data using main text equations (S.10) and (S.11).

	rsGreenF	rsGreen1	GMars-Q	rsFastLime	ffDronpaF	SkylanS
$\lambda_{\text{abs, initial state}}$ (nm) at pH 7.5	485	487	470	394/496	393/503	499
$\lambda_{\text{abs, PS 488 nm}}$ (nm) at pH 7.5	391	394	399	386/503	391/	387/503
$\lambda_{\text{em, max}}$ (nm)	511	509	498	518	520	514
$\epsilon^{C^-}_{\lambda_{\text{abs, anionic}}}$ (10^3 M^{-1} cm^{-1}) at pH 11	72	71	55	55.5	79.5	91
$\epsilon^{TH}_{\lambda_{\text{abs, neutral}}}$ (10^3 M^{-1} cm^{-1}) at pH 6.5	22.5	26	20.5	22	20	23
$\epsilon^{CH}_{\lambda_{\text{abs, neutral}}}$ (10^3 M^{-1} cm^{-1})	43.5	42	26	25	28.5	29
$\epsilon^{T^-}_{\lambda_{\text{abs, anionic}}}$ (10^3 M^{-1} cm^{-1})	49	ND	ND	59.5	ND	ND
$\epsilon^{C^-}_{\lambda_{\text{abs, 488}}}$ (10^3 M^{-1} cm^{-1}) at pH 11	71	71	20	53	54	70.5
$\epsilon^{TH}_{\lambda_{\text{abs, 405}}}$ (10^3 M^{-1} cm^{-1}) at pH 6.5	19.5	24	20	16.5	17	19
$\epsilon^{CH}_{\lambda_{\text{abs, 405}}}$ (10^3 M^{-1} cm^{-1})	43.5	38.5	14.5	21	19	20.5
$\epsilon^{T^-}_{\lambda_{\text{abs, 488}}}$ (10^3 M^{-1} cm^{-1})	43.5	ND	ND	59	ND	ND
pKa cis (K_c)	6.7	6.0	6.5	6.4	5.4	5.6
pKa trans (K_t)	10.2	ND	ND	10.9	ND	ND

Table S 1: Spectroscopic properties of the FPs used in this work. $\lambda_{\text{abs, initial state}}$: absorption peak of the neutral and anionic chromophore at pH 7.5 of the initial state (unirradiated sample). $\lambda_{\text{abs, PS 488 nm}}$: absorption peak of the neutral and anionic chromophore at pH 7.5 after off-switching by 488 nm light (PS 488nm). $\lambda_{\text{em, max}}$: fluorescence emission peak. $\epsilon^{C^-}_{\lambda_{\text{abs, anionic}}}$: extinction coefficient of the deprotonated cis chromophore and $\epsilon^{TH}_{\lambda_{\text{abs, neutral}}}$: extinction coefficient of the protonated trans chromophore calculated with the alkanine denaturation method. $\epsilon^{CH}_{\lambda_{\text{abs, neutral}}}$: extinction coefficient of the protonated cis chromophore and $\epsilon^{T^-}_{\lambda_{\text{abs, anionic}}}$: extinction coefficient of the deprotonated trans chromophore were calculated as described in the Methods section. $\epsilon^{C^-}_{\lambda_{\text{abs, 488}}}$, $\epsilon^{TH}_{\lambda_{\text{abs, 405}}}$, $\epsilon^{CH}_{\lambda_{\text{abs, 405}}}$ and $\epsilon^{T^-}_{\lambda_{\text{abs, 488}}}$ extinction coefficients at the wavelength 405 or 488 nm. ND = not determined due to the very high value of K_t.



Laser λ and power	$QY_{TH \rightarrow CH}$	$QY_{CH \rightarrow TH}$
405 nm / 0.8 mW	0.204	0.102
405 nm / 3.5 mW	0.183	0.102
405 nm / 7 mW	0.171	0.089
Laser λ and power	**$QY_{C- \rightarrow T-}$**	
488 nm / 15 mW	0.0059	
488 nm / 81 mW	0.0044	
488 nm / 145 mW	0.0041	

Figure S 6: Photoswitching curves of rsGreen1 at different laser powers. (A) Normalized fluorescence intensity traces acquired on rsGreen1 for different 488 nm irradiation (2 s irradiation steps at 15, 81 or 145 mW) and 405 nm irradiation (3 s irradiation steps at 0.8, 3.5 and 7 mW). Fits of these curves are displayed in Figure 4 in the main text. (B) 3 out of 4 quantum efficiencies were determined as described in methods section for the different laser powers.

