Electronic Supplementary Material (ESI) for Integrative Biology.

Supporting information

Fig. s1 Concentric circle bleaching protocol. (A) Concentric circle protocol of the plate bleaching configuration. Red circle represents the edge of a Petri dish. Blue line shows the path of the center of the illumination spot. Inset table show the time required on each circle. Each sequentially larger circle's radius increases in size by the illumination spot size's diameter. (B) Image of fluorescein Petri dish before photobleaching. (C) Image of fluorescein Petri dish after photobleaching. (D) Theoretical and observed photobleaching profiles. Blue represents theoretical photobleaching. Red observed photobleaching.

Fig. s2 Concentration dependence of photostability. All samples were bleached for 20 min in the tube bleach configuration under the white light illumination of a 300 W xenon arc lamp. Black is mCitrine, orange is Citrine 1.7, grey is Citrine 1.9, yellow is Citrine 1.10, blue is Citrine 1.11, and green is Citrine 2.

Fig. s3 Parts for Tube and plate bleach configurations. (A) Support device for tube bleach configuration. (B) Lab jack (C) Stepping motor for plate bleach configuration. (D) Spinning motor module for tube bleach and plate bleach configurations. (E) Petri Dish. (F) Petri Dish to spinner motor module adaptor. (G) PCR tube holder. (H) PCR tube holder to spinner motor module adaptor. (I) Lego Mindstorms intelligent brick. Bottom image shows the same components flipped over.

Fig. s4 Plate bleach configuration. (A) Top view. (B) Plate side view. (C) Brick side view (D) Bottom view.

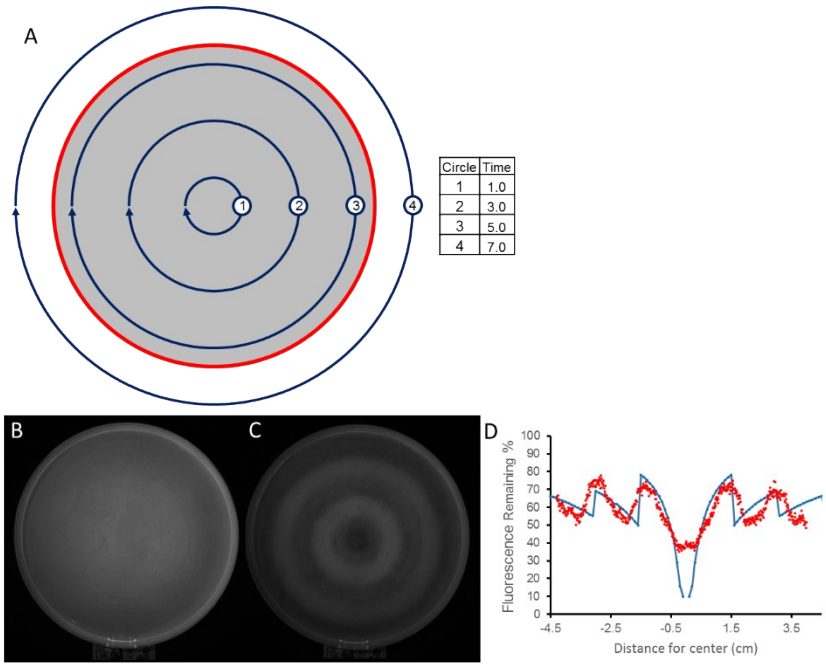
Fig. s5 Tube bleach configuration. (A) Right side view. (B) Front view. (C) Left side view (D) Bottom view.

Fig. s6 Validation of the tube bleach configuration. Each point is a single sample's set and each color identifies sample sets run at the same time. (e.g. the blue run contained six sample sets, three mCitrine and three Citrine2.)

Table s1 mCitrine variant mutations.

Table s2 Fluorescence lifetime.

Table s3 HeLa cell photobleaching.



