Supplementary Information

Chemogenetic Tags with Probe Exchange for Live-Cell Fluorescence Microscopy

Aditya Iyer[§*], Maxim Baranov[¥], Alexander J Foster[§], Shreyans Chordia[‖], Gerard Roelfes[‖], Rifka Vlijm[‡], Geert van den Bogaart[¥], Bert Poolman[§*]

[§]Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

[‡]Molecular Biophysics, Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

[¥]Department of Molecular Immunology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, the Netherlands

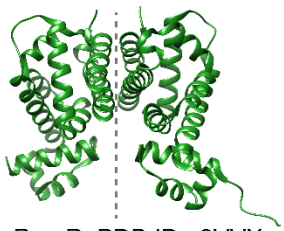
[‖]Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

*Email for correspondence: a.s.iyer@rug.nl; b.poolman@rug.nl

This PDF file includes:

Figures S1 to S26
Tables S1 to S3
Appendix 1: DNA sequences (5' to 3') of constructs used in the study
Appendix 2. Gating strategy for flow cytometry studies
Appendix 3. Sources and nomenclature of dyes
SI References

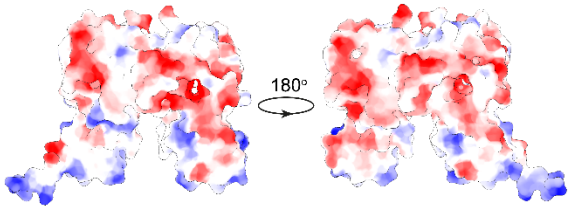
a



RamR: PDB ID : 3VVX

LmrR: PDB ID : 3F8C

b



c

Primary sequence: RamR

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NAGVAEGTLFRYFATKDELINTLYLHLKQDLCQSMI
MELDRSITDAKMMTRFIWNSYISWGLNHPARHRAI
RQLAVSEKLTKETEQRADDMFPELRDLCHRSVLM
VFMSDEYRAFGDGLFLALAETTMDFAARDPARAG
EYIALGFEAMWRALTREEQ

Primary sequence: LmrR

MGAEIPKEMLRAQTNVILLNVLKQGDNYVYGIIKQV
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QGGRRKYYRLTEIGHENMRLAFESWSRVDKIIENLE
ANKKSEAIKAA

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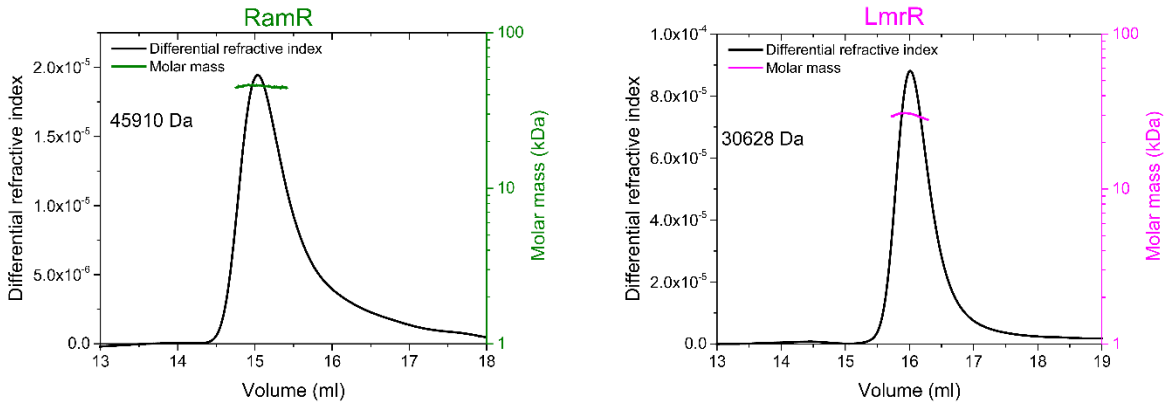

Fig. S1. (a) Protein structures by x-ray crystallography of transcription factor-based chemogenetic CTPEs: RamR and LmrR. (b) Coulombic surface mapping of the electrostatic potential of CTPEs depicted red for negative potential, white near neutral, and blue for positive potential. (c) The primary sequences of the CTPEs used in the study. (d) Size-exclusion chromatography coupled the multi-angle light scattering (SEC-MALS) analysis of CTPEs to determine the native molecular weight of the proteins. Black lines, signals from the refractive index detector (left-hand Y-axis); solid colored lines, calculated protein molecular weights (right–hand Y-axis). RamR (green) and LmrR (magenta) were observed to have a molecular weight of ~45.9 kDa and ~30.6 kDa, respectively, which correspond to dimeric structures. All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

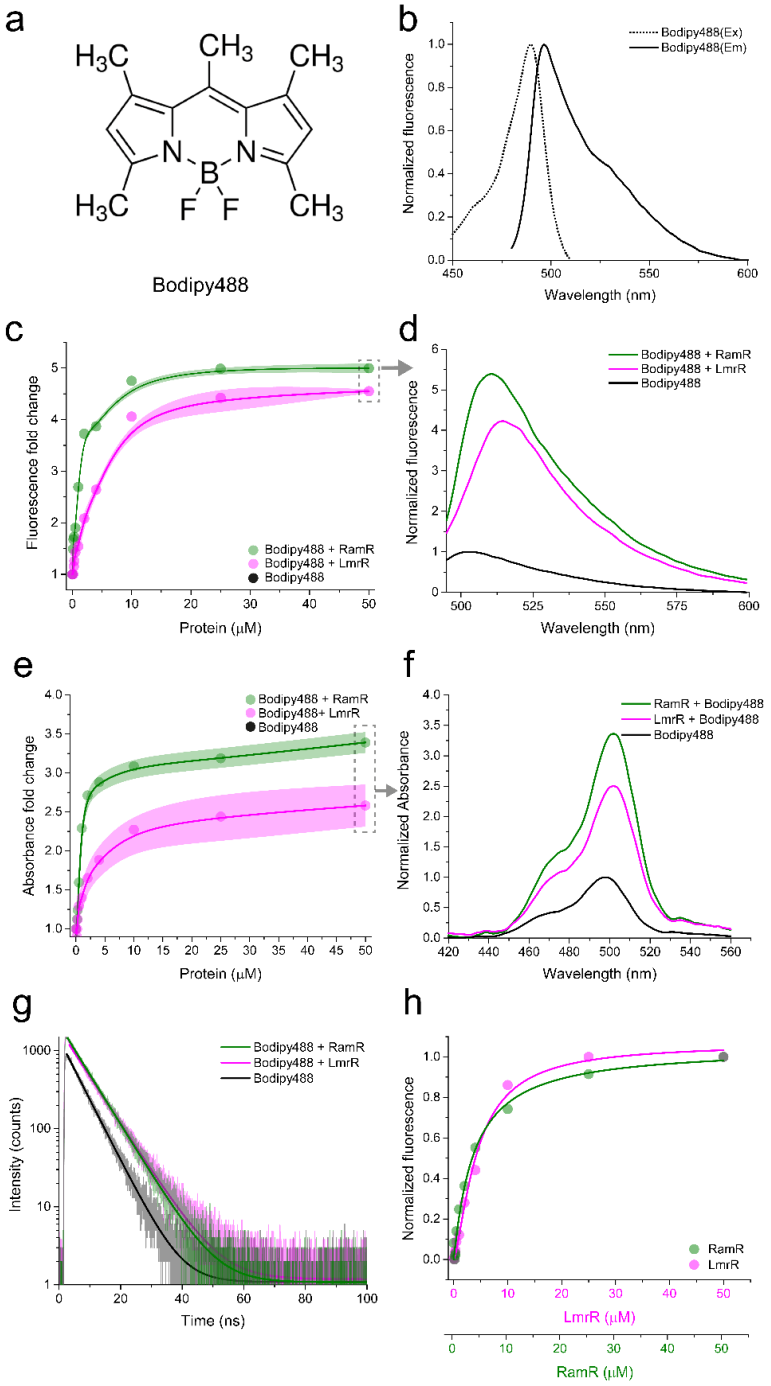

Fig. S2. Characterization of CTPEs (RamR: green and LmrR: magenta) with Bodipy488. (a) Structure of Bodipy488. (b) Excitation (dotted line) and emission (solid line) spectra of Bodipy488. (c) Fluorescence fold change of Bodipy488 on titration with CTPEs. (d) Fluorescence emission spectra of Bodipy488 with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Bodipy488 on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of Bodipy488 with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Bodipy488 with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). (h) Bound fraction of Bodipy488 with CTPEs ascertained from a Hill fit. All experiments were performed at 30 ^{o}C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

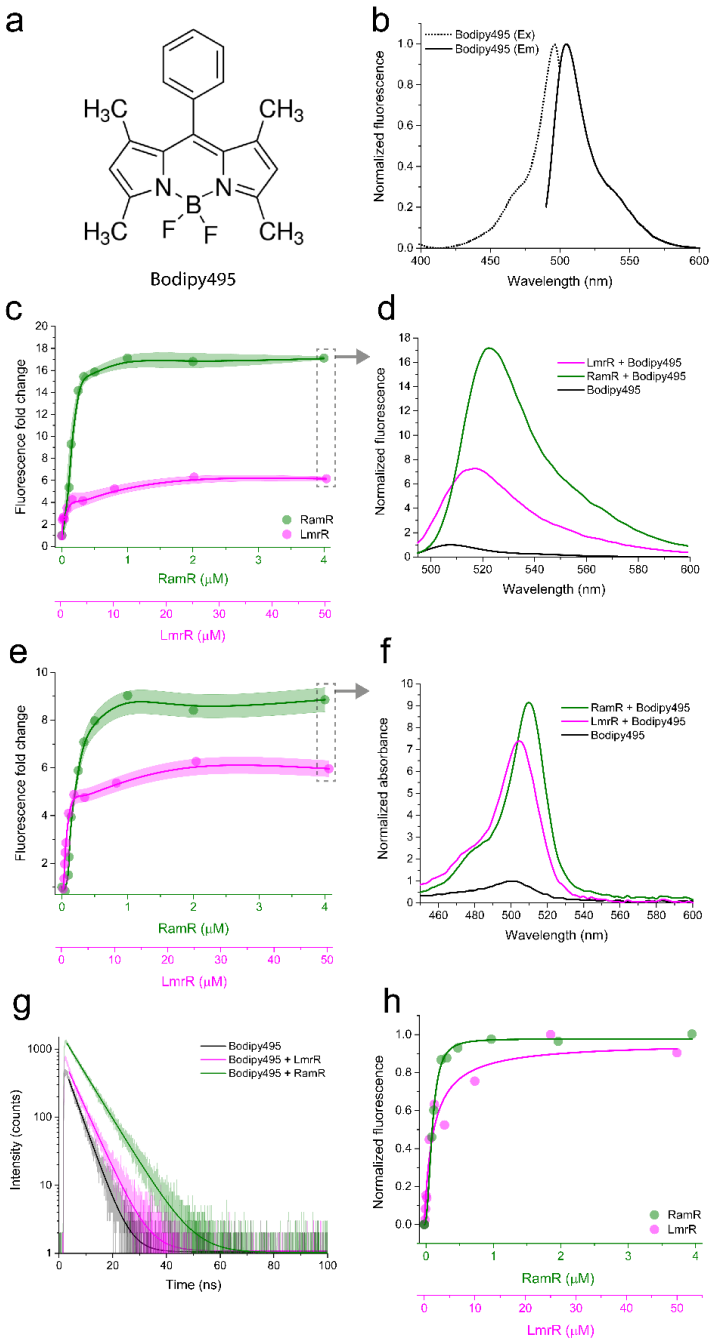

Fig. S3. Characterization of CTPEs (RamR: green and LmrR: magenta) with Bodipy495. (a) Structure of Bodipy495. (b) Excitation (dotted line) and emission (solid line) spectra of Bodipy495. (c) Fluorescence fold change of Bodipy495 on titration with CTPEs. (d) Fluorescence emission spectra of Bodipy495 with RamR and LmrR at a protein:dye molar ratio of 4:1 and 50:1, respectively. (e) Absorbance fold-change of Bodipy495 on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of Bodipy495 with RamR and LmrR at a protein:dye molar ratio of 4:1 and 50:1, respectively. (g) Fluorescence lifetime spectra of Bodipy495 with RamR and LmrR at a protein:dye molar ratio of 4:1 and 50:1 respectively fit with a mono-exponential decay function (solid lines). (h) Bound fraction of Bodipy495 with CTPEs ascertained from a Hill fit. All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

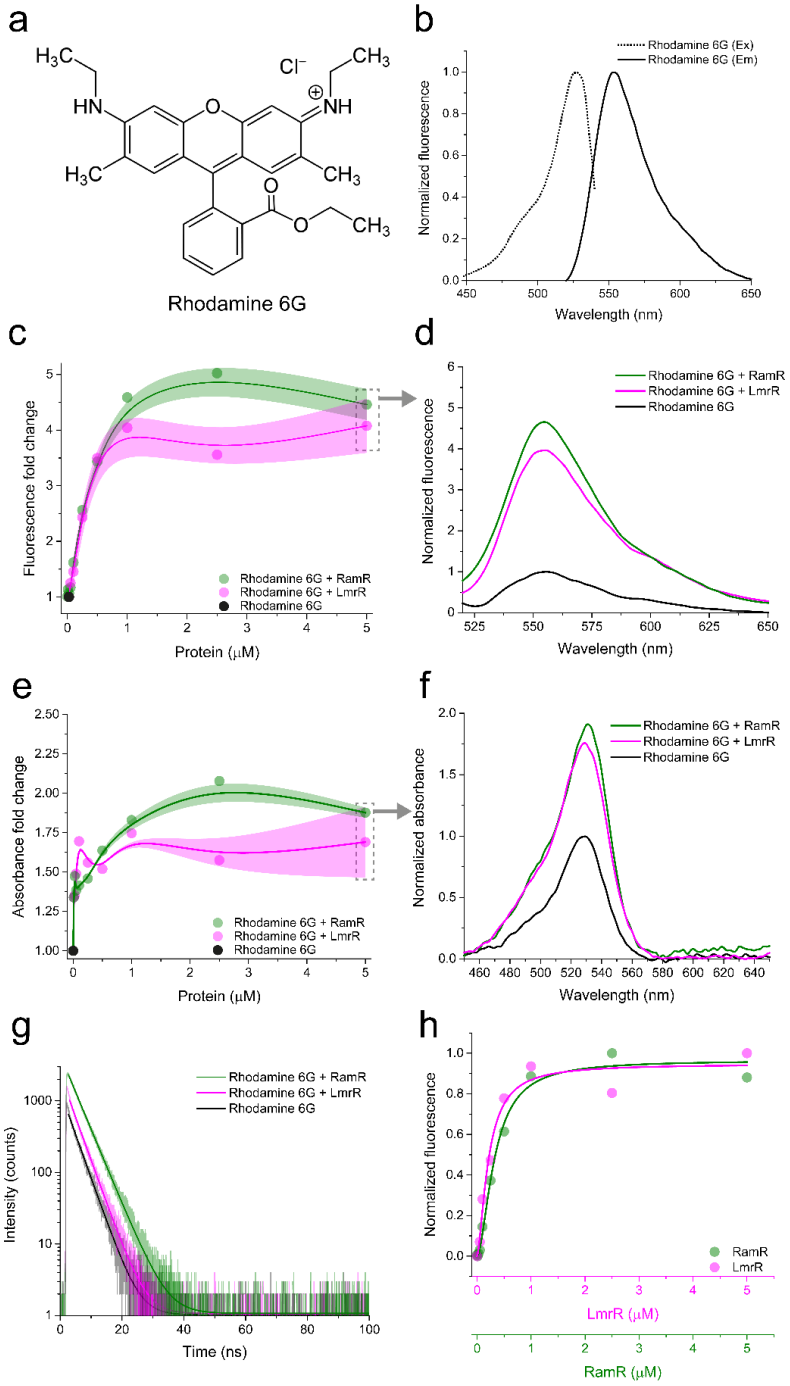

Fig. S4. Characterization of CTPEs (RamR: green and LmrR: magenta) with Rhodamine 6G. (a) Structure of Rhodamine 6G. (b) Excitation (dotted line) and emission (solid line) spectra of Rhodamine 6G. (c) Fluorescence fold change of Rhodamine 6G on titration with CTPEs. (d) Fluorescence emission spectra of Rhodamine 6G with CTPEs at a protein:dye molar ratio of 5:1. (e) Absorbance fold-change of Rhodamine 6G on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of Rhodamine 6G with CTPEs at a protein:dye molar ratio of 5:1. (g) Fluorescence lifetime spectra of Rhodamine 6G with CTPEs at a protein:dye molar ratio of 5:1 fit with a mono-exponential decay function (solid lines). (h) Bound fraction of Rhodamine 6G with CTPEs ascertained from a Hill fit. All experiments were performed at 30 ^{o}C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

