Electronic Supplementary Information
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Materials and Methods

Construction of His-CFP and His-YFP

The protein design is analogous to the one reported by Ye et al.[1]. The e*CFP* gene (1-229aa) was isolated by PCR using the primers X170 (5'GGAATTC**CATATG**GTGAGCAAGGGCGAGGAGCTG-3') and X171 (5'-GGAATTC**CATATG**TCCGGACTTGTACAGCTCGTCC-3') and the template pECFP-C1 (Clontech). The resulting product was digested with *Nde*I and was cloned to the same site of pET16b (Novagen) downstream from the T7 promoter. The e*YFP* gene (1-238aa) was isolated by PCR using primers X170 (5'GGAATTC**CATATG**GTGAGCAAGGGCGAGGAGCTG-3') and X171 (5'-GGAATTC**CATATG**TCCGGACTTGTACAGCTCGTCC-3') and the template pEYFP-C1 (Clontech). The resulting product was digested with *Nde*I and was cloned to the same site of pET16b (Novagen) downstream from the T7 promoter. Introduced restriction sites are in bold and underlined.

Construction of His-CFP-GBP-YFP

The e*CFP* gene (1-229aa) was isolated by PCR pECFP-C1 (CLONTECH) using the primers X388 (5'-GCG**GGATCC**GGTGAGCAAGGGCGAGGAGCTG-3') and X389 (5'-TGCG**GTCGAC**GATCCCGGCGGCGGTCACGAACTC-3') (introduced restriction sites are in bold and underlined) and template pIMBB430. The resulting product was digested with *BamHI – SalI* and was cloned into the corresponding sites of pETDuet-1 (Novagen) in the multi-cloning site 1 (MCS1) downstream from the T7 promoter 1. The e*YFP* gene (7-238aa) was isolated by PCR using primers X390 (5'-GGA**AGATCT**CGAGCTGTTCACCGGGGTGGTGC-3') and X391 (5'-CC**GGTACC**TTACTTGTACAGCTCGTCCATGCCGAG-3') and as template plasmid pIMBB504. The resulting PCR product was digested with *BglII-KpnI* and was cloned into the corresponding sites of multi-cloning site 2 (MCS2) of pIMBB652 [pETDuet-1 His-CFP (N1-229)] downstream of the T7 promoter 2, resulting in construct pIMBB654, in which each fluorescent molecule is expressed separately. The gene encoding galactose/glucose binding protein of *E.coli* (GBP) was isolated by PCR using primers X392 (5'-ACGC**GTCGAC**ACTCGCATTGGTGTAACAATC-3') and X393 (5'-CCT**AGATCT**TCAGCCAGGTTGTCTTTATCTACGC-3') and as template chromosomal DNA that was isolated from JM109 (Promega) cells. The resulting product encodes GBP that misses the 5 carboxy-terminal amino acids. The PCR product was digested with *SalI-BglII* and was cloned into the corresponding sites of pIMBB657 [pETDuet-1 His CFP (N1-229) YFP (N7-238)] from which construct the second T7 promoter and the second RBS had first been removed by digestion of pIMBB654 with *NotI-BglII*. In the final construct (pIMBB648) the triple fusion protein His CFP-GBP-YFP is expressed as a single polypeptide under the control of T7 promoter 1. The His_6 is inserted at the N-terminus of the protein by the cloning vector pETDuet-1. pIMBB648 was digested with *BamHI-KpnI* and the gene encoding the triple fusion was subcloned to the same sites of vector pRSETb (Invitrogen) resulting in construct pIMBB666. The pRSETb construct ensured higher and more stable expression of HisCFP-GBP-YFP in *E.coli* BL21*/pLysS (Novagen) cells and therefore it was used in all subsequent experiments.

