Supporting information

The influence of fluorescent protein maturation on FRET measurements in living cells

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Derivation of equation 1.

Our recently developed FRET-based crowding sensor (crGE, containing mCerulean3 and mCitrine) is excited at 405nm under the microscope and the emission is split at 505 nm. The 405-505 nm channel gives the emission of the donor (I_{donor}, mCerulean3), and the 505-786 nm channel gives the emission of the acceptor ($I_{acceptor}$, mCitrine). The emission in the acceptor channel is composed of three components: the cross-excitation of mCitrine (Fig. S1B), the bleed through of mCerulean3 (Fig. S1C), and the actual FRET signal. The spectrum of the sensor is complicated because it is a sum of the three species. When sensor with only matured mCitrine ($crGE_{cit}$) is present in excess, the intensity of $I_{acceptor}$ is relatively high due to cross-excitation of mCitrine, which causes an apparent increase in ratiometric FRET. When mature mCerulean3 ($crGE_{cer}$) is in excess, $I_{acceptor}$ is relatively low due to a fraction of sensor that does not have acceptors and there is no FRET possible, which causes a lower ratiometric FRET. Hence, the maturation of fluorescent protein has an influence on the measured ratiometric FRET15,18.

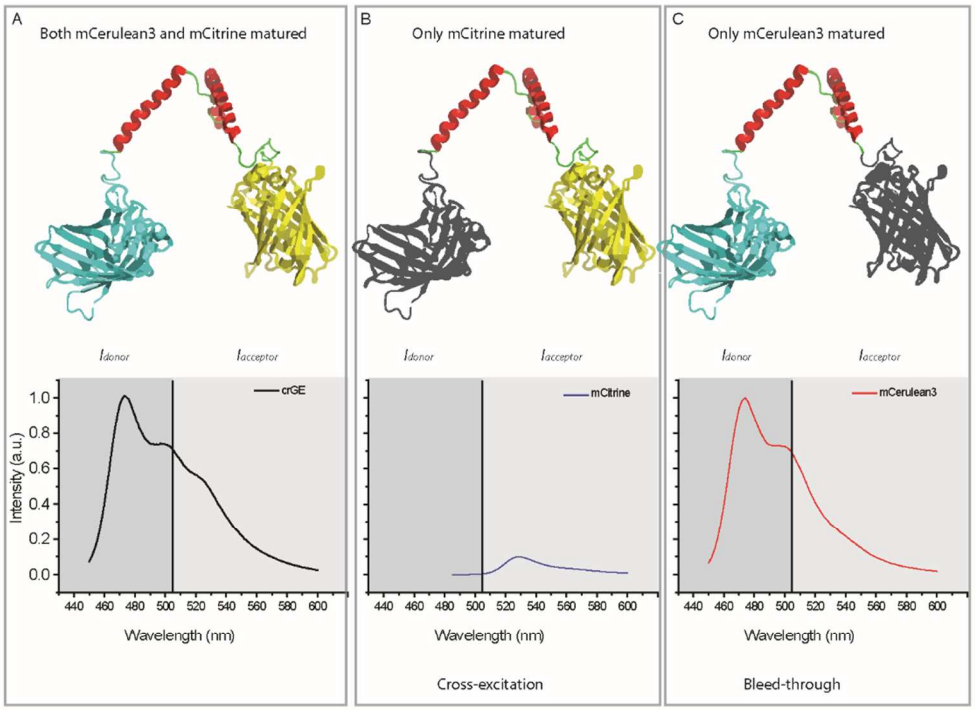

Fig. S1. Structures of FRET sensors with either the donor or acceptor or both fluorophores fully matured. **A**: Structure and fluorescence emission spectra of both mCerulean3 and mCitrine matured ($crGE_{cercit}$) (λ_{Ex} = 420 nm). **B**: Structure and fluorescence emission spectra of mCitrine matured ($crGE_{cit}$) (λ_{Ex} = 405 nm). **C**: Structure and fluorescence emission spectra of mCerulean3

matured ($crGE_{cer}$) (λ_{Ex}=405nm), which shows the bleed-through. The 450-505 nm channel is the donor emission channel (I_{donor}). The 505-786 nm channel is the acceptor emission channel ($I_{acceptor}$). The spectra show that $I_{acceptor}$ of crGE can be influenced by cross-excitation and bleed-through. The spectra serve as examples and are qualitative.

To quantify the influence of maturation on the observed ratiometric FRET, a model consisting of the maturation efficiency of the donor and the acceptor was build. The model is based on Eq. S1:

$$Ratiometric\ FRET = \frac{I_{acceptor}}{I_{donor}} \quad \text{Eq. S1}$$

Ratiometric FRET is the ratio of intensity of acceptor ($I_{acceptor}$) divided by intensity of donor (I_{donor}). For crGE sensor, I_{donor} is the fluorescent emission from the 450-505 nm channel and $I_{acceptor}$ is the fluorescent emission from the 505-797nm channel.

