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pH sensitivity of YFPs is reduced upon AlphaRep binding: proof of concept *in vitro* and in living cells

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Abstract (max 200 words)

Yellow fluorescent proteins (YFPs) are commonly used in biology to track cellular processes, particularly as acceptors in experiments using the F orster Resonant Energy Transfer (FRET) phenomenon. However, their fluorescence intensity is strongly pH-dependent, limiting their utility in acidic environments. Here, we explore the pH sensitivity of YFPs upon binding with an artificial repeat protein (α Rep) both *in vitro* and in living cells. We show that α Rep binds to Citrine, with high affinity in the nanomolar range at physiological and acidic pHs, leading to increased thermal stability of the complex. Moreover, α Rep binding reduces Citrine's pK_a by 0.75 pH units, leading to a decreased sensitivity to pH fluctuations. This effect can be generalized to other YFPs as Venus and EYFP *in vitro*.

An efficient binding of α Rep to Citrine has also been observed in living cells both at pH 7.4 and pH 6. This interaction leads to reduced variations of Citrine fluorescence intensity in response to pH variations in cells.

Overall, the study highlights the potential of α Reps as a tool to modulate the pH sensitivity of YFPs, paving the way for future exploration of biological events in acidic environments by FRET in combination with a pH-insensitive cyan donor.

Keywords

Fluorescent protein, YFP, alphaRep (α Rep), pH sensitivity, FRET, FLIM

Introduction

Fluorescent proteins (FPs) serve as genetically encoded fluorescent reporters and have been invaluable tools in cell biology for more than 25 years, enabling researchers to visualize and track various cellular processes with remarkable spatial and temporal resolution.^[1] Their chromophore is formed by the autocatalytic reaction of three residues embedded in their β -barrel structure at positions 65-66-67. Typically, position 66 is occupied by a tyrosine, and upon chromophore formation, its electron delocalization expands on two additional double bonds leading to a moiety capable of absorbing and fluorescing in the visible. Depending on the pH and its environment in the β -barrel, the chromophore may adopt an anionic or a protonated form, both having different photophysical properties (Figure 1a). Fluorescent proteins with an excitation maximum of 515 ± 5 nm are usually considered yellow fluorescent proteins (YFP). Those YFPs are an advantageous label combined with more blue or red FPs for multicolor labeling and the usual acceptor in combination with a cyan donor in Förster Resonant Energy Transfer (FRET) experiments.^[2] Despite their good brightness (Epsilon above $75\,000 \text{ mol.L}^{-1}.\text{cm}^{-1}$ et QY generally exceeding 0.65)^[3], their absorption and fluorescence strongly depend on pH due to the equilibrium between the anionic and fluorescent form of the chromophore and its protonated but nonfluorescent form.^[1] The pK_a is the pH at which 50% of the protein fluoresces when they are excited in their anionic absorption band. All YFPs derived from *av*GFP have a $\text{pK}_a > 5$ ^[3], even Citrine, one of the best, as far as the pH sensitivity is concerned^[4]. This strongly limits their use in the acidic compartments present in many cell types, such as the granules in the secretion pathway in eukaryotic cells^[5] or the vacuole in plant cells^[6] or in a cytoplasmic context where local proton concentration may be high or fluctuating due to intense enzymatic activity for example. In addition, the pH sensitivity of YFPs impacts their use as a FRET acceptor in acidic conditions: the modification of their absorption band at a low pH decreases the spectral overlap with the donor fluorescent band leading to the apparent decrease of the FRET efficiency independently from the monitoring of any biological event.^[7] Intense engineering has been undertaken to limit and control the sensitivity of YFPs -and, more generally, FPs- to pH fluctuations.^[8] Recently, tdLanYFP, a YFP with a very low $\text{pK}_a = 3.9$ was characterized.^[9] Other strategies rely on the interaction with a small partner protein, such as a nanobody.^[10-12] They modulate the FP properties such as brightness, switching rate between two fluorescent states, and also their pK_a .^[10-12]

In this manuscript, we focus on artificial repeat proteins (α Rep).^[13] Their alpha-helical HEAT-like repeat sequence is coding for a motif of 31 residues containing five highly variable positions. A library of α Reps with diversity in the number of inserted repeats and the sequence at the variable positions was built as a large source of protein binders (>1 billion clones).^[14] Here, we focus on bGFP-C, an α Rep selected to bind specifically *in vitro* and *in cellulo* to EGFP, a FP belonging to the *av*GFP family.^[15] The X-ray structure of this α Rep in interaction with EGFP has been solved (Figure 1b). bGFP-C binds EGFP on the edge of the β -barrel, mainly on strands 7, 8, and 9. The 7th strand (in red Figure 1b) is in front of the phenol moiety of the chromophore and mutations in this strand may impact the biochemical and photophysical behavior of the chromophore.^[12] In particular, this strand is usually chosen to insert a sensing protein in cpFPs to build biosensors,^[16] showing the importance of its residues in modulating the properties of the chromophore itself. In addition, the dissociation constant of the bGFP-C / EGFP complex is in the nM range and bGFP-C displays interactions with similar

affinities for other members of the *av*GFP family such as EYFP and ECFP, which share a very close sequence and protein surface.^[3] bGFP-C is called simply α Rep hereafter.

We hypothesized that the binding of α Rep to YFPs could lead to altered properties that may improve their pK_a for fluorescence imaging in acidic compartments. In this work, we, therefore, set out experiments to examine the effect of α Rep on YFPs. We chose the popular Citrine as a model, and our observations will be generalized *in vitro* to other YFPs from the same family, namely EYFP and Venus (Figure S1). A proof of concept in living cells is presented with Citrine.

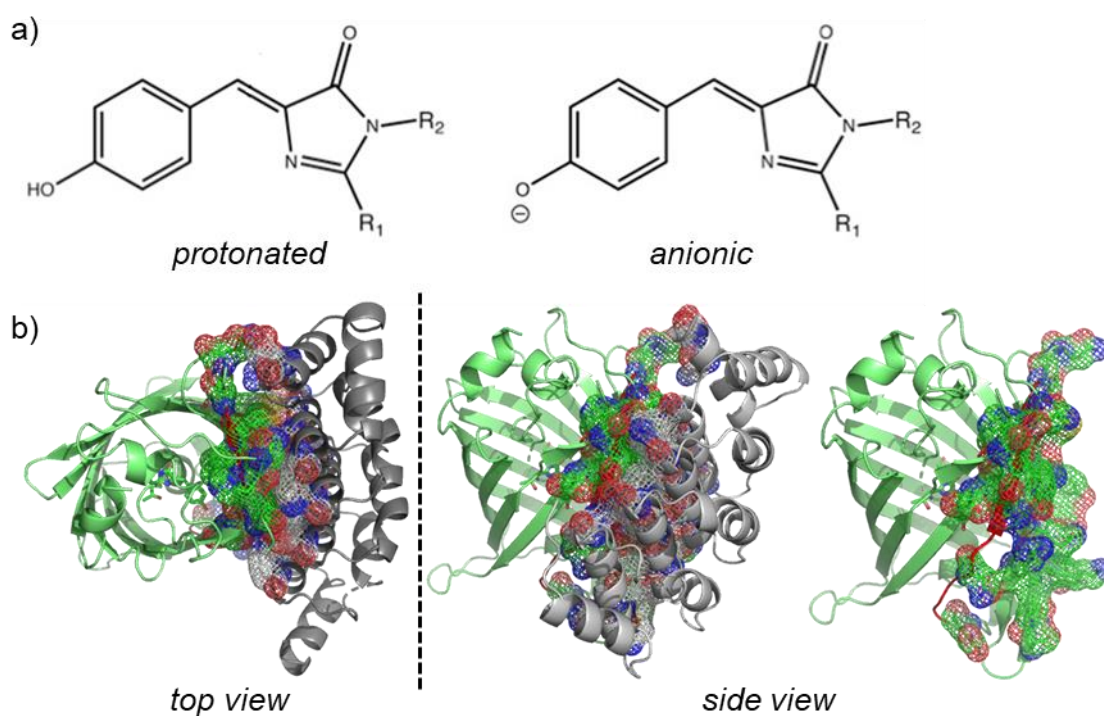


Figure 1: (a) Protonated nonfluorescent and anionic fluorescent chromophores of YFPs. (b) Structure of EGFP (green) - α Rep complex, top and side views (pdb 4XVP). The residues in interaction are represented in a mesh. The 7th strand of the β -barrel, in front of the chromophore, is in red on the side view mostly hidden by residues in interaction.

Experimental section

Plasmids

EYFP, Citrine, and Venus refer to three yellow fluorescent proteins (YFPs).^[3] For bacterial expression, YFPs were cloned in pProExHTa. α Rep variants bGFPC-GFP (called **α Rep**)^[15] and bNCS324-16 (called **control α Rep**)^[14] genes were subcloned in pQE81L vectors (Qiagen). All proteins are produced as His-tagged proteins. For eukaryotic expression, pECitrine-N1 (Clontech) was used. α Rep was subcloned in a modified pmCherry-N1 vector (Clontech).^[15] All the protein sequences are in Figure S1.

Protein expression and purification

The production and the purification of His-tagged recombinant YFPs were performed as previously described.^[17] The elution buffer is replaced by a buffer composed of a mixture of 2 mM CAPS, 2 mM MES, and 2 mM Bis–tris propane, pH 7.4. As the values of the epsilons for FPs vary from one publication to the other in a 10–20% range,^[3,9,18] we used an average epsilon of 80 000 mol⁻¹.L.cm⁻¹ at the maximum of the chromophore's absorption spectrum to determine the concentration of all YFPs before each experiment when needed.