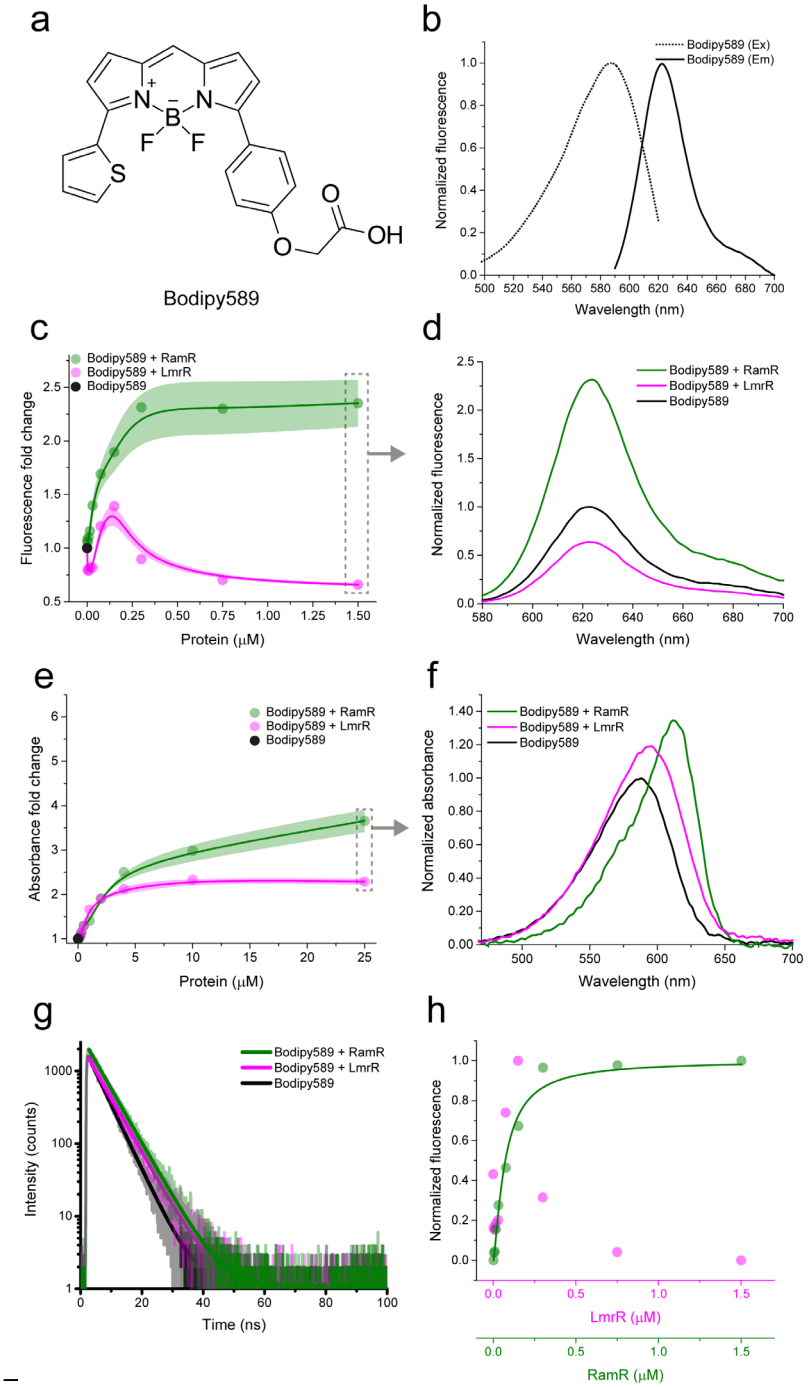

Fig. S5. Characterization of CTPEs (RamR: green and LmrR: magenta) with Bodipy589. (a) Structure of Bodipy589. (b) Excitation (dotted line) and emission (solid line) spectra of Bodipy589. (c) Fluorescence fold change of Bodipy589 on titration with CTPEs. (d) Fluorescence emission spectra of Bodipy589 with CTPEs at a protein:dye molar ratio of 1.5:1. (e) Absorbance fold-change of Bodipy589 on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of Bodipy589 with CTPEs at a protein:dye molar ratio of 1.5:1. (g) Fluorescence lifetime spectra of Bodipy589 with CTPEs at a protein:dye molar ratio of 1.5:1 fit with a mono-exponential decay function (solid lines). (h) Bound fraction of Bodipy589 with CTPEs ascertained from a Hill fit. All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

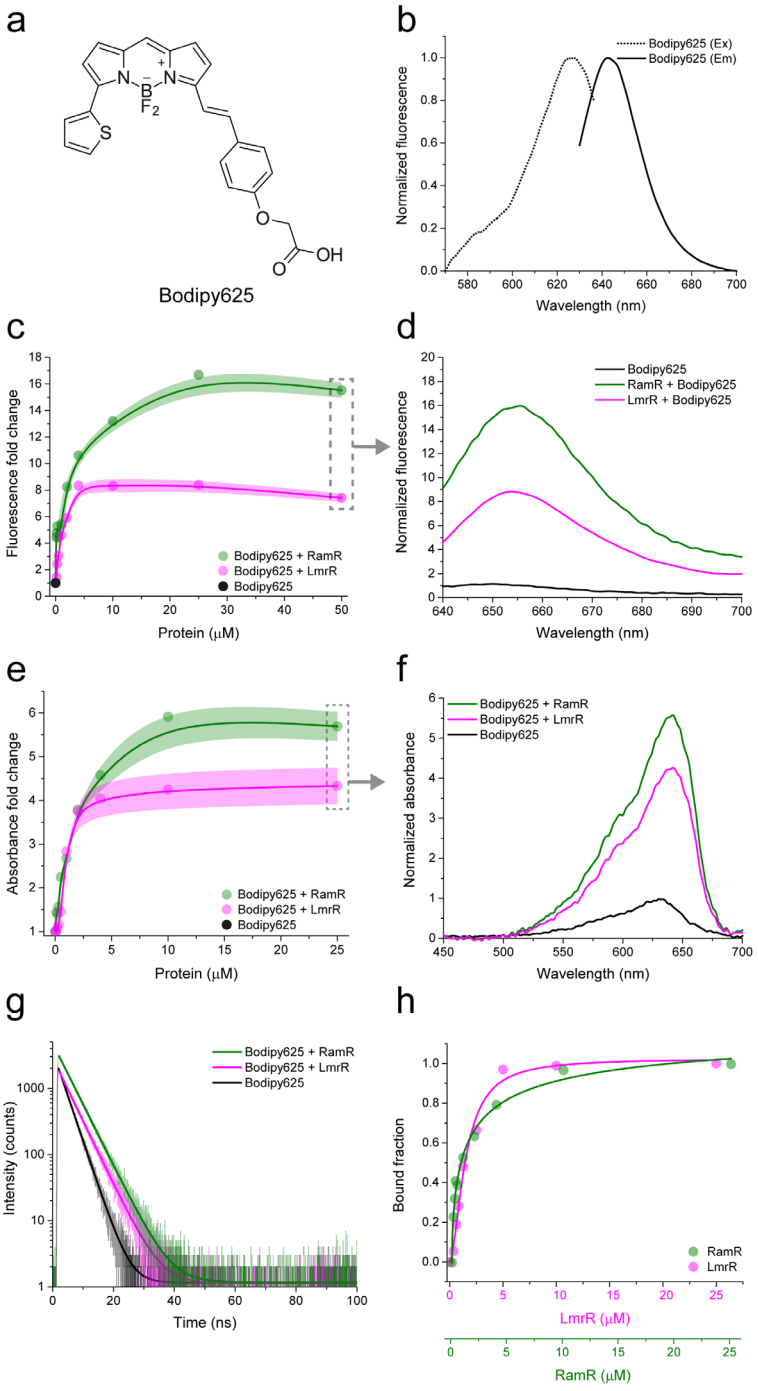

Fig. S6. Characterization of CTPEs (RamR: green and LmrR: magenta) with Bodipy625. (a) Structure of Bodipy625. (b) Excitation (dotted line) and emission (solid line) spectra of Bodipy625. (c) Fluorescence fold change of Bodipy625 on titration with CTPEs. (d) Fluorescence emission spectra of Bodipy625 with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Bodipy625 on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of Bodipy625 with CTPEs at a protein:dye molar ratio of 25:1. (g) Fluorescence lifetime spectra of Bodipy625 with CTPEs at a protein:dye molar ratio of 25:1 fit with a mono-exponential decay function (solid lines). (h) Bound fraction of Bodipy625 with CTPEs ascertained from a Hill fit. All experiments were performed at 30 ^{o}C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

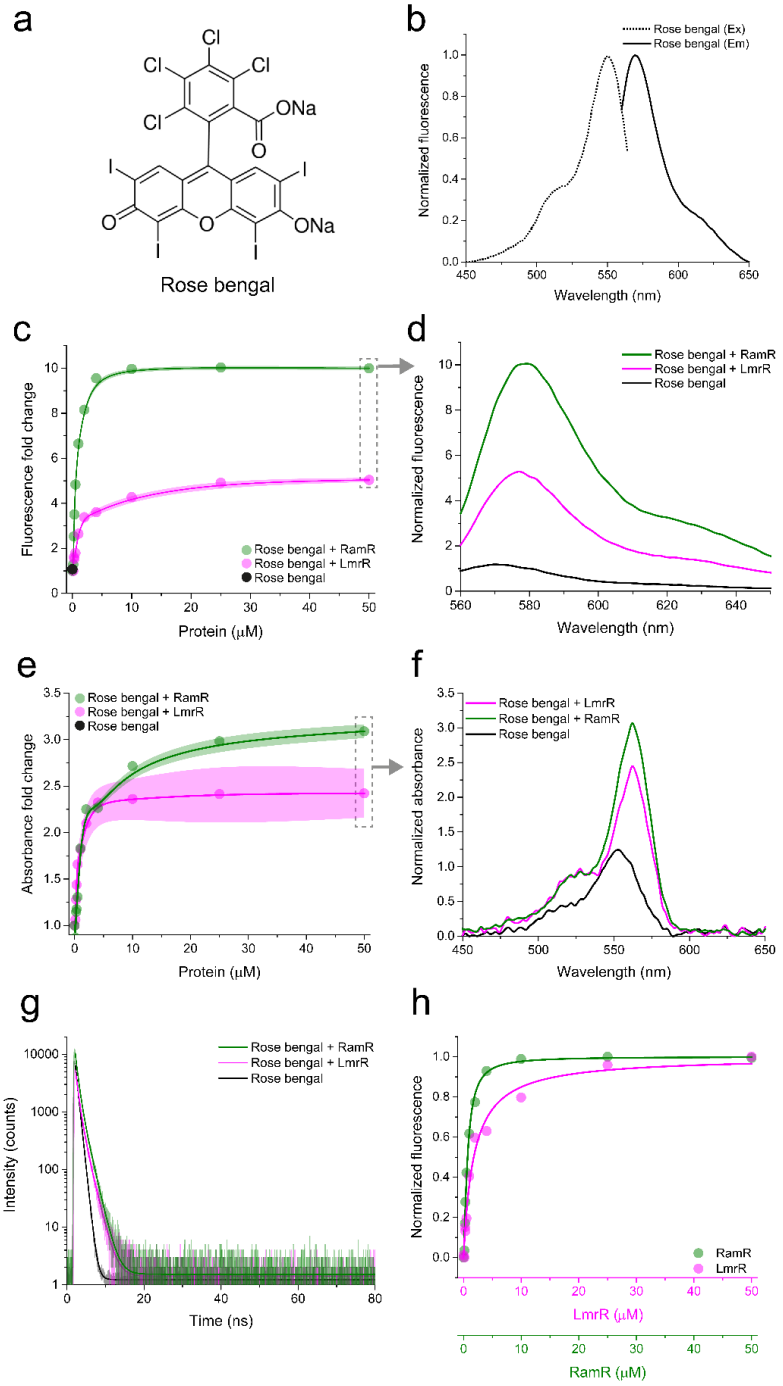

Fig. S7. Characterization of CTPEs (RamR: green and LmrR: magenta) with Rose bengal. (a) Structure of Rose bengal. (b) Excitation (dotted line) and emission (solid line) spectra of Rose bengal. (c) Fluorescence fold change of Rose bengal on titration with CTPEs. (d) Fluorescence emission spectra of Rose bengal with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Rose bengal on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of Rose bengal with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Rose bengal with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). (h) Bound fraction of Rose bengal with CTPEs ascertained from a Hill fit. All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

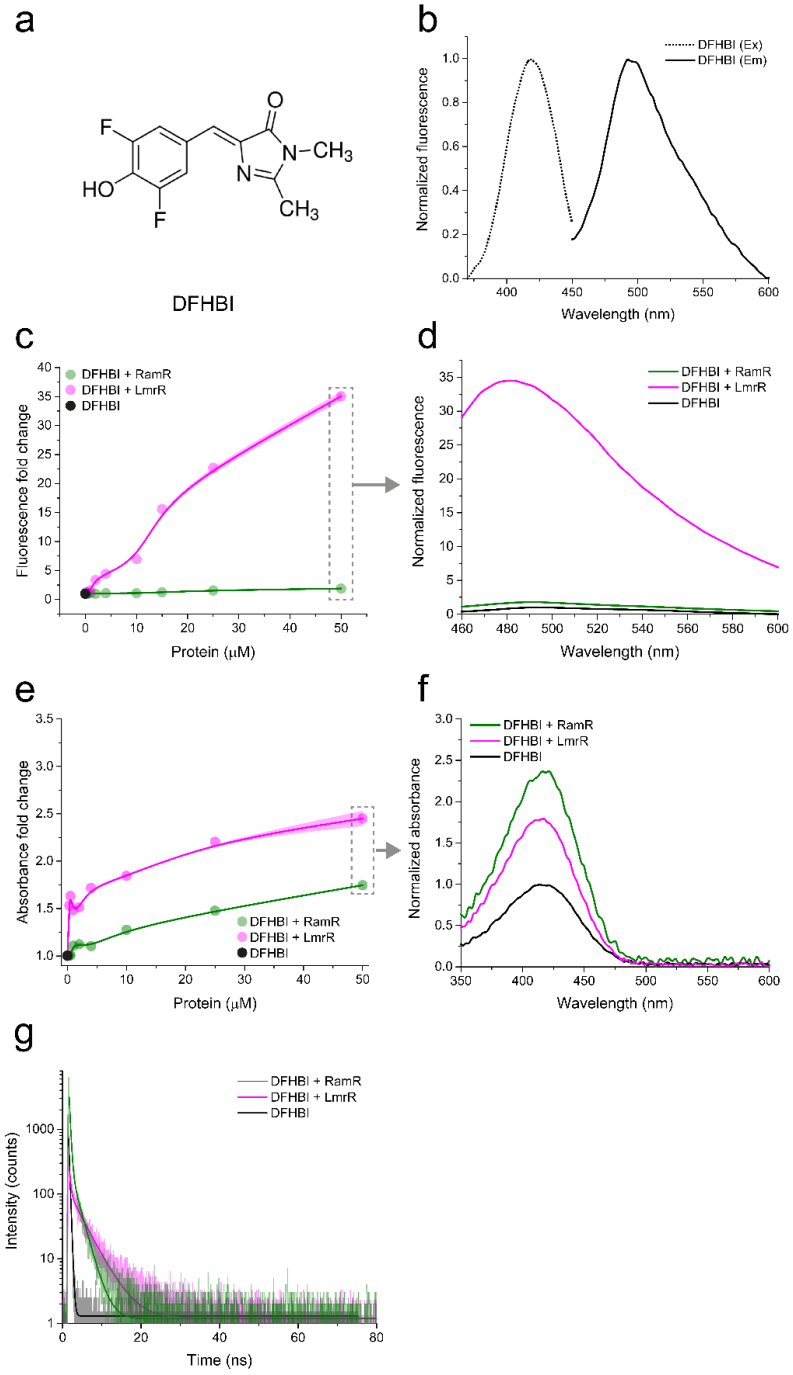

Fig. S8. Characterization of CTPEs (RamR: green and LmrR: magenta) with DFHBI. (a) Structure of DFHBI. (b) Excitation (dotted line) and emission (solid line) spectra of DFHBI. (c) Fluorescence fold change of DFHBI on titration with CTPEs. (d) Fluorescence emission spectra of DFHBI with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of DFHBI on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of DFHBI with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of DFHBI with CTPEs at a protein:dye molar ratio of 50:1 fit with a bi-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