We then dissected the composition of Eq. S1. I_{donor} depends on the donor fluorescent protein and is decreased by FRET (Fig. S1A and C). $I_{acceptor}$ includes three parts (Eq. S2, Fig. S1): $I_{donor\ bleed\ through}$ is bleed through from donor. I_{FRET} is the donor's fluorescence emission from FRET by FRET. $I_{acceptor\ cross\text{-}excitation}$ is the direct excitation of the acceptor at 405 nm.

$$I_{acceptor} = I_{donor\ bleed\ through} + I_{FRET} + I_{acceptor\ cross\ \text{excitation}} \quad \text{Eq. S2}$$

Now we quantify these three parts, with the assumption that crGE is fully matured. First, we quantify the donor bleed through ($I_{donor\ bleed\ through}$), which is proportional to the intensity in the donor channel (Eq. S3).

$$I_{donor\ bleed\ through} = \alpha \cdot I_{donor} \quad \text{Eq. S3}$$

To calculate I_{FRET}, we start with the FRET efficiency, Eq. S4.[1] I_{donor} is the intensity of donor in the presence of acceptor. $I_{donor\ only}$ is the intensity of donor in the absence of acceptor. The difference between I_{donor} and $I_{donor\ only}$ is αI_{donor} (Eq. S5), which is due to FRET. With Eq. S5, we can rewrite Eq. S4 into Eq. S6.

$$E = 1 - \frac{I_{donor}}{I_{donor\ only}} \quad \text{Eq. S4}$$

$$\Delta I_{donor} = I_{donor\ only} - I_{donor} \quad \text{Eq. S5}$$

[1] Lakowicz, J. R. & Masters, B. R. Principles of Fluorescence Spectroscopy. 3rd edn, (2006).

$$\Delta I_{donor} = \frac{E}{1-E} \cdot I_{donor} \quad \text{Eq. S6}$$

When considering the difference in brightness between donor and acceptor, we introduce coefficient γ (Eq. S7).

$$I_{FRET} = \gamma \cdot \Delta I_{donor} \quad \text{Eq. S7}$$

We quantified $I_{acceptor\ cross\text{-}excitation}$, which is proportional to the amount of acceptor. The number of donors and acceptors is the same in fully matured sensor, which means that the cross-excitation part ($I_{acceptor\ cross\text{-}excitation}$) is proportional to the intensity of the donor in the absence of acceptor ($I_{donor\ only}$). Hence, we introduce β to obtain the relation between cross excitation of acceptor and $I_{donor\ only}$. The relation is shown in Eq. S8. With Eq. S4, we can rewrite Eq. S8 to Eq. S9.

$$I_{acceptor\ cross\ excitation} = \beta \cdot I_{donor\ only} \quad \text{Eq. S8}$$

$$I_{acceptor\ cross\ excitation} = \frac{\beta \cdot I_{donor}}{1-E} \quad \text{Eq. S9}$$

Now we can insert Eq. S1, S3, S7, and S9 into Eq. S1, resulting in Eq. S10:

$$Ratiometric\ FRET = \frac{I_{acceptor}}{I_{donor}} = \frac{\alpha \cdot I_{donor} + \gamma \cdot \frac{E}{1-E} \cdot I_{donor} + \frac{1}{(1-E)} \cdot \beta \cdot I_{donor}}{I_{donor}} \quad \text{Eq. S10}$$

Next, we introduced the degree of maturation to obtain Eq. S11, in which m_{donor} is the maturation percentage of donor, $m_{acceptor}$ is the maturation percentage of acceptor, and I^0 indicates the intensity of fully matured protein. The bleed through only relates to the maturation of donor and can be calculated with Eq. S12. FRET requires that both donor and acceptor are matured. Cross-excitation is only related to the maturation of acceptor.

Lastly, to predict the effect of maturation on ratiometric FRET, we determined the coefficients α, β and γ, and FRET efficiency (*E*). We quantified these parameters with purified fluorescent proteins (mCerulean3 and mCitrine) and purified, fully matured crGE. Coefficient α is related to the emission of mCerulean3 (Fig. S1C). It was determined by excitation of purified mCerulean3 (λ_{Ex} = 405nm) and measurement of the emission in channel 405-505 and channel 505-797nm. The coefficient β is employed to build the relation between the ratio of emission upon cross excitation of acceptor, and donor emission in the absence of acceptor ($I_{donor\ only}$). To determine coefficient β, we need to know the emission intensity of mCitrine (Ex 405 and 488 nm) and the emission intensity of mCerulean3 (λ_{Ex} = 405 nm). We excited purified mCitrine and mCerulean at 405nm and 488 nm, respectively. We did not observe emission of mCerulean3 when excited

at 488 nm, which indicates that we can quantify the amount of mCitrine in crGE with 488nm excitation. We excited crGE sensor at 405 and 488nm separately and recorded the intensity.