The production and the purification of His-tagged recombinant α Rep were adapted from a previous work.^[15] In the first step, the plasmid coding for each protein was transformed into the expression strain (*E.coli* M15). An overnight pre-culture of a clone was grown at 37°C in 50 mL of 2YT medium containing 100 μ g/mL ampicillin and 1% of glucose. For expression, 25 mL of bacteria were grown at 37°C in 1 L of 2YT medium containing 100 μ g/mL ampicillin, at 200 rpm, up to an OD of 0.6 at 600 nm. The addition of 0.5 mM IPTG induced protein expression. The cells were further incubated for four hours at 37°C. Then the culture was centrifuged for 30 minutes at 5000 g, 4°C. The bacterial pellet was harvested, resuspended in 20 mL of a buffer containing antiprotease tablet (cComplete Protease Inhibitor Cocktail, Roche), Tris 20 mM, and NaCl 200 mM at pH 7.5 and frozen at -80°C for several days. The bacterial pellet was resuspended in buffer (40 mL, Tris 20 mM, NaCl 200 mM, pH 7.5, DNase 0.5 mg/mL) and lysed with a cell disruptor (Constant Systems Ltd). The cell debris was removed by centrifugation for 30 minutes at 10 000 g, 4°C. The supernatant was filtered and then applied to a chromatography column containing nickel-nitriloacetic acid (Ni–NTA) agarose beads (His Trap FF crude, GE Healthcare) for 1 hour. The protein was eluted with a phosphate buffer at pH 8 containing 300 mM NaCl and 300 mM imidazole. This purification was further carried out with size exclusion chromatography (Hiload 16/600 SuperdexTM 75 Cytivia) using a buffer composed of Tris 10 mM, NaCl 150 mM at pH 7.5. SDS-PAGE of the purified protein shows a single band between 15 kDa and 18 kDa for bGFPC and bNCS324-16.

Differential Scanning Calorimetry and Isothermal Titration Calorimetry

For differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC), proteins have been prepared in buffers containing the same mixture of CAPS 33 mM, MES 33 mM, Bis–tris propane 33 mM, and NaCl 50 mM adjusted at pH 7.4 or pH 5.5. To avoid any aggregation of Citrine at pH 5.5, a pH close to the pI, four short-time dialysis (two hours) of the stock solution (concentration ~350 μ M) at pH 5.5 were preferred instead of a single one

overnight. The α Rep followed the same process. At pH 7.4, two long-time dialyses were performed. The final pH of the protein solutions and their concentration were accurately monitored just before each experiment.

Thermal stability of Citrine at 20 μ M, α Rep at 20 μ M and complex Citrine - α Rep at 1:1 molar ratio (20 μ M:20 μ M) was performed by DSC on an auto PEAQ DSC (Malvern Panalytical, France). Each measurement was completed by a baseline scan with the sample buffer.

Scans were performed at 1°C min⁻¹ between 35 and 120°C. The heat capacity of the buffer was subtracted from that of the protein sample before analysis. Analysis of the raw data was performed using the Microcal PEAQ-DSC software v1.63. T_m is the temperature at which half of the protein is denaturated.

The binding parameters were assessed at 25°C with a MicroCal PEAQ-ITC instrument (Malvern Panalytical, France). Aliquots (2 μ L) of Citrine at high concentration were placed in the 40 μ L injection syringe and were injected every 180 s into a 20 μ M α Rep solution (cell volume, 0.24 mL), under a stirring speed of 500rpm, using the Microcal PEAQ-ITC Control software v.1.41. Citrine's concentrations were 320 μ M at pH 7.4 and 230 μ M at pH 5.7. Analysis of the raw data was performed using the Microcal PEAQ-ITC analysis software v.1.41 according to the one set of sites binding model.

Absorption and fluorescence spectroscopies and pK_a determination

The spectra were recorded with a SynergyH1 plate reader (Biotek) in 96-transparent-well plates and spectra were processed as previously described.^[7] Absorption spectra were recorded from 350 nm to 560 nm and fluorescence emission spectra from 515 nm to 670 nm upon excitation at 480 nm. Fluorescence lifetimes were recorded on a TCSPC setup described elsewhere,^[19] with an excitation at 515 \pm 10 nm and an emission at 535 \pm 3 nm. The decays were fitted with SymPhoTime software with a single exponential. For pH levels ranging from pH 11 to pH 5.5, buffer solutions contained 33 mM CAPS, 33 mM MES, and 33 mM Bis-tris propane and were adjusted to the appropriate pH by the addition of H₂SO₄ or NaOH. For pH levels ranging from 5.5 to 2.5, buffer solutions consisted of 50 mM citric acid with an appropriate volume of Na₂HPO₄ to adjust the pH. In those buffers, the residual chloride concentrations were below 20 mM depending on the final concentration of α Rep. The molar ratio YFP: α Rep in the solution is 1:1. Aliquots from a concentrated stock protein solution were diluted into the different buffers at least 12 h before measurements. After a first series of measurements at low chloride concentration, a solution at 6 M NaCl was added to adjust the [Cl⁻] concentration to 200 mM. For the determination of pK_a, the variation of the absorbance with the pH was fitted with IgorProV6 Software (Wavemetrics) according to the following equation (1):

$$A(pH) = \frac{A_{max}^{Basic} + A_{min}^{Acid} 10^{n_{Hill} \times (pK_a - pH)}}{1 + 10^{n_{Hill} \times (pK_a - pH)}} \quad (1)$$

Live cell imaging and cytometry

Live cell experiments were performed in COS7 cells transiently transfected with XTremeGene HT (ThermoFisher) following the manufacturer's protocol. The cytosolic pH of COS7 cells was modified by a method based on the K⁺/H⁺ ionophore nigericin (final concentration 15 μ M) in

the presence of a buffer (HEPES 15 mM and MES 15 mM) at pH 8 to pH 5.5 containing external potassium ions (140 mM KCl). FLIM experiments were performed on a homemade TCSPC setup described previously.^[20] Citrine was excited at 466 nm with a pulsed diode (Picoquant) and its fluorescence was detected with a 535/40 nm bandpass filter. SymPhoTime64 software (Picoquant) was used for data acquisition and processing. The fluorescence intensity of α Rep-mCherry was monitored with a CCD camera (Orca, Hamamatsu) with a Chroma filter set for RFPs (ET580/25x for excitation, ET625/30m for emission and ZT594rdc for the dichroic mirror). FastFLIM lifetime images and histograms were calculated for all pixels above 500 cps by SymPhoTime64 software. For each cell, a single TCSPC fluorescence decay gathers all the photons collected from cytosolic Citrine. For cells expressing Citrine alone, the decays are fitted with a mono-exponential fit function (2) to compute the lifetime of Citrine alone, $\tau_{Citrine}$:

$$I(t) = Background + I_0 e^{-\frac{t}{\tau_{Citrine}}} \quad (2)$$

For cells expressing both Citrine and α Rep-mCherry, bi-exponential fit function (3) was used:

$$I(t) = Background + I_0 \times (\alpha_1 e^{-\frac{t}{\tau_1}} + \alpha_2 e^{-\frac{t}{\tau_2}}) \quad (3)$$

The average lifetime is computed as $\langle \tau_{Citrine \text{ with } \alpha Rep - mCherry} \rangle = \alpha_1 \tau_1 + \alpha_2 \tau_2$. The quality of the fit is evaluated by the weighted residual distribution and Pearson's χ^2 test. The apparent FRET efficiency is then calculated with equation (4) for each cell:

$$E_{FRET}^{Apparent} = 1 - \frac{\langle \tau_{Citrine \text{ with } \alpha Rep - mCherry} \rangle}{\tau_{Citrine}} \quad (4)$$

For wide-field fluorescence microscopy and flow cytometry experiments, cells were transfected either with plasmids coding for Citrine or for both Citrine and α Rep-mCherry with a 1:3 DNA ratio. For wide-field microscopy, cells were observed successively with Citrine and mCherry filters (filters YFP2427B Semrock for Citrine and filters ET580/25X and ET625/30 Semrock for mCherry) at pH 7.4 and 15 min after the addition of an excess of buffer at pH 5.5 in the presence of 15 μ M nigericin, leading to a final pH below pH6. For flow cytometry, cells were trypsinized 24 h after transfection. 5 min before the analysis, the intracellular pH was modified with a buffer at the appropriate pH in the presence of nigericin. Flow-cytometry measurements were done on a Cytoflex S instrument with the CytExpert software (Beckman Coulter), using a 488 nm laser excitation and a 525/40 BP emission filter for Citrine and a 561 nm laser excitation and a 610/20 BP emission filter for mCherry. The data processing was performed using FloJow software (BDbiosciences). Living cells were identified with the Forward Scatter (FCS) and Side Scatter (SSC) channels and fluorescent cells in both, Citrine and mCherry, channels were chosen to calculate the averaged Citrine fluorescence intensity.

Results

α Rep binds Citrine at physiological and acidic pHs

DSC experiments were first performed to probe the Citrine - α Rep interaction at physiological and acidic pHs (Figure 2, Table 1). The melting temperature (T_m) of the heating thermogram of Citrine alone slightly decreases from 78°C at pH 7.4 to 64°C at pH 5.5 showing that Citrine is a less structured protein at low pH. On the other hand, the T_m of α Rep increases from 83°C to 88°C from pH 7.4 to pH 5.5 indicating a slightly higher stability at the lower pH value. At pH 7.4, the equimolar mixture of Citrine and α Rep presents a single transition centered at 83°C associated with an increased ΔH of 386 kcal.mol⁻¹. The shift of the mixture thermogram compared to one of Citrine alone is consistent with the formation of a stabilized complex between both proteins. At pH 5.5, two principal peaks, at $T_{m1} = 74^\circ\text{C}$ and $T_{m2} = 88^\circ\text{C}$, are observed for the mixture that starts to melt around 50°C, a temperature lower than that at pH 7.4. The interaction between Citrine and α Rep is thus less efficient at lower pHs. Nevertheless, T_{m1} is still higher than the one for Citrine alone: the interaction of α Rep with Citrine leads to a stabilized structure, also at low pHs, down to pH 5.5. T_{m2} is likely the melting temperature of free α Rep. The shoulder at 65°C could be attributed to a small fraction of free Citrine at the lower pH.

