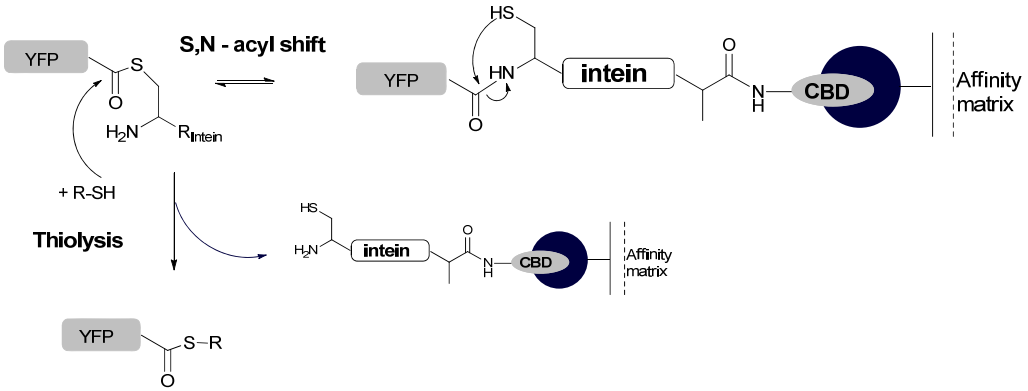
Construction of plasmids, protein expression and purification:

To construct plasmids, pHT477 (His-ECFP-Intein-CBD-Strep) and pHT482 (Strep-EYFP-Intein-CBD-His), DNA encoding for Strep-tag and His-tag were introduced to the fusion protein at the C-terminus by applying point mutagenesis method using two pairs of oligonucleotides for Strep-tag, ON061, 5′-gcaagctggagccacccgcagttcgaaaagtgactgcaggaaggggatccgg-3′ and ON062, 5′-cttttcgaactgcgggtggctccagcttgcttgaagctgccacaaggcaggaac-3′ and for His-tag, ON059, 5′-gaagcgagccaccatcaccatcaccatcaccattgactgcaggaaggggatccgg-3′ and ON060, 5′-atggtgatggtgatggtgatggtggctcgcttcttgaagctgccacaaggcaggaac-3′ with pHT409 and pHT423 as templates, respectively. Plasmids, pHT409 (His-ECFP-Intein-CBD) and pHT423 (Strep-EYFP-Intein-CBD) were constructed by inserting two pairs of complementary oligonucleotides for His-tag, ON031, 5′-tatggaagcgagccaccatcaccatcaccatcaccattc-3′ and ON032, 5′-tagaatggtgatggtgatggtgatggtggctcgcttcca-3′ and for Strep-tag, ON047, 5′-tatggcaagctggagccacccgcagttcgaaaagtctgc-3′ and ON048, 5′-tagcagacttttcgaactgcgggtggctccagcttgcca-3′ into plasmids, pTWIN1-ECFP and pTWIN1-EYFP [1] at *Nde*I.

Plasmids pHT477 and pHT482 were transformed into *E.coli* Rosetta 2 (DE3) cells and transformants were selected on Chloramphenicol (34 mg/L) and Ampicillin (125 mg/L) containing Agar plates. Protein expression and purification were performed as described previously[2] leading to MESNA-protein thioesters of eCFP (from pHT477) and eYFP (from pHT482). LC/MS: eYFP calculated: 28538, found: 28539; eCFP calculated: 29898, found: 29896.