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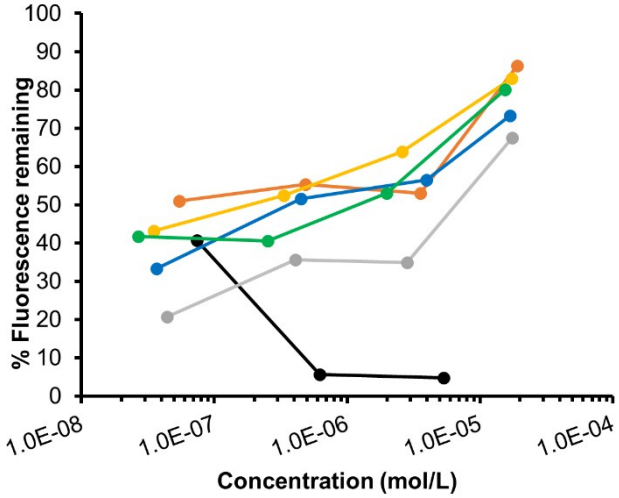

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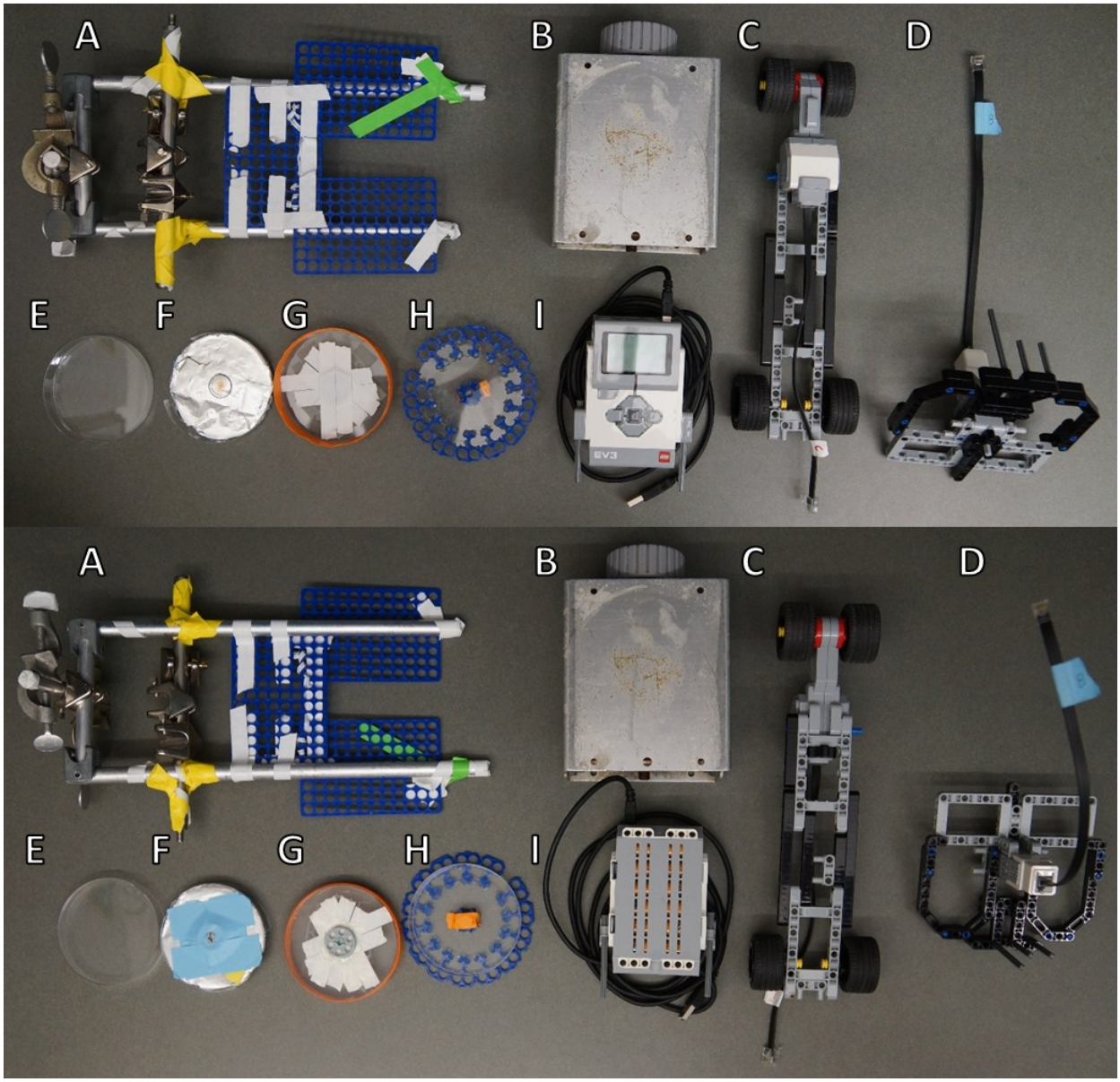