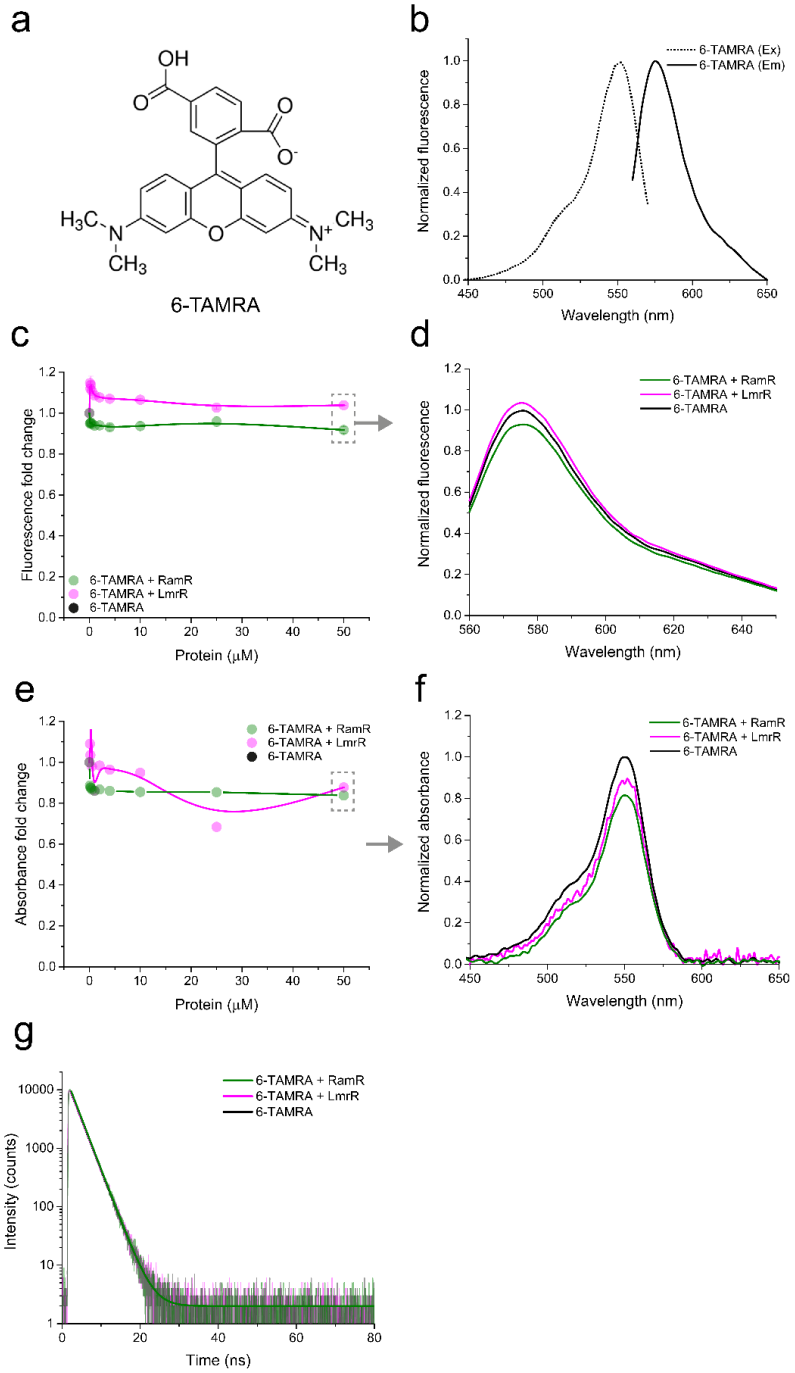

Fig. S9. Characterization of CTPEs (RamR: green and LmrR: magenta) with 6-TAMRA. (a) Structure of 6-TAMRA. (b) Excitation (dotted line) and emission (solid line) spectra of 6-TAMRA. (c) Fluorescence fold change of 6-TAMRA on titration with CTPEs. (d) Fluorescence emission spectra of 6-TAMRA with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of 6-TAMRA on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of 6-TAMRA with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of 6-TAMRA with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

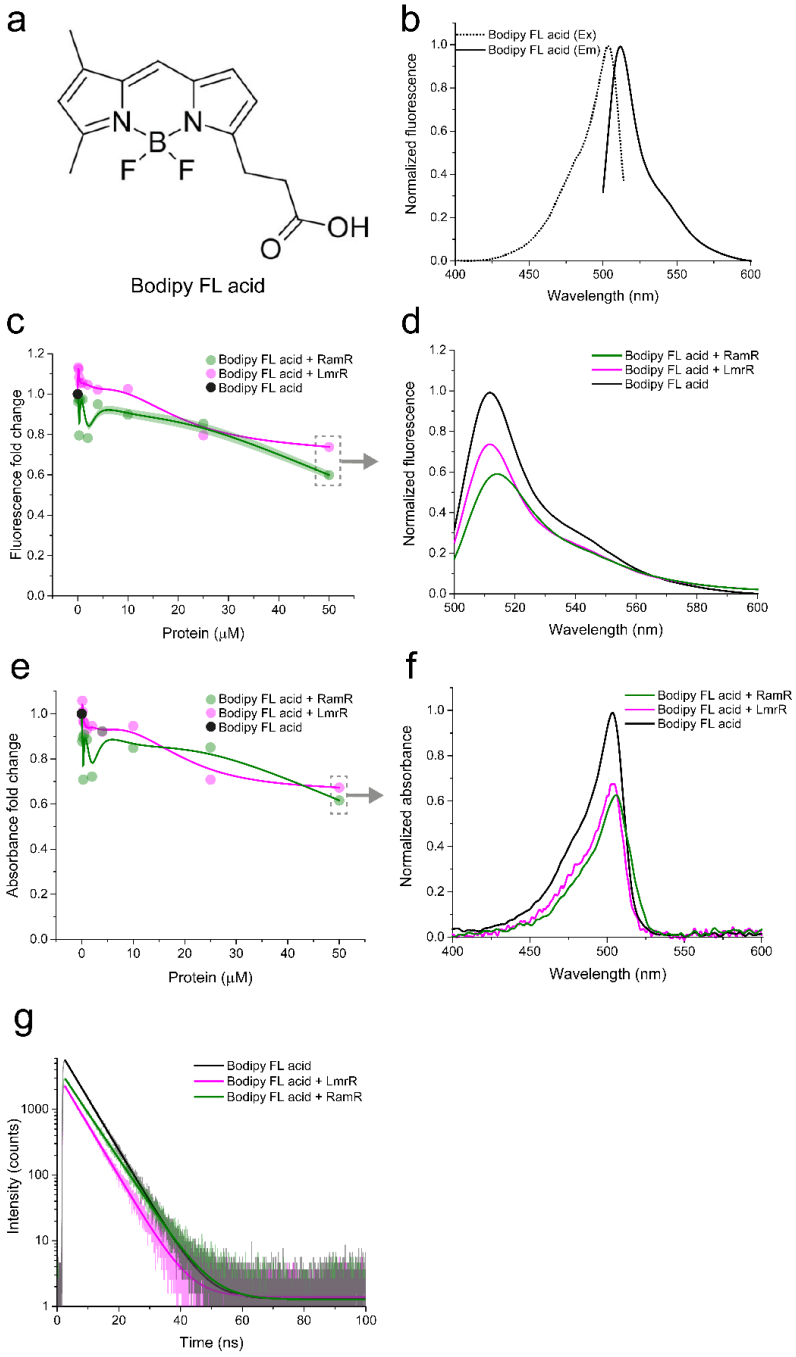

Fig. S10. Characterization of CTPEs (RamR: green and LmrR: magenta) with Bodipy FL acid. (a) Structure of Bodipy FL acid. (b) Excitation (dotted line) and emission (solid line) spectra of Bodipy FL acid. (c) Fluorescence fold change of Bodipy FL acid on titration with CTPEs. (d) Fluorescence emission spectra of Bodipy FL acid with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Bodipy FL acid on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel (c) and (e). (f) Absorption spectra of Bodipy FL acid with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Bodipy FL acid with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). All experiments were performed at 30 ^{o}C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

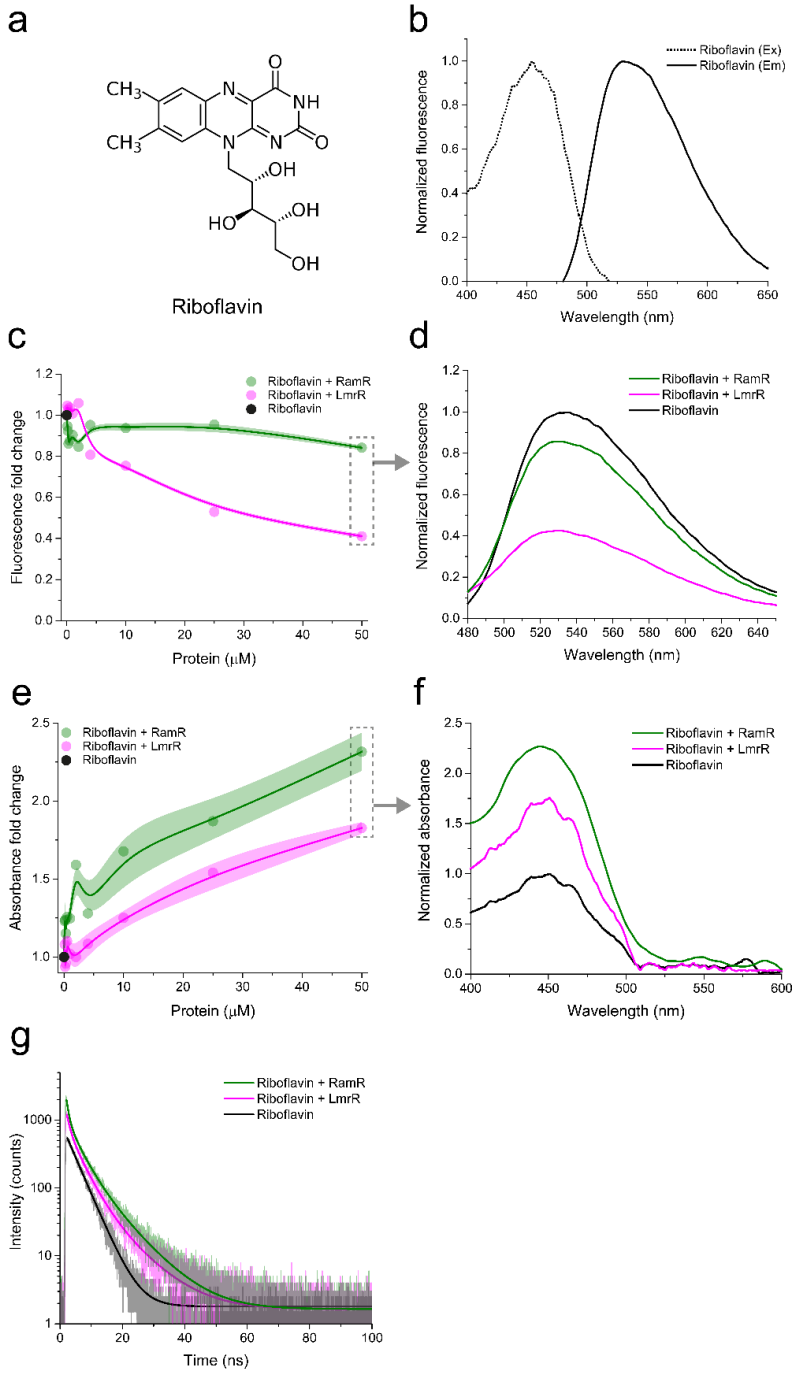

Fig. S11. Characterization of CTPEs (RamR: green and LmrR: magenta) with Riboflavin. (a) Structure of Riboflavin. (b) Excitation (dotted line) and emission (solid line) spectra of Riboflavin. (c) Fluorescence fold change of Riboflavin on titration with CTPEs. (d) Fluorescence emission spectra of Riboflavin with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Riboflavin on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel c) and e). (f) Absorption spectra of Riboflavin with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Riboflavin with CTPEs at a protein:dye molar ratio of 50:1 fit with a bi-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

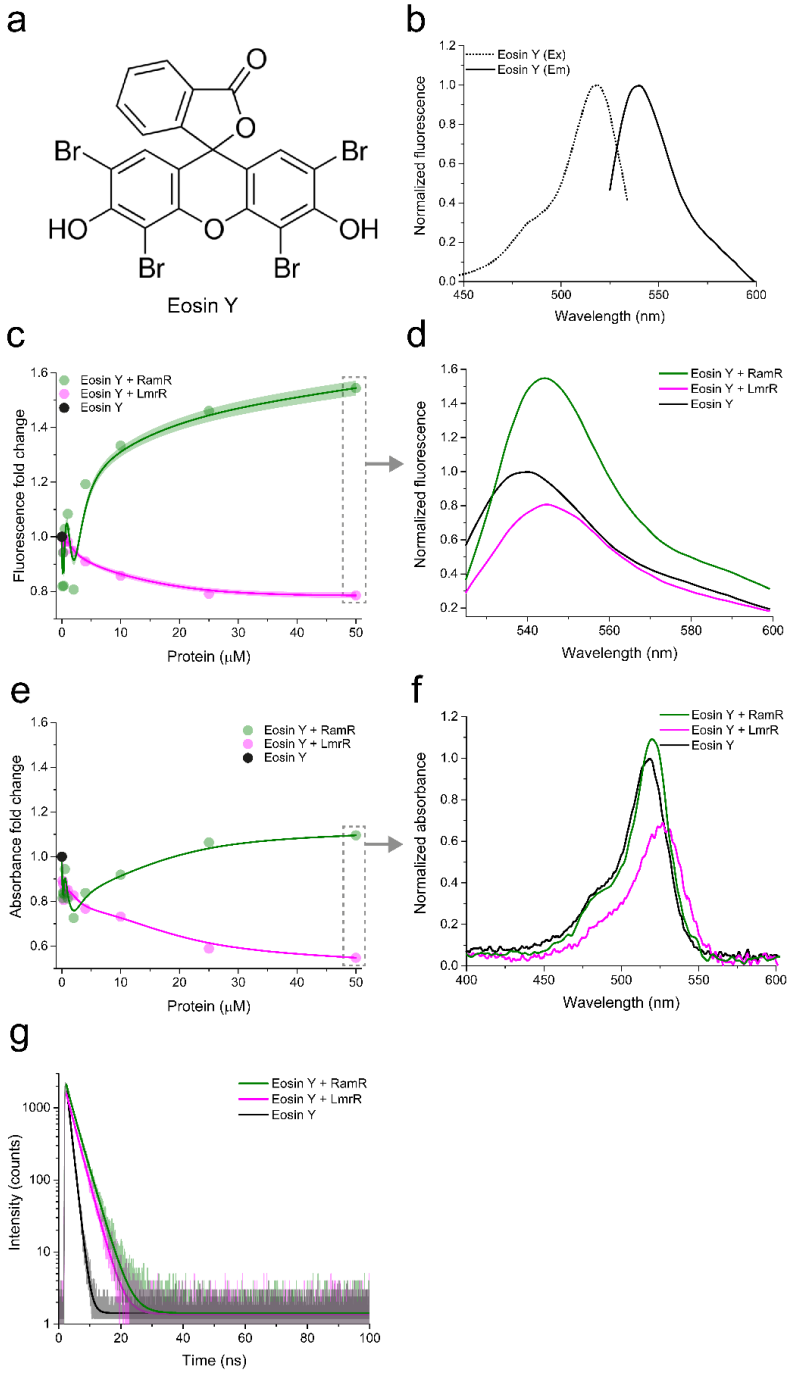

Fig. S12. Characterization of CTPEs (RamR: green and LmrR: magenta) with Eosin Y. (a) Structure of Eosin Y. (b) Excitation (dotted line) and emission (solid line) spectra of Eosin Y. (c) Fluorescence fold change of Eosin Y on titration with CTPEs. (d) Fluorescence emission spectra of Eosin Y with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Eosin Y on titration with CTPEs. Solid lines represent spline fits, and shaded regions represent s.d. over three independent measurements in panel c) and e). (f) Absorption spectra of Eosin Y with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Eosin Y with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

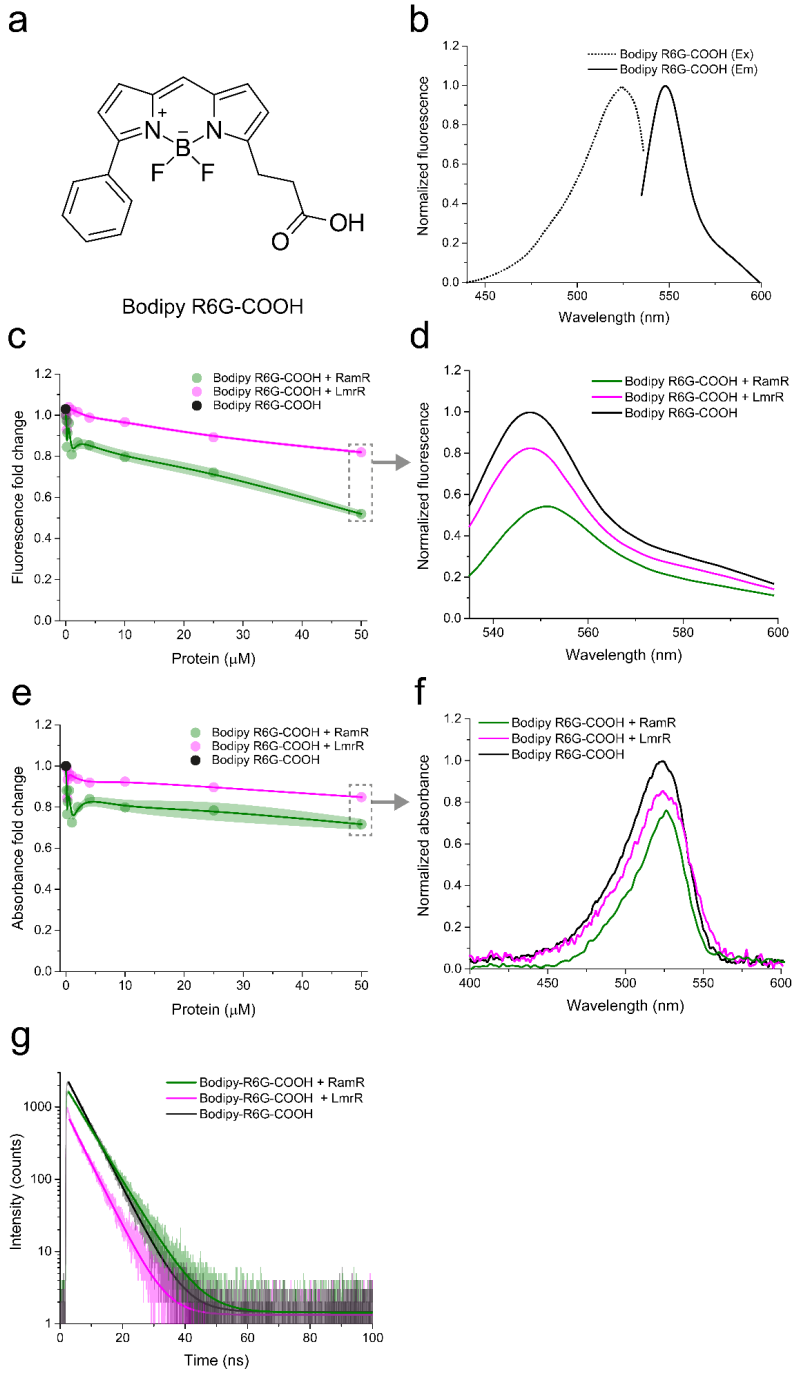

Fig. S13. Characterization of CTPEs (RamR: green and LmrR: magenta) with Bodipy R6G. (a) Structure of Bodipy R6G. (b) Excitation (dotted line) and emission (solid line) spectra of Bodipy R6G. (c) Fluorescence fold change of Bodipy R6G on titration with CTPEs. (d) Fluorescence emission spectra of Bodipy R6G with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Bodipy R6G on titration with CTPEs. Shaded regions in panel c) and e) represent s.d. over three independent measurements. (f) Absorption spectra of Bodipy R6G with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Bodipy R6G with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