$$Ratiometric\ FRET = \frac{m_{donor} \cdot \alpha \cdot I^0_{donor} + m_{donor} \cdot m_{acceptor} \cdot \gamma \cdot I^0_{donor} \cdot \frac{E}{1-E} + \frac{1}{(1-E)} \cdot m_{acceptor} \cdot \beta \cdot I^0_{donor}}{m_{donor} \cdot I^0_{donor}}$$

$$= \frac{m_{donor} \cdot \alpha \cdot I^0_{donor}}{m_{donor} \cdot I^0_{donor}} + \frac{m_{donor} \cdot m_{acceptor} \cdot \gamma \cdot I^0_{donor} \cdot \frac{E}{1-E}}{m_{donor} \cdot I^0_{donor}} + \frac{\frac{1}{(1-E)} \cdot m_{acceptor} \cdot \beta \cdot I^0_{donor}}{m_{donor} \cdot I^0_{donor}} \quad \text{Eq. S11}$$

$$I_{donor} = m_{donor} \cdot I^0_{donor} \quad \text{Eq. S12}$$

$$I_{acceptor} = m_{acceptor} \cdot I^0_{acceptor} \quad \text{Eq. S13}$$

Equation S11 can be simplified to Equation S14:

$$Ratiometric\ FRET = \alpha + m_{acceptor} \cdot \gamma \cdot \frac{E}{1-E} + \frac{1}{(1-E)} \cdot \frac{m_{acceptor} \cdot \beta}{m_{donor}} \quad \text{Eq. S14}$$

We use Eq. S14 and Figure S8 to quantify the effect of maturation on the FRET ratio. To determine how the bleed-through, the FRET, and the cross-excitation influence the observed ratiometric FRET signal, we quantified the coefficients α, β and γ with purified mCerulean3, mCitrine, and crGE. The value of α, which is only related to the emission of mCerulean3, is 0.66. Coefficient γ is the brightness difference between mCerulean3 and mCitrine, and it is ~2. The coefficient β, which is employed to build the relation between the ratio of emission upon cross excitation of acceptor, and donor emission in the absence of acceptor ($I_{donor\ only}$), is 0.05. We know that the FRET efficiency (*E*) of crGE in pRSET A is about 12±2%[2] in exponentially growing *E. coli* cells in MOPS minimal medium. Inserting all parameters into Eq. S14, yields the following relationship.

$$Ratiometric\ FRET = 0.66 + 0.34 \cdot m_{acceptor} + 0.06 \cdot \frac{m_{acceptor}}{m_{donor}} \quad \text{Eq. S15}$$

[2] Liu, B. et al. Design and Properties of Genetically Encoded Probes for Sensing Macromolecular Crowding. *Biophys. J.* **2017**, *112*, 1929-1939.