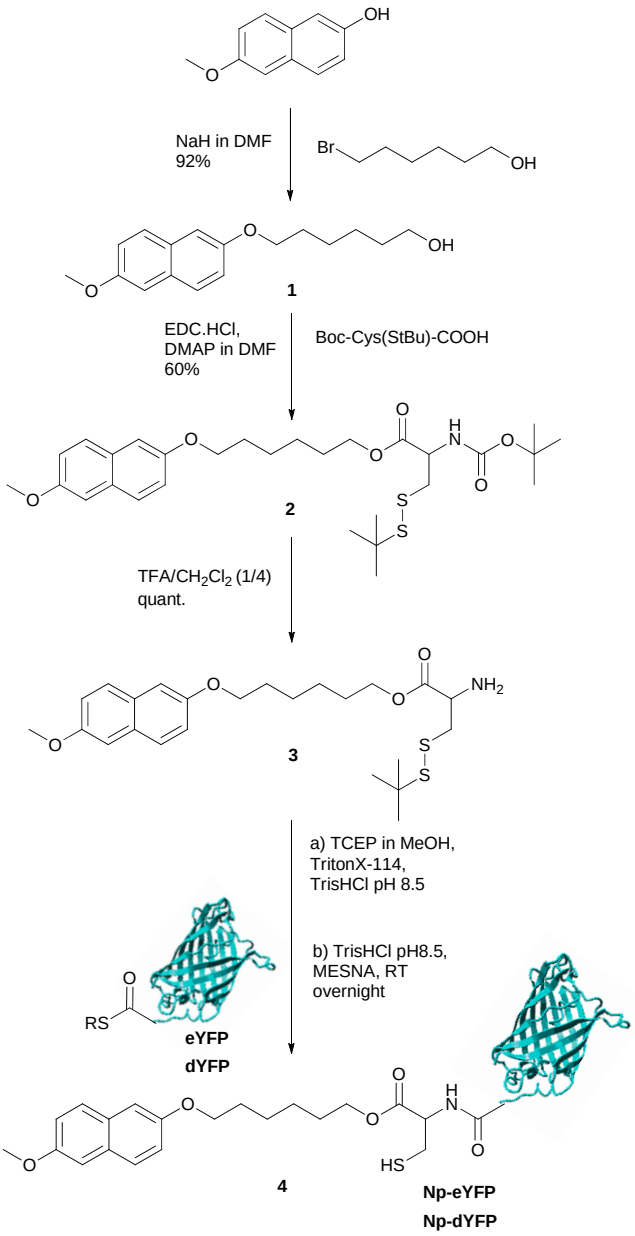
Dimerizing variants, dYFP and dCFP have been expressed and purified as described previously[2].

The generation of a protein thioester using the pTWIN system from NEB is depicted in Scheme 1. The protein is expressed in fusion with an intein domain followed by a chitin binding domain (CBD) for purification. Upon addition of a thiol, intein cleavage is induced and the protein is cleaved from the intein which binds to the chitin beads used for purification. Like this the pure C-terminal thioester can be isolated.

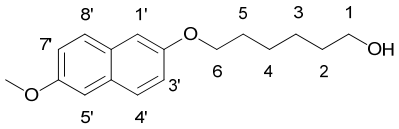


Supporting Scheme 1 Generation of a YFP-thioester using the pTWIN-system from NEB.

Synthesis of Naphthalene derivatives:



6-[(6-methoxy-2-naphthyl)oxy]hexan-1-ol **(1)**

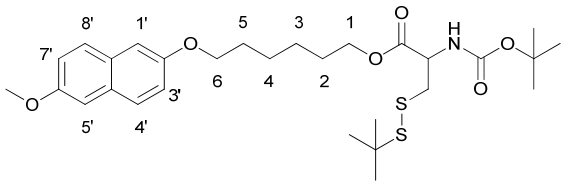


(1)

NaH (110 mg, 2.8 mmol) was added to a cold (0 ºC) solution of 6-methoxynaphthalen-2-ol (400 mg, 2.3 mmol) in DMF (6 mL) and stirred at 0 ºC for 30 min then at room temperature for a further 30 min. A solution of 6-bromo-hexan-1-