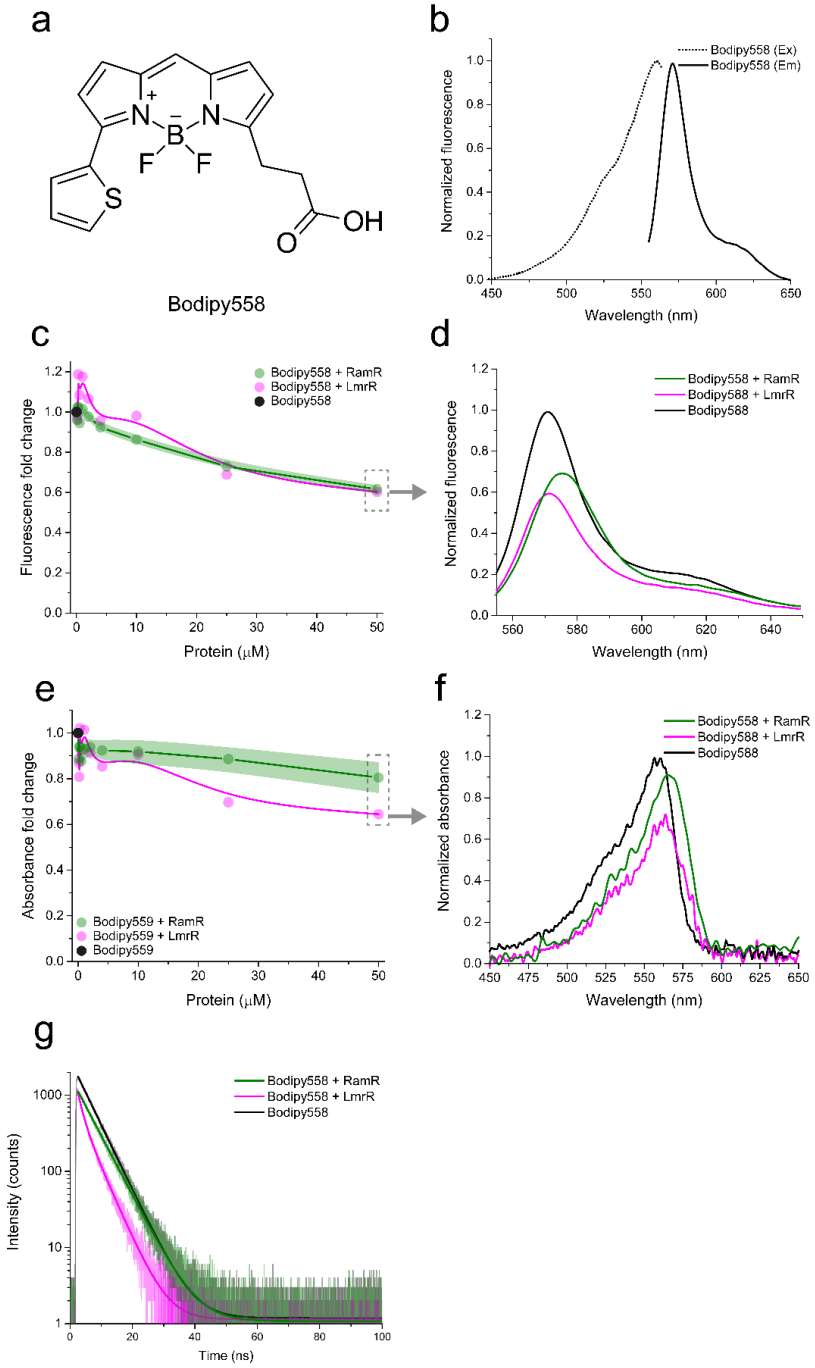

Fig. S14. Characterization of CTPEs (RamR: green and LmrR: magenta) with Bodipy558. (a) Structure of Bodipy558. (b) Excitation (dotted line) and emission (solid line) spectra of Bodipy558. (c) Fluorescence fold change of Bodipy558 on titration with CTPEs. (d) Fluorescence emission spectra of Bodipy558 with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Bodipy558 on titration with CTPEs. Shaded regions in panel c) and e) represent s.d. over three independent measurements. (f) Absorption spectra of Bodipy558 with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Bodipy558 with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

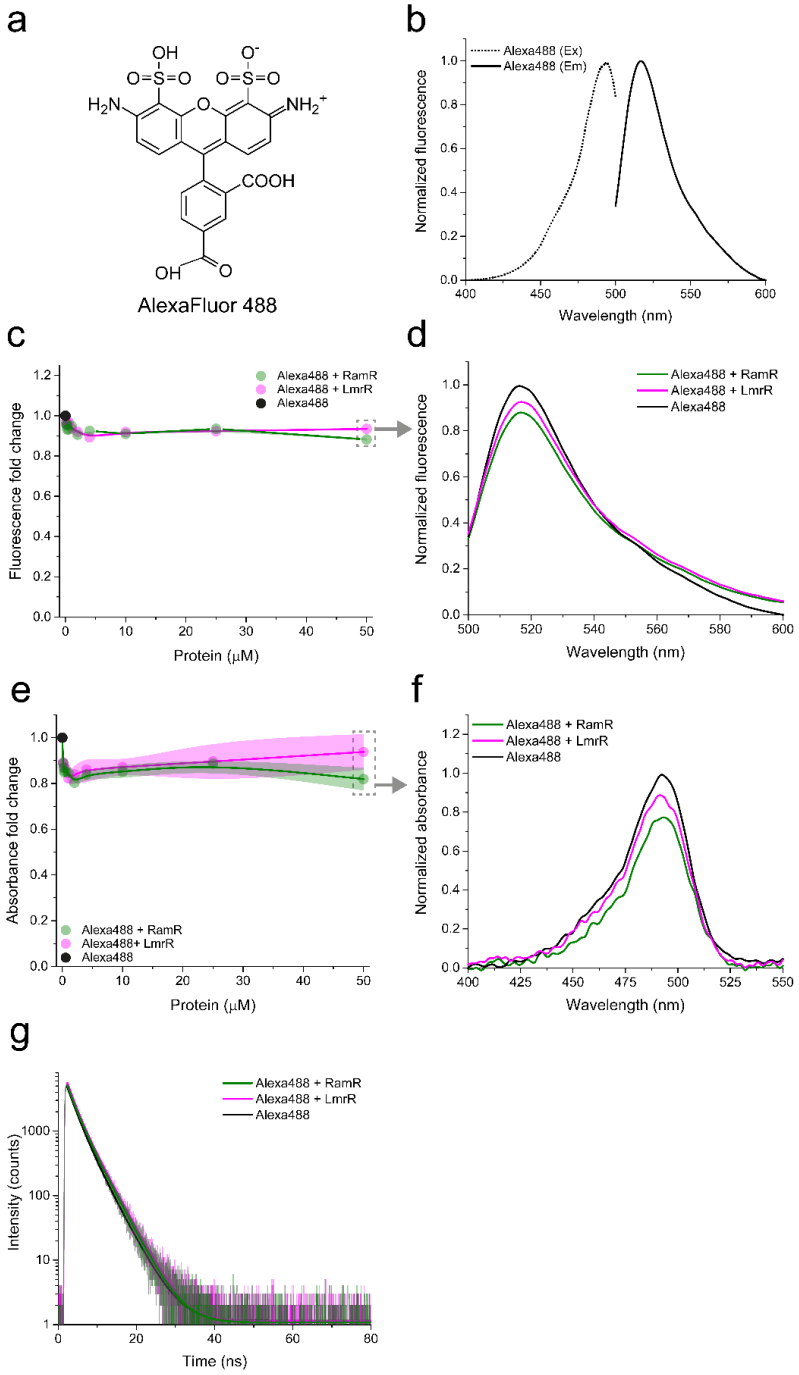

Fig. S15. Characterization of CTPEs (RamR: green and LmrR: magenta) with Alexa488. (a) Structure of Alexa488. (b) Excitation (dotted line) and emission (solid line) spectra of Alexa488. (c) Fluorescence fold change of Alexa488 on titration with CTPEs. (d) Fluorescence emission spectra of Alexa488 with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Alexa488 on titration with CTPEs. Shaded regions in panel c) and e) represent s.d. over three independent measurements. (f) Absorption spectra of Alexa488 with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Alexa488 with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

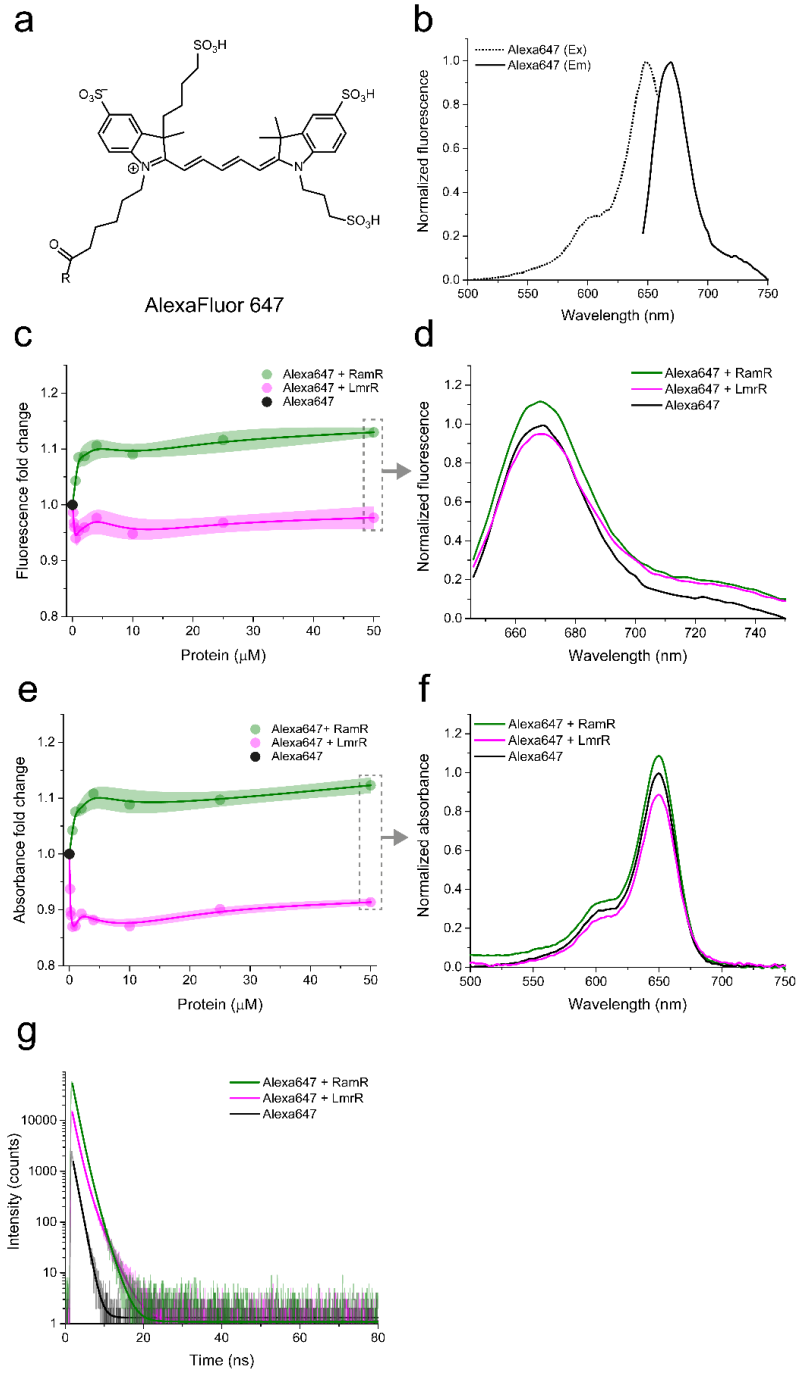

Fig. S16. Characterization of CTPEs (RamR: green and LmrR: magenta) with Alexa647. (a) Structure of Alexa647. (b) Excitation (dotted line) and emission (solid line) spectra of Alexa647. (c) Fluorescence fold change of Alexa647 on titration with CTPEs. (d) Fluorescence emission spectra of Alexa647 with CTPEs at a protein:dye molar ratio of 50:1. (e) Absorbance fold-change of Alexa647 on titration with CTPEs. Shaded regions in panel c) and e) represent s.d. over three independent measurements. (f) Absorption spectra of Alexa647 with CTPEs at a protein:dye molar ratio of 50:1. (g) Fluorescence lifetime spectra of Alexa647 with CTPEs at a protein:dye molar ratio of 50:1 fit with a mono-exponential decay function (solid lines). All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

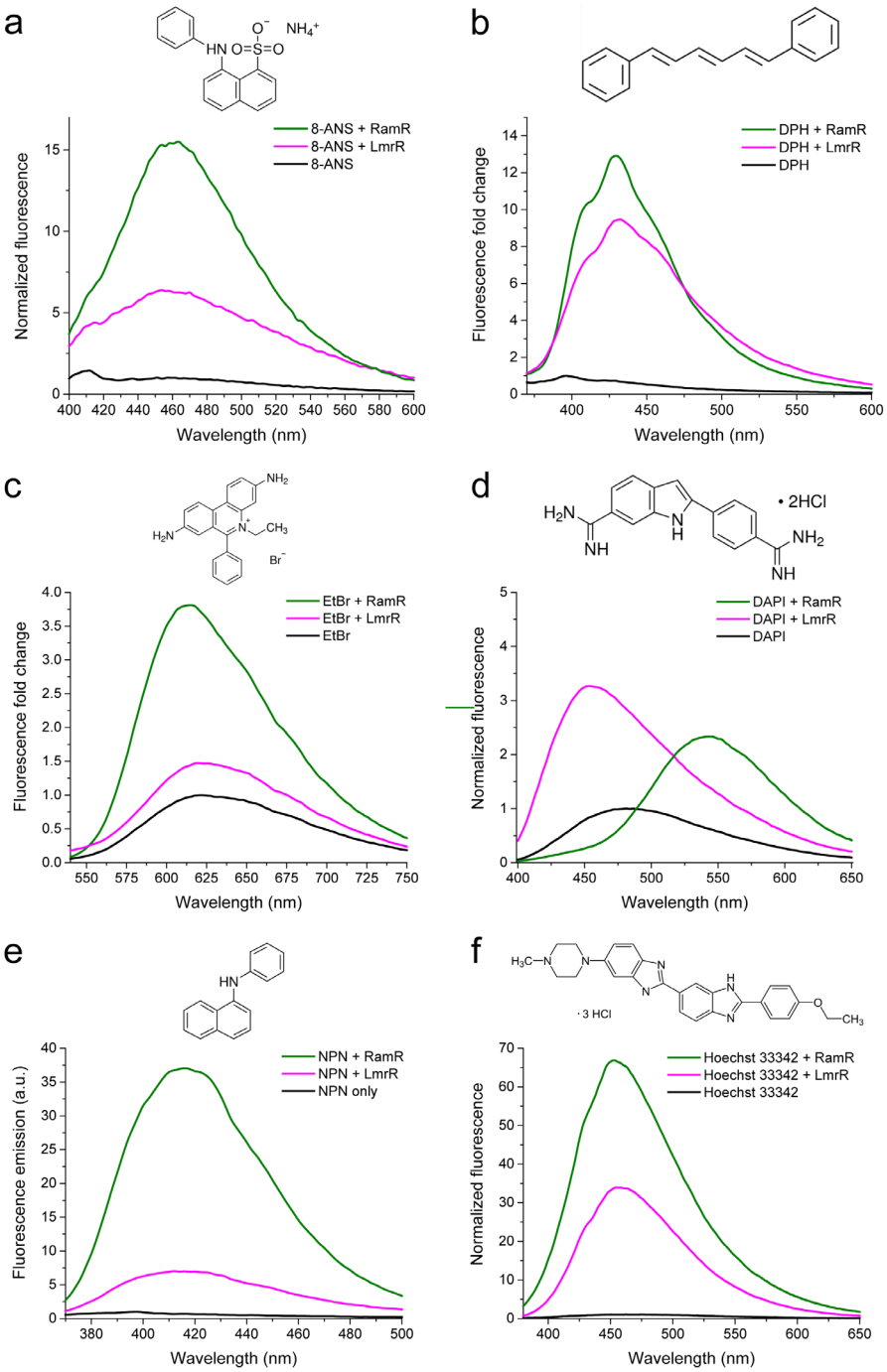

Fig. S17. Fluorescence emission spectra of nonspecific intercalating dyes with CTPEs (RamR: green and LmrR: magenta) with (a) *8*-Anilinonaphthalene-1-sulfonic acid (ANS), (b) 1,6-diphenyl-1,3,5-hexatriene (DPH), (c) ethidium bromide (EtBr), (d) 4′,6-diamidino-2-phenylindole (DAPI), (e) 1-N-phenylnaphthylamine (NPN), and (f) Hoechst 33342 at protein:dye molar ratio of 50:1. The corresponding dye structures are indicated above each panel. All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