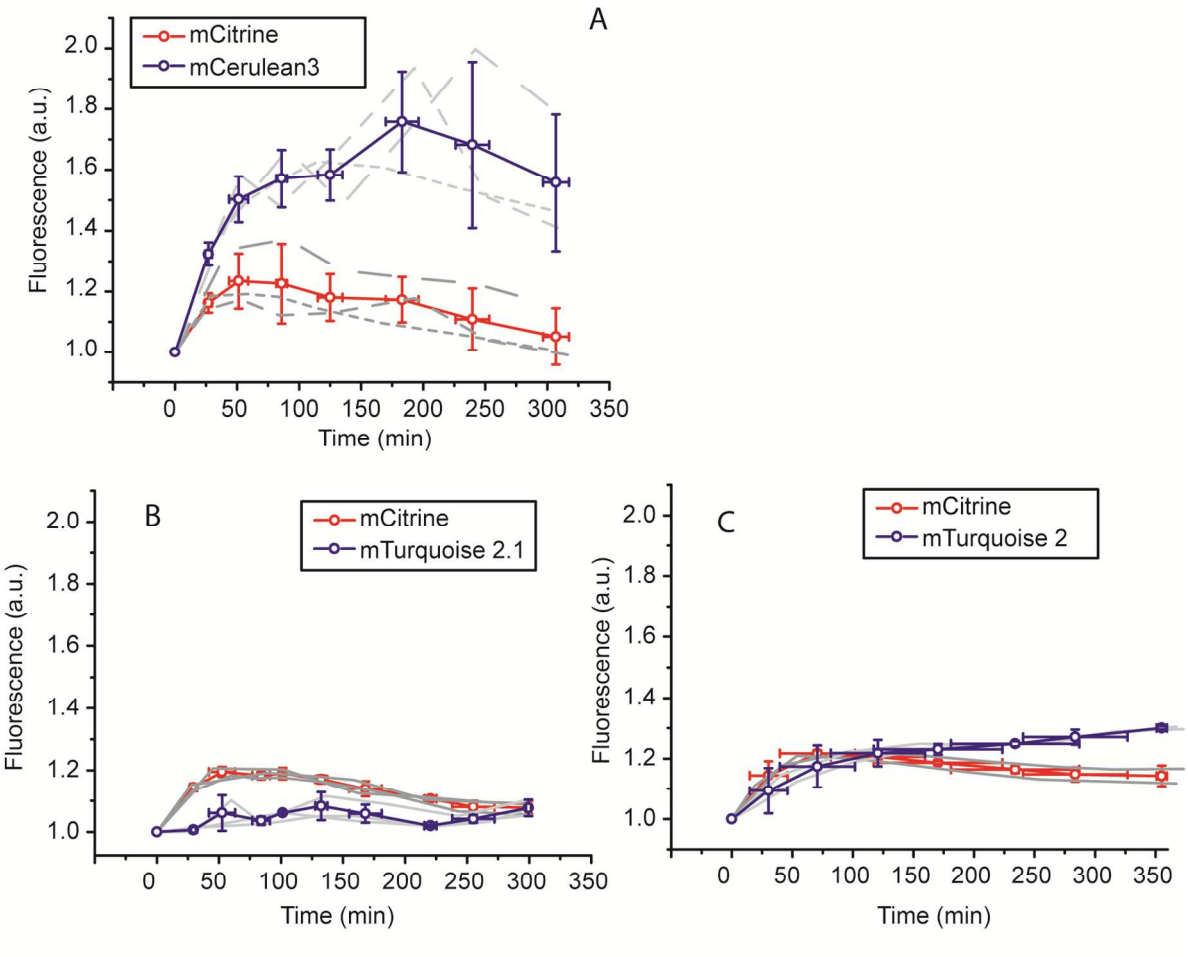

Figure S2. Fluorescence increase upon addition of chloramphenicol for the different fluorophores, for A. crGE containing mCitrine and mCerulean3, B. crTC2.1 containing mCitrine and mTurquoise 2.1, and C. crTC containing mCitrine and mTurquoise 2. Dashed lines connect the points (average of about 100 cells for each timepoint) from biological replicates. The colored lines show the averages + standard deviations of the biological replicates.

