

SUPPLEMENTARY MATERIALS

Structure-based rational design of an enhanced fluorogen-activating protein for fluorogens based on GFP chromophore

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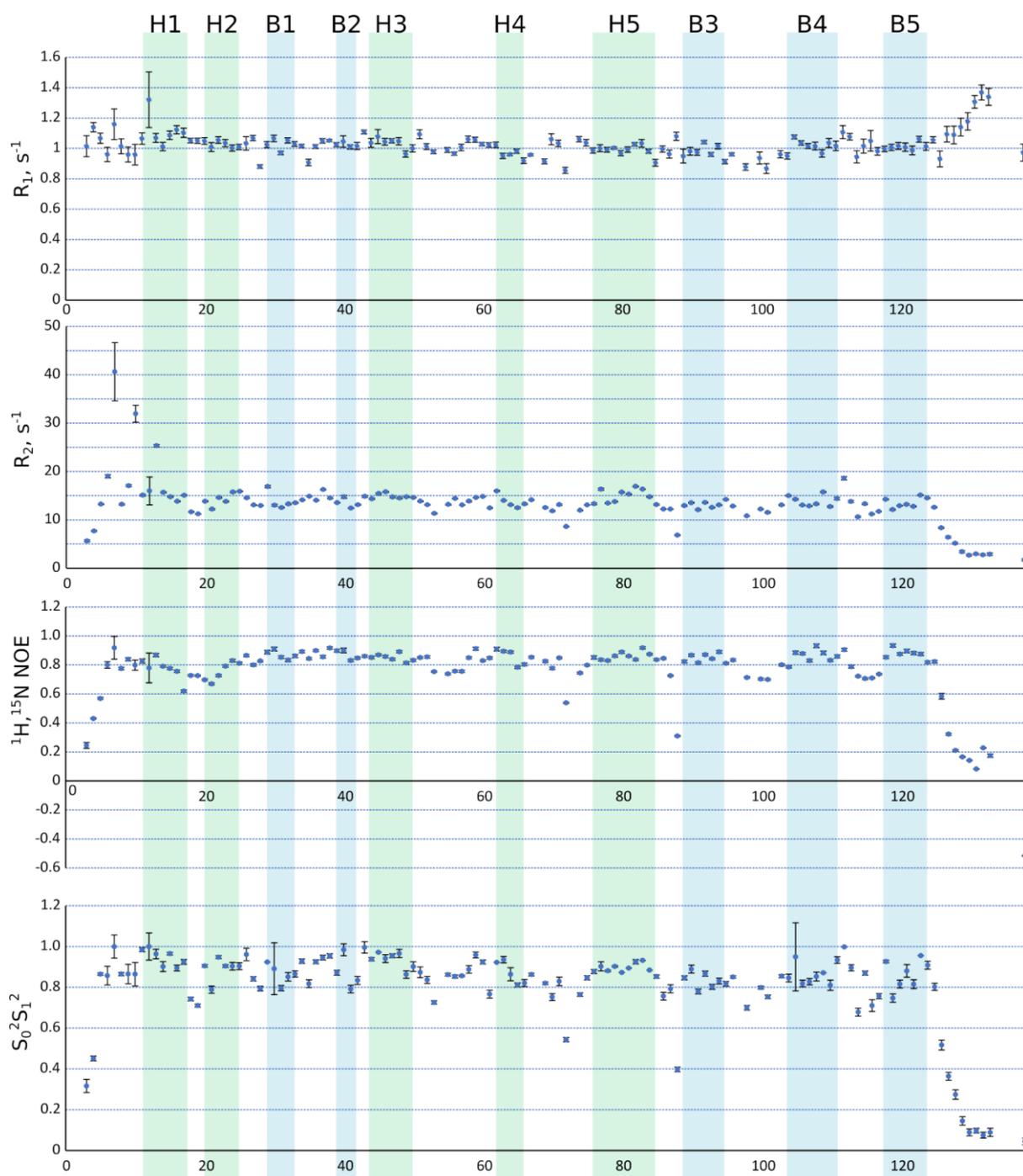


Figure S1. ^{15}N NMR relaxation parameters of FAST/N871b. Rates of longitudinal (R_1) and transverse (R_2) relaxation, $^1\text{H}, ^{15}\text{N}$ heteronuclear steady-state NOE and order parameters of NH groups are plotted for the individual residues of γ FAST in complex with N871b. All parameters were measured at 25 °C at 700 MHz. S_0^2 and S_1^2 are the order parameters of subnanosecond and nanosecond motions, respectively.