Purification of His-CFP-GBP-YFP

Plasmid pIMBB666 expressing CFP-GBP-YFP under the control of T7 promoter was transformed into the *E.coli* strain BL21*/pLysS. A single transformed colony was inoculated in Luria-Broth medium, supplemented with 100μg/ml ampicillin and chloramphenicol and was left to grow for 9 hours at 30°C. Protein expression was induced at OD_{600} equal to 0.5 with 0.05mM isopropyl-β-D-thiogalactopyranoside (Anatrace) at 17°C for 12 hours. The cells expressing GBP-Fluo1 were collected by centrifugation (15 minutes; 4°C; 5.000 rpm). The cell pellet was resuspended in a solution of 1M NaCl, 50mM Tris-HCl pH 8.0, 5mM imidazole, 10% glycerol. The cell suspension was disrupted by sonication (for a total of 30min) and freezing the suspension every one minute of sonication, followed by centrifugation (30 min; 4°C; 30,000 rpm (40,000g). HisGBP-Fluo1 was purified from the cell lysate supernatant by Ni^{+2}-NTA (Qiagen) affinity chromatography. The resin was washed once with a buffer containing 1M NaCl, 50mM Tris-HCl pH 8.0, 5mM imidazole, 10% glycerol and once with a buffer containing 50mM NaCl, 50mM Tris-HCl pH 8.0, 5mM imidazole, 10% glycerol and the protein was eluted with 150mM imidazole. Protein was dialyzed (12h; 4°C) in buffer containing 50mM KCl, 50mM Tris-HCl pH 8.0, 10% glycerol and was then stored in 50mM KCl, 50mM Tris-HCl pH 8.0, 50% glycerol. Protein yield was around 17mg protein per 1 l culture. Purity and intactness of the purified protein was determined by SDS-PAGE (12% acrylamide).

Sample preparation

The proteins were stored at -18°C or at -80°C in buffer containing 50 mM Tris-HCl pH 8.0, 50 mM KCl and 5 mM EDTA mixed with 50% glycerol. Solutions of the protein were prepared as follows: Fluorescent protein (CFP, YFP or CFP-GBP-YFP) in storage buffer was added to a buffer containing 50 mM Tris-HCl pH 8.0 and 50 mM KCl, followed by addition of 2 mM of EDTA. Final concentration of fluorescent proteins was 2 μM, corresponding to a molecular concentration of $1.2\ 10^{15}$ cm^{-3}. If not mentioned otherwise, all experiments were performed two minutes after the addition of EDTA.

Steady-state optical measurements

Transmission measurements of fluorescent proteins in solution were performed in polystyrene cuvettes, using a Cary 300 spectrophotometer operating in double beam mode. The transmission was referenced against identical buffer solution without fluorescent proteins. The measurements were corrected

for differences in the transmission of the individual cuvettes and converted to absorbance using Lambert-Beer's law. The absorption cross section σ was calculated via the following relation:

$$Abs = -\ln\frac{P}{P_{ref}} = \sigma N d$$

where Abs stands for the sample absorbance, and P and P_{ref} for the optical powers transmitted through the sample and the reference, respectively. N represents the molecular concentration and d the absorption length. The absorption length inside the cuvettes was 1 cm.

Steady-state fluorescence was excited with a continuous wave laser diode (Sanyo DL-3146-151) emitting at 405 nm. The optical power on the sample was 1.72 mW. The excitation light was focused into a spot with a diameter of approximately 1 mm, close to the edge of the sample cuvette. Fluorescence emission was collected with an optical fiber (core diameter 1000 µm), mounted in contact with the cuvette using an SMA connector and oriented perpendicularly to the direction of the excitation beam. The optical fiber guided the fluorescence light to an Ocean Optics 2000 SD fiber spectrometer with internal CCD array for parallel acquisition of the fluorescence spectrum.

Excitation spectra of the fluorescent proteins were obtained using a Fluorolog 3-11 spectrofluorimeter (Jobin-Yvon SPEX), using a Xenon lamp for sample excitation. Excitation wavelength was selected with a 300 mm monochromator. The excitation bandwidth was 2.5 nm. The fluorescence light was dispersed again in a 300 mm monochromator and detected with a R928 photomultiplier from Hamamatsu in photon counting mode. The detection bandwidth was set to 5 nm. The spectra were collected with 1 nm step width and the sampling time varied between 0.2 s and 0.5 s depending on the protein under investigation. Spectra were corrected for dark noise, lamp profile and instrument response.