	Physiological pH			Acidic pH		
	$T_m / ^\circ\text{C}$	$\Delta H / \text{kcal.mol}^{-1}$	K_D / nM	$T_m / ^\circ\text{C}$	$\Delta H / \text{kcal.mol}^{-1}$	K_D / nM
Citrine	78	159		64	160	
α Rep	83	157		88	165	
Citrine - α Rep	83	386	48 ± 7	(1) 74 (2) 88	(1) 253 (2) 173	137 ± 9

Table 1: Melting temperatures (T_m) and variations of melting enthalpy (ΔH) measured by DLS, and K_D measured by ITC of Citrine, α Rep, and Citrine – α Rep complex at physiological and acidic pHs. For acidic pHs, T_m and ΔH were estimated at pH 5.5 and K_D at pH 5.7.

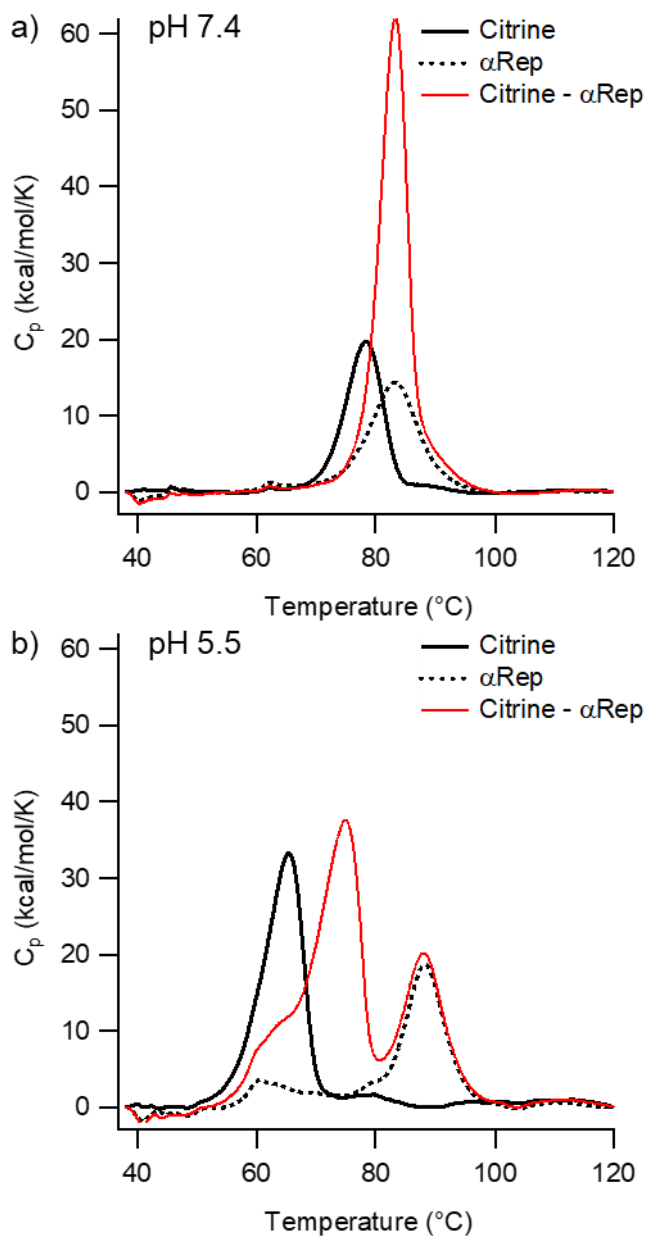


Figure 2: Heating thermograms of Citrine, α Rep, and an equimolar mixture of Citrine and α Rep at pH 7.4 (a) and pH 5.5 (b), proteins concentration: 20 μ M.

To confirm those first observations, ITC experiments were performed to determine the affinity parameters of Citrine for α Rep in physiological (pH 7.4) and acidic conditions (pH 5.7) (Figure 3). The dissociation constants are in the nanomolar range, with K_D values of 48 ± 7 nM and 137 ± 9 nM and stoichiometry values (n) of 1.3 and 0.8 at pH 7.4 and pH 5.7 respectively. A clear exothermic interaction with the formation of nearly equimolar complexes was observed at both pHs.

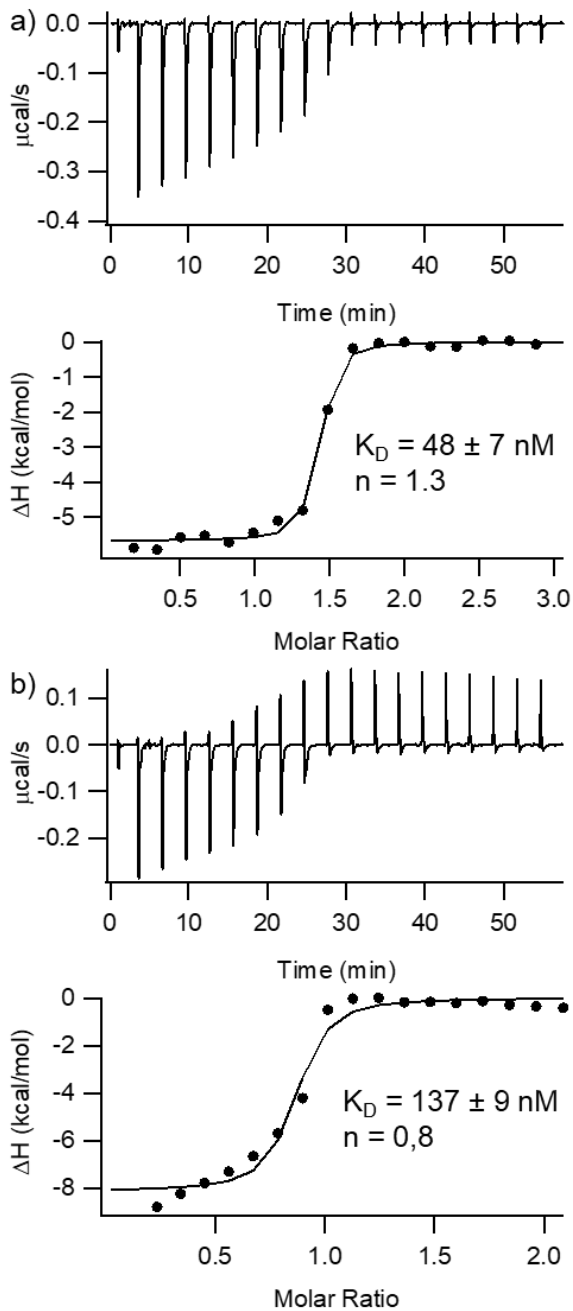


Figure 3: Isothermal calorimetric titration (ITC) of α Rep (20 μ M), with Citrine at 320 μ M or at 230 μ M for pH 7.4 (a) and pH 5.7 (b) respectively.

At physiological pH, the results are very close to previous measurements with another fluorescent protein, the EGFP ($K_D = 19 \pm 12$ nM, $n = 1.2$, Figure 3a).^[15] This consistency is expected as EGFP and Citrine differ by only six residues in positions 64, 65, 68, 69, 72, and 203,^[3] none of those were found in interaction with the α Rep, most of them being located or pointing in the barrel (Figure S1). The specific interaction was also confirmed using two negative controls. In the first one, the Citrine was replaced by mCherry, a fluorescent protein that shares the same β -barrel fold as Citrine but with a completely different residue sequence and protein surface (Figure S1). In the second one, the α Rep binder used in this study has been exchanged for another binder selected against another protein target (Figure S1).^[14] In both

cases, no binding was observed by ITC (Figure S2). At acidic pH, an interaction between Citrine and α Rep is observed with a K_D value still in the nM range ($K_D = 137 \pm 9$ nM, $n = 0.8$, Figure 3b), but 3 - 4 times higher than at physiological pH showing that the interaction is slightly less efficient.

The interaction Citrine - α Rep reduces the pH sensitivity of Citrine

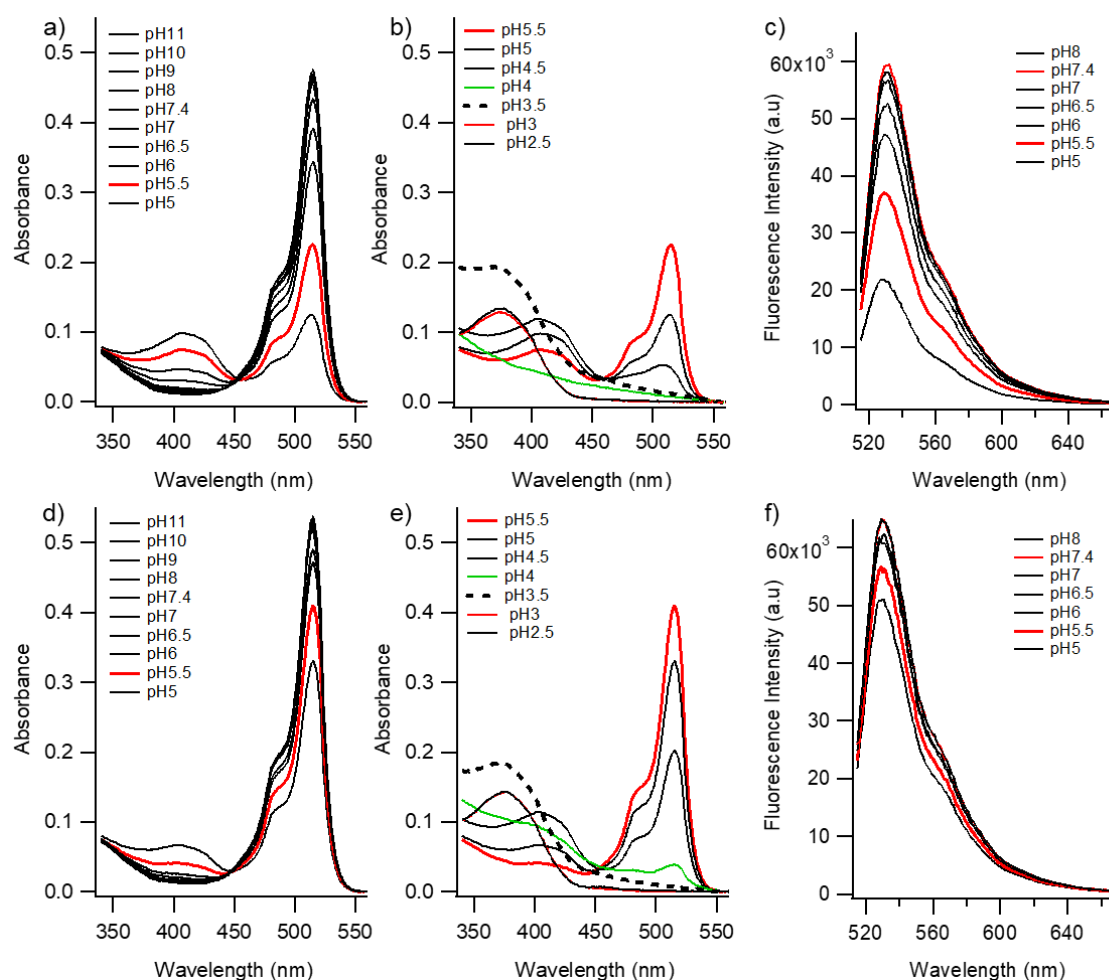


Figure 4: Absorption spectra from pH 2.5 to pH 5.5 (a and d) and from pH 5 to pH 11 (b and e) and fluorescence emission spectra upon excitation at 480 nm from pH 5 to 8 (c and e) for Citrine at 10 μ M (first row) and an equimolar mixture of Citrine and α Rep, 10 μ M each (second row). Data set of a representative experiment.