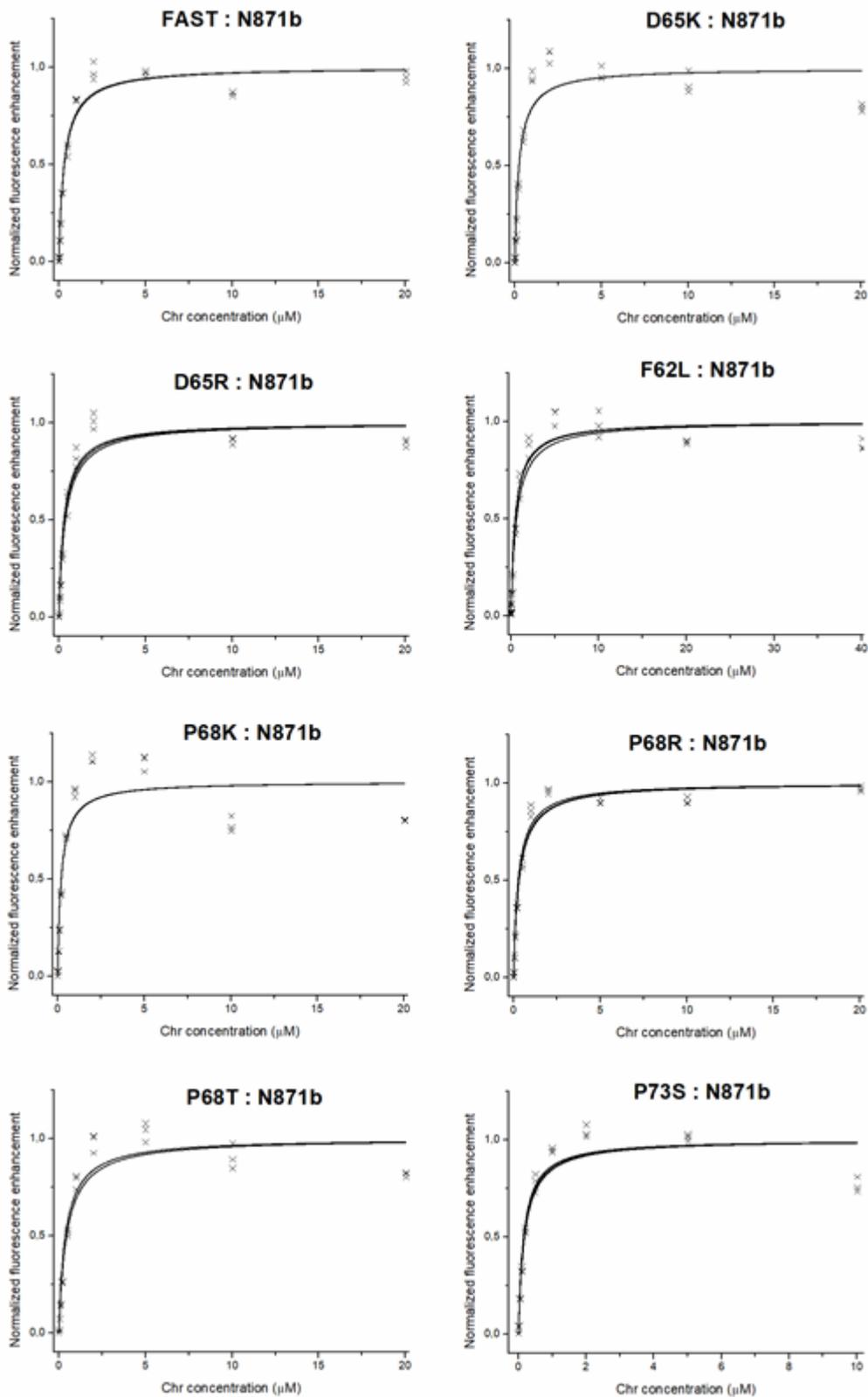


Figure S2. Titration curves observed for **N871b** complexes with FAST mutants

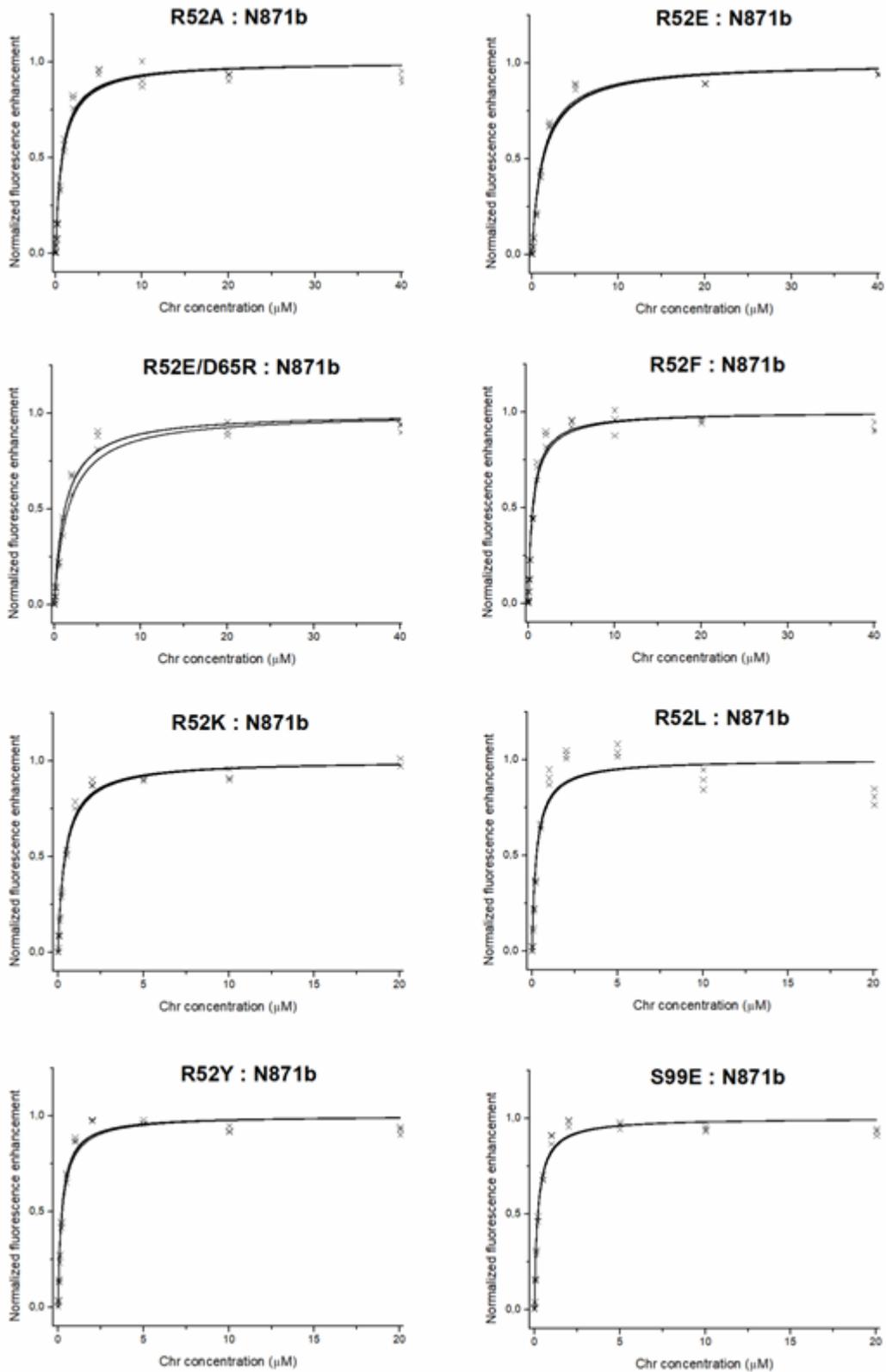


Figure S3. Titration curves observed for **N871b** complexes with FAST mutants

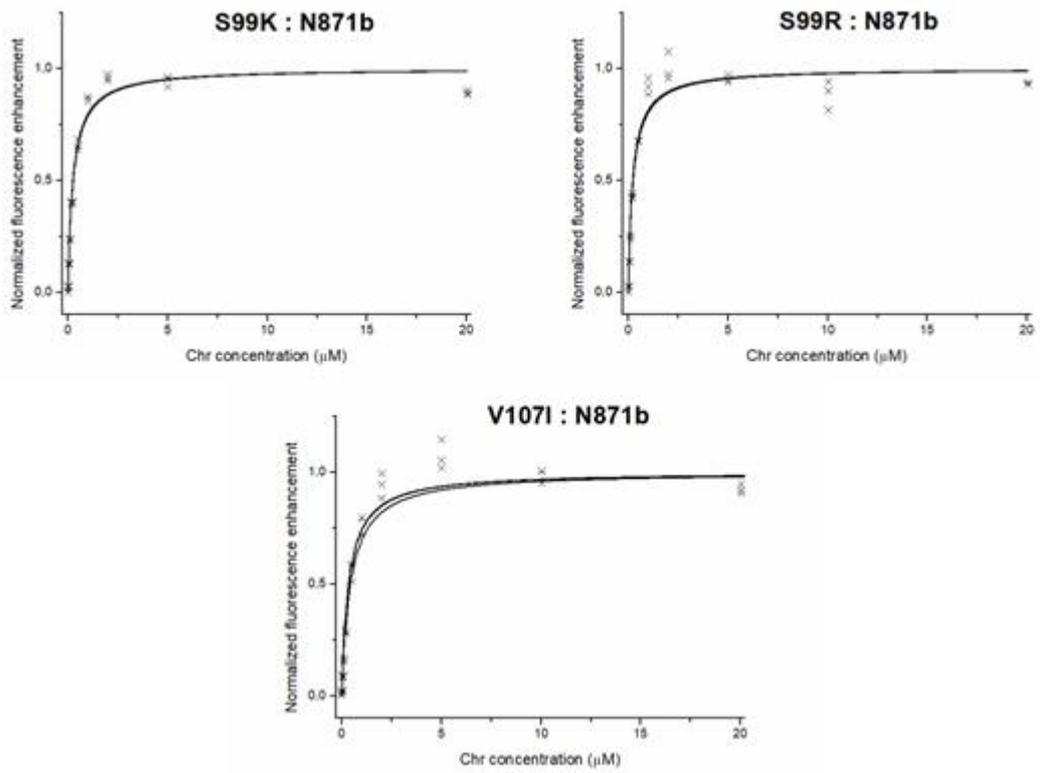


Figure S4. Titration curves observed for **N871b** complexes with FAST mutants

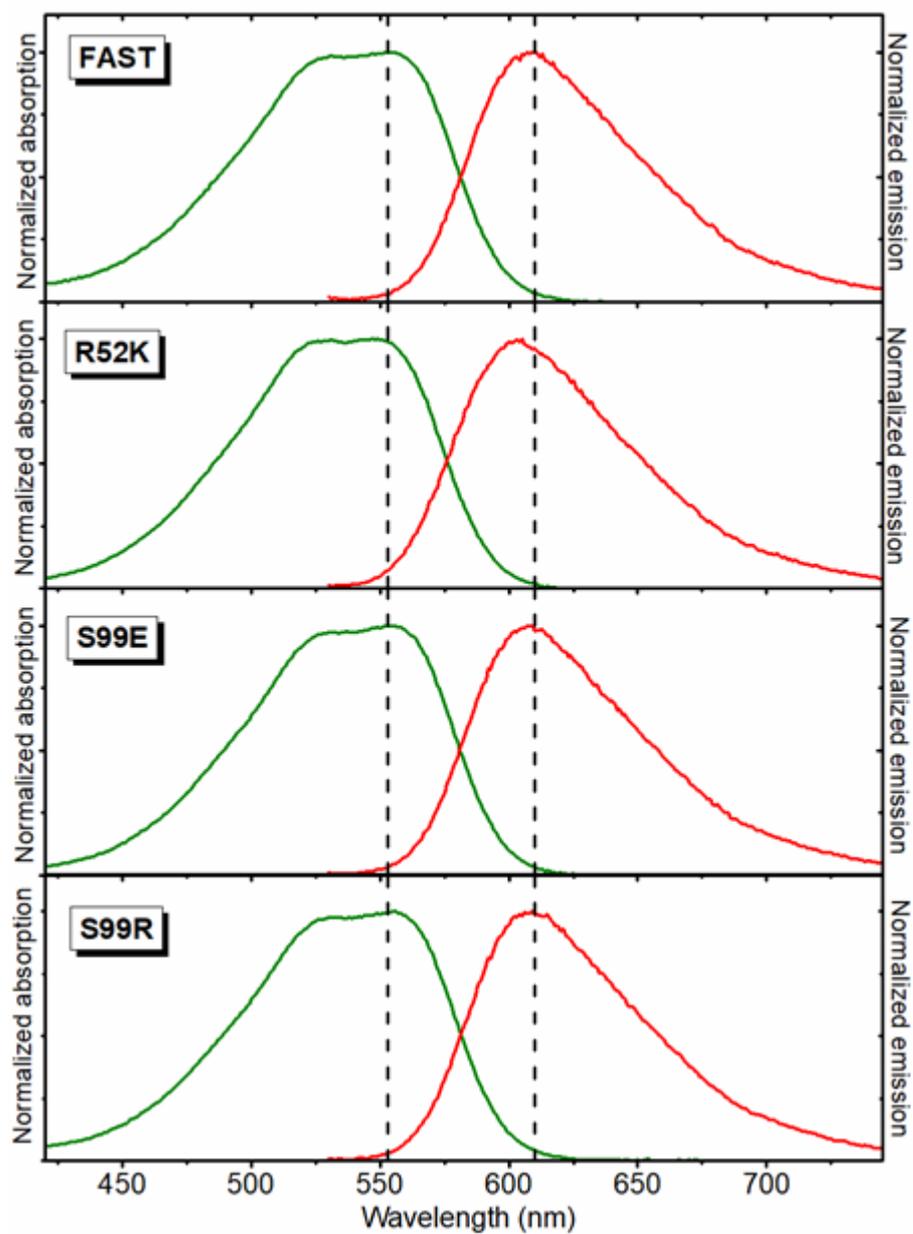


Figure S5. Fluorescence (red) and absorption (green) spectra of **N871b** complexes with FAST mutants