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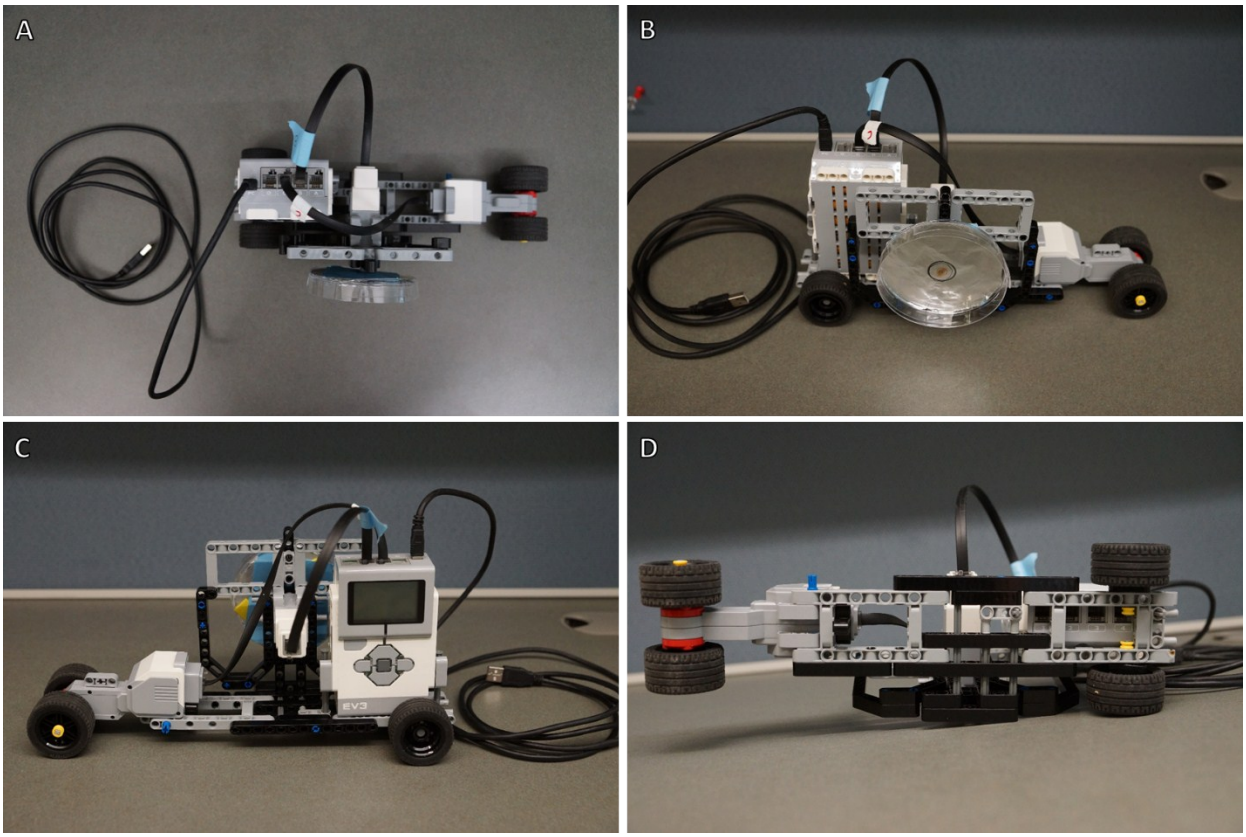