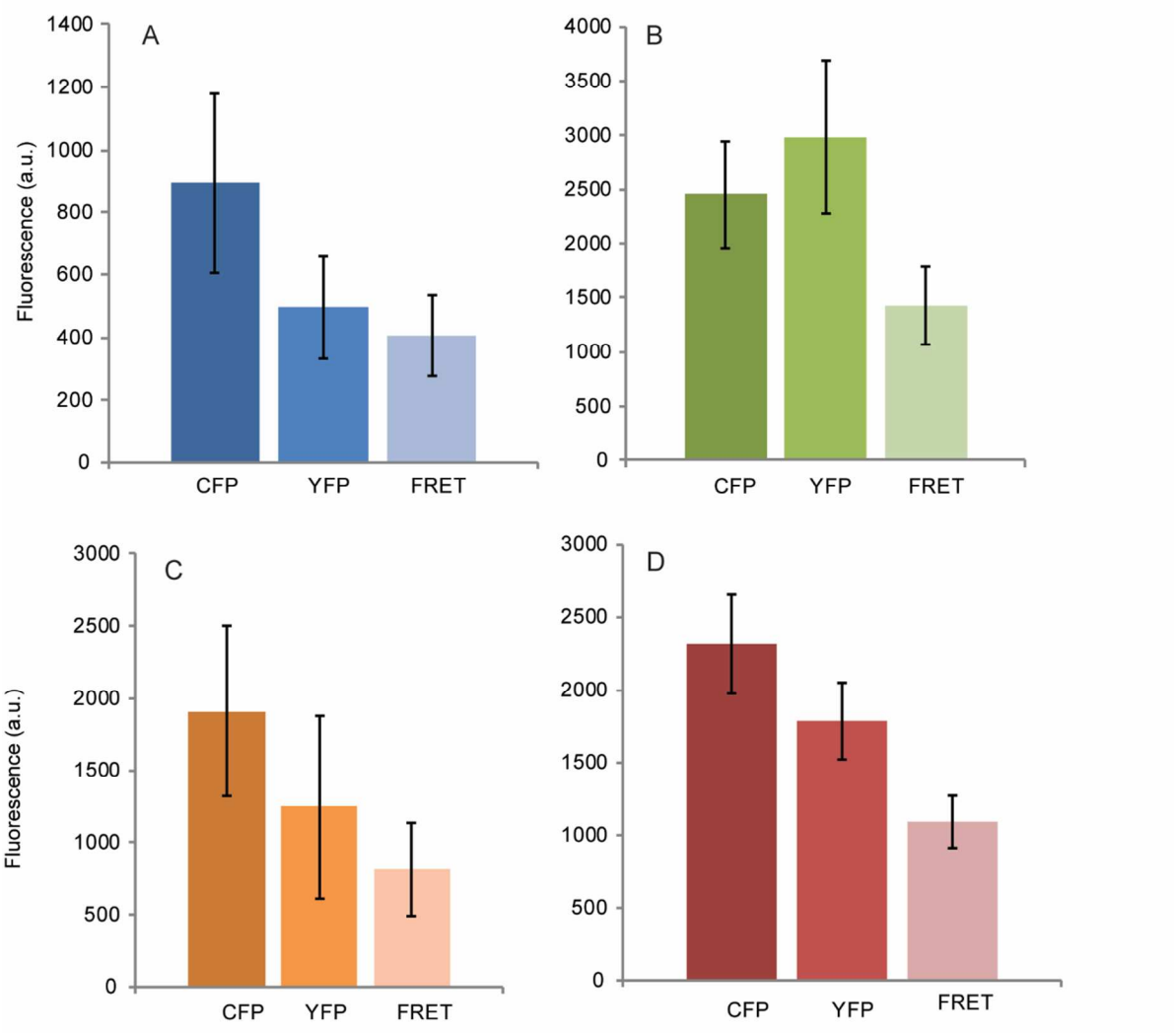

Figure S3: Fluorescence emission of the fluorophores under the different expression conditions in yeast: **A**. Purified crGE sensor, which has maximally matured fluorophores at 80% maturation. **B** to **E** *in vivo* sensor: **B**. expressed from the GAL1 promoter, induction with 0.2% galactose. The data is showing a large portion of immature CFP. **C**. Cells grown in mixed medium of 2% galactose and 0.2% glucose. **D**. Constitutive expression of crGE sensor from the TEF1 promoter. Error bars are standard deviations over at least 20 cells.

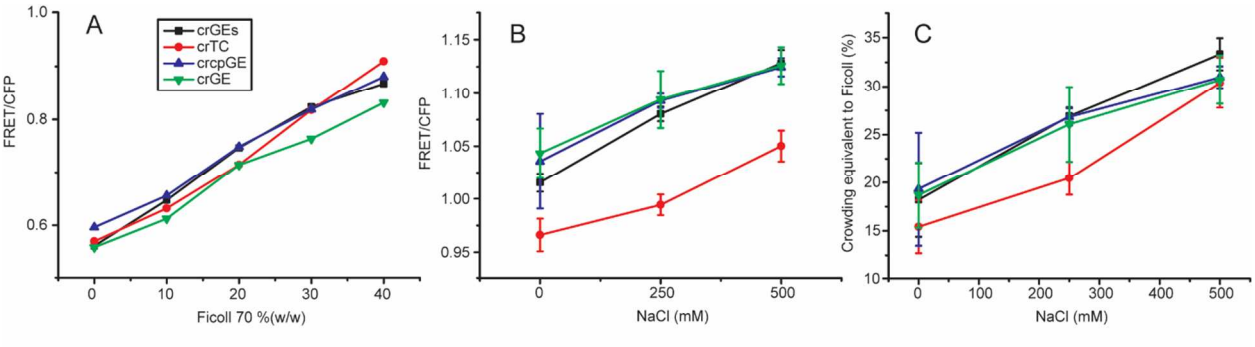

Figure S4. Sensor function is not dependent on the fluorescent protein identity, allowing facile exchange of fluorescent proteins **A**: Effect of Ficoll 70 on the FRET/CFP (525 nm / 475 nm emission after excitation at 420 nm, of purified crowding sensors measured by fluorometry. **B**: FRET (505-795 nm) /CFP (450-505 nm) ratio after excitation at 405 nm of different sensors in *E. coli*, before and after NaCl-induced (250 and 500 mM) osmotic upshift, and determined by confocal fluorescence microscopy. Osmotic upshifts were performed in media without potassium and glucose to prevent (rapid) recovery. **C**: Weight percentage Ficoll equivalents for the different sensors expressed in *E. coli*, calculated after calibration with purified sensor. Error bars are the standard deviation of >100 cells.