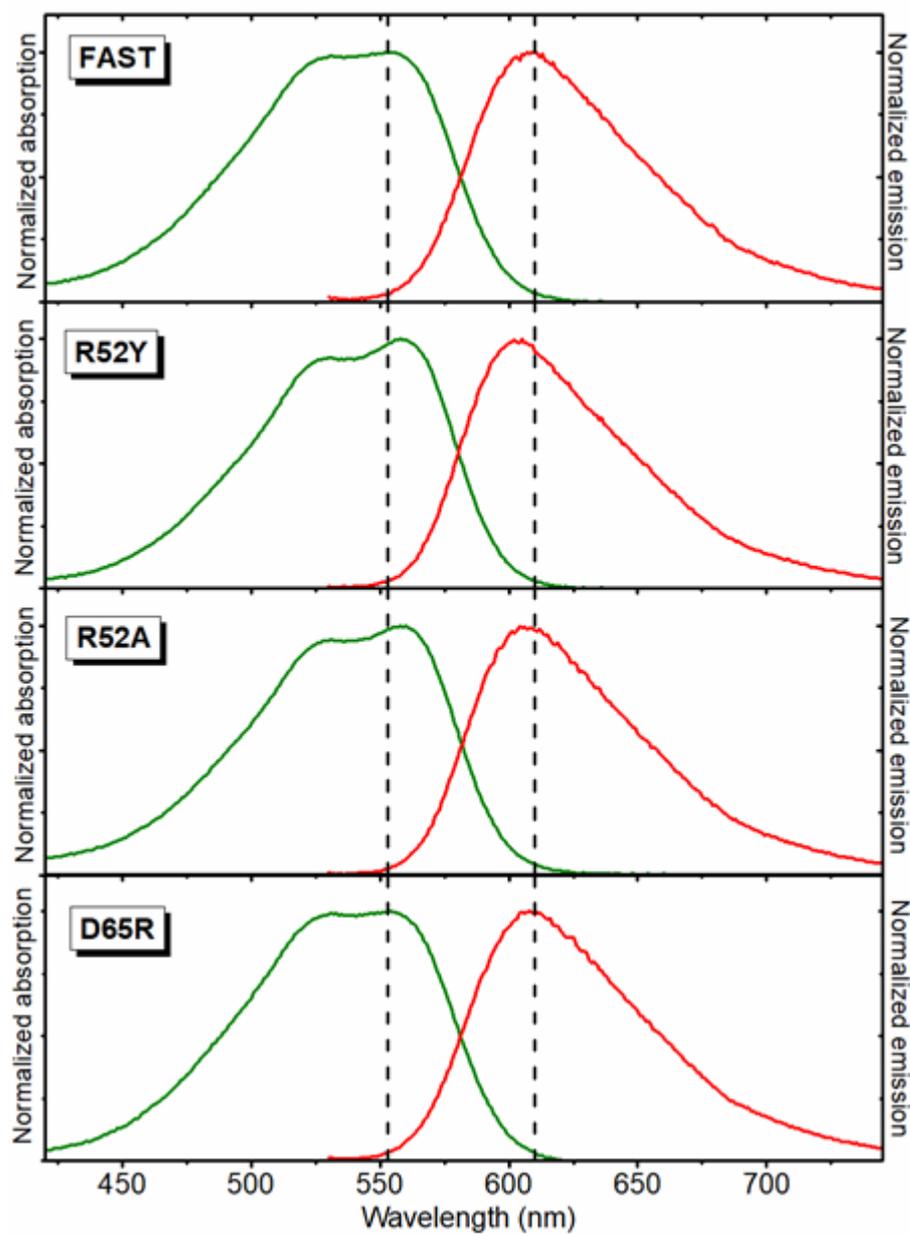


Figure S6. Fluorescence (red) and absorption (green) spectra of **N871b** complexes with FAST mutants

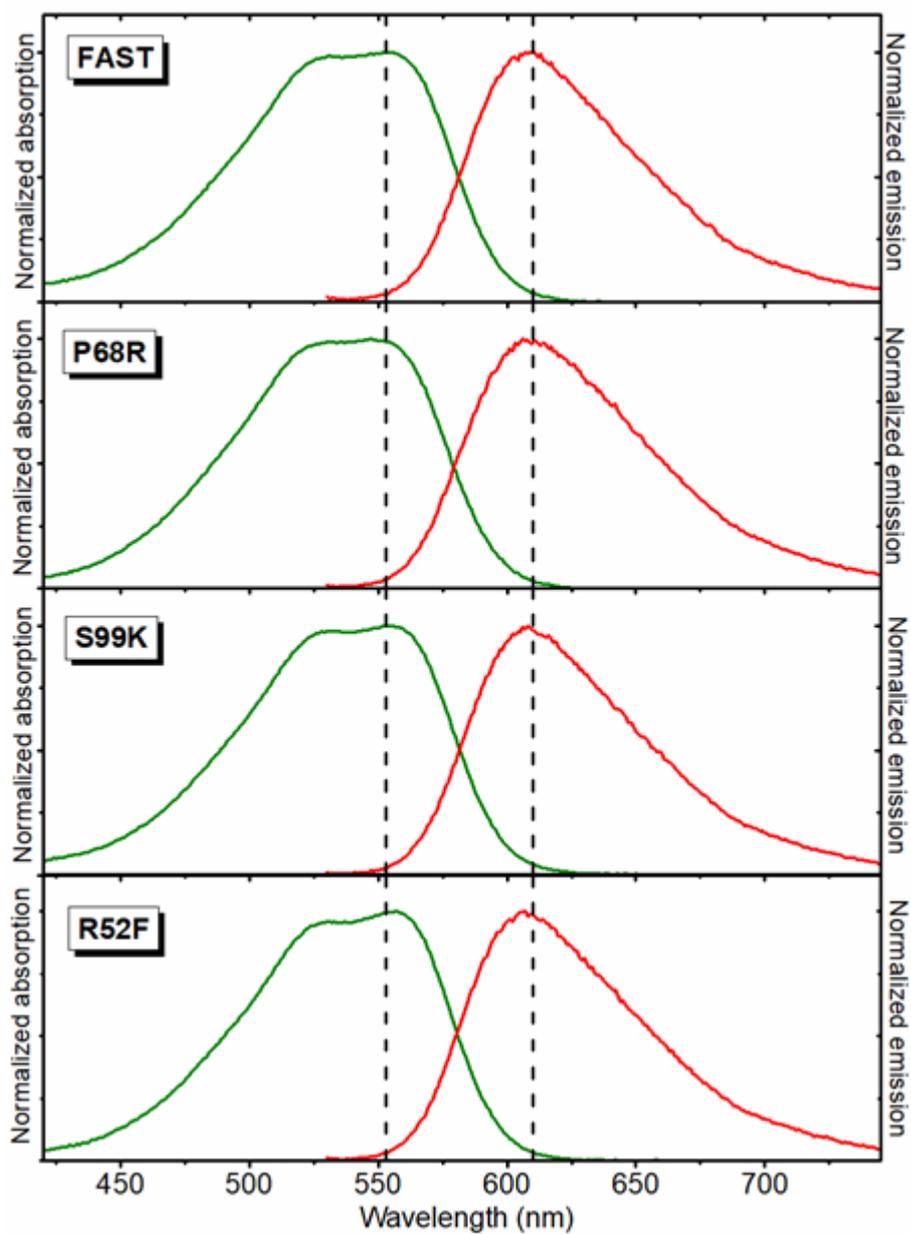


Figure S7. Fluorescence (red) and absorption (green) spectra of **N871b** complexes with FAST mutants

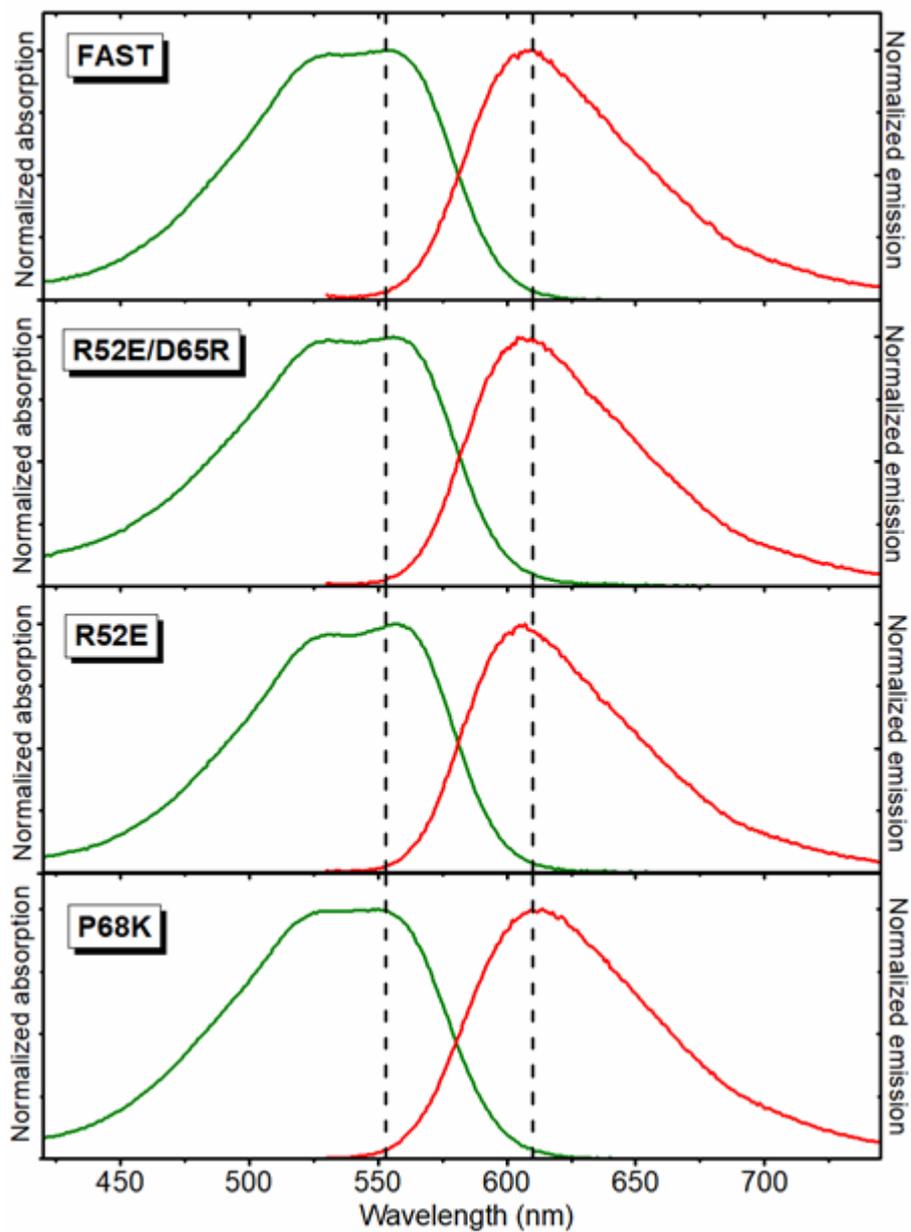


Figure S8. Fluorescence (red) and absorption (green) spectra of **N871b** complexes with FAST mutants