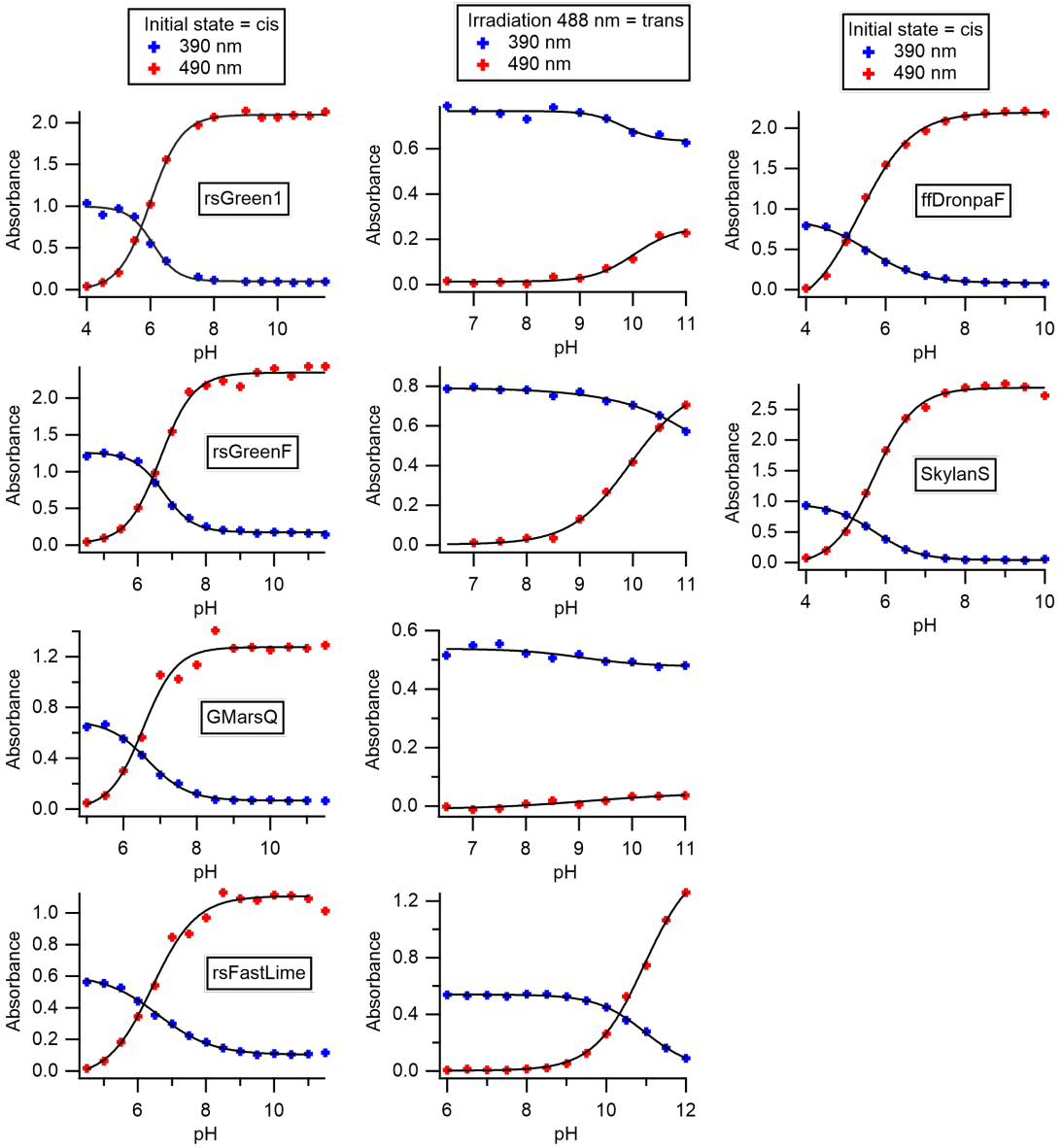


Figure S 7: Extinction coefficient determination of the chromophore states. 'Cis' means that the sample consisted of unirradiated proteins. 'Trans' means that the sample was irradiated with 488 nm light pH 7.4 until fluorescence extinction, then diluted into the appropriate buffer. '390' and '490' nm refer to the maximun absorbance of the protonated and deprotonated species, respectively, because the exact peak wavelengths differ between the different proteins. The solid lines show a fit with a sigmodial function (see methods section).