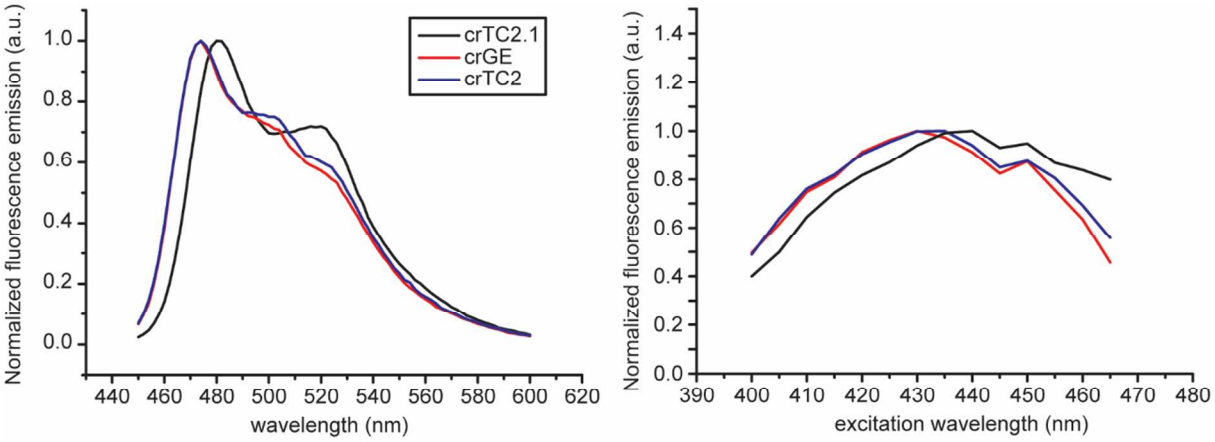

Figure S5. Emission (left) and excitation (right) spectra of purified crGE, crTC2, and crTC2.1, recorded by fluorometry after excitation at 420 nm (left) and at emission 475 nm (right) in 10 mM NaP_i, pH 7.4. Data was normalized to the maximum intensities.

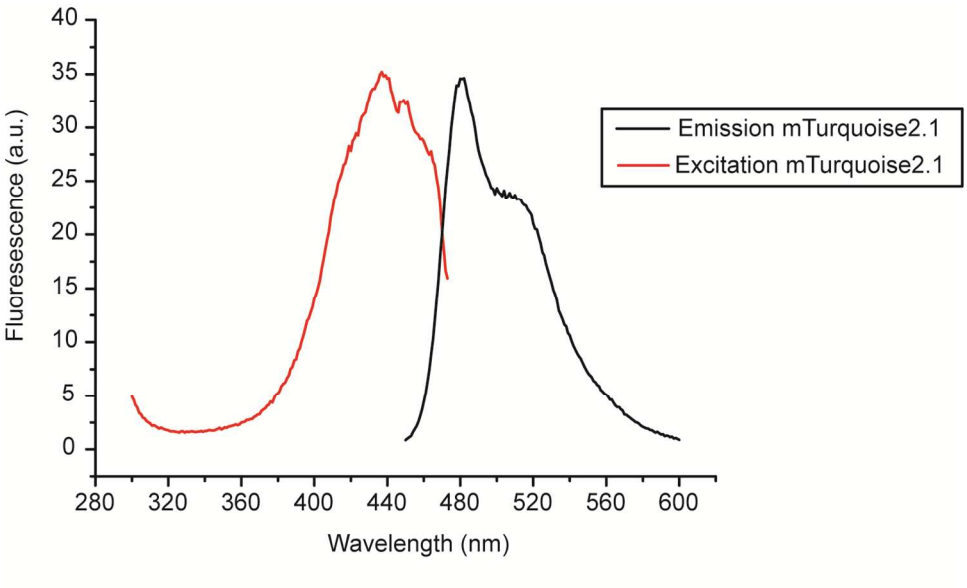

Figure S6. Excitation and emission spectrum of mTurquoise2.1 (without mCitrine). Excitation spectrum was recorded by fluorometer at emission wavelength of 480 nm, and emission spectrum was recorded at excitation wavelength of 440 nm, in 10 mM NaP_i, pH 7.4.