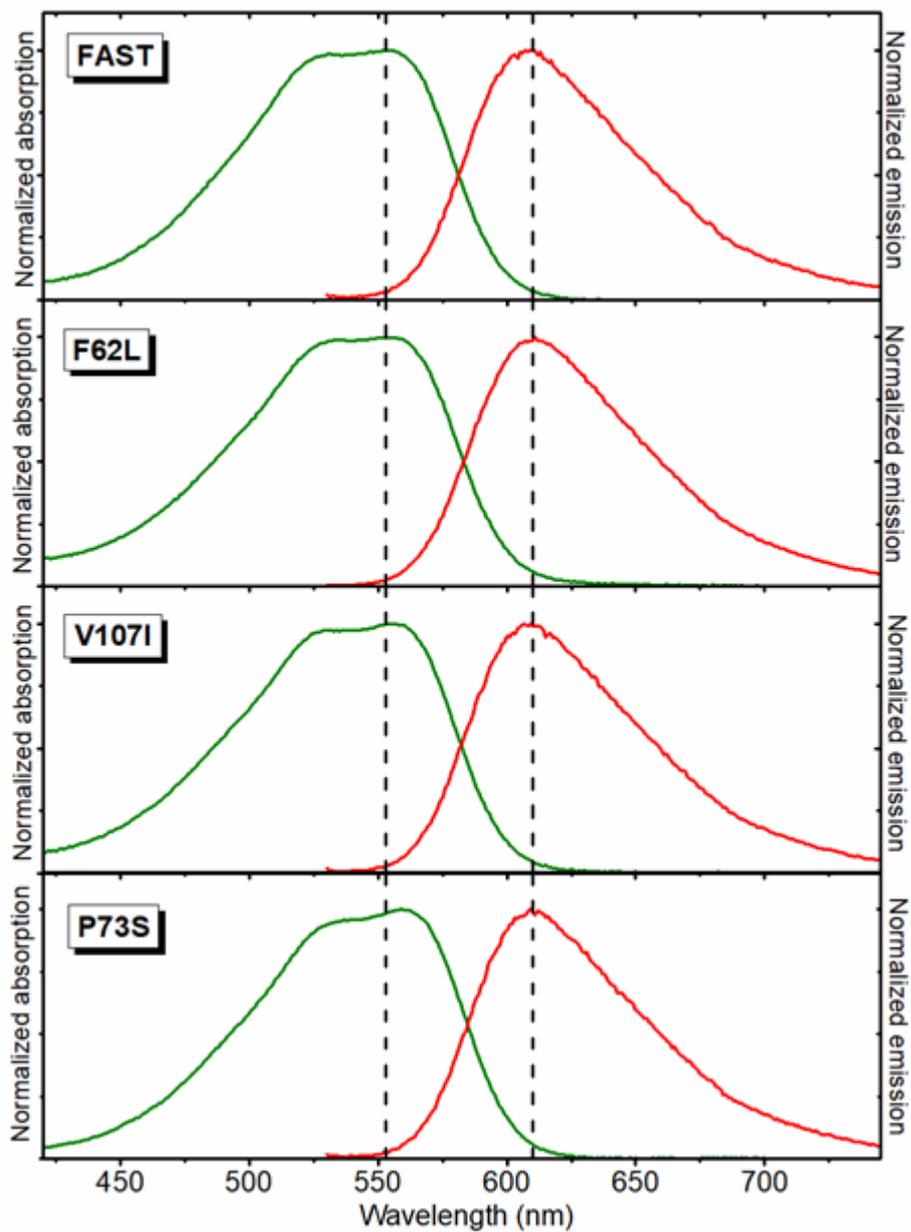


Figure S9. Fluorescence (red) and absorption (green) spectra of **N871b** complexes with FAST mutants

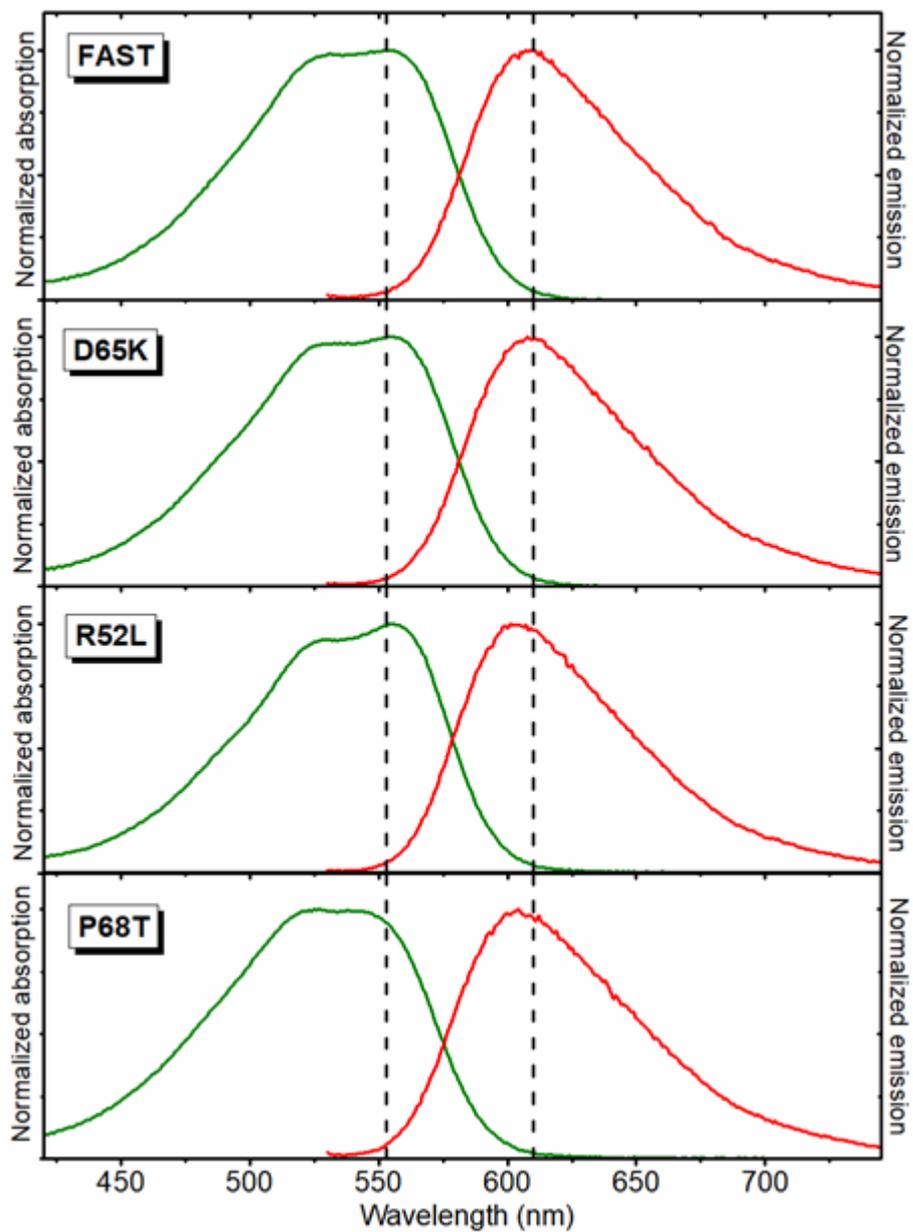


Figure S10.

Fluorescence (red) and absorption (green) spectra of **N871b** complexes with FAST mutants