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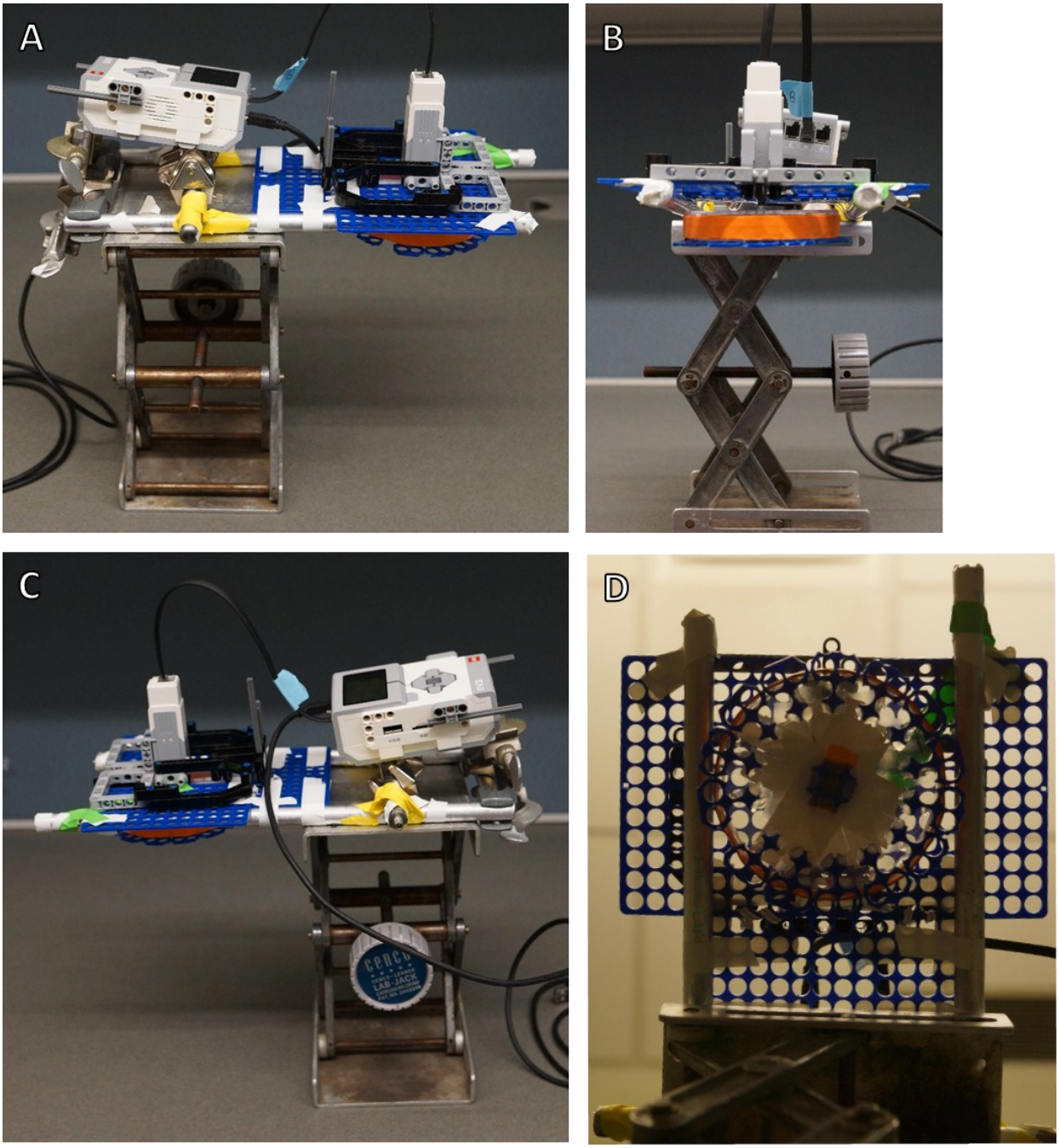