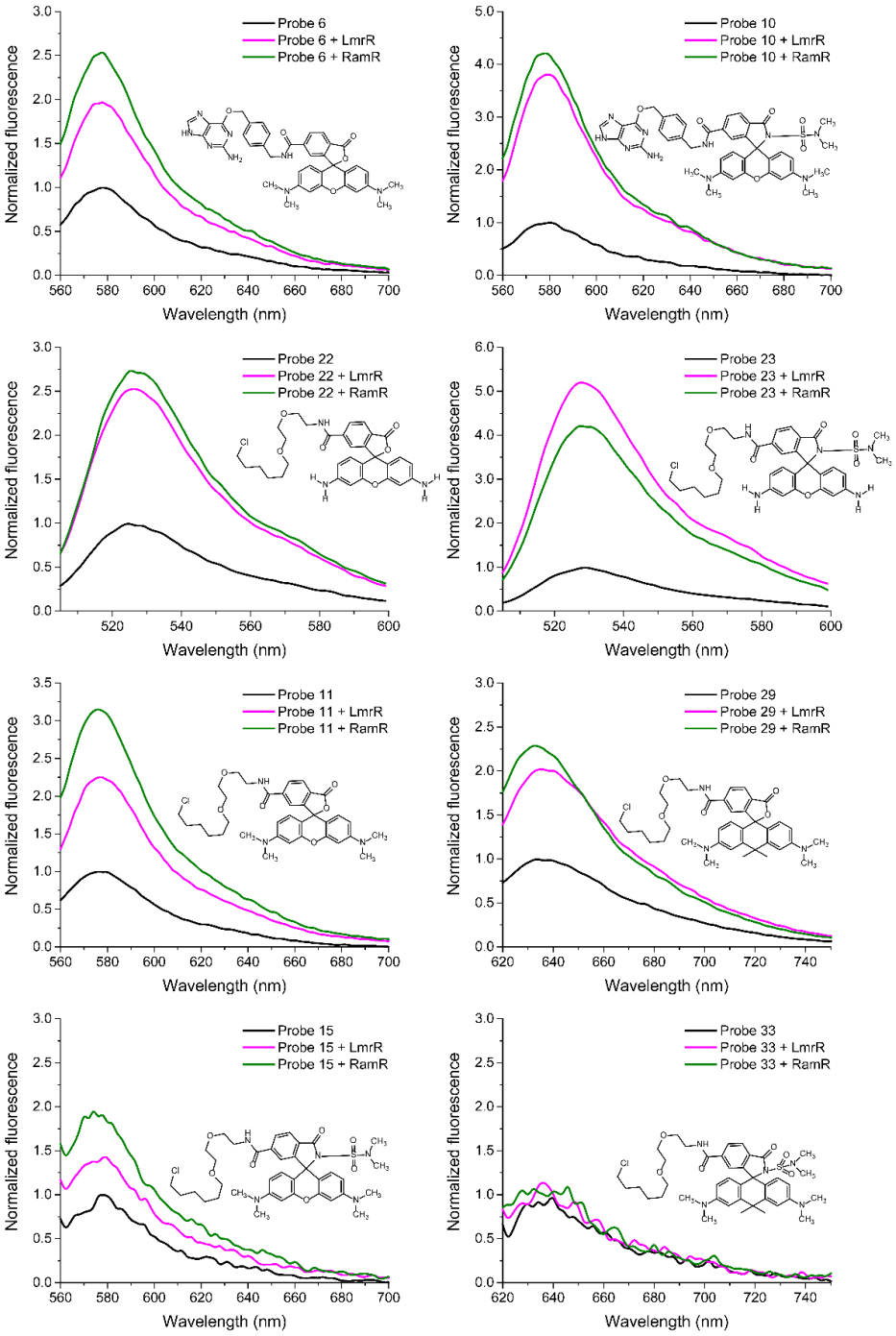

Fig. S18. Fluorescence emission spectra of 6-TAMRA modified (MaP) probes (Wang et al. 2020) with CTPEs at a protein:dye molar ratio of 50:1. MaP probes comprise of 6-TAMRA covalently linked to the SNAP-Tag ligand: O^6-benzyl guanidine (Probe 6 and Probe 10) or to the Halo-Tag ligand (Probe 11, 15, 22, 23, 29 and 33). All experiments were performed at 30 ^{o}C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

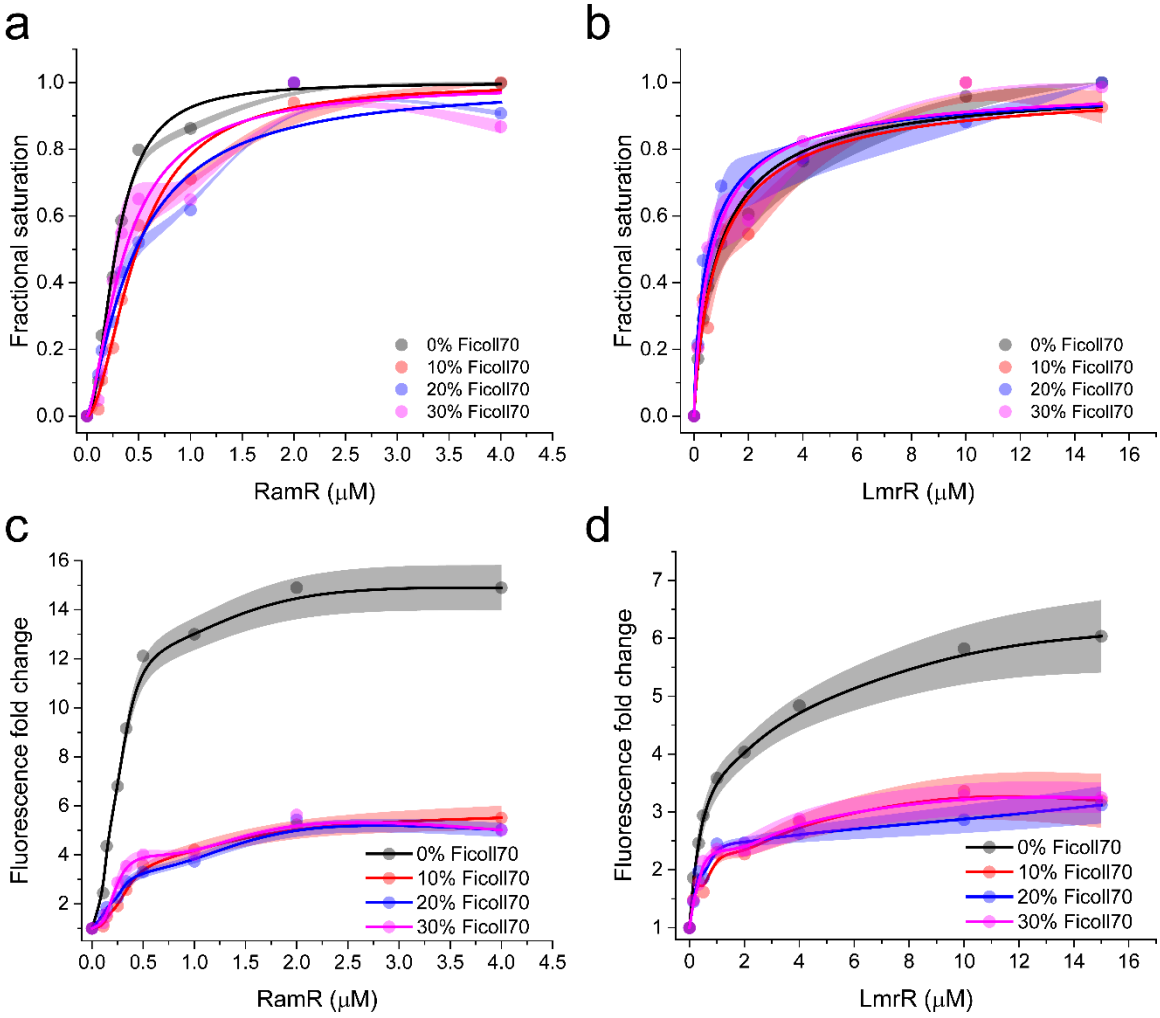

Fig. S19. Effect of Ficoll70 on the fluorescence of Bodipy495 in the presence of CTPEs (a,c) RamR and (b,d) LmrR. Saturation experiments were performed by titrating the respective CTPE tag with 1 μM Bodipy495. The fold change in emission fluorescence of Bodipy495 with increasing concentrations of Ficoll70 is shown in panel c and d. All experiments were performed at 30 °C in 20 mM K-MOPS, 150 mM NaCl buffered at pH 7.0.

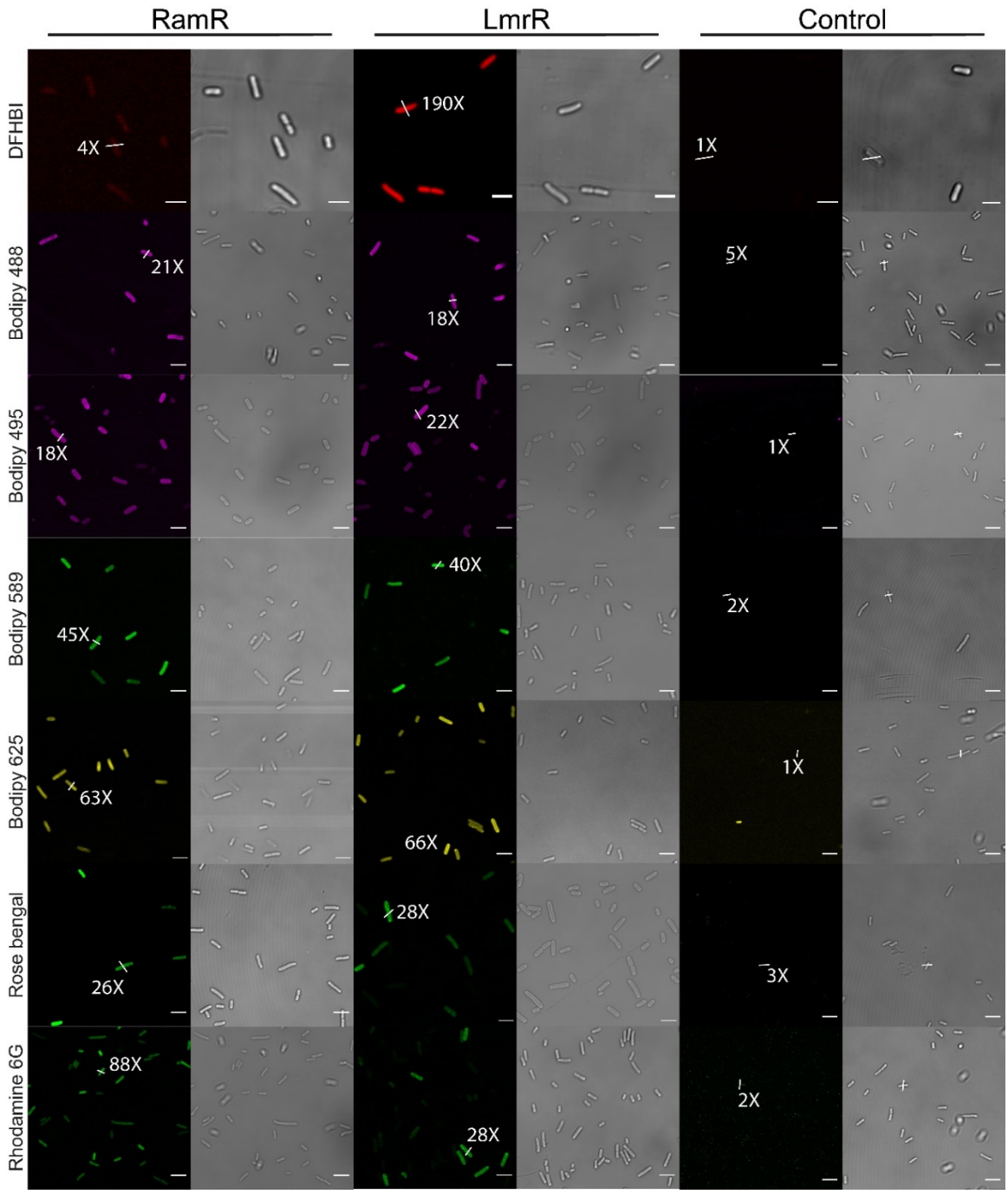

Fig. S20. Overview of labeled *E. coli* cells expressing cytoplasmic CTPEs from a pBAD vector. The signal to background fluorescence ratios for arbitrarily picked cells for each dye is mentioned next to white overlays. All measurements were performed at 30 °C using 15 μM of corresponding dye. For controls, the signal to background ratio was minimal for all samples. The scale bar is 3 μm.

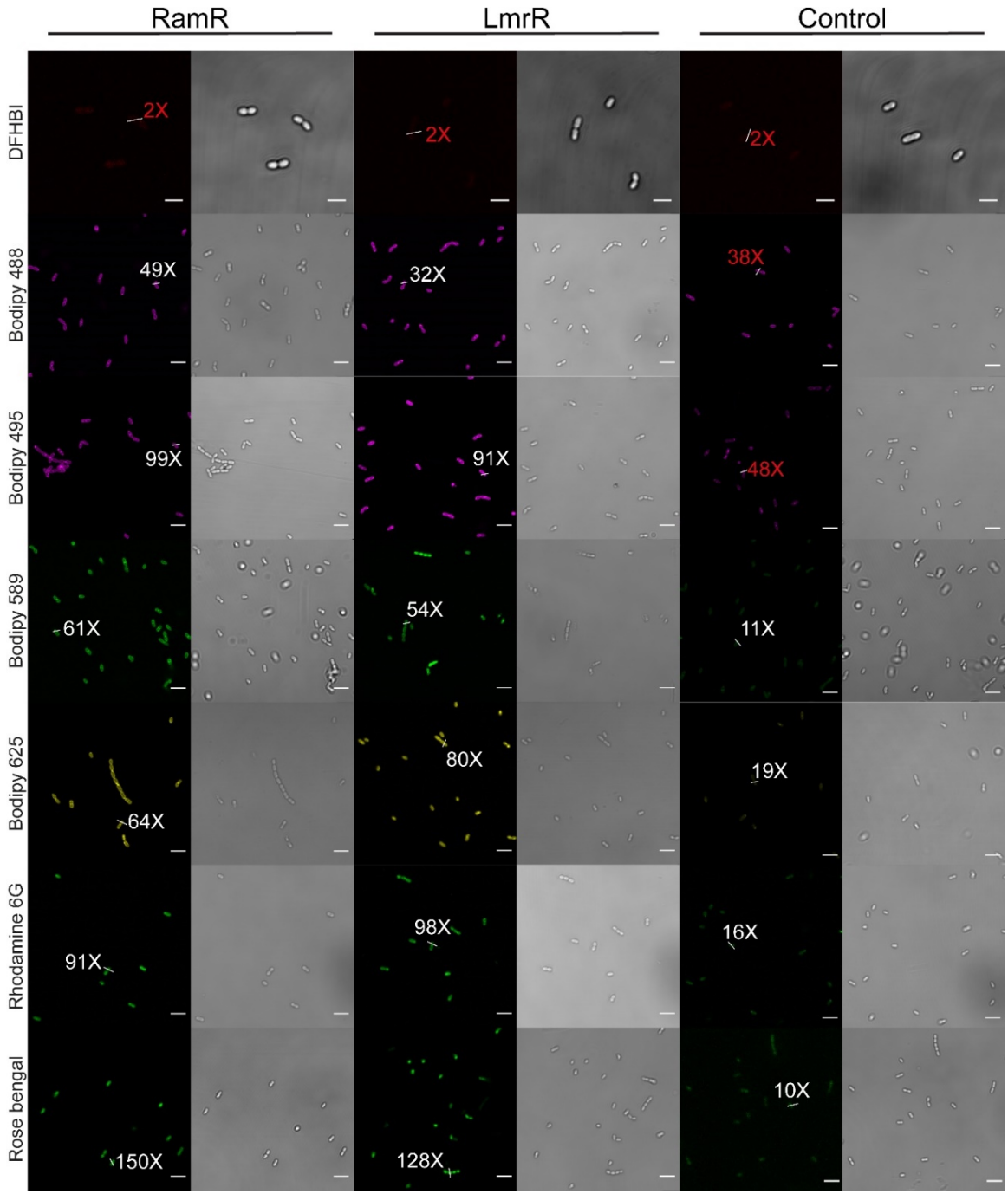

Fig. S21. Overview of labeled *L. lactis* cells expressing cytoplasmic CTPEs from a pNSC8048 (LmrR) and pNZC3GH vector (RamR) respectively. The signal to background fluorescence ratios for arbitrarily picked cells for each dye is mentioned next to white overlays and non-specific staining is depicted as red overlays. All measurements were performed at 30 ^{o}C using 15 µM of corresponding dye. For controls, Bodipy495 and Bodipy488 showed significant non-specific staining and, DFHBI was impermeable suggesting their inapplicability under our experimental conditions. All samples indicating some background staining. Scale bar is 3 µm.