Absorption and fluorescence emission spectra of Citrine were recorded with and without α Rep in a range of pH from 2.5 to 11 (Figure 4). When the pH of the solution is reduced, the decrease in the absorption band of the anionic chromophore at 515 nm is concomitant with the increase in one of the protonated chromophore at 410 nm (Figure 4a and b). At pHs around pH 4.5, the baseline tends to increase in the UV, as the consequence of a strong diffusion likely due to aggregation at a pH slightly lower than the isoelectric point of the Citrine ($pI = 5.5$). If the pH is even lowered, a single band at 380 nm, characteristic of the denatured form of the fluorescent protein, remains (Figure 4b). In the presence of α Rep, the evolution of the spectra is similar but shifted at lower pHs (Figure 4d and e). Upon excitation of the anionic chromophore at 480 nm,

the amplitude of the fluorescence emission spectra follows the same evolution as the absorption spectra without or with α Rep (Figure 4c and f). We did not observe changes in the shape or the position of the spectra (Figure S3). In addition, absorbance and fluorescence intensity at the maximum of the chromophore's absorption and emission bands vary similarly with the pH showing that the change in fluorescence emission is mostly due to the decrease of the proportion of the fluorescent anionic chromophore in the solution (Figure 5a). The variations of the absorbance of the anionic chromophore with pH will be used for quantitative analysis (see below). The fluorescence lifetime of the Citrine's excited state has also been monitored at pH 7.4 and pH 5.5 (Figure S4). Without α Rep, it remains constant at 3.47 ± 0.03 ns at both pHs. In the presence of two equivalents of α Rep, it decreases from 3.65 ± 0.03 ns at pH 7.4 to 3.50 ± 0.03 ns at pH 5.5 (Figure S4).

From a quantitative point of view, for each condition, the pK_a , was calculated from the experimental data using a titration model (Figure 5b and c). Anions such as chloride can bind to the chromophore pocket of YFPs, influencing the equilibrium between the anionic fluorescent and protonated nonfluorescent forms of the chromophore. In most of the experiments, the chloride concentration was kept below 20 mM and the pK_a for Citrine alone ($pK_a = 5.50 \pm 0.03$) is close to the ones of the literature without chloride.^[9] The interaction of Citrine with α Rep decreases its pK_a by 0.75 pH unit. In the presence of 200 mM chloride, as expected, the pK_a for Citrine alone is increased (from 5.50 to 5.70) as the one for the complex (from 4.75 to 5.25) Citrine – α Rep that remains nevertheless still lower. As a control, the α Rep binder is replaced by the control α Rep protein that does not interact with Citrine (Figure S5). In that case, the pK_a of the mixture is the same as for the Citrine alone confirming that the observed variations of the pK_a are due to the direct interaction Citrine – α Rep.

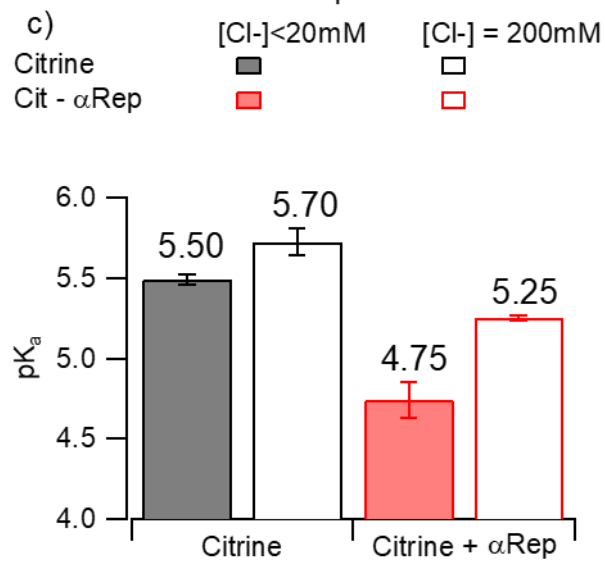
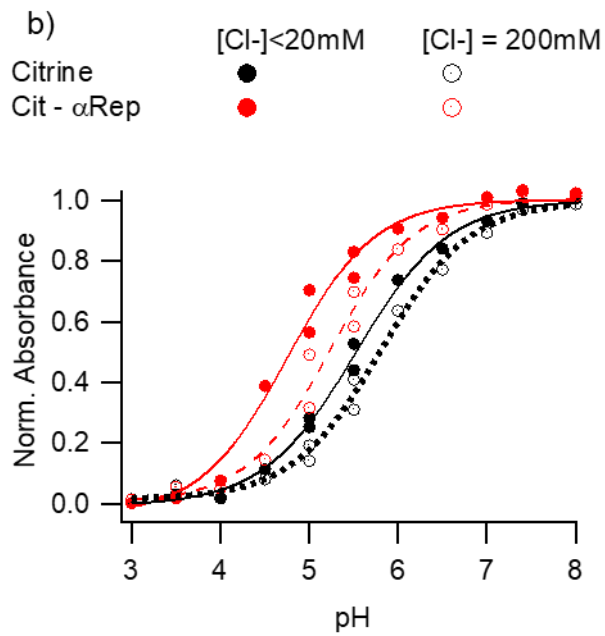
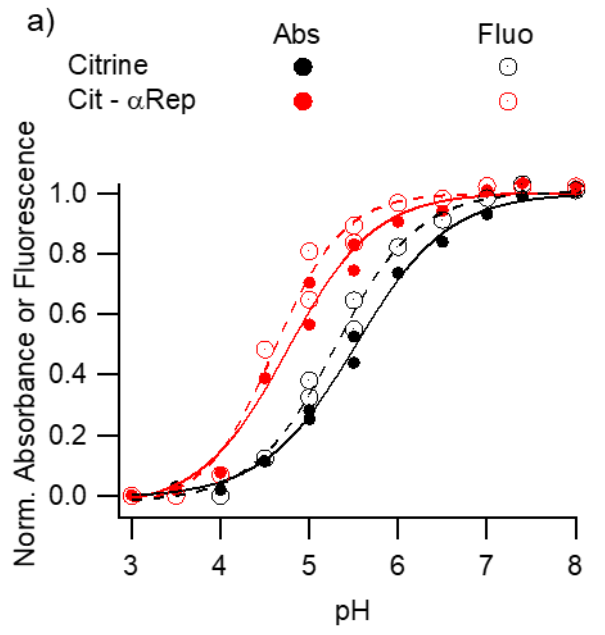


Figure 5: (a) Comparison of the pH dependence of the absorbance at the maximum of the anionic absorption band and of the maximum fluorescence intensity at a chloride concentration below 20 mM (data set of Figure 4). (b) Comparison of the pH dependence of the absorbance at the maximum of the anionic absorption band at low and high chloride concentrations. Data with the same markers are identical in (a) and (b), all data were normalized at pH 8. (c) Average pK_a values of Citrine and a 1:1 mixture of Citrine – α Rep for 3 independent experiments at $[Cl^-] < 20$ mM and 2 independent experiments at $[Cl^-] = 200$ mM.

α Rep reduces the pK_a of other YFPs with close sequences

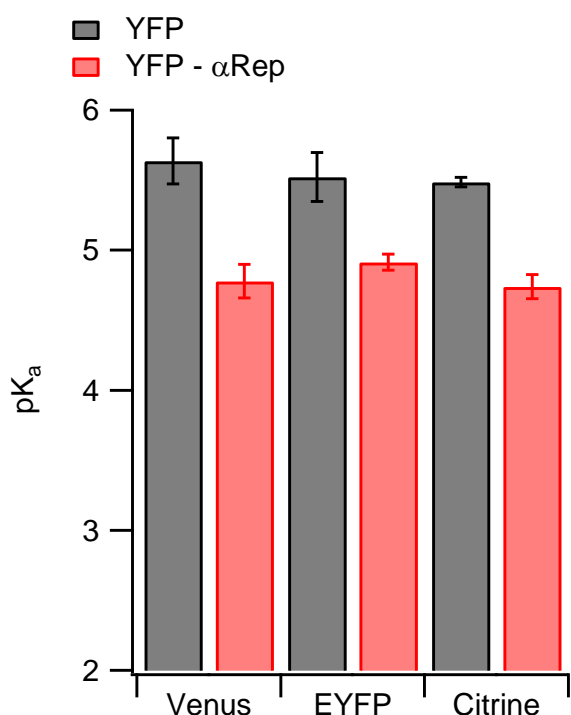


Figure 6: Average pK_a values of YFPs and an equimolar mixture YFP – α Rep ($n = 3$, $[Cl^-] < 20$ mM). The data for Citrine are the same as in Figure 5 and added for comparison.

EYFP and Venus are popular yellow fluorescent proteins with sequences close to Citrine (Figure S1). The pK_a values of those YFPs have been measured at a low chloride concentration, alone or in interaction with α Rep. As for Citrine, the interaction with α Rep decreases the pK_a of the FPs showing that the effect of the interaction on pK_a can be generalized to FPs of the same family *in vitro* (Figure 6).