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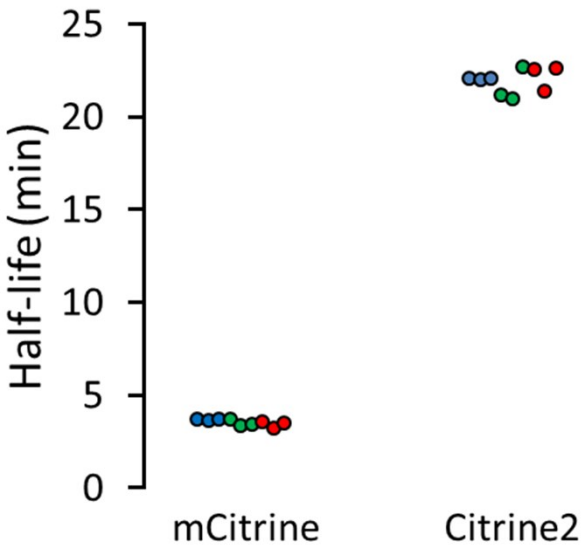

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Table S1 mCitrine variant mutations

Citrine 1.7		Y145H		V163A		K214E	M218T	D234G
Citrine 1.9		Y145H	N149Y	V163A		K214E	M218T	D234G
Citrine 1.10		Y145H	N149Y	V163A	K206Q	K214E	M218T	D234G
Citrine 1.11	M69T	Y145H	N149Y	V163A	K206Q	K214E	M218T	D234G
Citrine 2	S30T	M69T	Y145H	N149Y	V163A	K206Q	K214E	M218T	D234G

Table S2 Fluorescence lifetime

	Monoexponential fitting			**Biexponential fitting**								
	τ[a]	±[b]	χ^2	τ1	±	A1[c]	±	τ2	±	A2	±	χ^2
mCitrine	3.63	0.02	1.15	-	-	-	-	-	-	-	-	-
Citrine 1.7	2.58	0.02	0.89	3.36	0.56	0.32	2.6E-01	1.92	4.0E-01	0.68	0.202	0.78
Citrine 1.9	2.90	0.03	1.06	3.17	0.26	0.61	2.7E-01	1.86	7.6E-01	0.39	0.19	1.05
Citrine 1.10	2.75	0.02	0.93	-	-	-	-	-	-	-	-	-
Citrine 1.11	3.24	0.03	0.95	-	-	-	-	-	-	-	-	-
Citrine 2	3.31	0.02	0.88	1.44	0.04	0.26	1.2E-02	3.49	6.0E-03	0.74	0.003	0.86

[a] All lifetimes (τ) are reported in ns. [b] ± are the standard deviations. [c] A1 and A2 are fractions of each lifetime making up the biexponential decay curves.

Table S3 HeLa cell photobleaching

Imaging mode		**$t_{1/2}$[a] (s)**	**±[b]**	**InPhO fold change[c]**
widefield	mCitrine	34.18	2.38	-
	Citrine 2	59.63	5.90	1.72
confocal	mCitrine	2.57	0.23	-
	Citrine 2	5.34	1.97	2.05

[a] The $t_{1/2}$ are HeLa photobleaching half-lives. [b] ± is the standard error. [c] InPhO fold change was compared to the InPhO of mCitrine in the same imaging mode.