ol (250 μL, 1.9 mmol) in DMF (6 mL) was slowly added to the solution (over 15 min). The solution was stirred at room temperature under an argon atmosphere for 5 h then solvent removed *in vacuo* to give the crude product as a green solid. This was purified by column chromatography (SiO_2, ethyl acetate: cyclohexane 1:1) to give the pure product as a white solid (481 mg, 92 %). ^{1}H NMR ($CDCl_3$, 400 MHz): 1.43-1.58 (m, 4H, H3, H4); 1.62 (m, 2H, H2); 1.85 (m, 2H, H5); 3.66 (t, *J* = 6.6 Hz, 2H, H1); 3.89 (s, 3H, OCH_3); 4.05 (t, *J* = 6.5 Hz, 2H, H6); 7.10 (m, 2H, H1', H5'); 7.13 (dt, *J* = 8.8, 2.4 Hz, 2H, H3', H7'); 7.63 (dd, *J* = 8.9, 5.2 Hz, 2H, H4', H8'). ^{13}C NMR ($CDCl_3$, 100 MHz): 25.75, 26.14, C3, C4; 29.45, C5; 32.86 C2; 55.47, OCH_3; 63.03, C1; 68.09, C6; 106.27, 107.19, C1', C5'; 119.01, 119.38, C3', C7'; 128.25, 128.28, C4', C8'; 129.84, 129.97, C9', C10'; 155.73, 156.22, C2', C6'. LC/MS: *m/z*: 275.07 $(M + H)^+$.

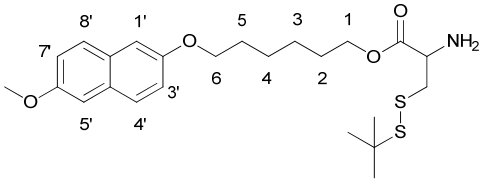
6[(6-methoxy-2-naphthyl)oxy]hexyl *N*-Boc-3-(t-butyl-disulfanyl)alaninate **(2)**



(2)

Boc-Cys(StBu)-COOH (113 mg, 0.37 mmol) and EDC.HCl (70 mg, 0.37 mmol) were dissolved in DMF (4 mL) and stirred at room temperature for 15 min. This solution was then added to a stirring solution of **1** (25 mg, 0.09 mmol) in DMF (1 mL). DMAP (4.5 mg, 0.04 mmol) was added to the solution and the resulting solution was stirred at room temperature under an argon balloon for 6.5 h. The solvent was removed *in vacuo* to give a yellow oil, which was redissolved in CH_2Cl_2. Water was added to the solution and the product was extracted twice with fresh CH_2Cl_2. The organic layers were combined, dried over Na_2SO_4, filtered and dried *in vacuo* to give the crude product as an orange oil. Following column chromatography (SiO_2, ethyl acetate: cyclohexane 1:4), the desired product **(2)** was obtained as a clear oil (31 mg, 60 %). ^{1}H NMR ($CDCl_3$, 400 MHz): 1.31 (s, 9H, StBu); 1.45 (s, 9H, $C(CH_3)_3$); 1.46-1.58 (m, 4H, H3, H4); 1.72 (m, 2H, H2 or H5); 1.85 (m, 2H, H2 or H5); 3.16 (m, 2H, CH_2S); 3.89 (s, 3H, OCH_3); 4.05 (t, *J* = 6.4 Hz, 2H, H1 or H6); 4.19 (t, *J* = 6.7 Hz, 2H, H1 or H6); 4.57 (m, 1H, CH); 5.38 (t, *J* = 7.9 Hz, 1H, NH); 7.09-7.13 (m, 4H, H1', H3', H5', H7'); 7.63 (dd, *J* = 8.9, 5.4 Hz, 2H, H4', H8'). ^{13}C NMR ($CDCl_3$, 100 MHz): 25.95, 26.02, C3, C4; 28.54, $C(\underline{C}H_3)_3$; 28.67, 29.39, C2, C5; 29.97, $C(\underline{C}H_3)_3$; 43.18, CH_2S; 48.33, $S\underline{C}(CH_3)_3$; 55.61, CH, 55.51, OCH_3; 65.92, 68.02, C1, C6; 80.26, $O\underline{C}(CH_3)_3$; 106.29, 107.21, C1', C5'; 119.05, 119.40, C3', C7'; 128.28, 128.31, C4', C8'; 129.88, 129.99, C9', C10'; 155.23, HNCOO; 155.73, 156.26, C2', C6'; 170.99, $CH_2\underline{C}OO$. LC/MS: calculated for $C_{29}H_{43}NO_6S_2Na$: 588.2424 $(M+Na)^+$; found: 588.13 $(M + Na)^+$.