The interaction Citrine - α Rep reduces the pK_a of Citrine in living cells

The interaction between α Rep-mCherry and EGFP was proven by the colocalization of a targeted EGFP construct in a previous study.^[15] Here we chose to assess the interaction between Citrine and α Rep in living cells by Förster Resonance Energy Transfer (FRET) using Citrine as a fluorescent donor and mCherry fused to α Rep as an acceptor. The Förster distance, at which 50% of Citrine proteins in the excited state will transfer their energy to mCherry is 59.9 Å.^[3] Such a short distance is likely reached only if Citrine and α Rep-mCherry form a complex. A

consequence of FRET is the decrease in the lifetime of the Citrine excited state. Here this lifetime was monitored by Fluorescence Lifetime Imaging and displayed as a color code in the images (Figure 7).

At physiological pH, when Citrine is expressed in COS7 cells, its fluorescence lifetime is 3.3 ns on average, with a low dispersion (Figure 7a and e). This value is slightly lower than recorded *in vitro* for a purified protein (see above). The intracellular pH was manipulated using nigericin, an antiporter H^+/K^+ that facilitates the exchange of K^+ for H^+ across membranes of living cells following a protocol optimized and validated previously.^[7,21,22] As a consequence, there is a dissipation of the proton gradient (ΔpH) across the cell membranes and the pH inside cells equilibrate with the one outside. When the intracellular pH is decreased using a KCl-rich buffer at pH 6 in the presence of nigericin,^[7] the lifetime is kept at a close value, consistent with *in vitro* data above (Figure 7b and e). When Citrine and a fusion αRep -mCherry are co-expressed in the COS7 cells with two independent plasmids, the lifetimes were shorter and their distribution was spread over 1.5 ns (Figure 7c, d and e). There is a clear FRET between Citrine and mCherry fused to αRep : Citrine and αRep interact in live cells at physiological and acidic pH. The spreading of the lifetime is cell-dependent and strongly related to the relative amount of αRep -mCherry. The fluorescence intensity in the acceptor channel, proportional to αRep -mCherry expression levels in the cell, has been monitored. The evolution of the FRET efficiency with the fluorescence intensity of αRep -mCherry follows a saturation curve (Figure 7f). For lower fluorescence values, the amount of αRep -mCherry in the cell is too low to interact with all the Citrine proteins. Some Citrine are still free leading to a low apparent FRET efficiency. For higher fluorescence values, all Citrine are in interaction with αRep -mCherry, and the apparent FRET efficiency is reaching a maximum. Altogether, these results show an efficient interaction of Citrine with αRep -mCherry in the living cell consistently with the first observations of interactions between EGFP and αRep .^[15] In addition, they also show that the FRET efficiency is the same at neutral and acidic pHs. In consequence, the decrease of fluorescence intensity due to FRET from Citrine to mCherry is expected to be the same at all pHs tested in the next experiments in living cells.

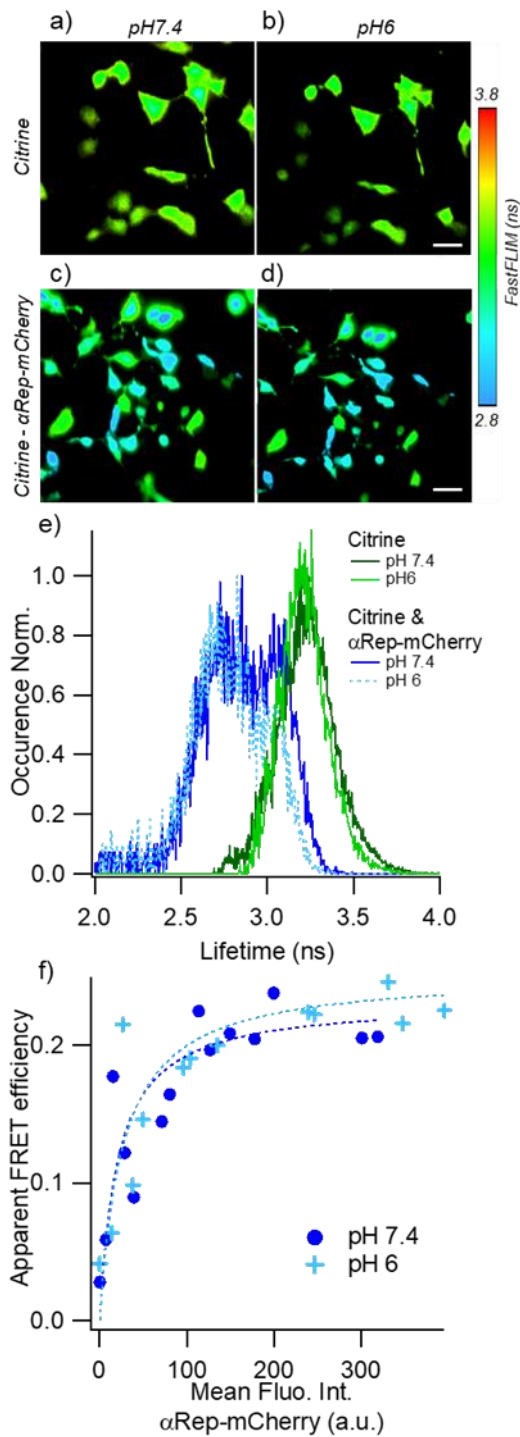


Figure 7: Fluorescence lifetime images (FastFLIM) of COS7 cells expressing either Citrine (first row) or Citrine and α Rep-mCherry (second row) at pH 7.4 in PBS (a, c) or at pH 6 (b, d), scale bar 15 μ m. (e) Lifetime distribution in all pixels in the four FastFLIM images. (f) Variation of the apparent FRET efficiency calculated for 16 cells in images c and d with the fluorescence intensity of α Rep-mCherry in the same cells (one point represents one cell).

In a second step, to monitor the effect of the binding of α Rep-mCherry on the pH sensitivity of Citrine's fluorescence properties, it is necessary to monitor the fluorescence intensity of Citrine in living cells at various pHs. An example of intensity variations between neutral and acidic pHs is shown in Figure 8a-d, and α Rep clearly limits those variations (Figure S6 for quantitative analysis of intensity images:). Nevertheless, flow cytometry is the method of choice to explore a large cell population and have a statistical and accurate approach to the average fluorescence intensities. In addition, among the cell population co-expressing Citrine and α Rep-mCherry, flow cytometry offers the possibility to choose cells expressing both proteins, based on their fluorescence intensity in the yellow (Citrine) and in the red channels (mCherry) (Figure S7). The amounts of plasmids for transfection have been chosen to have, on average, Citrine and α Rep-mCherry expression levels at which the FRET efficiency is maximum in FLIM, most of Citrine being in a bound form. The average fluorescence intensity of Citrine was calculated for various intracellular pHs (Figure 8e). First, it depends on the FRET efficiency that is pH-independent (Figure 7f). So, we could estimate that the FRET will impact the Citrine brightness in the same proportion at all pHs. Second, it is also proportional to the fraction of the proteins with an anionic and fluorescent chromophore, which is pKa dependent. At pH 6.5, the fluorescence starts to decrease with a drop of 35% at pH 5.5 for Citrine alone. In the presence of α Rep-mCherry, at pH 5.5, the relative drop of fluorescence to pH 7.4 is limited to 15%. This shows that the interaction Citrine – α Rep limits the protonation of the Citrine's chromophore in living cells, as *in vitro*.

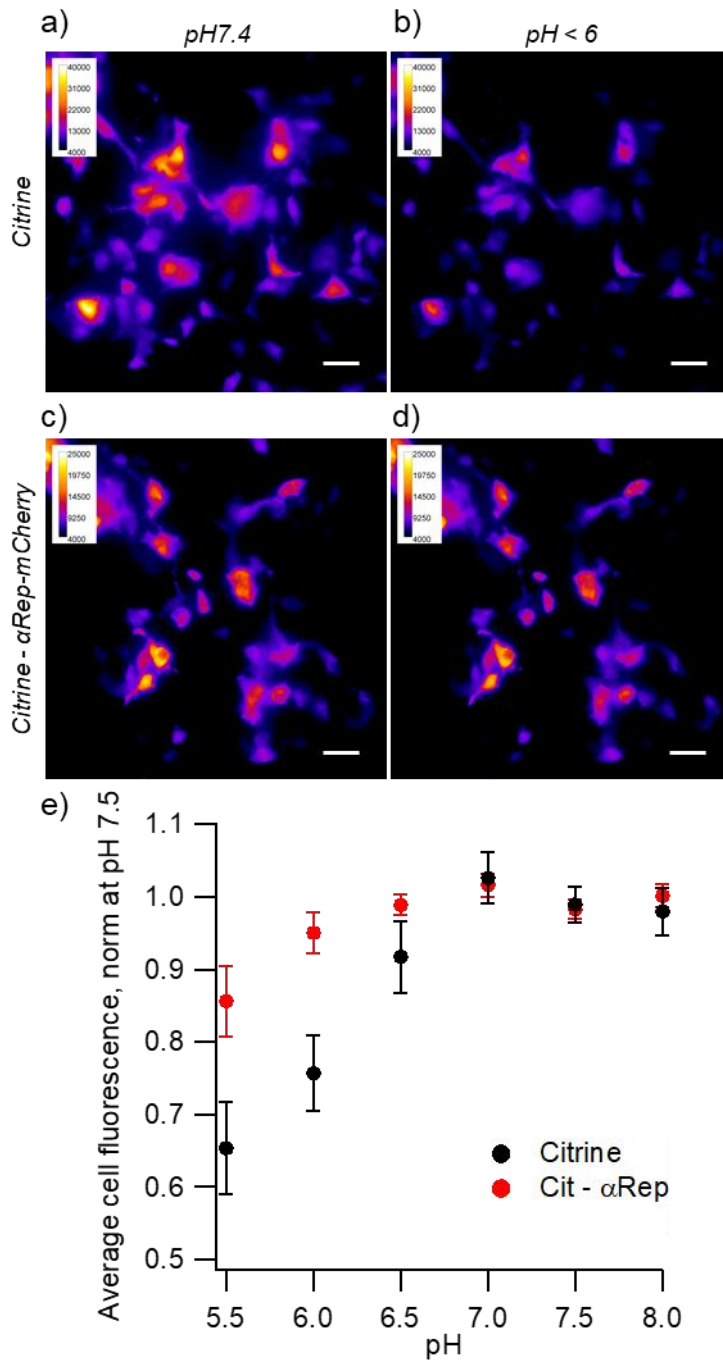


Figure 8: Wide-field fluorescence microscopy images of COS7 cell expressing Citrine (first row) or Citrine and α Rep – mCherry (second row) at pH 7.4 (a, c) and pH < 6 (b, d), scale bar 20 μ m. Histograms of fluorescence intensities in each image and the α Rep – mCherry intensity image are in Figure S6. (e) Average cellular fluorescence of Citrine for COS7 cells expression of either Citrine or both Citrine and α Rep – mCherry. In the latter case, only cells expressing both Citrine and α Rep – mCherry were chosen based on their fluorescence intensities. Data from each experiment were normalized at pH 7.5 and averaged (n = 4).