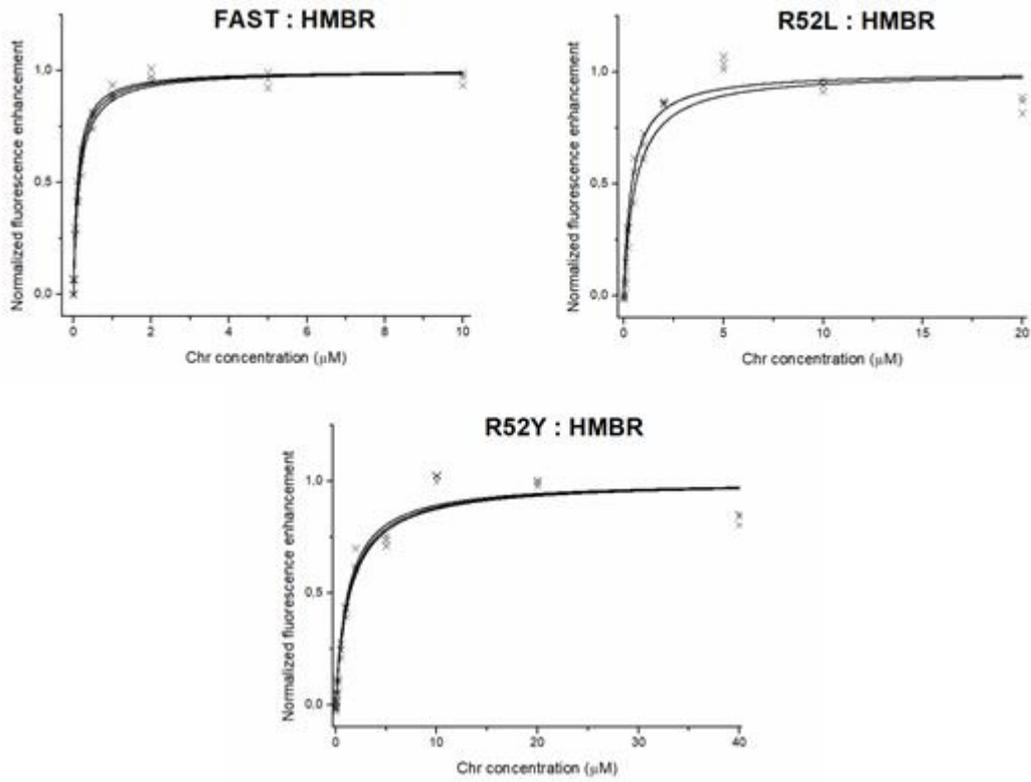


Figure S11. Titration curves observed for **HMBR** complexes with FAST mutants

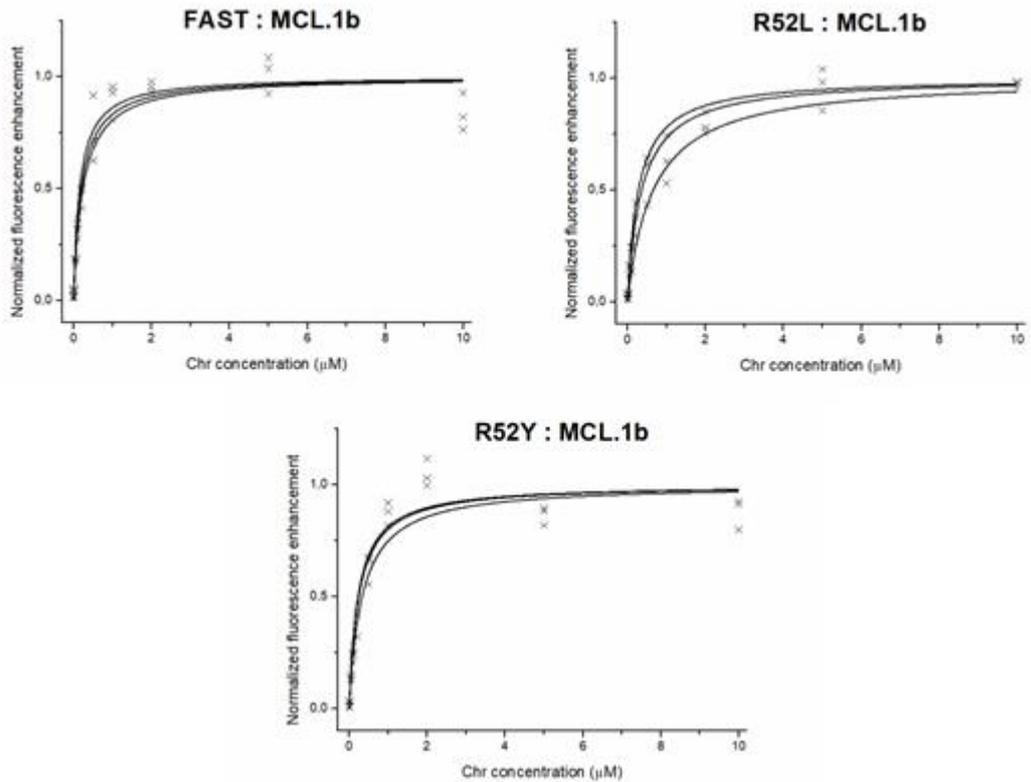


Figure S12. Titration curves observed for **MCL.1b** complexes with FAST mutants

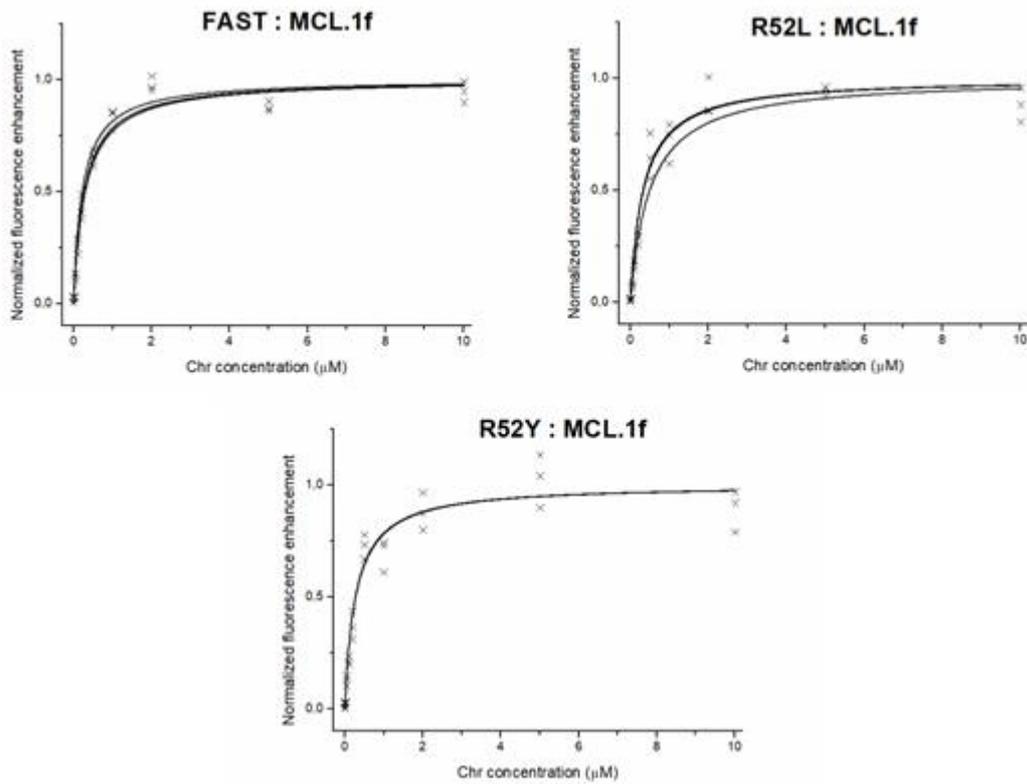


Figure S13. Titration curves observed for **MCL.1f** complexes with FAST mutants

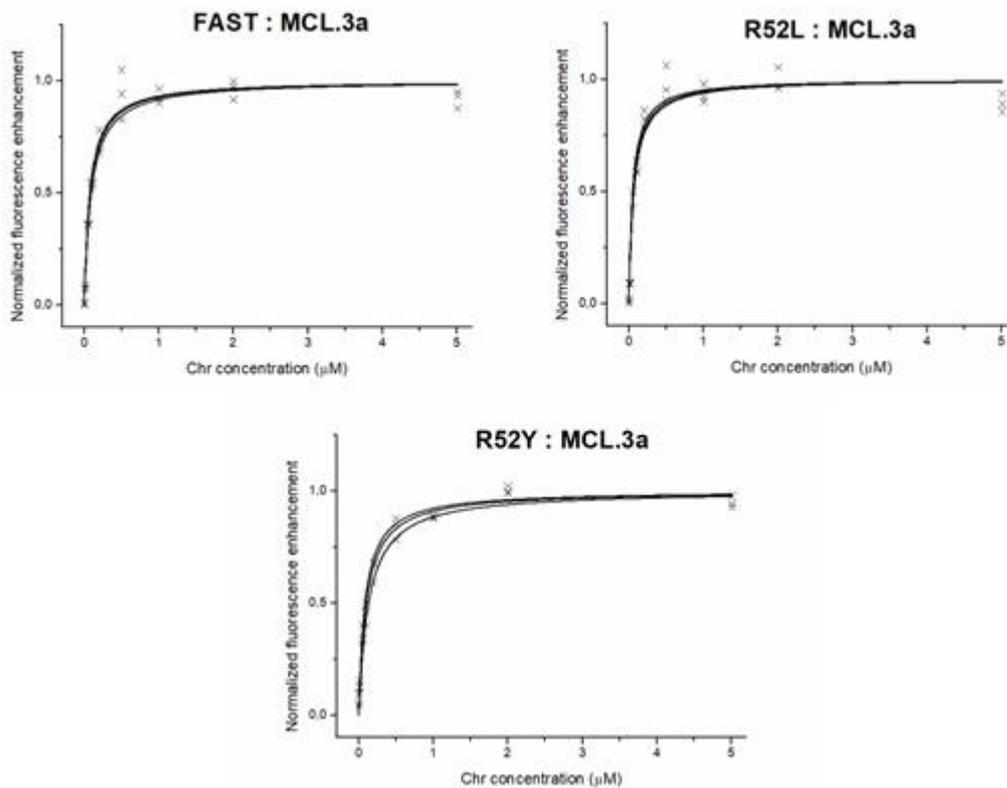


Figure S14. Titration curves observed for **MCL.3a** complexes with FAST mutants

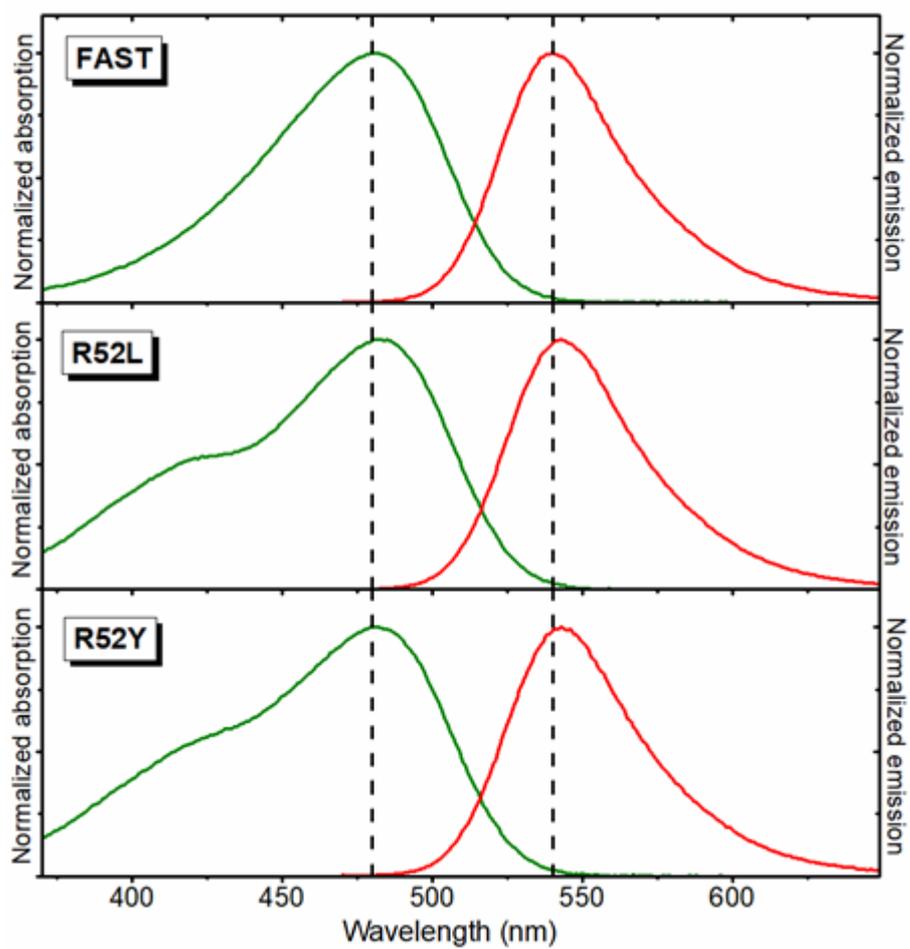