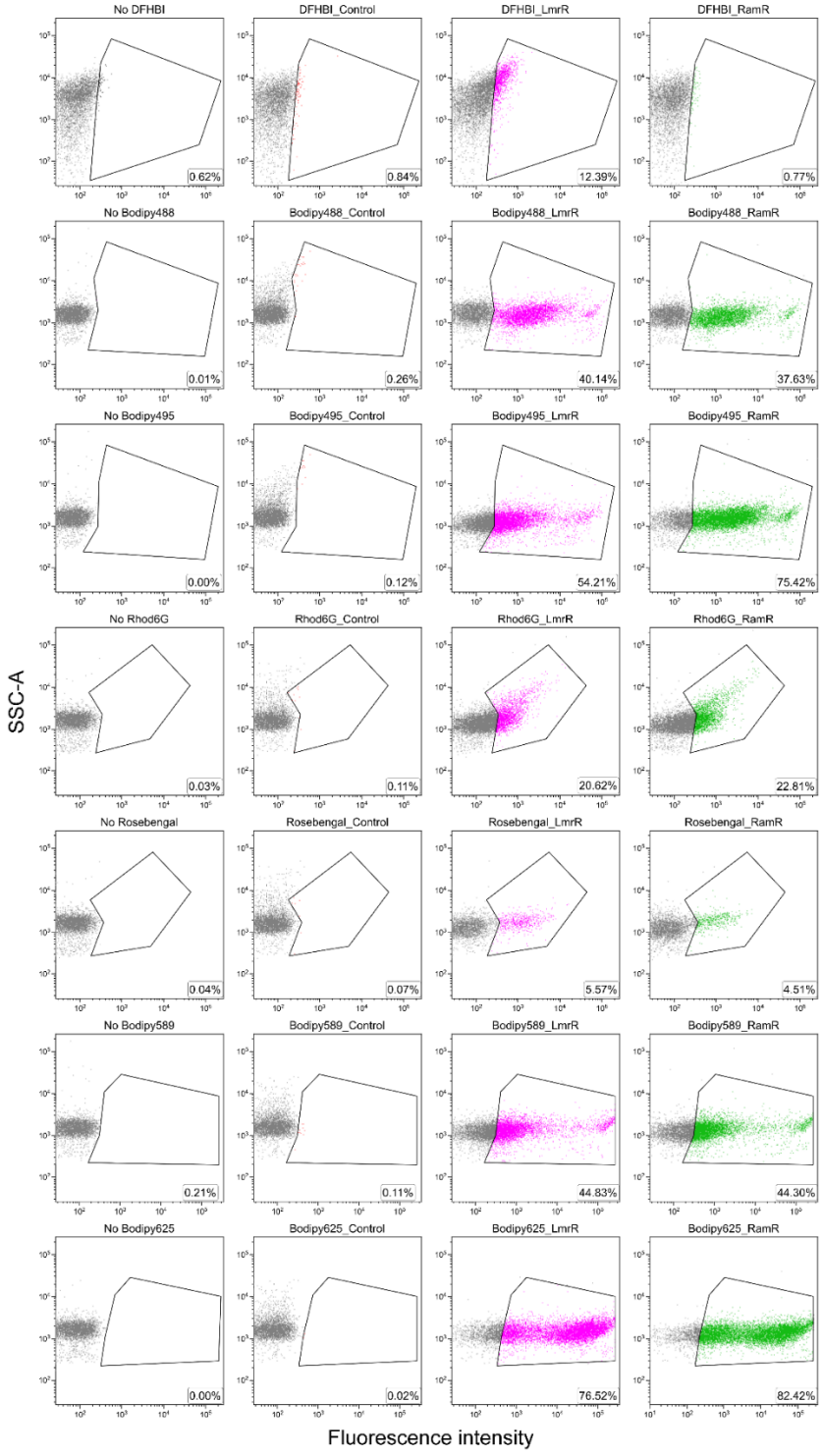

Fig. S22. Flow cytometry side scatter area plots (SSC-A) of *E. coli* cells without any dye (gated black), expressing the cytoplasmic protein OsmY with corresponding dye (control: gated red), expressing LmrR (gated magenta) and expressing RamR (gated green). The low fraction of DFHBI labeled cells with LmrR is likely due to inefficient excitation and collection channels (See methods) as the percentages are significantly lower than estimations of the number of fluorescent cells from microscopy images (**see Supplementary Fig. 20**). The bottom right in each panel shows the % of gated (labeled) cells from a total population of 10,000 cells.

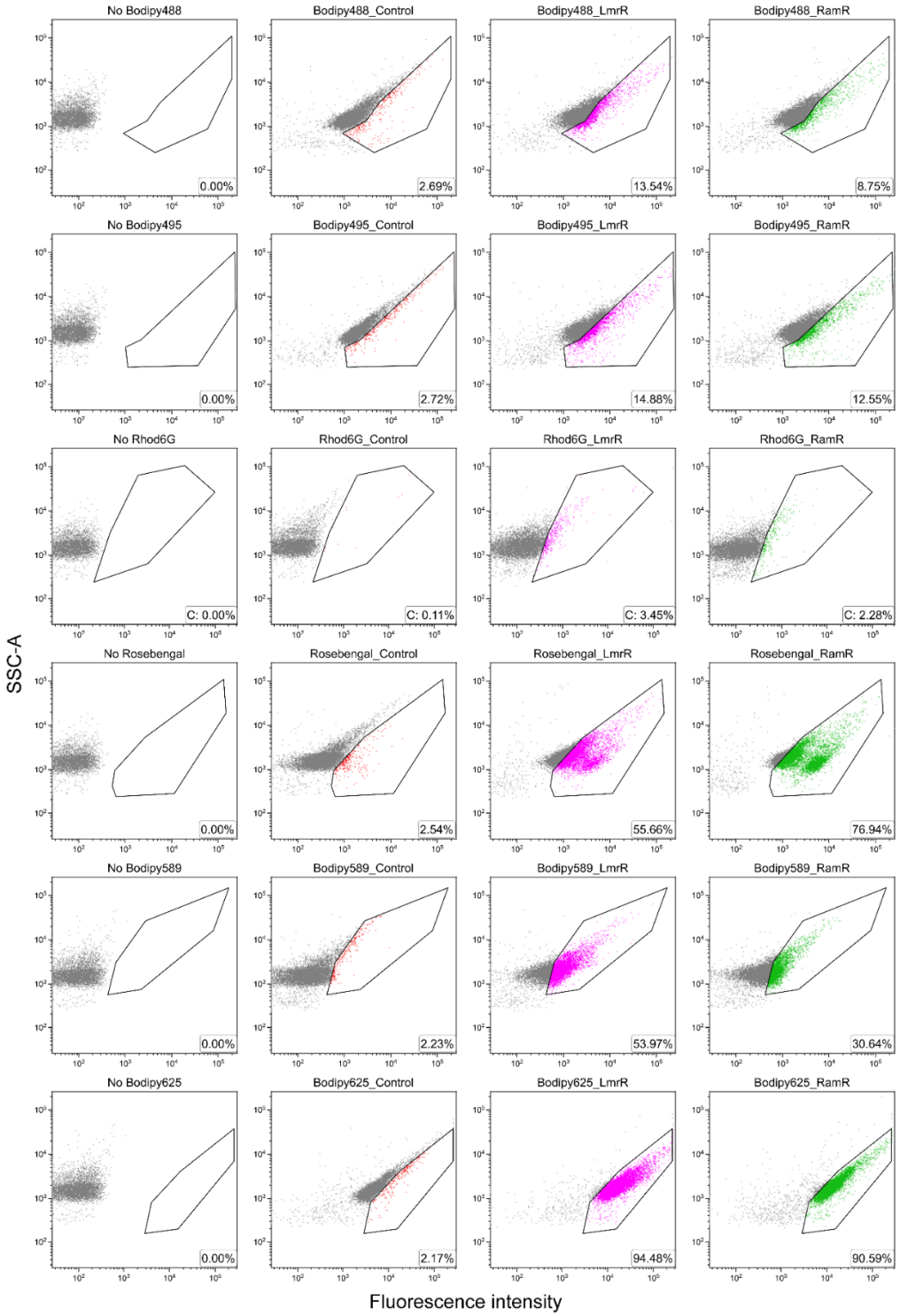

Fig. S23. Flow cytometry side scatter area plots (SSC-A) of Δ*lmrR L. lactis* cells without any dye (gated black), with corresponding dye (control; gated red), expressing LmrR (gated magenta) and RamR (gated green). The bottom right in each panel shows the % of gated (labeled) cells from a total population of 10,000 cells.

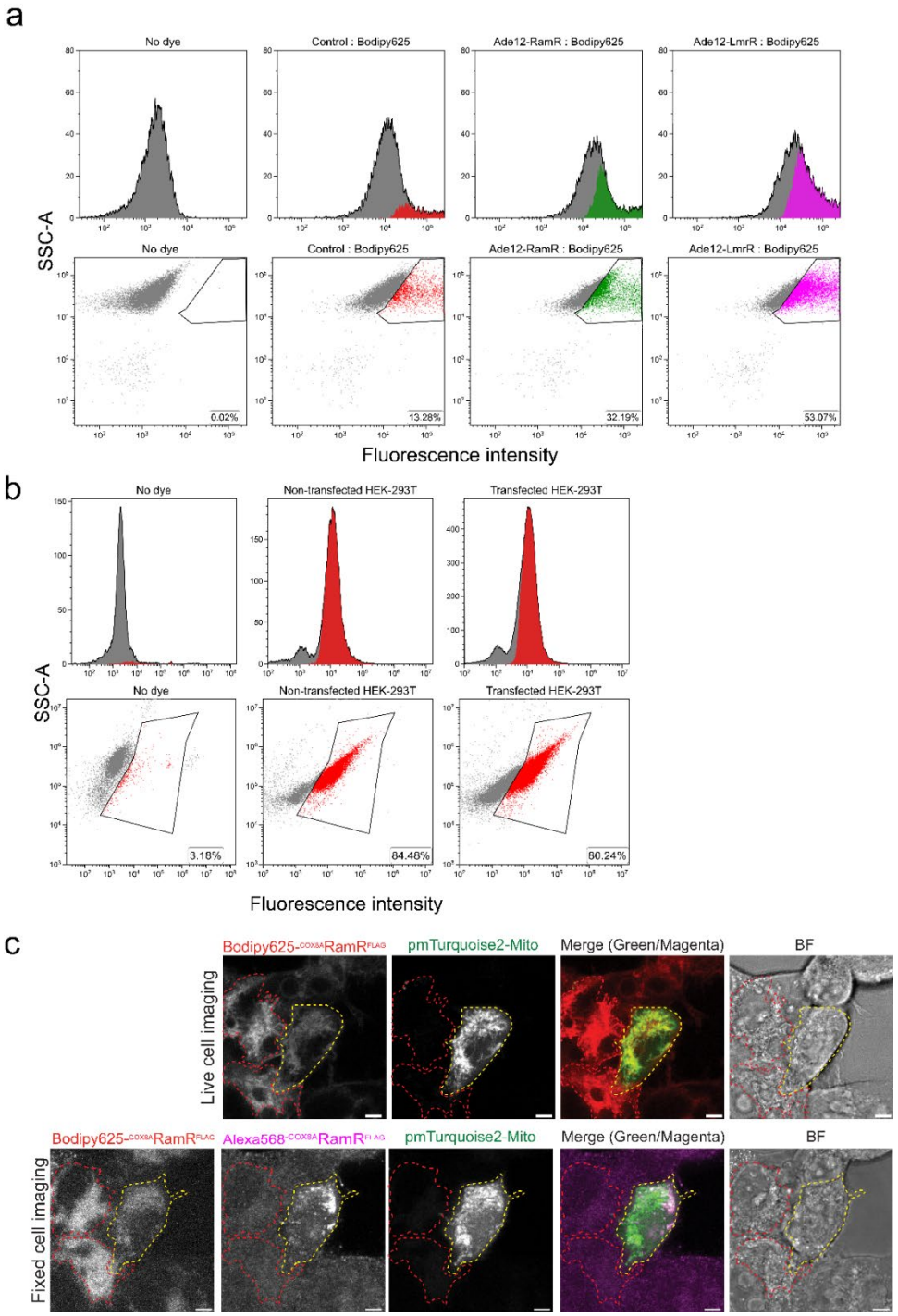

Fig. S24. Flow cytometry side scatter area plots (SSC-A) of *Saccharomyces cerevisiae* (panel a) and HEK-293T cells with Bodipy625 (panel b). Control *S. cerevisiae* (SR80) cells exhibit some background staining that possibly emanates from either non-specific interaction with the cell-wall and/or lipid membrane. *S. cerevisiae* cells expressing Ade-LmrR or Ade-RamR have enhanced fluorescence compared to untransformed *S. cerevisiae* (SR80) cells. FACS analysis of unspecific uptake of Bodipy625 by untransfected HEK-293T cells with no Bodipy625 added and those after the addition of Bodipy625. (b) Untransfected HEK-293T cells exhibit significant uptake (~ 84% labeled cells) of Bodipy625 compared to transfected HEK-293T cells. (c) High background staining was observed in both untransfected (red borders) and transfected (yellow borders) cells.

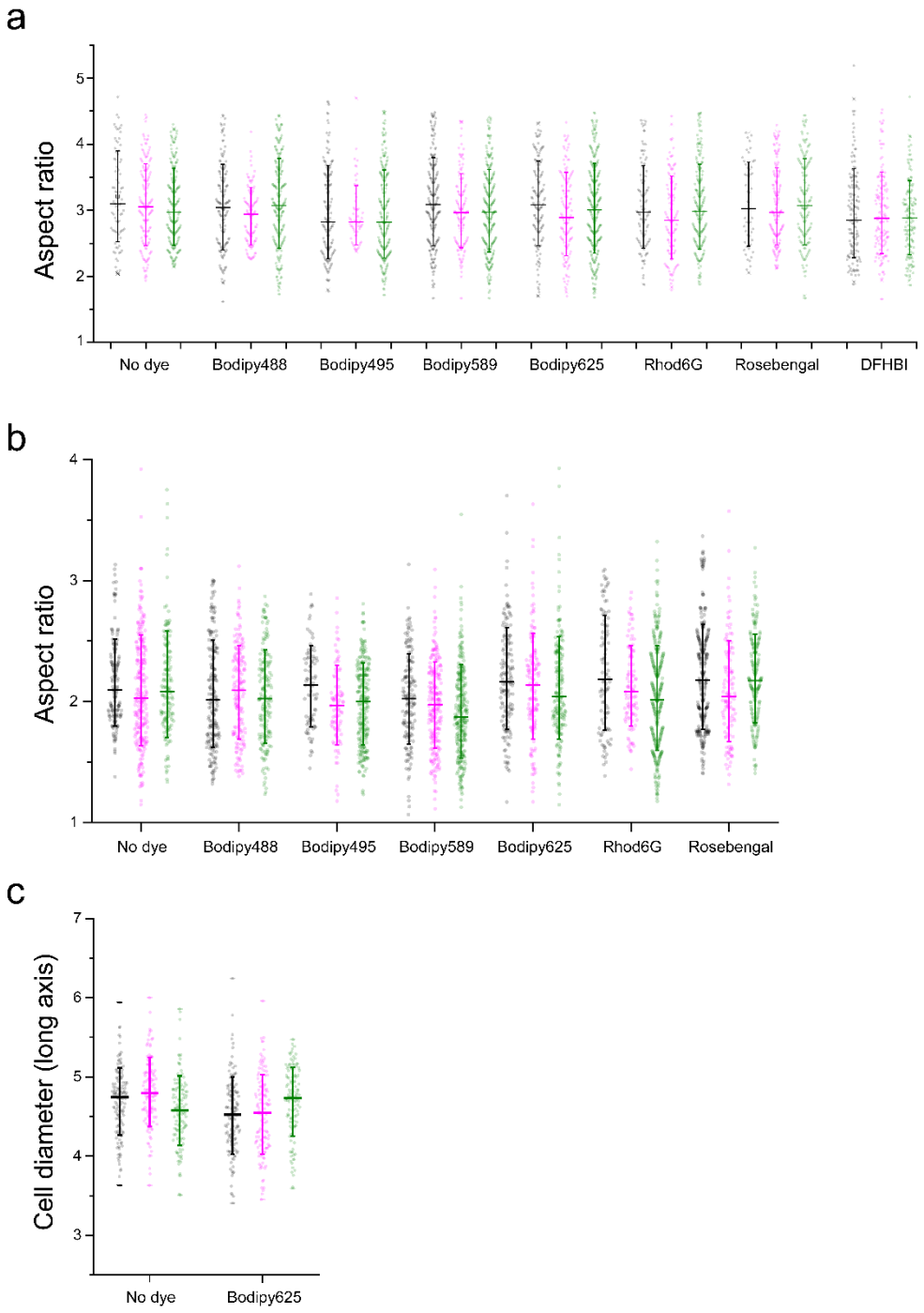

Fig. S25. Effect of dye labeling on CTPEs on cell morphology ascertained by phase-contrast microscopy. The aspect ratio (length divided by width) plotted for unlabeled and dye-labeled (a) *E. coli*, (b) *L. lactis,* and (c) cell diameter along the long axis for spherical *S. cerevisiae* cells represented in black (control), magenta (LmrR) and green (RamR) as measured from phase-contrast microscopy in the mid-exponential growth phase. The impact of cell diameters for *S. cerevisiae* cells was assessed only with Bodipy625 dye since other dyes did not permeate through the cell-wall. The horizontal bars indicate median values, and the vertical bars indicate standard deviation in the sample of at least 100 cells in each case. Images were analyzed using the MicrobeJ plugin[1] in Fiji software.