Discussion

Our results show that α Rep binds Citrine at physiological pH with a very high affinity and this binding stabilizes the Citrine's as shown by an increased T_m . Furthermore, as the shape and position of the absorption and emission spectra do not change, this suggests that the binding of α Rep does not induce significant modifications in the electrostatic field in the cavity around the chromophore.^[23] In order to understand the modification induced by the binding of α Rep on Citrine's structure, the available X-ray structures of EGFP and EGFP- α Rep complex can be compared. The RMSD profile of each C α atom of EGFP in the EGFP- α Rep complex vs. EGFP shows that there is no substantial global influence on the overall fold of EGFP as it has already been observed for interaction with nanobodies (Figure S8).^[12] The binding rather influences the strength of intra-molecular bonds in the β -barrel as shown by the T_m variation. Nevertheless, the small fluctuations on the RMSD profile are observed in the flexible turns mainly between the 7th and 8th as well as the 8th and 9th strands, with positions Q157 and G174 being mainly affected. This is consistent with the direct interaction of Q157 with N160 and E163 in the α Rep.^[15] Also, G174 is nearby S175 and V176 both interacting with α Rep's Y60 and L63.^[15] In addition, the fluorescence lifetime of the chromophore in the bound form is higher than for the free Citrine (3.65 ± 0.03 ns and 3.47 ± 0.03 ns respectively). This is a direct consequence of the rigidification of the chromophore in its pocket upon binding either through an increase of the compaction around the chromophore or a strengthening of the H-bond network.^[19,24] The X-ray structures for the complex and EGFP have different resolutions, 3.40 Å and 1.65 Å, respectively. Moreover, the resolution of the complex structure is too low to see the water molecules and thus follow the extended H-bond network around the chromophore. So, the changes observed by comparing the two structures are too small to provide any conclusions that would pinpoint the residues in the rigidification (Figure S9). As a consequence, we postulate, that the presence of α Rep mainly induces a cooperative effect that limits the global dynamics of the protein and of its chromophore. At pH 5.5, the T_m of Citrine is decreased and the one of α Rep is increased: the intramolecular forces in both proteins are modified. The structural changes in both partners have a direct impact on the stability of the complex that unfolds at a lower T_m at acidic pH, the binding being also slightly less efficient but still in the nM range. Nevertheless, the bound form has a higher T_m than Citrine alone, showing again a global structuring effect of α Rep on Citrine. We know from the absorption measurements, that at pH 5.5, some Citrine have a protonated and nonfluorescent chromophore. The ones remaining anionic and fluorescent have the same fluorescence lifetime as the fluorescent form of the Citrine alone at pH 5.5. At this low pH, our results point out that the binding has no major impact on the dynamic of the chromophore in its pocket. So, the structuring effect is not as strong as at physiological pH. To conclude, all these effects are likely behind the lowering of their pK_a , making Citrine still fluorescent and thus usable at significantly more acidic pHs. The good expression and solubility of α Rep in living cells enabled us to confirm this effect in COS7 cells. We also observed that the chloride sensitivity of Citrine is not reduced in the Citrine - α Rep complex. Thus, the latter does not significantly interfere with the diffusion pathway of chloride ions toward the well-known halogen cavity near the chromophore of YFPs.^[25,26] Indeed, chloride ions are likely to use the channel formed by the network of water molecules observed in KillerRed^[27] or avGFP and TurboGFP^[28] and connecting the lids of the

β -barrel to the chromophore vicinity. As expected from the crystal structure of the complex, the lid remains accessible to chloride in the presence of α Rep. (Figure 1).

Conclusion

The binding of α Rep to Citrine is efficient, even in acidic solutions and at acidic pHs in living cells. It increases the stiffness around the chromophore at physiological pH and influences its protonation, likely by stabilizing the H-bond networks around the anionic chromophore. As a consequence, the pK_a of the Citrine is decreased, maintaining high fluorescence even at acid pH down to 5.5 in living cells, allowing a brighter label at lower pH values. More generally, this study demonstrates the feasibility of modulating the properties of YFPs through the binding of an α Rep. The next step will be the design of FRET-based biosensors to monitor biochemical events in acidic environments like the phagosomes of phagocytic cells, such as neutrophils' cellular modal.^[29,30] Using as an acceptor a Citrine- α Rep fusion in pair with Aquamarine or mTurquoise2 will pave the way for the next generation of such biosensors adapted for acid environments. Finally, our work opens up the possibility of using both pK_a and lifetime measurements to screen for other α Reps with completely different binding modes, potentially leading to greater effects not only on pK_a and pH sensitivity but also on other parameters such as brightness or photostability.

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Supplementary figures.

a)

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mCherry      1 M V S K G E E D N M A I I K E F M R F K V H M E G S V N G H E F E I E G E G E G R P Y E G T Q T A K L K V T K G G P
EGFP        1 M V S K G E E . . . . L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K F . I C T T G K
Venus       1 M V S K G E E . . . . L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K L . I C T T G K
Citrine     1 M V S K G E E . . . . L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K F . I C T T G K
EYFP       1 M V S K G E E . . . . L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K F . I C T T G K

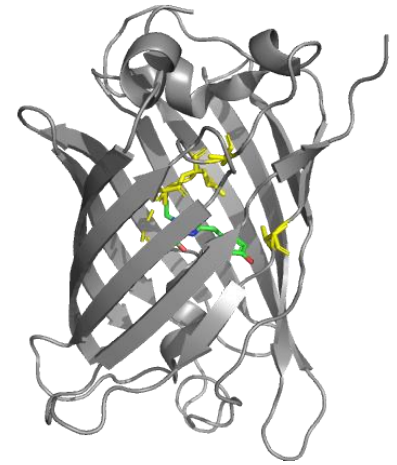
mCherry     59 L P F A W D I L S P Q F M Y G S K A Y V K H P A D I P . . D Y L K L S F P E G F K W E R V M N F E D G G V V T V T Q
EGFP       54 L P V P W P T L V T T L T Y G V Q C F S R Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A
Venus      54 L P V P W P T L V T T L S Y G L Q C F A R Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A
Citrine    54 L P V P W P T L V T T F S Y G L M C F A R Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A
EYFP      54 L P V P W P T L V T T F S Y G L Q C F A R Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A

mCherry    115 D S S L Q D G E F I Y K V K L R G T N F P S D G P V M Q K T M G W E A S S E R M Y . . . . P E D G A L K G E I K Q
EGFP      112 E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K . L E Y N Y N S H N V Y I M A D K Q K N G I K V N F K I
Venus     112 E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K . L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I
Citrine   112 E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K . L E Y N Y N S H N V Y I M A D K Q K N G I K V N F K I
EYFP     112 E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K . L E Y N Y N S H N V Y I M A D K Q K N G I K V N F K I

mCherry    169 R L K L K D G G . . . . . H Y D A E V K T T Y K A K P V Q L P G A Y N V N I K L D I T . . . . S H N E D Y T I V E Q
EGFP     169 R H N I E D G S V Q L A D H Y Q Q N T P I . . . . G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E
Venus    169 R H N I E D G S V Q L A D H Y Q Q N T P I . . . . G D G P V L L P D N H Y L S Y Q S A L S K D P N E K R D H M V L L E
Citrine  169 R H N I E D G S V Q L A D H Y Q Q N T P I . . . . G D G P V L L P D N H Y L S Y Q S A L S K D P N E K R D H M V L L E
EYFP    169 R H N I E D G S V Q L A D H Y Q Q N T P I . . . . G D G P V L L P D N H Y L S Y Q S A L S K D P N E K R D H M V L L E

mCherry    219 Y E R A E G R H S T G G M D E L Y K
EGFP     224 F V T A A G . . . I T L G M D E L Y K
Venus    224 F V T A A G . . . I T L G M D E L Y K
Citrine  224 F V T A A G . . . I T L G M D E L Y K
EYFP     224 F V T A A G . . . I T L G M D E L Y K
    
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b)



c)