Figure S15. Fluorescence (red) and absorption (green) spectra of **HMBR** complexes with FAST mutants

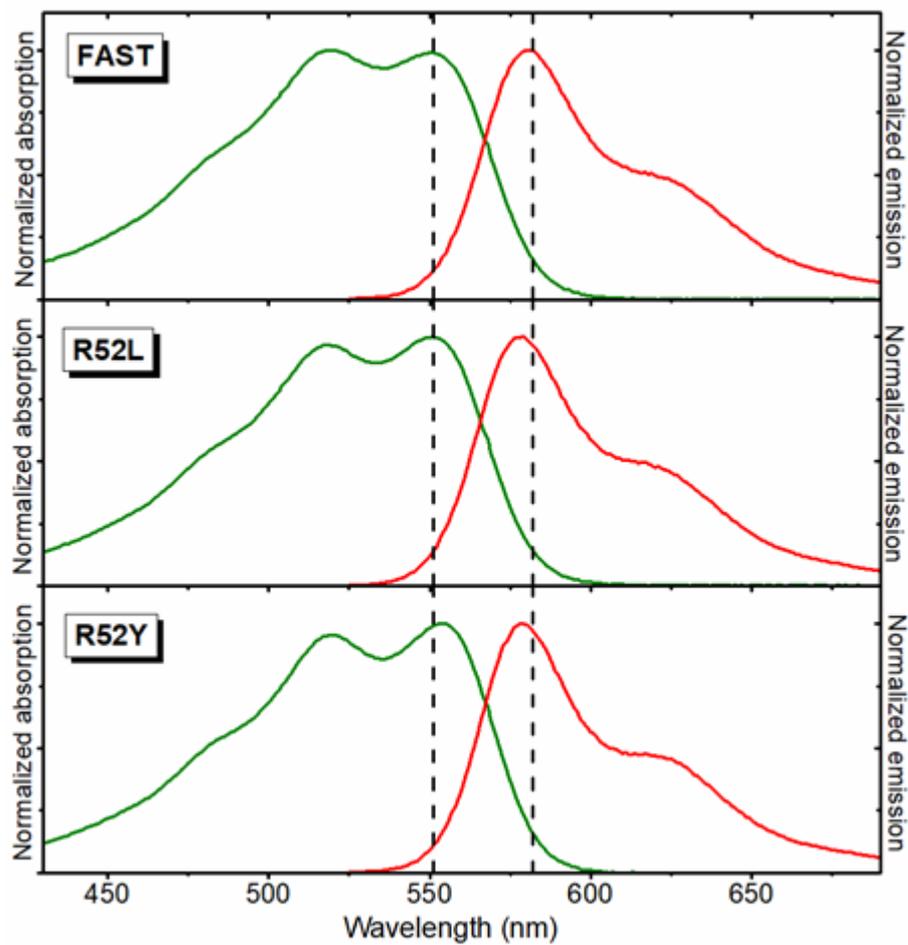


Figure S16. Fluorescence (red) and absorption (green) spectra of **MCL.1b** complexes with FAST mutants.

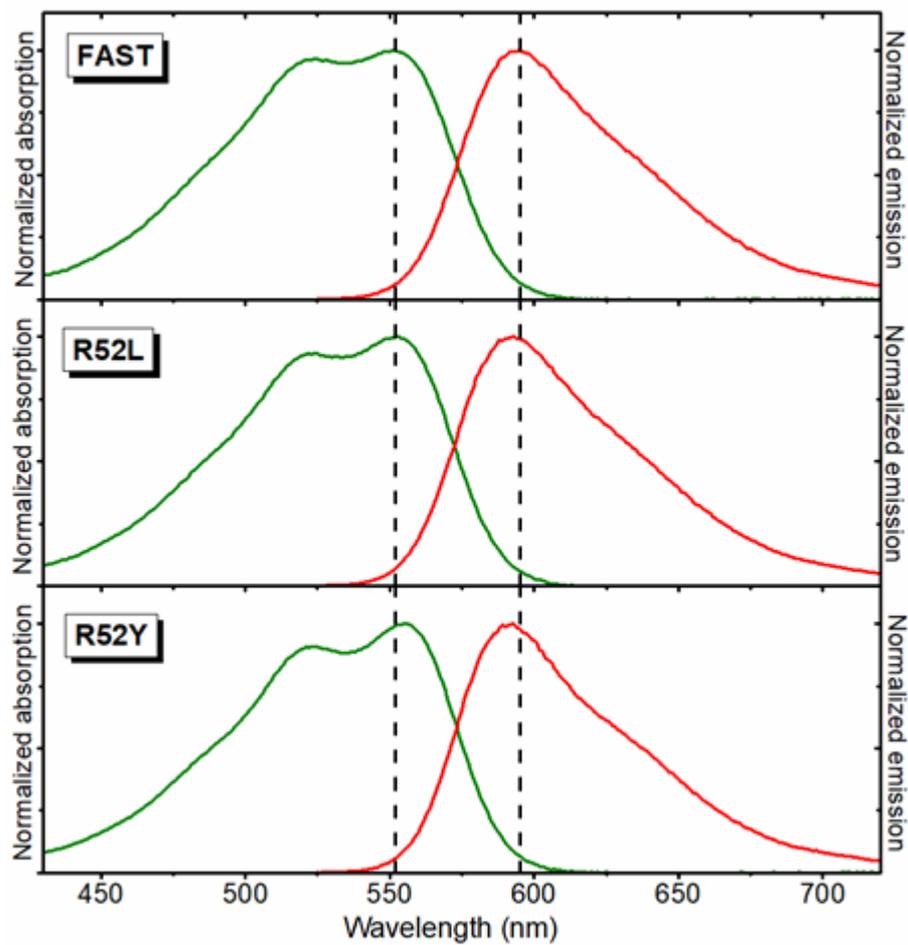


Figure S17. Fluorescence (red) and absorption (green) spectra of **MCL.1f** complexes with FAST mutants.

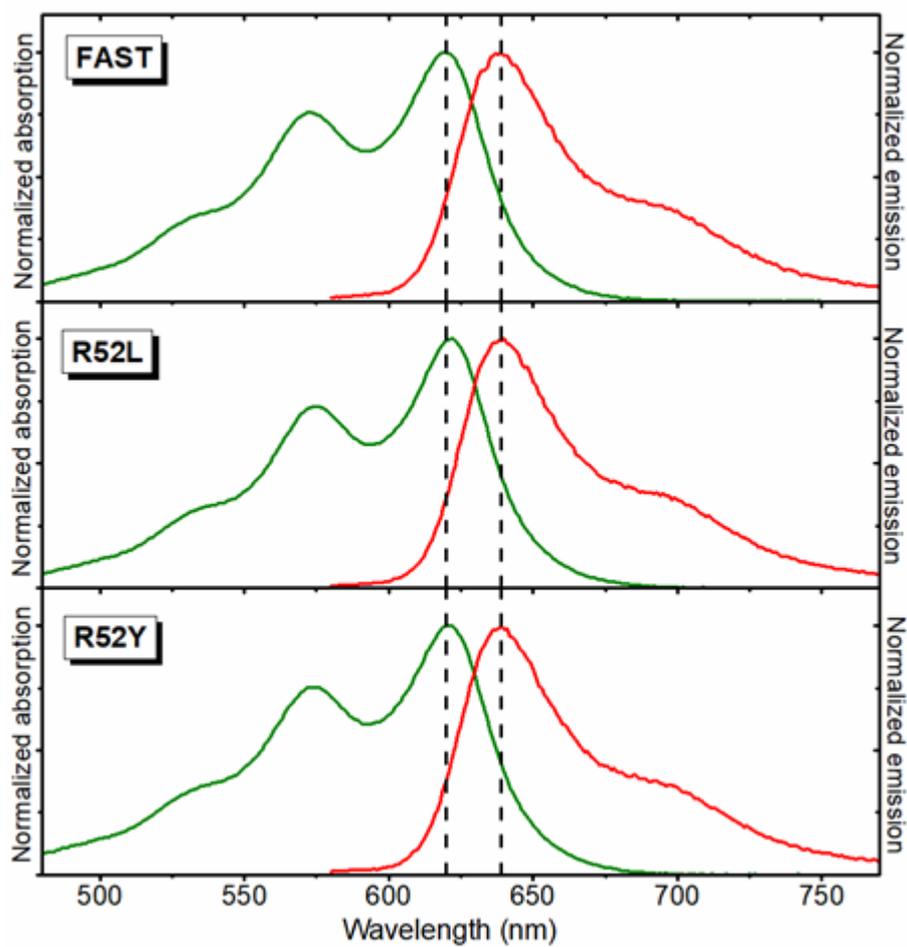


Figure S18. Fluorescence (red) and absorption (green) spectra of **MCL.3a** complexes with FAST mutants.