6-[(6-methoxy-2-naphthyl)oxy]hexyl 3-(t-butyl-disulfanyl)alaninate **(3)**

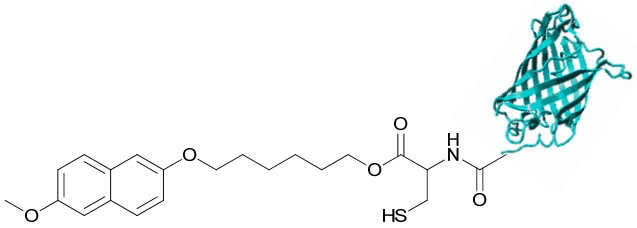


(3)

2 (14.8 mg, 26.2 µmol) was dissolved in a mixture of TFA:CH_2Cl_2 (1:4, 500 µL) and the solution stirred at room temperature for 3 h. The solvent was removed *in vacuo* to give the product as a brown oil (17.6 mg, quantitative). ^{1}H NMR ($CDCl_3$, 500 MHz): 1.32 (s, 9H, tBu); 1.43 (m, 2H, H3 or H4); 1.53 (m, 2H, H3 or H4); 1.72 (m, 2H, H2 or H5); 1.83 (m, 2H, H2 or H5); 3.20 (dd, *J* = 14.7, 7.2 Hz, 1H, CH_2S); 3.30 (dd, *J* = 14.6, 3.5 Hz, 1H, CH_2S); 3.89 (s, 3H, OCH_3); 4.03 (t, *J* = 6.3 Hz, 2H, H1 or H6); 4.25 (t, *J* = 6.6 Hz, 2H, H1 or H6); 4.42 (m, 1H, CH); 7.08-7.12 (m, 4H, H1', H3', H5', H7'); 7.62 (t, *J* = 8.4 Hz, 2H, H4', H8'); 7.70 (br, 3H, NH_3^+). ^{13}C NMR ($CDCl_3$, 125 MHz): 25.75, 25.95, C3, C4; 28.38, 29.33, C2, C5; 29.89, C($\underline{C}H_3)_3$; 39.10, CH_2S; 49.34, S$\underline{C}(CH_3)_3$; 52.96, CH, 55.53, OCH_3; 67.67, 67.97, C1, C6; 106.35, 107.28, C1', C5'; 119.09, 119.37, C3', C7'; 128.33, C4', C8'; 129.94, 130.01, C9', C10'; 155.71, 156.30, C2', C6'; 168.16, C=O. LC/MS: calcd for $C_{24}H_{36}NO_4S_2$: 466.2080 $(M+H)^+$; found: 466.20 $(M + H)^+$.

Protein Ligation:

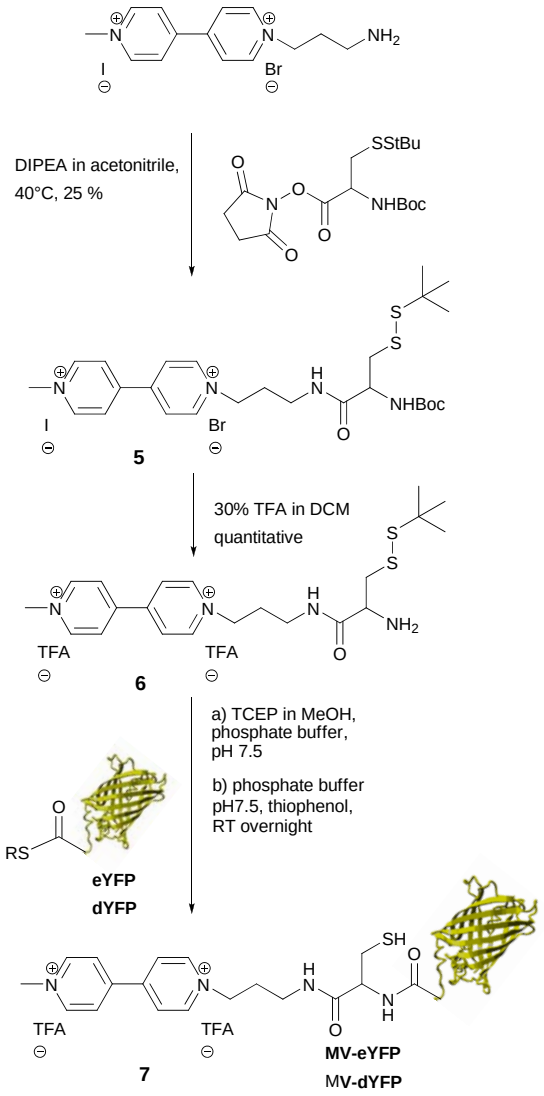
Np-eCFP and Np-dCFP (**4**):