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bGFPC      1 M R G S H H H H H T D P E K V E M Y I K N L Q D D S M R V R Y N A A T A L G K I G D E R A V E P L I K A L K D
bNCS324-16 1 M R G S H H H H H T D P E K V E M Y I K N L Q D D S Y F V R R A A A A A L G K I G D E R A V E P L I K A L K D

bGFPC      57 E D G Y V R L E A A A A L G E I G D E R A V E P L I K A L K D E D P D V R S E A A L A L G K I G D E R A V E P L
bNCS324-16 57 E D R F V R . . . . . S S A A Y A L G E I G D E R A V E P L

bGFPC      113 I K A L K D E D R Y V R M A A A W A L G K I G G E R V R A A M E K L A E T G T G F A R K V A V N Y L E T H K S L
bNCS324-16 82 I K A L K D E D W F V R R A A A V A L G E I G G E R V R A A M E K L A E T G T G F A R K V A V N Y L E T H K S L

bGFPC      169 I S
bNCS324-16 138 I S
    
```

Figure S1 Amino acid sequences of fluorescent proteins and binders: (a) Sequence alignment of mCherry, EGFP, Venus, Citrine, and EYFP, the chromophore sequence is in the green box. (b) EGFP structure (pdb 4XVP) is in grey and residues mutated in Citrine are in yellow, all facing the inside of the barrel. The residue 65 in the chromophores is also different. (c) Sequence alignment of binders, bGFPC called **α Rep** and bNCS324-16 called **control α Rep**

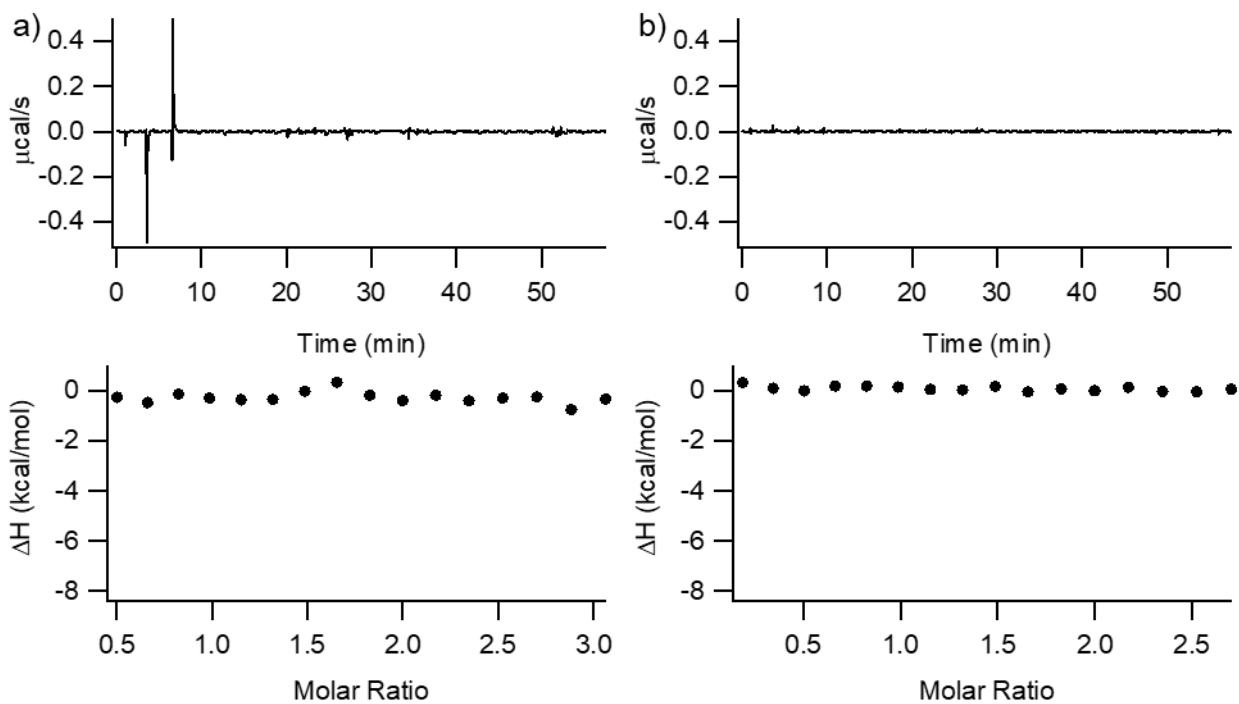


Figure S2: Raw signals of ITC obtained at pH 7.4 of Citrine (320 μM) in control αRep (16.5 μM) (a) and mCherry (320 μM) in αRep (14.3 μM) (b).

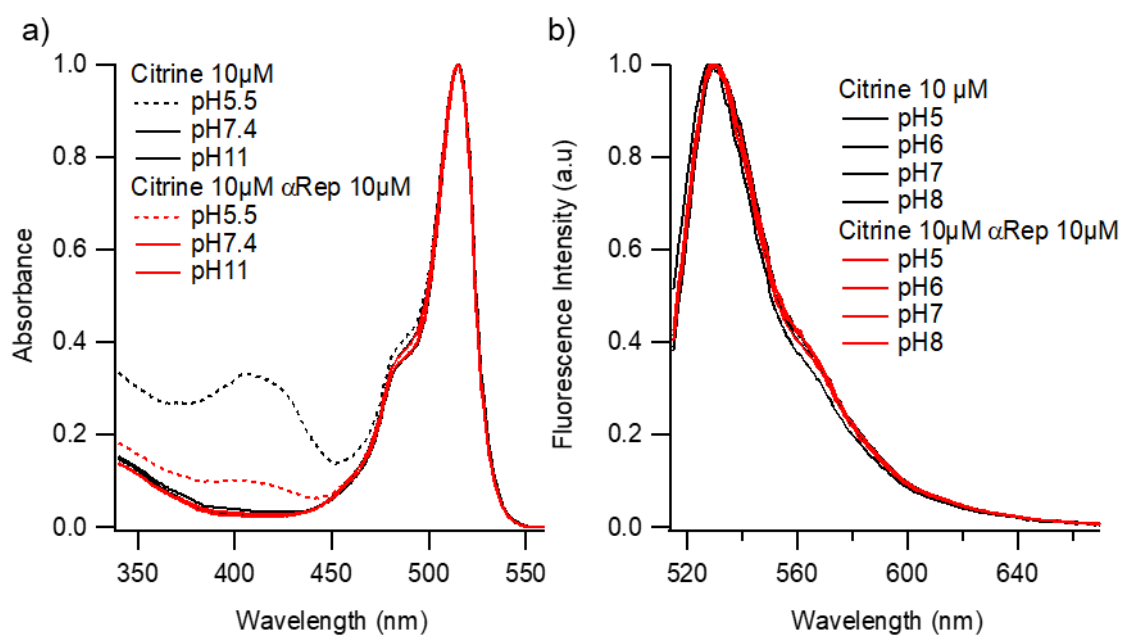