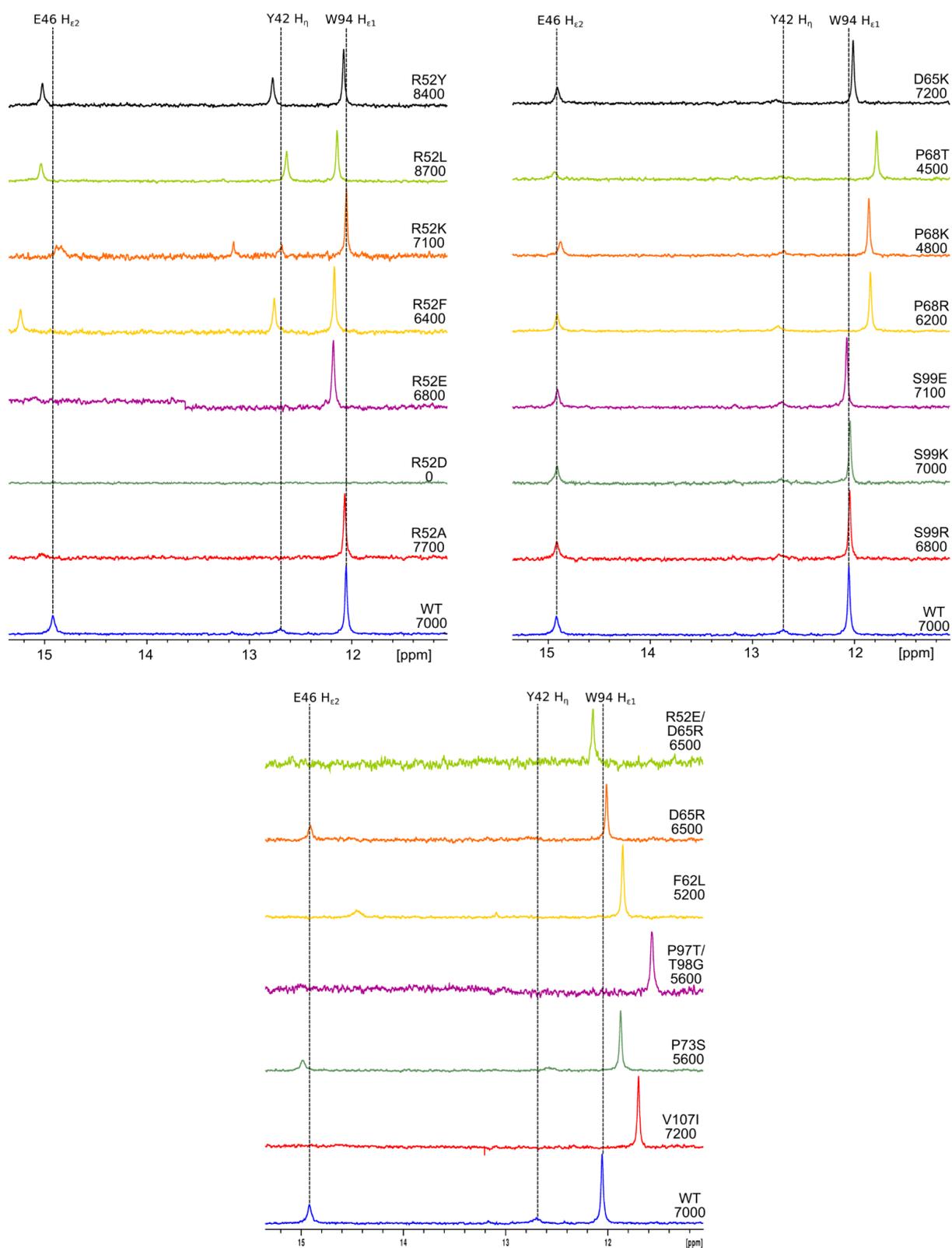


Figure S19. The overlay of ^1H NMR spectra of FAST/**N871b** complexes, recorded for the wild-type protein and its mutants. Spectra were obtained at 25 °C, molecular brightness of the complex is indicated.

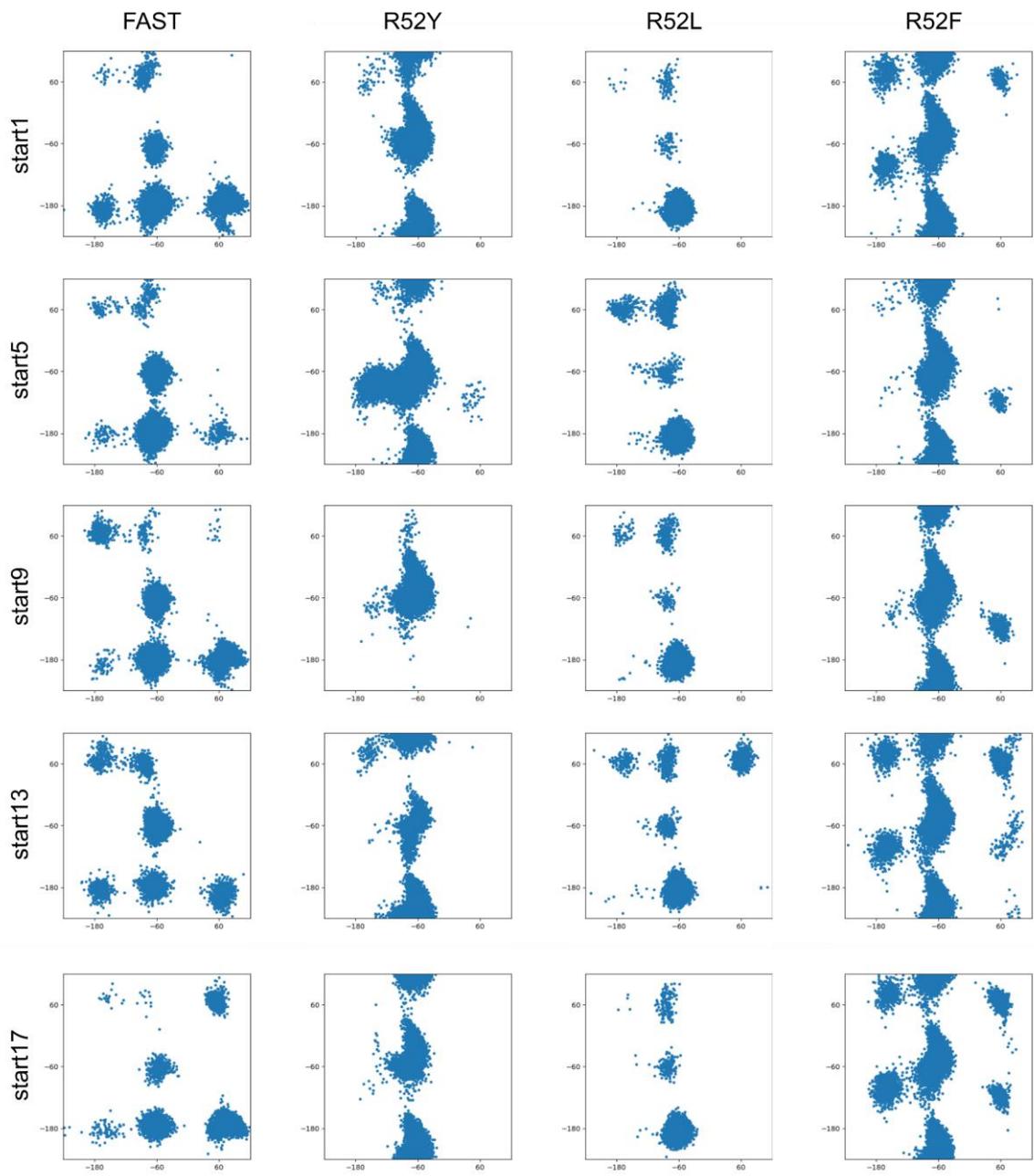


Figure S20. χ_1/χ_2 distributions obtained for the sidechain of #52 residue in the course of 5 independent MD simulations, started from various NMR models in PDB ID 7AVA (models 1, 5, 9, 13 and 17).