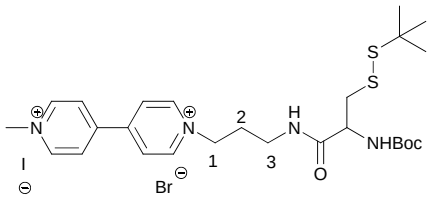
(4)

(3) (5 mg, 11 µmol) was dissolved in methanol (200 µL) and StBu-deprotected with tris(2-carboxyethyl)phosphine (TCEP) at mole ratios of 1:1.1 for 1 h at room temperature. TritonX-114 (100 µL of a 10% solution) and Tris.HCl buffer (pH = 8.5, 50 µL) were slowly added and the mixture allowed to sit at ambient temperature for 1.5 h. After this time, the mixture was slowly added to the CFP thioester (from above, 580 µL, 425 µM, ie. at a mole ratio of 1:45 (CFP: **3**)). NaCl and MESNA were added to final concentrations of 500 mM and 200 mM respectively. The ligation mixture was kept at room temperature in the dark overnight. Excess **(3)** was removed by size-exclusion chromatography and the remaining mixture of proteins was purified by triton exchange. The triton was removed using a Ni-NTA-column and ligated protein eluted using imidazole. The buffer was exchanged to storage buffer (25 mM sodium phosphate pH 7.5, 100 mM NaCl) and the protein was stored in small aliquots at low (25-40 µM) concentration at -80°C. The ligated protein was analysed by SDS-PAGE and LC/MS: Calculated for Np-dCFP: 30208, found: 30208; calculated for Np-eCFP: 30133, found 30131.

Synthesis of Methylviologen derivatives



4-(1-{3-[2-amino-3-(tert-butyldisulfanyl)propanamido]propyl}pyridin-1-ium-4-yl)-1-methylpyridin-1-ium (**5**)

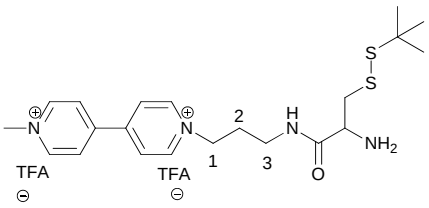


(**5**)

In a round bottom flask, a solution of 1-(3-aminopropyl)-1'-methyl-[4,4'-bipyridine]-1,1'-diium[3] (200 mg, 0.46 mmol) in 50 mL acetonitrile was prepared and DIPEA (90 mg, 0.69 mmol) was added. The hydroxysuccinimide ester of the Boc and StBu-protected cysteine[4] (214 mg, 0.55 mmol) was added to the mixture and stirred for two hours at 40 ºC. The solvent was evaporated and 100 mg of the crude mixture was

dissolved in H_2O and purified via preparative HPLC to isolate 15 mg (estimated yield: 25%) of compound **5**.
^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 1.34 (s, 9H, $SC(CH_3)_3$), 1.47 (s, 9H, $OC(CH_3)_3$), 2.31 (m, 2H, H2), 2.88 - 3.16 (m, 2H, CH_2S), 3.31 - 3.29 (m, 2H, H3), 4.24 (m, 1H, CH), 4.53 (s, 3H, CH_3), 4.77 (m, 2H, H1), 8.49-8.72 (m, 4H, ArCH), 9.23 (m, 4H, ArCH); LC/MS calculated for $C_{26}H_{40}N_4O_3S_2$: 520.25 $(M)^+$, found 519.3, 520.3 $(M)^+$.