Time-correlated single photon counting

Fluorescence decays of the proteins were recorded using time-correlated single photon counting (TCSPC). The experimental set-up for TCSPC comprised pulsed laser excitation and simultaneous detection of fluorescence decays at multiple wavelengths, using a multi-channel PMT array and multidimensional TCSPC readout[2]. It is optimally suited for simultaneous monitoring of donor and acceptor fluorescence decays in FRET constructs. The fluorescent proteins were excited with a 375 nm picosecond pulsed laser diode (BDL-375, Becker-Hickl, Germany). Pulse repetition rate of the laser was 50 MHz, average power < 1 mW and pulse duration below 100 ps. Fluorescence from the sample in disposable acrylic cuvettes (Sarstedt #67.755, Germany) was collected perpendicular to the excitation beam, focused with a quartz lens and dispersed by a 160mm optical spectrograph (Oriel M-125, Germany) fitted with a 16-channel photomultiplier array (PML-Spec, Becker-Hickl, Germany). The spectrograph had a 600 line-pairs per mm grating, providing a dispersion of 13nm per photomultiplier channel within the spectral region of 397-605nm. In this way the whole spectrum of the CFP-GBP-YFP protein is mapped onto the PMT array. The TCSPC board SPC-830 (Becker-Hickl, Germany) was synchronized with the laser diode driver in the reversed regime (stop signal was derived from the laser pulse). We used 1024-point sampling on a 25 ns time-base and a channel width of 24.4 ps. The instrument response function of the setup, measured as half-width of the spontaneous Raman scattering peak of water at 430nm, was 190 ps (cf. Fig. 6 of the main article). Fluorescence decays of proteins were measured with an integration time of 60 s.

Kinetic model of protein fluorescence based on rate equations

Model formalism

The time development of the population concentration in each energy level of the system depicted in Figure 2 of the main article is governed by the following set of equations:

$$\begin{aligned}
\frac{dN_1}{dt} &= \sigma_{CFP}\Phi N_0 - \left(W_{CFP} + W_t N_a\right)N_1 \\
N_{CFP} &= N_0 + N_1 \\
\frac{dN_b}{dt} &= \sigma_{YFP}\Phi N_a + W_{tr} N_a N_1 - W_{YFP} N_b \\
N_{YFP} &= N_a + N_b
\end{aligned} \qquad \text{(S1)}$$

The symbols, in addition to the ones already described in the main text, refer to incident intensity of the excitation light in units of photons per square centimetre and second (Φ); the population concentration in energy level i (N_i), where the subscripts 0 and 1 describe ground and excited state of CFP, and subscripts a and b describe ground and excited state of YFP; the total concentrations of CFP and YFP, N_{CFP} and N_{YFP}, respectively. All concentrations are in molecules of protein per cubic centimetre. Using different units for the concentration leads to parameter values different from those derived from experimental data in the main article.

The system of equations (1) can be simplified by assuming that measurements are made in the regime of low excitation density, i.e. the population in any of the excited states is negligible and the operation regime is far from population saturation. This is justified by the low excitation intensities used in the experiments and the fast depopulation kinetics of the first excited state of both fluorescent proteins, which lie in the nanosecond range. In addition, the fluorescence intensity behaved linearly as a function of excitation intensity in our experiments, which is generally regarded as an indication that the measurement is performed far from saturation. In this regime, the mass balance equations reduce to

$$\begin{aligned}
N_{CFP} &\approx N_0 \\
N_{YFP} &\approx N_a
\end{aligned} \qquad \text{(S2)}$$

Substituting for N_0 and N_a in the differential equations, and defining the energy transfer rate $W_{tr} = W_t N_{YFP}$ leads to a system of two coupled differential equations

$$\begin{aligned}
\frac{dN_1}{dt} &= \sigma_{CFP}\Phi N_{CFP} - \left(W_{CFP} + W_{tr}\right)N_1 \\
\frac{dN_b}{dt} &= \sigma_{YFP}\Phi N_{YFP} + W_{tr} N_1 - W_{YFP} N_b
\end{aligned} \qquad \text{(S3)}$$

The differential equations (S3) describe the time-dependent populations in the excited states of CFP and YFP completely, including energy transfer between them. The equations can be solved sequentially, since the equation determining the population concentration in the excited state of CFP, N_1, is independent of N_b.