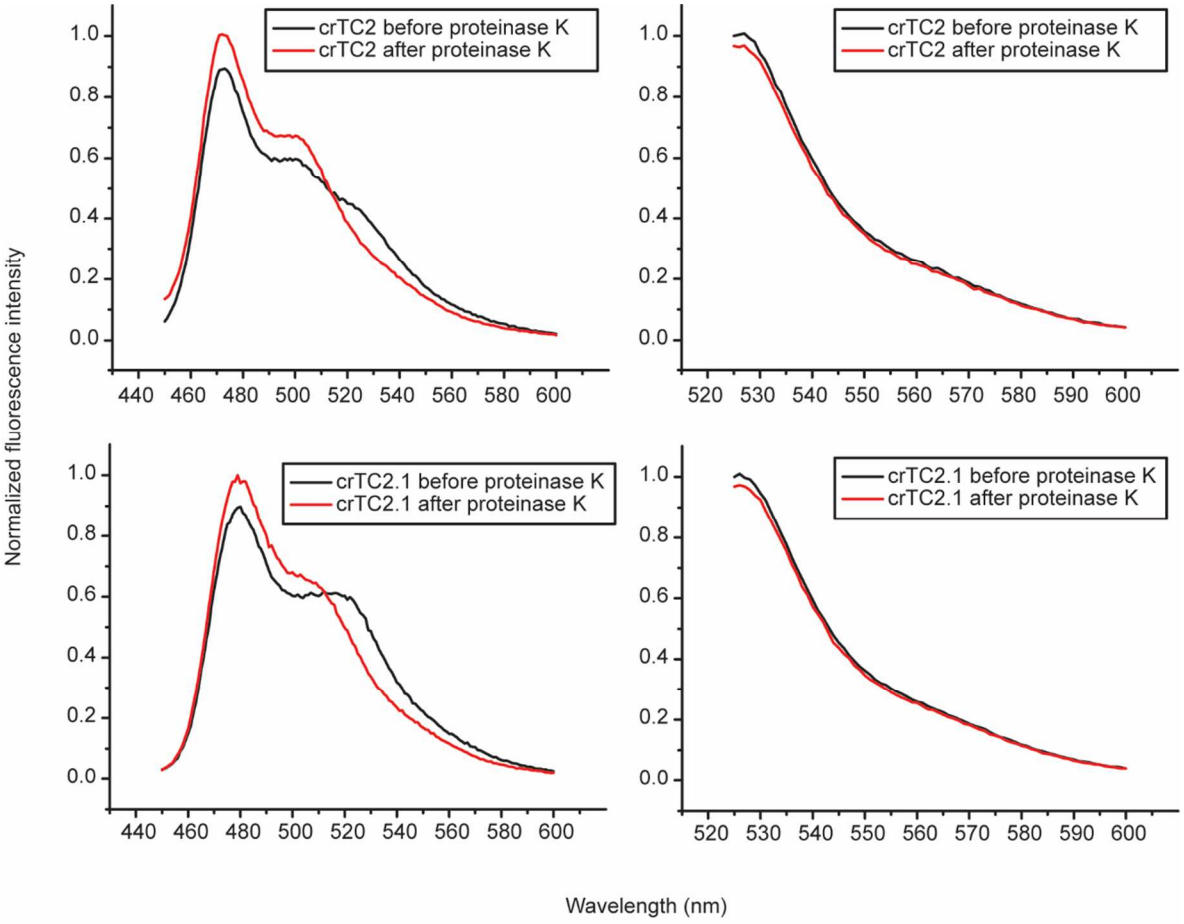

Figure S7: Proteinase K treatment for FRET determination. **A**. crTC2 before and after proteinase K treatment excited at 420 nm. **B**. crTC2 before and after proteinase K treatment excited at 505 nm. **C**. crTC2.1 before and after proteinase K excited at 420 nm. **D**. crTC2.1 before and after proteinase K excited at 505 nm. Spectra were normalized to the maximum emission prior proteinase K addition. Experiments were performed in 10 mM NaPi, pH 7.4, with 2 µL (5 mg/mL) proteinase K for 2 minutes, after which 2 µL (100 mM in isopropanol) PMSF was added to the cuvette.