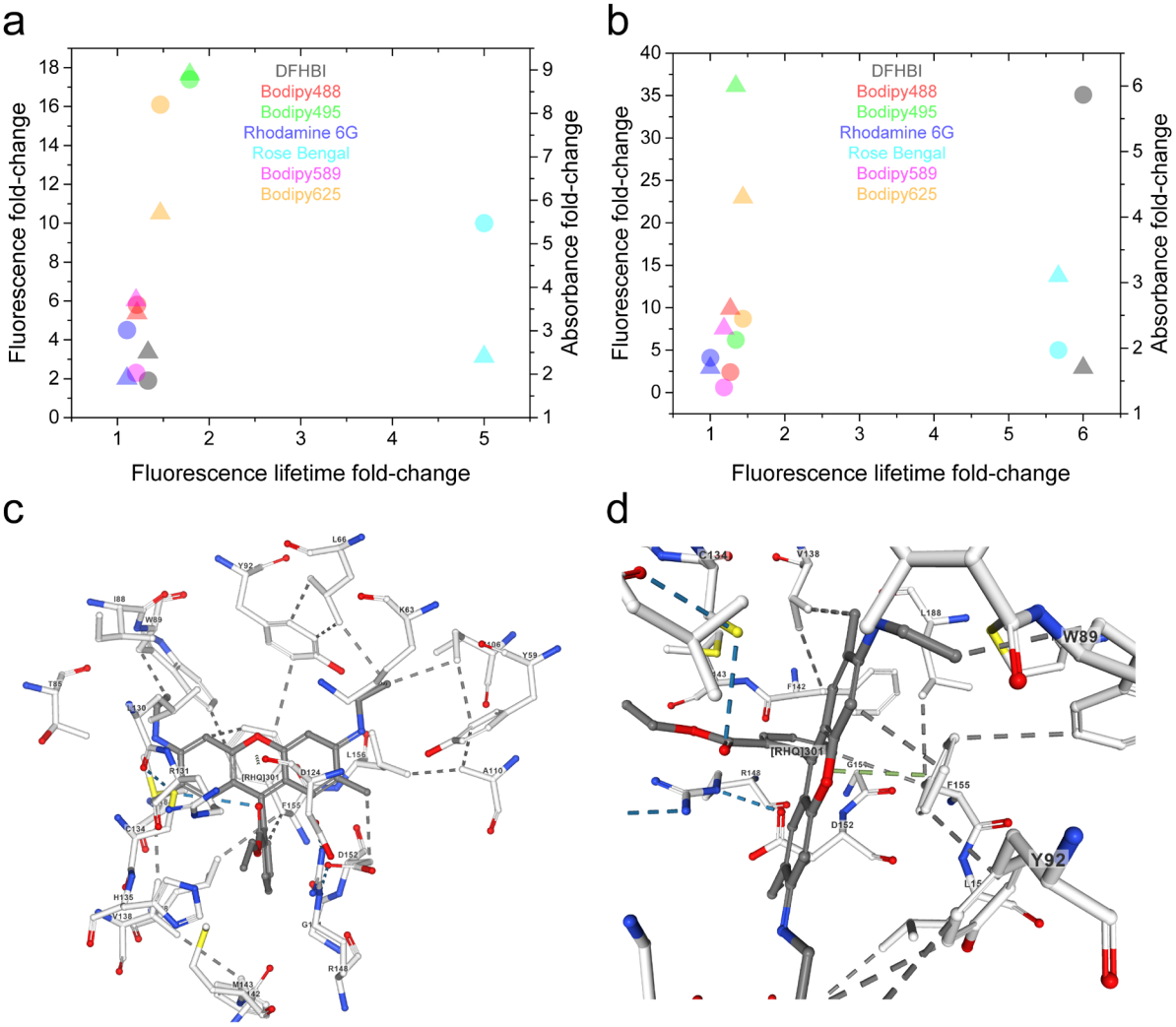

Fig. S26. a-b) Correlation plots of fold-changes in emission fluorescence and absorbance with fold-changes in fluorescence lifetimes for RamR (panel a) and LmrR (panel b). Identical colors depicting fluorescence fold-change (circles) and absorbance fold-changes (squares)indicate the same dye. c-d) An interaction map of a crystal structure of Rhodamine 6G-Bound form of RamR depicting hydrophobic contacts (grey lines) and hydrogen bonds (blue lines). The π-π stacking interactions between Phe155 and Rhodamine 6G are shown in light green (panel b). Interactions are determined by geometric criteria as described in PoseView[2]. Image created with Rhodamine 6G-Bound form of RamR using the PDB ID: 3VVZ on NGL Viewer[3].

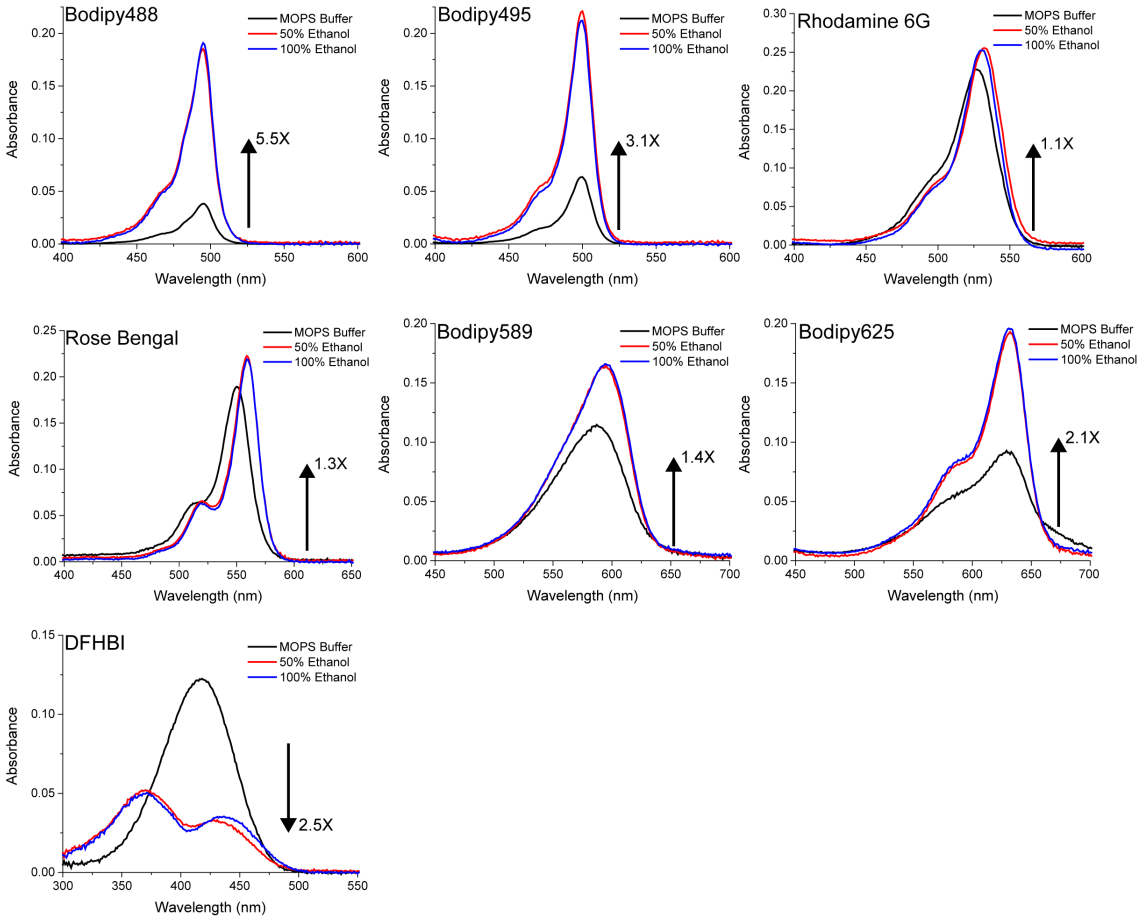

Fig. S27. Absorption spectra of seven fluorogenic dyes in different solvents. The absorption spectra were recorded at 5 µM dye in 20 mM K-MOPS, 150 mM NaCl buffer at pH 7.0 (black traces), in the same buffer with 50% ethanol (red traces), and 100% ethanol (blue traces) at room temperature. The black arrows next to the curves depict the fold-change in peak absorption. The spectra recorded in 50% ethanol and 100% ethanol are comparable. The depicted curves are the average from three independent measurements.

Table S1. Comparison of CTPEs to existing non-covalent fluorogenic tagging systems¶

Parent	Protein tag	Applicable dyes/ fluorogens	Emission wavelengths (nm)	Fluorescence fold increase	Applicable in bacteria?	Applicable in yeast?	Applicable in eukaryotes?	Fluorescence lifetime (ns)	Ref.	Kd (μM)	MW (Da)	Native state	Extinction coefficient (M^{-1} cm^{-1})
Transcription factors	CTPE	DFHBI, Bodipy488, Bodipy495, Rhod6G, Rose bengal, Bodipy589, Bodipy625, etc.	495-642	2.3-35	Yes	Yes	No	Enhanced	this study	0.17-5.0	28,000 - 44,000	Dimer	42,000-219,000
Photoactive Yellow Protein (PYP)	Y-FAST	HBR	530-540	22-191	Yes	Yes	Yes	Unknown	4,5	0.62	14,000	Monomer	44,000
		HMBR	530-541	82-550	Yes	Yes				0.13			45,000
		HBR-3,5-DOM	600	220	Unknown	Yes			6	0.97			39,000
	redFAST	HBR-3,5-DOM	554	6		Yes				1.30	13,583		43,000
	greenFAST		544	8		Yes				16.20	13,881		n.d.
	nanoFAST	HBR-DOM2	380-530	253		Unknown			7	0.85	10,948		25,500
UnaG	UnaG	Bilverdin	527	7-11	Yes	Yes			8	9.60E-05	16,500		77,300
Human single chain antibodies (scFVs)	HL1.0.1	Thiazole orange derivative (TO1)	530	2,600	Unknown	Yes			9,10	1.70E-03	25,942		60,000
	HL4	Malachite green derivative (MG)	649	15,700		Yes				0.59	27,610		133,000
	L5		658	4,100		Yes				0.32	16,362		103,000
	H6		656	18,000		Yes				0.04	16,317		105,000
	dL5*	MHN-ester	532	3,000		Yes			11,12	9.00E-05	28,700	Dimer	64,000
	DIR scFVs	TO-PRO-5, TO-PRO-3, TO-PRO-1, YO-PRO-1, PO-PRO-1*	450-750	100		Yes			13	0.01-0.7	25,228 - 27,426	Monomer	n.d.

¶ CTPE, Chemogenetic Tag with Probe Exchange; Y-FAST, Yellow Fluorescence-Activating and absorption-Shifting Tag (Y-FAST); DFHBI, 3,5-difluoro-4-hydroxybenzylidene imidazolinone; HBR, 4-hydroxybenzylidene-rhodanine; HMBR, 4-hydroxy-3-methylbenzylidene-rhodanine; n.d., not determined; HBR-3,5-DOM, 4-hydroxy-3,5-dimethoxybenzylidene rhodamine; HBR-DOM2, (Z)-5-(4-hydroxy-2,5-dimethoxybenzylidene)-2-thioxothiazolidin-4-one.

Table S2. Strains used in the study

Strain/type	Genotype	Reference
Escherichia coli		
BL21(DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	[14]
BW25113	Δ(araD-araB)567 Δ(rhaD-rhaB)568 ΔlacZ4787 (::rrnB-3) hsdR514 rph-1	[15]
Lactococcus lactis		
NZ9000	MG1363*; pepN::nisR/K*	[16]
NZ9000 *ΔlmrR*	MG1363; *pepN::nisR/K ; ΔlmrR*	[16]
NZ9000 *ΔlmrR pNSC8048-lmrR*	NZ9000 *ΔlmrR* harboring the plasmid pNSC8048-lmrR	[16]
NZ9000 *ΔlmrR pNZC3GH-ramR*	NZ9000 *ΔlmrR* harboring the plasmid pNZC3GH-ramR	This study
Saccharomyces cerevisiae		
BY4709	*MATα ura3Δ0*	[17]
SR80	BY4709 harboring the empty plasmid pDD-ADH	This study
SR80 Ade12-LmrR	BY4709 harboring the plasmid pDD-ADH-Ade12-lmrR	This study
SR80 Ade12-RamR	BY4709 harboring the plasmid pDD-ADH-Ade12-ramR	This study
Primary human embryonic kidney cells (HEK-293T)		
WT	Untransfected Wild-type HEK cells	
WT RamR	WT cells harboring the plasmid pcDNA3.1-Cox8A-RamR-FLAG	This study

Table S3. Plasmids used in the study

Plasmid name	Protein	Promoter	Affinity tag	Inducer	Sub-cellular location	Organism
pET17b-ramR	Cytoplasmic RamR	T7	Strep-Tag	IPTG	cytoplasm	*Escherichia coli BL21 (DE3)*
pET17b-lmrR	Cytoplasmic LmrR	T7	Strep-Tag	IPTG	cytoplasm	*Escherichia coli BL21 (DE3)*
pBAD-cyto-ramR	Cytoplasmic RamR	araBAD	His6-Tag	arabinose	cytoplasm	*Escherichia coli*
pBAD-cyto-lmrR	Cytoplasmic LmrR	araBAD	His6-Tag	arabinose	cytoplasm	*Escherichia coli*
pBAD-osmY-ramR	Osmotically inducible protein Y fused to RamR in the C-terminus	araBAD	-	arabinose	periplasm	*Escherichia coli*
pBAD-osmY-lmrR	Osmotically inducible protein Y fused to LmrR in the C-terminus	araBAD	His6-Tag; Strep-Tag	arabinose	periplasm	*Escherichia coli*
pNM077-ramR-pbP5	Penicillin-binding protein 5 fused to RamR in the N-terminus	ptrcdown	-	IPTG	inner membrane facing the periplasm	*Escherichia coli*
pNM077-lmrR-pbP5	Penicillin-binding protein 5 fused to LmrR in the N-terminus	ptrcdown	-	IPTG	inner membrane facing the periplasm	*Escherichia coli*
-	Control strain	-	-	-	-	*Lactococcus lactis* pNZ9000 *ΔlmrR*
pNSC8048-lmrR	Cytoplasmic LmrR	PnisA	-	nisin A	cytoplasm	*Lactococcus lactis* pNZ9000
pNZC3GH-ramR	Cytoplasmic RamR	PnisA	-	nisin A	cytoplasm	*Lactococcus lactis* pNZ9000 *ΔlmrR*
pDD-ADH	Prsii426: 2µ empty plasmid (multicopy) with URA3 marker, no protein expressed[18]	ADH1	His6-Tag	constitutive	cytoplasm	*Saccharomyces cerevisiae BY4709*
pDD-ADH-ade12-lmrR	Cytoplasmic adenylosuccinate synthase fused to LmrR in the C-terminus	ADH1	His6-Tag	constitutive	cytoplasm	*Saccharomyces cerevisiae BY4709*
pDD-ADH-ade12-ramR	Cytoplasmic adenylosuccinate synthase fused to RamR in the C-terminus	ADH1	His6-Tag	constitutive	cytoplasm	*Saccharomyces cerevisiae BY4709*
pcDNA3.1-Cox8A-RamR-FLAG	Codon optimized RamR with a mitochondrial targeting signal (Cox8A)	CMV	FLAG-Tag	constitutive	Inner mitochondrial membrane	*HEK-293T from primary embryonic human kidney*

Appendix 1: DNA sequences (5' to 3') of constructs used in the study

RamR (native)

ATGGTGGCGCGTCCGAAGAGCGAGGACAAGAAACAAGCGCTGCTGGAAGCGGCGACCCAG
GCGATTGCGCAAAGCGGTATTGCGGCGAGCACCGCGGTGATTGCGCGTAACGCGGGTGTT
GCGGAGGGTACCCTGTTCCGTTACTTTGCGACCAAGGACGAACTGATTAACACCCTGTATCT
GCACCTGAAACAGGATCTGAGCCAAAGCATGATCATGGAGCTGGACCGTAGCATTACCGAT
GCGAAAATGATGACCCGTTTCATCTGGAACAGCTACATTAGCTGGGGCCTGAACCATCCGG
CGCGTCACCGTGCGATCCGTCAGCTGGCGGTTAGCGAGAAGCTGACCAAAGAAACCGAAC
AACGTGCGGACGATATGTTCCCGGAACTGCGTGATCTGAGCCACCGTAGCGTGCTGATGGT
TTTTATGAGCGACGAGTACCGTGCGTTCGGTGATGGCCTGTTTCTGGCGCTGGCGGAAACC
ACCATGGATTTTGCGGCGCGTGATCCGGCGCGTGCGGGCGAGTATATTGCGCTGGGCTTT
GAAGCGATGTGGCGTGCGCTGACCCGTGAGGAACAGGCGGCGTGGAGCCACCCGCAATTT
GAAAAGTAA