Figure S3: Overlay of normalized absorption (a) and fluorescence emission (b) spectra of Citrine (10 μM) and a mixture 1:1 Citrine - αRep , 10 μM each.

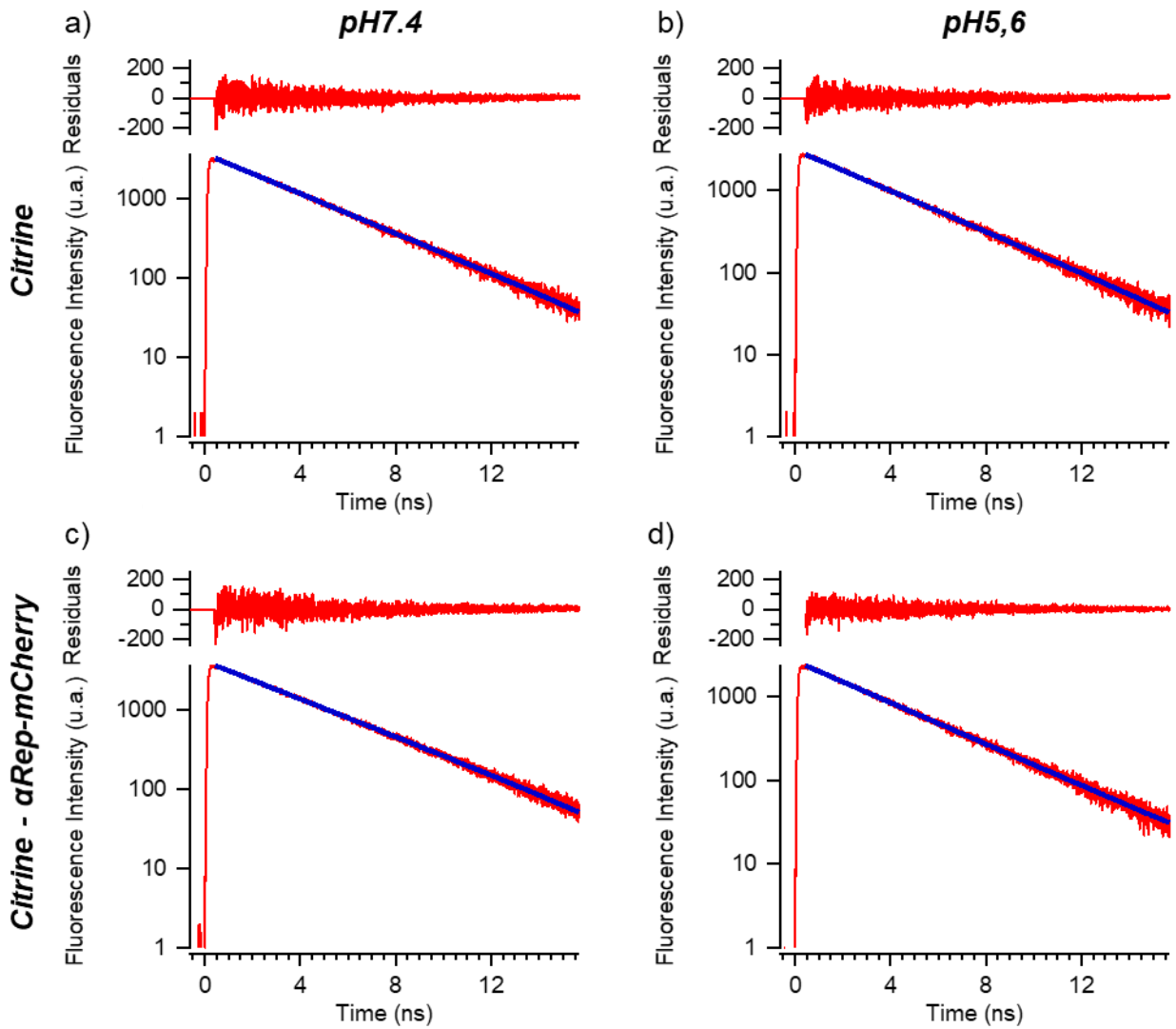


Figure S4: Fluorescence decays (red), monoexponential fit (blue), and residuals for Citrine (a, b) and a mixture 1:1 Citrine – α Rep (c,d) at pH 7.4 (a,c) and pH5.6 (b,d).

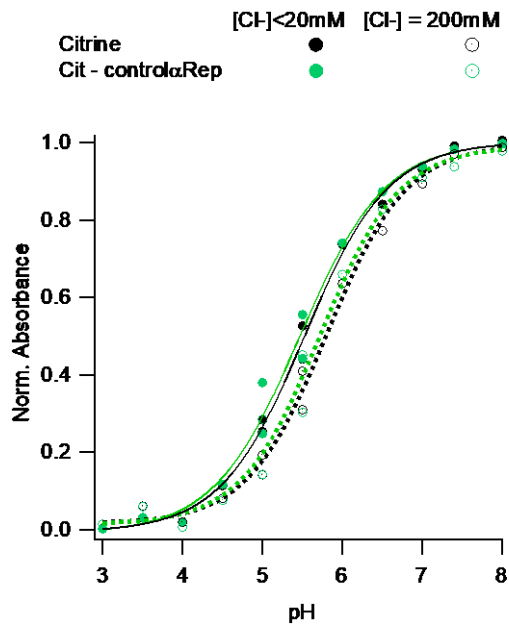


Figure S5: Chloride effect on the pH dependence of the absorbance at the maximum of the anionic band of the chromophore. Experimental data were normalized to 100% at the maximum of their analytical fits.

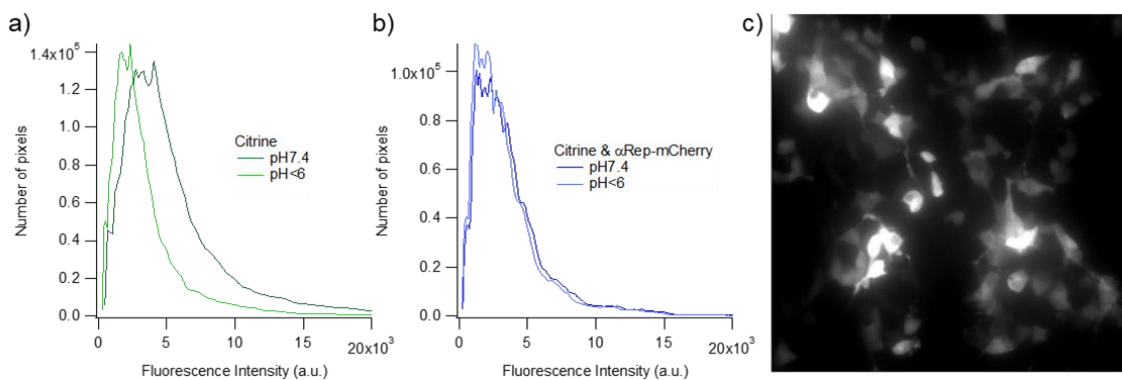


Figure S6: Histograms of fluorescence intensities in each image at pH 7.4 and pH < 6 for Citrine alone (a) and a co-expression of Citrine and α Rep – mCherry (b). Intensity image of mCherry at pH 7.4 for the same field of view as Figure 8c.

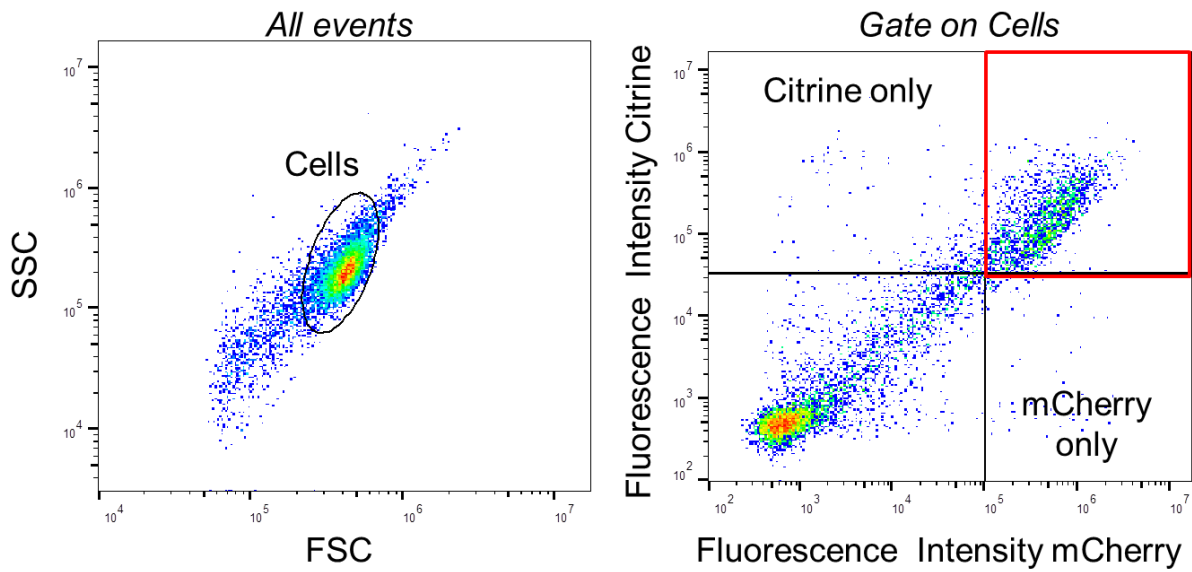


Figure S7: Gating strategy to cytometry experiments for a co-expression of Citrine and α Rep – mCherry (data at pH 7.4). The average Citrine fluorescence is calculated for the events in the red square.

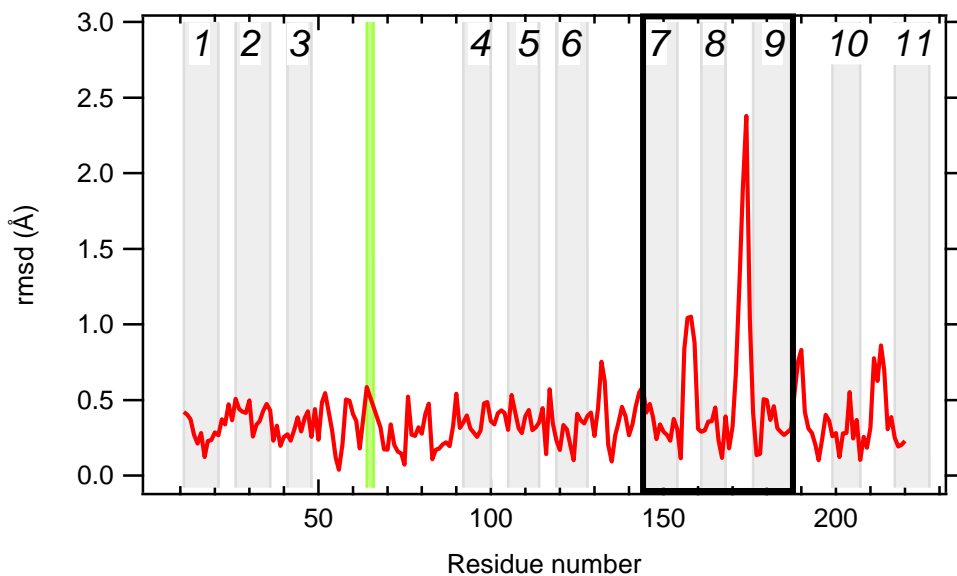


Figure S8: RMSD profile for each $C\alpha$ atoms of EGFP in the EGFP - α Rep complex (pdb 4XVP) vs. EGFP (pdb 6YLQ). The position of the eleven β -strands and of the chromophore are indicated in grey and green, respectively. The black box represents the binding area of α Rep.

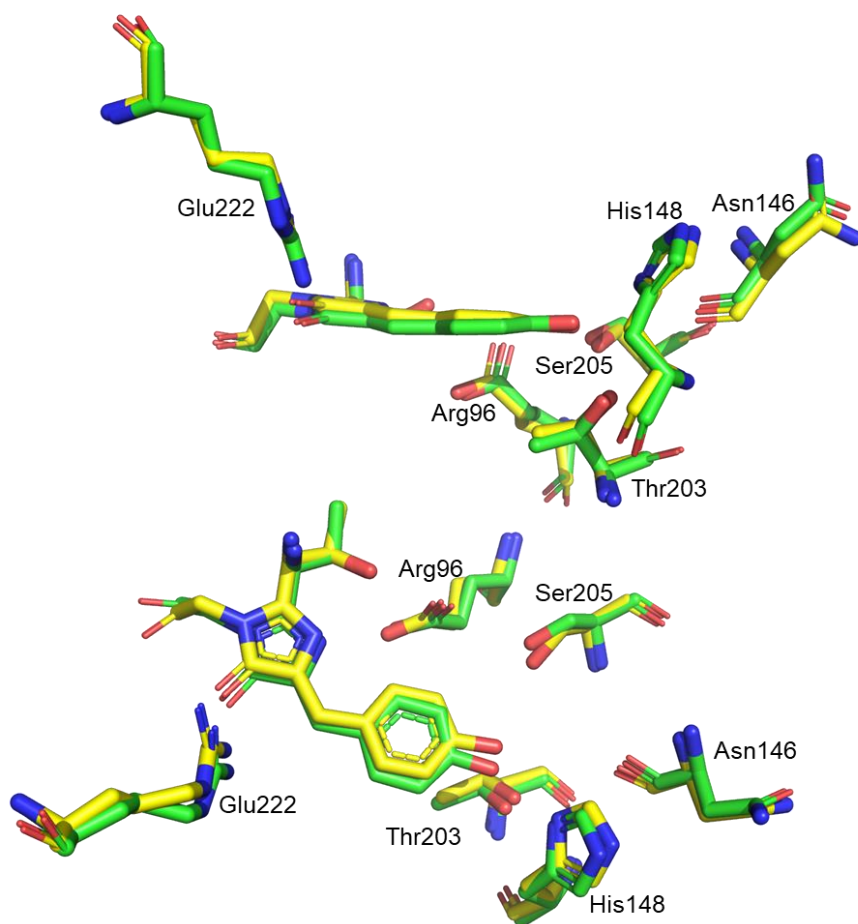


Figure S9: Structures of the chromophore in its pocket for EGFP (green, pdb 6YLQ) and EGFP - α Rep complex (yellow, pdb 4XVP).