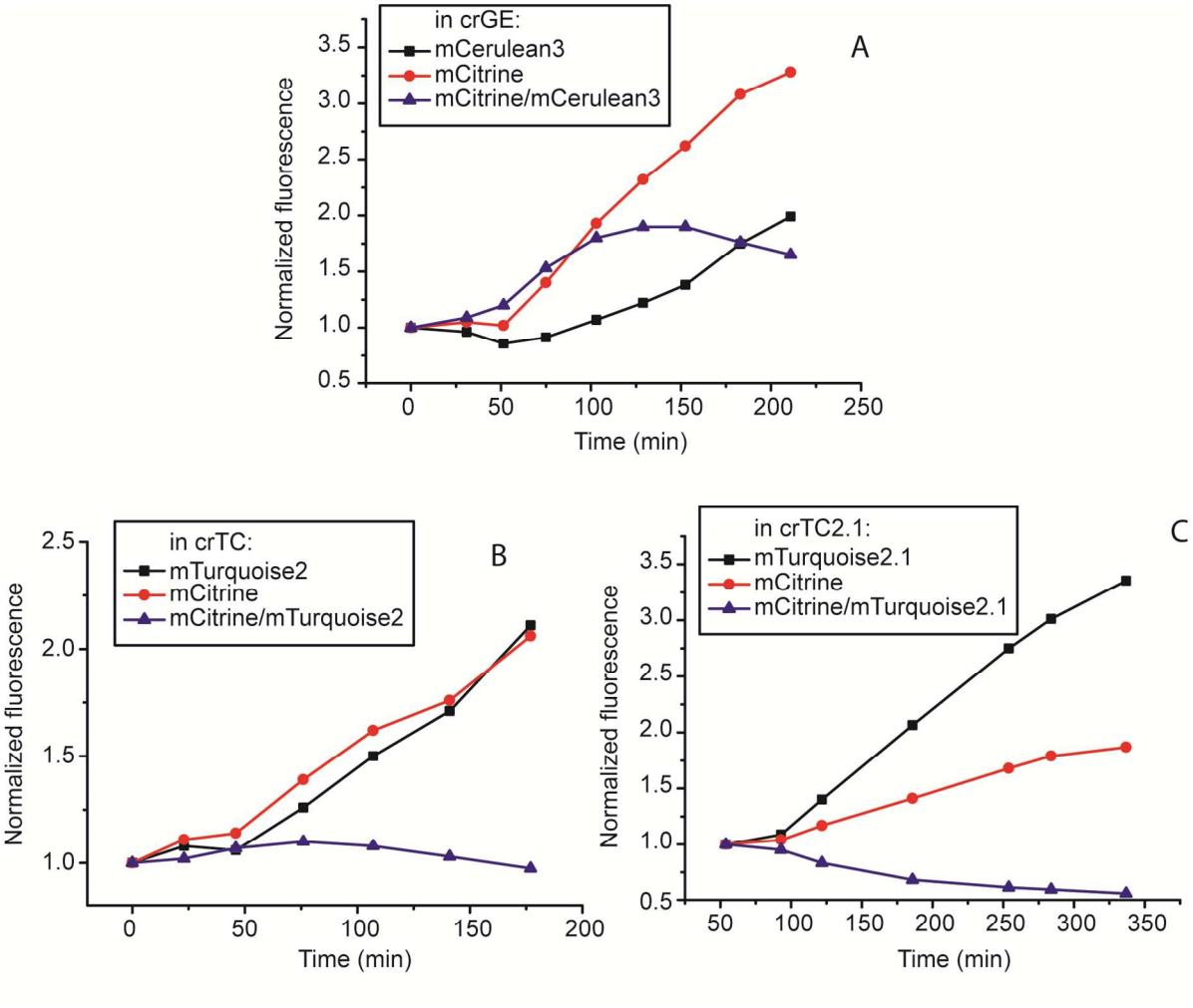

Figure S8: Observing the fluorescence increase upon exciting the individual fluorophores after addition of IPTG, by fluorometry. **A**. crGE with mCitrine and mCerulean3, **B**. crTC with mCitrine and mTurquoise2, and **C**. crTC2.1 with mCitrine and mTurquoise2.1. The data closely follows the trends that we observed with the chloramphenicol treatment.

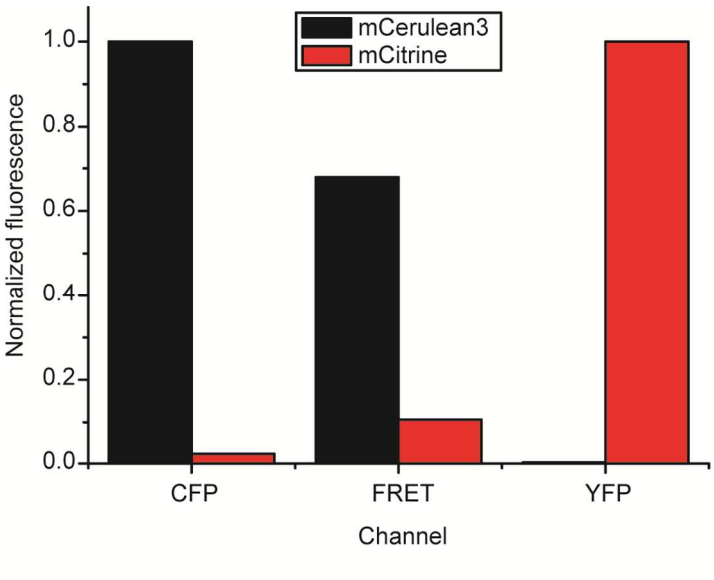

Figure S9: Comparison of fluorescence in the CFP (ex 405, em 450-505), FRET (ex 405, em 505-786), and YFP (ex 488, em 505-786) confocal microscopy channels of purified mCerulean3 and mCitrine. The proteins were dissolved in 10 mM NaPi, pH 7.4, and measurements were done in a droplet placed on a cover slide. Data are normalized to the highest intensity for each fluorophore.