Rise of excited state population

In this section, we solve equations (S3) assuming that initially all proteins are in their ground state. Excitation takes place starting at time t=0 with constant photon flux density Φ. The initial conditions to calculate the increase in fluorescence emission are $N_1(t=0)=N_b(t=0)=0$. Due to the large energy difference between ground and excited states in these proteins, the thermal population of the higher energy levels can be neglected. Solving equations (3) gives

$$\begin{aligned} N_1(t) &= \frac{\sigma_{CFP}\Phi N_{CFP}}{W_{CFP}+W_{tr}}\left(1-\exp\{-(W_{CFP}+W_{tr})t\}\right) \\ N_b(t) &= \frac{\sigma_{YFP}\Phi N_{YFP}}{W_{YFP}} + \frac{W_{tr}}{W_{CFP}+W_{tr}}\frac{\sigma_{CFP}\Phi N_{CFP}}{W_{YFP}} \\ &\quad - \frac{W_{tr}}{W_{CFP}+W_{tr}}\frac{\sigma_{CFP}\Phi N_{CFP}}{W_{CFP}+W_{tr}-W_{YFP}}\exp\{-(W_{CFP}+W_{tr})t\} \\ &\quad - \left[\begin{array}{l}\dfrac{\sigma_{YFP}\Phi N_{YFP}}{W_{YFP}} + \dfrac{W_{tr}}{W_{CFP}+W_{tr}}\sigma_{CFP}\Phi N_{CFP} \\ \times\left(\dfrac{1}{W_{YFP}} - \dfrac{1}{W_{CFP}+W_{tr}-W_{YFP}}\right)\end{array}\right]\exp\{-W_{YFP}t\} \end{aligned} \qquad (S4)$$

In order to solve for the population in the excited state of YFP, the solution to $N_1(t)$ was introduced into the rate equation for $N_b(t)$. The time dependence of population in the excited state of CFP shows an increase with an inverse single exponential function whose decay rate is the sum of the rates that describe energy loss from CFP, i.e. CFP spontaneous decay (W_{CFP}) and energy transfer to YFP (W_{tr}). The time dependence of the population of the first excited state of YFP depends on two mechanisms: the feeding of YFP by the energy transfer from CFP and the spontaneous decay of the first excited state of YFP. The energy transfer increases the speed with which the fluorescence signal rises over that expected when YFP is excited through direct absorption.

Decay of excited state population

The inverse process to excitation is the decay of excited state population in the absence of excitation light. For convenience in notation, we define the time, at which the excitation light is switched off, again as t=0. At this time, the populations of the proteins' excited states are

$$\begin{aligned} N_1(t=0) &= N_1^0 \\ N_b(t=0) &= N_b^0 \end{aligned}$$

where N_1^0 and N_b^0 are constants.

These equations also represent the initial conditions for the solution of equations (S3). The constants N_1^0 and N_b^0 can be obtained from equations (S4) with knowledge of the excitation regime preceding t=0. Further, to determine the time dependence of the population density in the excited states from equations (S3), Φ here is set to 0. We obtain the solutions

$$\begin{aligned} N_1(t) &= N_1^0\exp\{-(W_{CFP}+W_{tr})t\} \\ N_b(t) &= \left(N_b^0 + \frac{W_{tr}N_1^0}{W_{CFP}+W_{tr}-W_{YFP}}\right)\exp\{-W_{YFP}t\} \\ &\quad - \frac{W_{tr}N_1^0}{W_{CFP}+W_{tr}-W_{YFP}}\exp\{-(W_{CFP}+W_{tr})t\} \end{aligned} \qquad (S5)$$

The fluorescence decay of the excited state of CFP in this model is single exponential. The decay rate is the sum of the spontaneous CFP decay rate and the energy transfer rate to YFP. In contrast, fluorescence decay from the excited state of YFP is governed by two exponential functions. One describes the decrease in fluorescence emission due to spontaneous decay of the population in the excited state of YFP. The other, entering the equation with a negative prefactor, is a feed term which decreases with the same rate that the CFP fluorescence is decaying with. This term actually slows down the intrinsic population decay of YFP's excited state by delivering additional energy via the energy transfer process from CFP.