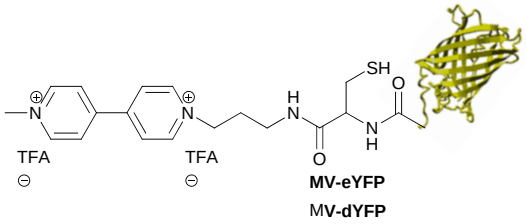
4-{1-[3-(2-amino-3-sulfanylpropanamido)propyl]pyridin-1-ium-4-yl}-1-methylpyridin-1-ium (**6**)



(**6**)

Compound **5** (3 mg, 5 µmol) was dissolved in 1 mL of 30 % TFA in dichloromethane (v/v). The mixture was stirred for three hours. After co-evaporation with toluene, compound **6** was obtained as an orange solid (quantitative).
^{1}H NMR (400 MHz, CD_3OD) δ = 1.37 (s, 9H, $SC(CH_3)_3$), 2.42 – 2.24 (m, 2H, H2), 3.29 - 3.09 (m, 2H, CH_2S), 3.35 - 3.50 (m, 2H, H3), 4.14 (m, 1H, CH), 4.53 (s, 3H, CH_3), 4.81 (m, 2H, H1), 8.55 – 8.78 (m, 4H, ArCH), 9.11 – 9.39 (m, 4H, ArCH).
^{13}C NMR (100 MHz, CD_3OD) δ = 30.08, $SC(\underline{C}H_3)_3$; 32.32, C2; 37.08, C3; 42.12, CH_2S; 54.01, CH; 60.67, C1; 127.85, 128.23, 147.36, 148.02, ArCH. LC/MS calculated for $C_{21}H_{32}N_4OS_2$: 420.20 $(M)^+$, found 420.1 $(M)^+$.

Ligation to protein thioesters eYFP and dYFP (**7**)

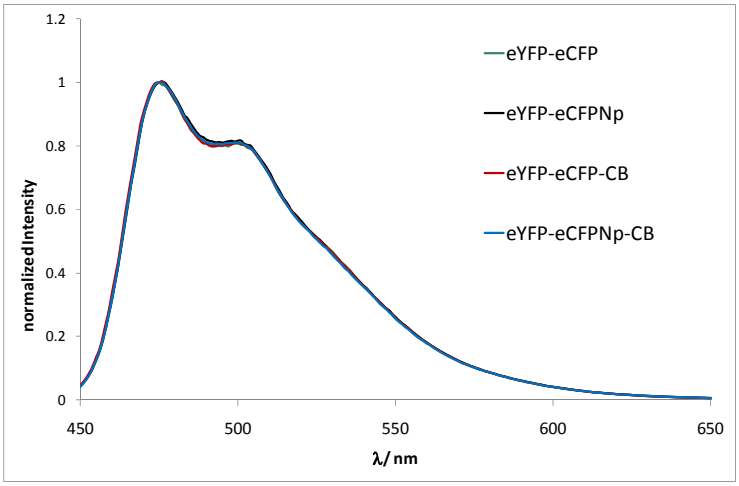


(**7**)

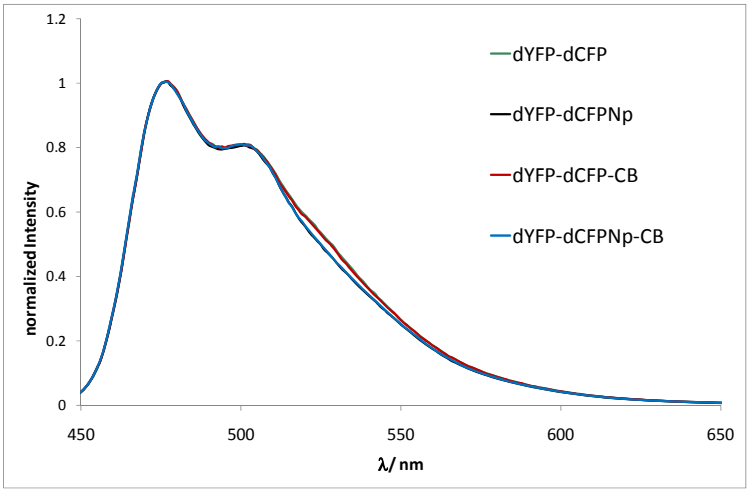
Compound **6** (10 mg, 15µmol) was dissolved in a mixture of 60 µL methanol and 240 µL ligation buffer (Na-phosphate 25 mM, NaCl 50 mM, pH7.5). For StBu deprotection, 60 µL of a 0.5 M solution of TCEP was added and the mixture was incubated for 30 min at room temperature. The mixture was then added to the YFP thioester (200 µM) at a molar ratio of 1:20 (YFP : **6**) and thiophenol was added to a final concentration of 300 mM. The ligation mixture was incubated on a rotating wheel in the dark at room temperature overnight. The mixture was then centrifuged to remove any precipitate. To remove excess **6,** the buffer was exchanged to ligation buffer using centrifugal filters with a molecular weight cutoff of 10 kDa. LC/MS: MV-eYFP calculated: 28745, found: 28751; MV-dYFP calculated: 28828, found: 28822.