RamR (codon-optimized for *L. lactis*)

ATGGGTGGCATGGTGGCGCGTCCGAAGAGCGAGGATAAGAAACAAGCTCTACTTGAAGCTG
CTACACAAGCCATTGCGCAAAGTGGGATTGCCGCATCGACAGCTGTAATTGCTAGAAATGCC
GGTGTTGCAGAAGGTACCCTTTTCAGATATTTTGCTACCAAAGACGAATTAATTAATACACTG
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CACGTCATCGTGCAATCCGTCAGCTTGCTGTTTCTGAGAAATTAACAAAAGAAACAGAACAA
CGTGCTGACGATATGTTTCCAGAACTACGTGATTTATCACATCGTTCAGTCCTTATGGTTTTT
ATGAGTGACGAGTATCGTGCATTCGGAGATGGATTATTTCTGGCACTAGCTGAAACTACTAT
GGATTTTGCTGCGCGTGATCCCGCGCGTGCAGGAGAGTATATTGCTCTTGGCTTTGAAGCT
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LmrR(K55D/K59Q)

ATGGGTGCCGAAATCCCGAAAGAAATGCTGCGTGCTCAAACCAATGTCATCCTGCTGAATGT
CCTGAAACAAGGCGATAACTATGTGTATGGCATTATCAAACAGGTGAAAGAAGCGAGCAACG
GTGAAATGGAACTGAATGAAGCCACCCTGTATACGATTTTTGATCGTCTGGAACAGGACGGC
ATTATCAGCTCTTACTGGGGTGATGAAAGTCAAGGCGGTCGTCGCAAATATTACCGTCTGAC
CGAAATCGGCCATGAAAACATGCGCCTGGCGTTCGAATCCTGGAGTCGTGTGGACAAAATC
ATTGAAAATCTGGAAGCAAACAAAAAATCTGAAGCGATCAAAGCCGCCTGGAGCCACCCGC
AGTTCGAAAAA**CATCATCATCATCATCAT**$^{\text{His6-Tag}}$TGA

OsmY-RamR

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CGCGACCGGCTCTGCCTACGCGGAAAACAACGCGCAGACTACCAATGAAAGCGCAGGGCA
AAAAGTCGATAGCTCTATGAATAAAGTCGGTAATTTCATGGATGACAGCGCCATCACCGCGA
AAGTGAAGGCGGCCCTGGTGGATCATGACAACATCAAGAGCACCGATATCTCTGTAAAAAC
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TATCGCCAAAGCGGTAGATGGTGTGAAAAGCGTTAAAAATGATCTGAAAACTAAGGGCGGTG
GCATGGTGGCGCGTCCGAAGAGCGAGGACAAGAAACAAGCGCTGCTGGAAGCGGCGACCC
AGGCGATTGCGCAAAGCGGTATTGCGGCGAGCACCGCGGTGATTGCGCGTAACGCGGGTG
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ATGCGAAAATGATGACCCGTTTCATCTGGAACAGCTACATTAGCTGGGGCCTGAACCATCCG
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CAACGTGCGGACGATATGTTCCCGGAACTGCGTGATCTGAGCCACCGTAGCGTGCTGATGG
TTTTTATGAGCGACGAGTACCGTGCGTTCGGTGATGGCCTGTTTCTGGCGCTGGCGGAAAC
CACCATGGATTTTGCGGCGCGTGATCCGGCGCGTGCGGGCGAGTATATTGCGCTGGGCTTT
GAAGCGATGTGGCGTGCGCTGACCCGTGAGGAACAGGCGGCGTGA

OsmY-LmrR

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GTCCTGAAACAAGGCGATAACTATGTGTATGGCATTATCAAACAGGTGAAAGAAGCGAGCAA
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GCATTATCAGCTCTTACTGGGGTGATGAAAGTCAAGGCGGTCGTCGCAAATATTACCGTCTG
ACCGAAATCGGCCATGAAAACATGCGCCTGGCGTTCGAATCCTGGAGTCGTGTGGACAAAA
TCATTGAAAATCTGGAAGCAAACAAAAAATCTGAAGCGATCAAAGCCGCCTGGAGCCACCCG
CAGTTCGAAAAA**CATCATCATCATCATCAT**$^{His6\text{-}Tag}$TGA

RamR-PBP5 with DsbA signal peptide

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ACAAGAAACAAGCGCTGCTGGAAGCGGCGACCCAGGCGATTGCGCAAAGCGGTATTGCGG
CGAGCACCGCGGTGATTGCGCGTAACGCGGGTGTTGCGGAGGGTACCCTGTTCCGTTACTT
TGCGACCAAGGACGAACTGATTAACACCCTGTATCTGCACCTGAAACAGGATCTGAGCCAAA
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CGGTTAGCGAGAAGCTGACCAAAGAAACCGAACAACGTGCGGACGATATGTTCCCGGAACT
GCGTGATCTGAGCCACCGTAGCGTGCTGATGGTTTTTATGAGCGACGAGTACCGTGCGTTC
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GCGCGTGCGGGCGAGTATATTGCGCTGGGCTTTGAAGCGATGTGGCGTGCGCTGACCCGT
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GATCGAGCAACGCCCGCTGGTTGTGTTGCAAGAAATCCCGGAAGGTAACTTCTTCGGCAAA
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LmrR-PBP5 with DsbA signal peptide

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GCTGCGTGCTCAAACCAATGTCATCCTGCTGAATGTCCTGAAACAAGGCGATAACTATGTGT
ATGGCATTATCAAACAGGTGAAAGAAGCGAGCAACGGTGAAATGGAACTGAATGAAGCCAC
CCTGTATACGATTTTTGATCGTCTGGAACAGGACGGCATTATCAGCTCTTACTGGGGTGATG
AAAGTCAAGGCGGTCGTCGCAAATATTACCGTCTGACCGAAATCGGCCATGAAAACATGCG
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TGAAGCCGAAAGTAAAAAACTGCTAACCTGGGGCTTCCGTTTCTTTGAAACCGTTAACCCAC
TGAAAGTAGGTAAAGAGTTCGCCTCTGAACCGGTTTGGTTTGGTGATTCTGATCGCGCTTCG
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GTTTGGTTAA

Ade12-RamR-6His

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TTGTTGTAGACGGTGTTAAGTATGATTTCCATATGTTACCATCTGGTTTAGTCAACCCAAACT
GCCAAAACCTTTTGGGTAATGGTGTTGTTATTCATGTTCCATCTTTTTTCAAAGAGTTGGAAA
CCTTGGAAGCTAAAGGTTTGAAGAACGCAAGGAGTAGATTATTTGTTTCTTCCAGAGCACATT
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TACAAAGTTTTGCCAGGTTGGGATCAAGATATTACCAAAATTACAAAGTATGAAGATTTGCCG
GAAAACGCAAAGAAGTACTTAAAATATATTGAAGATTTTGTTGGCGTTCCTGTTGAATGGGTT
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CCGTCAGCTGGCGGTTAGCGAGAAGCTGACCAAAGAAACCGAACAACGTGCGGACGATATG
TTCCCGGAACTGCGTGATCTGAGCCACCGTAGCGTGCTGATGGTTTTTATGAGCGACGAGT
ACCGTGCGTTCGGTGATGGCCTGTTTCTGGCGCTGGCGGAAACCACCATGGATTTTGCGGC
GCGTGATCCGGCGCGTGCGGGCGAGTATATTGCGCTGGGCTTTGAAGCGATGTGGCGTGC
GCTGACCCGTGAGGAACAGGCGGCG**CATCATCATCATCATCAT**$^{\text{His6-Tag}}$TAA

Ade12-LmrR-6His

ATGGTTAACGTTGTATTGGGCTCCCAATGGGGTGATGAGGGTAAAGGTAAACTAGTTGATTT
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TTGTTGTAGACGGTGTTAAGTATGATTTCCATATGTTACCATCTGGTTTAGTCAACCCAAACT
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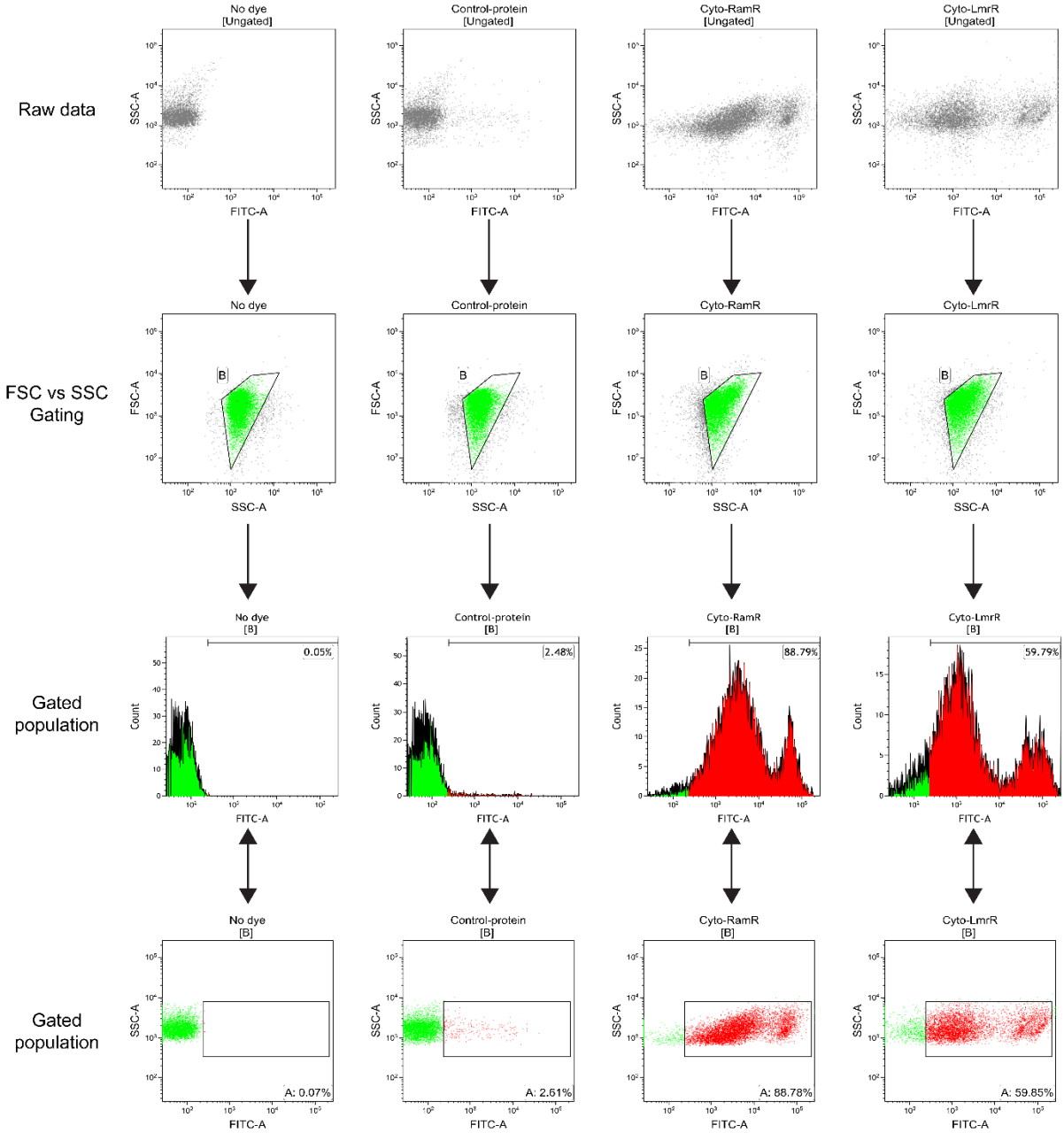
GGGGTGATGAAAGTCAAGGCGGTCGTCGCAAATATTACCGTCTGACCGAAATCGGCCATGA
AAACATGCGCCTGGCGTTCGAATCCTGGAGTCGTGTGGACAAAATCATTGAAAATCTGGAAG
CAAACAAAAAATCTGAAGCGATCAAAGCC**CATCATCATCATCATCAT**$^{His6\text{-}Tag}$TAA

^{Cox8A}RamR^{FLAG} with *HindII* and *EcoRI* restriction sites (red)

AAGCTTATGAGCGTGCTGACCCCCCTGCTGCTGCGCGGCCTGACCGGCAGCGCC
CGCCGCCTGCCCGTGCCCCGCGCCAAGATCCACAGCCTGATGGTGGCCCGCCCC
AAGAGCGAGGACAAGAAGCAGGCCCTGCTGGAGGCCGCCACCCAGGCCATCGCC
CAGAGCGGCATCGCCGCCAGCACCGCCGTGATCGCCCGCAACGCCGGCGTGGCC
GAGGGCACCCTGTTCCGCTACTTCGCCACCAAGGACGAGCTGATCAACACCCTGT
ACCTGCACCTGAAGCAGGACCTGAGCCAGAGCATGATCATGGAGCTGGACCGCAG
CATCACCGACGCCAAGATGATGACCCGCTTCATCTGGAACAGCTACATCAGCTGGG
GCCTGAACCACCCCGCCCGCCACCGCGCCATCCGCCAGCTGGCCGTGAGCGAGA
AGCTGACCAAGGAGACCGAGCAGCGCGCCGACGACATGTTCCCCGAGCTGCGCG
ACCTGAGCCACCGCAGCGTGCTGATGGTGTTCATGAGCGACGAGTACCGCGCCTT
CGGCGACGGCCTGTTCCTGGCCCTGGCCGAGACCACCATGGACTTCGCCGCCCG
CGACCCCGCCCGCGCCGGCGAGTACATCGCCCTGGGCTTCGAGGCCATGTGGCG
CGCCCTGACCCGCGAGGAGCAGGCCGCCTGGAGCCACCCCGACTACAAGGACGA
CGACGACAAGTAGGAATTC

Appendix 2. Gating strategy for flow cytometry studies



Gating strategy: For *E. coli*, *L. lactis,* and *S. cerevisiae* cells, firstly using the FSC/SSC gating, cell debris was removed from the main cell population. A positivity threshold gate for each sample was defined based on unlabeled (0%) and control cells expressing no or control protein yet labeled (< 3%). An identical positivity threshold gate was applied to all samples for a given organic dye. 10000 events were collected for each sample. For HEK293T cells, a starting cell population per sample was collected with the stopping rule of 30000 events per preliminary gate drawn in FSC/SSC. Next, the cells were analyzed, placing gates on PE population indicating FLAG expression labeled with Alexa Fluor 568. APC filter was used to detect Bodipy-625 fluorescence. Negative/high background populations were defined by unstained cells visible in PE channel and untransfected but Bodipy-625 pulsed cells in APC channel.

Appendix 3. Sources and nomenclature of dyes

Name	Commercial Name	CAS	Pubchem CID	Source
DFHBI	DFHBI	1241390-29-3	70808995	Sigma Aldrich/Merck
Bodipy488	BODIPY™ 493/503	121207-31-6	14766991	ThermoFischer Scientific
Bodipy495	BODIPY dye	194235-40-0	25058170	Sigma Aldrich/Merck
Rhodamine 6G	Rhodamine 6G	989-38-8	13806	Sigma Aldrich/Merck
Rose Bengal	Rose Bengal	632-69-9	34343	Sigma Aldrich/Merck
Bodipy 589	BDP TR carboxylic acid	150152-64-0	131954291	Lumiprobe GmbH
Bodipy 625	BDP 630/650 carboxylic acid	2183512-02-7	123784196	BroadPharm/ Lumiprobe GmbH
Riboflavin	Riboflavin	83-88-5	493570	Sigma Aldrich/Merck
AlexaFluor488	AlexaFluor489	500004-82-0	135627723	ThermoFischer Scientific
Bodipy FL COOH	BDP FL carboxylic acid	165599-63-3	9817511	Lumiprobe GmbH
Eosin Y	Eosin Y	15086-94-9	27020	Sigma Aldrich/Merck
Bodipy R6G COOH	BDP R6G carboxylic acid	174881-57-3	21982947	Lumiprobe GmbH
6-TAMRA	6-TAMRA	91809-67-5	2762604	Sigma Aldrich/Merck
Bodipy558	BDP 558/568 carboxylic acid	150173-72-1	131954294	Lumiprobe GmbH
AlexaFluor647	AlexaFluor648	400051-23-2	102227060	ThermoFischer Scientific
Probe 6	Probe 6	-	-	Wang et. al. 2020
Probe 10	Probe 10	-	-	Wang et. al. 2020
Probe 11	Probe 11	-	-	Wang et. al. 2020
Probe 15	Probe 15	-	-	Wang et. al. 2020
Probe 22	Probe 22	-	-	Wang et. al. 2020
Probe 23	Probe 23	-	-	Wang et. al. 2020
Probe 29	Probe 29	-	-	Wang et. al. 2020
Probe 33	Probe 33	-	-	Wang et. al. 2020
Ethidium bromide	Ethidium bromide	1239-45-8	14710	Sigma Aldrich/Merck
Hoechst33342	Hoechst33343	23491-52-3	1464	ThermoFischer Scientific
DPH	DPH	115534-33-3	5706757	Sigma Aldrich/Merck
NPN	NPN	90-30-2	7013	Sigma Aldrich/Merck
DAPI	DAPI	47165-04-8	2954	Sigma Aldrich/Merck
ANS	ANS	82-76-8	1369	Sigma Aldrich/Merck
Thioflavin T	Thioflavin T	2390-54-7	16953	Sigma Aldrich/Merck

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