Solutions for free fluorescent proteins

From equations (4) and (5), we can derive the time dependence of the excited state populations of the individual proteins. In the case that there is no energy transfer between CFP and YFP (W_{tr}=0), the fluorescent proteins act independent of each other. The equations for the population rise reduce to

$$\begin{aligned} N_1(t) &= \frac{\sigma_{CFP}\Phi N_{CFP}}{W_{CFP}}\left(1-\exp\{-W_{CFP}t\}\right) \\ N_b(t) &= \frac{\sigma_{YFP}\Phi N_{YFP}}{W_{YFP}}\left(1-\exp\{-W_{YFP}t\}\right) \end{aligned} \qquad (S6)$$

and the equations for the population decay become

$$\begin{aligned} N_1(t) &= N_1^0\exp\{-W_{CFP}t\} \\ N_b(t) &= N_b^0\exp\{-W_{YFP}t\} \end{aligned} \qquad (S7)$$

Both are governed exclusively by the spontaneous decay rates of the two proteins, W_{CFP} and W_{YFP}.

Solutions for steady-state excitation

The population densities in the excited states of CFP and YFP in CFP-GBP-YFP under steady state excitation can be determined from equations (4) in the limit of $t\to\infty$. We obtain

$$\begin{aligned} N_1 &= \frac{\sigma_{CFP}\Phi N_{CFP}}{W_{CFP}+W_{tr}} \\ N_b &= \frac{\sigma_{YFP}\Phi N_{YFP}}{W_{YFP}} + \frac{W_{tr}}{W_{CFP}+W_{tr}}\frac{\sigma_{CFP}\Phi N_{CFP}}{W_{YFP}} \end{aligned} \qquad (S8)$$

To obtain the steady-state population densities of CFP and YFP as free proteins, we again set W_{tr}=0 and obtain

$$\begin{aligned} N_1 &= \frac{\sigma_{CFP}\Phi N_{CFP}}{W_{CFP}} \\ N_b &= \frac{\sigma_{YFP}\Phi N_{YFP}}{W_{YFP}} \end{aligned} \qquad ()9)$$

Fluorescence emission

Fluorescence emission density (in units of photons per unit volume and time) is proportional to the population density in a given excited state. The proportionality constant is the radiative decay rate W^{rad}_i. The photon emission densities from CFP and YFP, Φ_{CFP} and Φ_{YFP}, can thus be written as

$$\begin{aligned} \Phi_{CFP} &= W^{rad}_{CFP} N_1 \quad \left(= W^{rad}_{CFP} \frac{\sigma_{CFP} \Phi N_{CFP}}{W_{CFP}} \right) \\ \Phi_{YFP} &= W^{rad}_{YFP} N_b \quad \left(= W^{rad}_{YFP} \frac{\sigma_{YFP} \Phi N_{YFP}}{W_{YFP}} \right) \end{aligned} \qquad \text{(S10)}$$

The terms in brackets describe the photon emission density from the free proteins in steady state, and are valid under these conditions only.

In an analogous fashion, the photon emission densities for CFP-GBP-YFP can be derived combining equations (S10) with equations (S8). It is obvious that for CFP-GBP-YFP the fluorescence emission from CFP is always lower, that from YFP is always higher than their counterparts from free individual CFP and YFP proteins. This demonstrates the expected effect of energy transfer.

Equations (S4) – (S10) completely describe the photon flow through the fluorescent proteins, from absorption of the excitation photon to emission of the fluorescence photon.

References

1 Ye, K.; Schultz, J.S. *Anal. Chem.* **2003**, 75, 3451 – 3459.
2 Becker, W. *Advanced Time-Correlated Single Photon Counting Techniques:* Springer Ser. Chem. Phys. Vol. 81, 2005.