Fluorescence spectroscopy

All samples for fluorescence spectroscopy measurements were prepared under ambient conditions in quartz cuvettes. Samples were prepared in phosphate buffer (25 mM Na-phosphate, 50 mM NaCl, pH 7.5) with 10 µM TCEP. The concentration of the proteins was determined by UV/Vis spectroscopy on a NanoDrop ND-1000 spectrophotometer using the absorbance at 435 nm and ε_{435}= 32500 $M^{-1}cm^{-1}$ for CFP and the absorbance at 515 nm and ε_{515} = 84000 $M^{-1}cm^{-1}$ for YFP[2]. Fluorescence data were recorded on a Varian Cary Eclipse photoluminescence spectrometer. Fluorescence emission spectra were recorded at an excitation wavelength of 410 nm. Samples were prepared by premixing the YFP compound with a stock solution of CB[8] in water (50 µM) to a final concentration of 2 µM YFP and 20 µM CB[8]. This solution was mixed with a 2 µM solution of CFP compound to yield final protein concentrations of 1 µM each and after a short incubation time, the fluorescence spectrum was recorded. To ensure the data was comparable, all samples were prepared in the same way.



Supporting Figure 1 Normalized fluorescence spectra of normal fluorescent proteins, protein concentration 1 µM, CB[8] 10 µM.



Supporting Figure 2 Normalized fluorescence spectra of dimerizing fluorescent proteins, protein concentration 1 µM, CB[8] 10 µM.

Fluorescence anisotropy measurements

To show that CB[8] does not act as an inducer of protein homodimerization for the modified fluorescent proteins, homo-FRET measurements were performed with eCFPNp and eYFPMV. Homodimerization of two fluorescent proteins leads to a decrease of fluorescence anisotropy due to intermolecular energy transfer[5]. This technique has been used before to study the cucurbit[8]uril-induced homodimerization of FGG-mYFP[6]. In the case of FGG-modified proteins, the anisotropy value decreases from 0.32 without CB[8] down to 0.27 depending on the concentration of CB[8][6]. For the proteins studied here, no changes in anisotropy upon addition of CB[8] were detected. The fluorescent proteins modified with naphthalene and methyl-viologen show no decrease in anisotropy (Table 1). These results confirm the need for both a naphthalene element and a methyl-viologen element for CB[8] induced protein dimerization and concommitant absence of protein homodimerization upon addition of CB[8].

Protein + Supramolecule	eCFPNp -	eCFPNp CB[8]	eYFPMV -	eYFPMV CB[8]
Anisotropy	0.314	0.315	0.320	0.326

Supporting Table 1 Anisotropy values for modified fluorescent proteins, concentration 1 μM.

Fluorescence anisotropy data were recorded on a Varian Cary Eclipse photoluminescence spectrometer. YFP was excited at 500 nm and emission was recorded from 524 nm to 532 nm. CFP was excited at 435 nm and emission was recorded from 472 nm to 478 nm. Anisotropy values were averaged over each wavelength.

References:

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[6] H. D. Nguyen, D. T. Dang, J. L. J. van Dongen, L. Brunsveld, *Angew. Chem. Int. Ed.* 2010, **49**, 895–898.