Theoretical bleaching

The theoretical bleaching model used here assumes a single line of illumination with a length equal to the diameter of the real illumination spot. This approximates the complex donut-like spot shape of the xenon arc lamp light source. To calculate the irradiance, or total illumination time(s), a circumference is calculated for every 1 mm across the diameter of the theoretical Petri Dish based around the center of the Petri Dish. For each circle of illumination (3 mm steps in the overlapping circle protocol) that would touch a given 1 mm point, the time spent on that circle of illumination is divided by the circumference for that 1 mm point, giving the irradiance for than 1 mm point from that circle of illumination. Where multiple illumination circles touch the same 1 mm point, their irradiances are summed for that 1 mm point. This irradiance is then used as the time variable (t) in the half-life equation with N_0 starting at 100 (100% fluorescence remaining). The half-life ($t_{1/2}$) is fit to the average % fluorescence remaining (59%) from the experiments.

$$N(t) = N_0(1/2)^{\frac{t}{t_{1/2}}}$$

Experimental minutia

Initial testing was performed using the non-overlapping concentric circle protocol (Fig. s1) with 30 s on the inner-most circle. After Citrine 1.7, we switched to the overlapping circle protocol, where the time spent on the inner-most circle was 6 s during the first rounds of screening but after Citrine 1.10, a longer time of 10 s was used to differentiate between the more photostable variants. The distance that the robot drives should be calibrated periodically to ensure that the robot drives 3 mm (1/5th of the illumination diameter) each step (the battery power seems to change the step size). Fig. s1 shows the equivalent theoretical and fluorescein plate bleach data for the non-overlapping concentric circle protocol as Fig. 1 does for the overlapping concentric circle protocol. For the non-overlapping circle protocol, bleaching of a fluorescein plate (58% average fluorescence remaining), caused uneven fluorescence across the Petri Dish and a line profile through the center revealed a distinct saw-tooth pattern (Fig. s1B-D). The difference between the maximum and minimum remaining fluorescence was 43% ± 10% std. dev. (Fig. s1D).

Extinction coefficient and QY

Extinction coefficients were determined by measuring the absorption spectrum using a UV/Vis spectrometer (Beckman Coulter DU 800) at pH 7.25 in 1×TBS and the same concentration of the FP in 1 M NaOH. In 1 M NaOH, the chromophore has an extinction coefficient of 44 000 $M^{-1}cm^{-1}$ at their absorption peak near 450 nm.[1] Application of the Beer-Lambert law gives the concentration of the alkaline denatured chromophore, which can be used to calculate the extinction coefficient for the intact protein at pH 7.25.

Quantum yields were determined by creating a dilution series of the FPs ranging in absorbance from approximately 0.02 to 0.05, in 1×TBS at pH 7.0. An emission scan from 450 nm to 800 nm with an excitation wavelength of 440 nm was used. The sum of the emission fluorescence values over this range were then plotted against their absorbance values, creating a linearly increasing set of data points. The slope is directly related to the QY. To convert the slope into QY, a standard sample with a known QY (In our case mCitrine with its known QY of 0.76)[2] must be prepared and measured under the same conditions as the unknown samples, resulting in its own slope. The slopes for the Citrine variants were then divided by the slope of mCitrine and multiplied by the QY of mCitrine, resulting in the QY of the mCitrine variant. All fluorescence spectra were obtained using a fluorescence plate reader (Tecan Safire2).

1 L. A. Gross, G. S. Baird, R. C. Hoffman, K. K. Baldridge and R. Y. Tsien, *Proc. Natl. Acad. Sci.*, 2000, **97**, 11990–11995.

2 P. J. Cranfill, B. R. Sell, M. A. Baird, J. R. Allen, Z. Lavagnino, H. M. de Gruiter, G.-J. Kremers, M. W. Davidson, A. Ustione and D. W. Piston, *Nat. Methods*, 2016, 1–7.