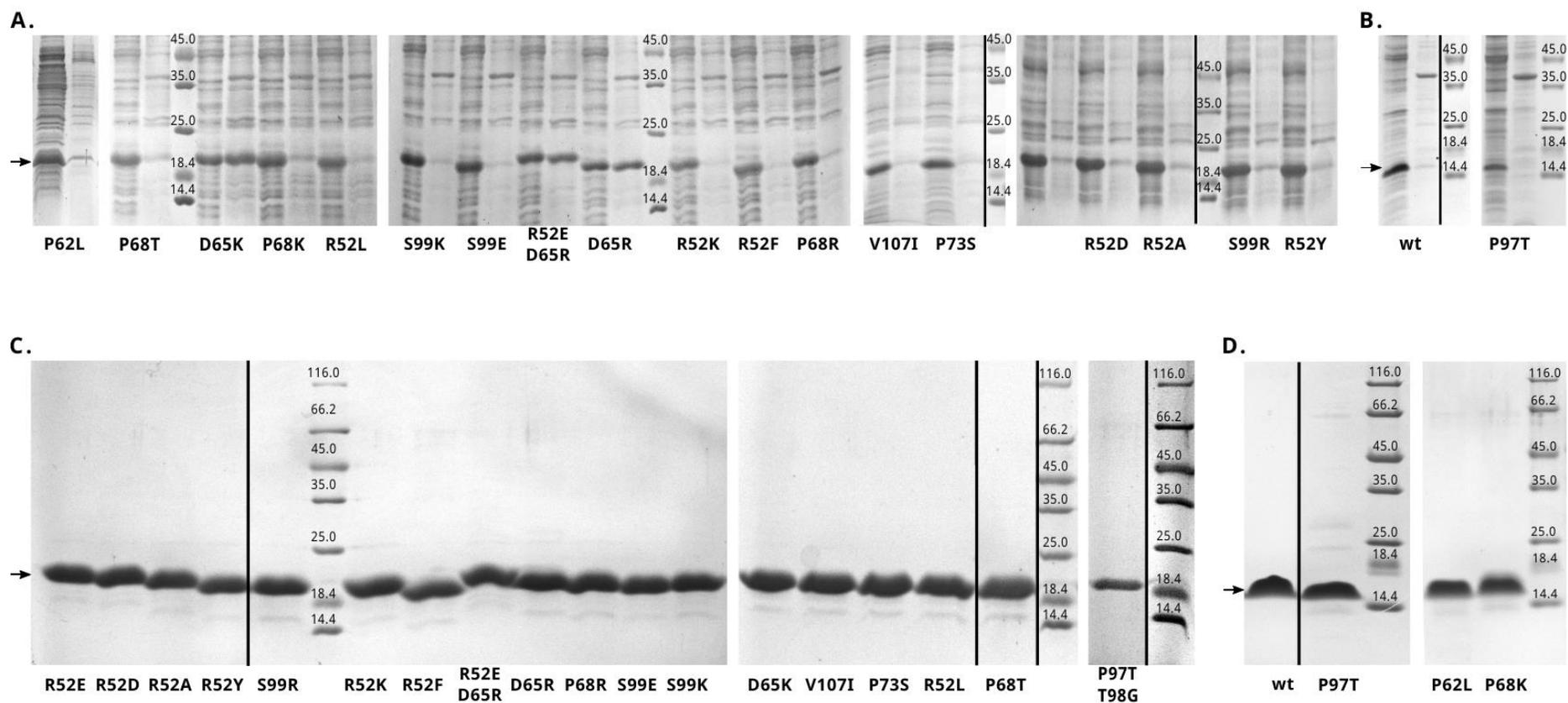


Figure S21. Bacterially expressed and purified mutant variants of FAST used for fluorogenic properties tests. **A, B** - Cellular accumulation of FAST mutants. For each mutant a pair of soluble and insoluble cellular protein samples is applied to Tris-Glycine-SDS-PAGE (**A**) or Tris-Tricine-SDS-PAGE (**B**). An equivalent to 25 μ l of M9 medium is loaded into each line. **C, D** - Purified FAST mutants. An aliquot of 2.5 μ g of each variant (except for P97T-T98G) is applied on Tris-Glycine-SDS-PAGE (**C**) or Tris-Tricine-SDS-PAGE (**D**). The protein bands corresponding to the FAST variants are indicated with an arrow. Protein molecular weight marker: 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kDa. Molecular weight of the wild type of FAST (wt) is 14.7kDa.

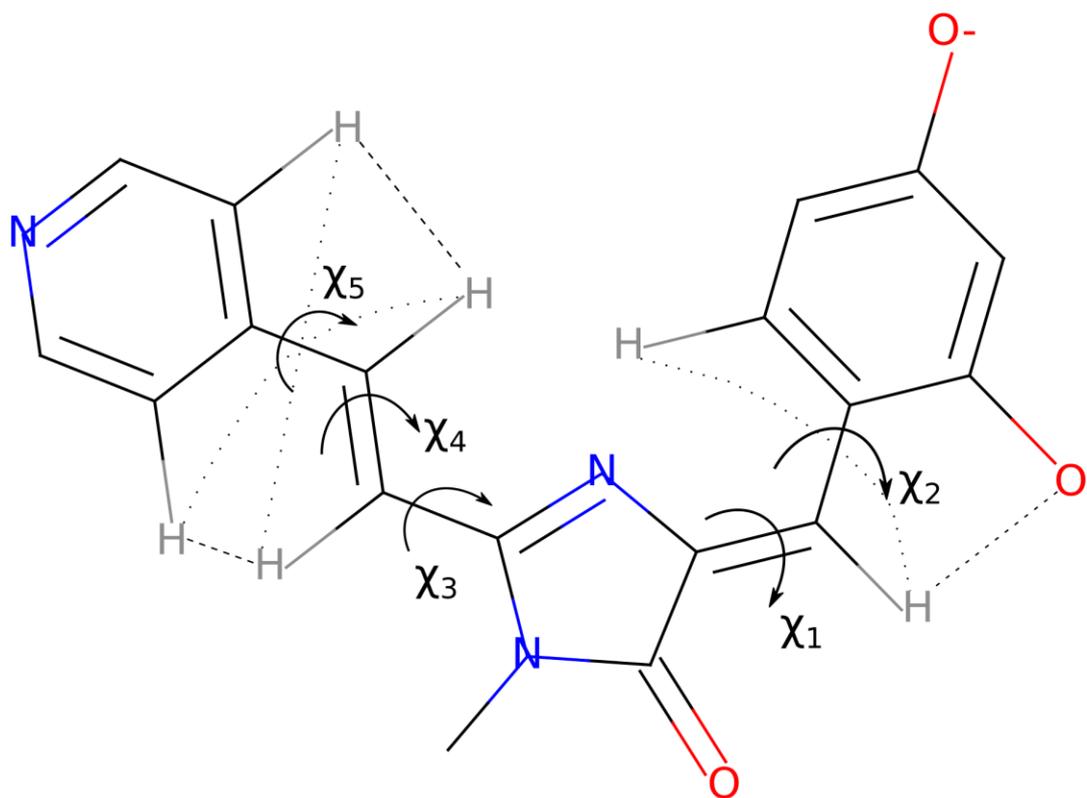


Figure S22. The chemical structure of **N871b**. Torsion angles χ_1 - χ_5 and excluded nonbonded interactions are depicted. Nonbonded interactions that shift energy minimum from planar conformation are shown by dashed (this conformation) or dotted (after rotation of χ_2 or χ_5 by 180°) lines. Hydrogen atoms not included in excluded nonbonded list are not shown for clarity.

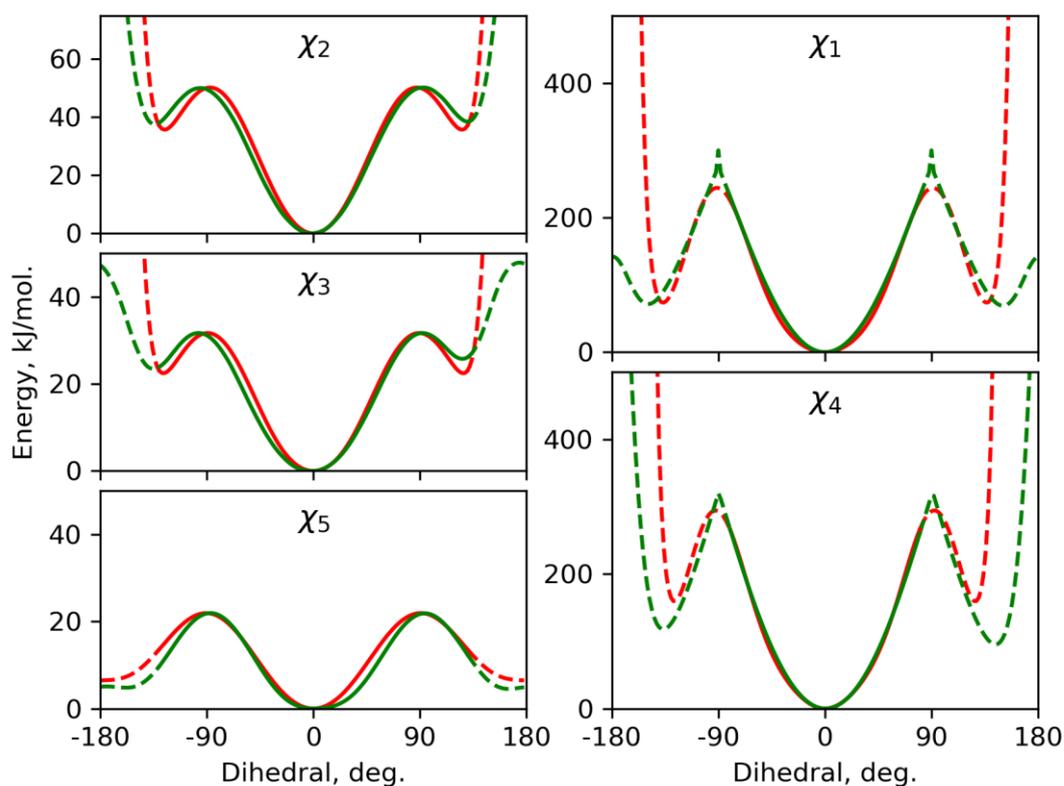


Figure S23. The Gromacs (red) and GAMESS (green) energies of **N871b** plotted versus dihedral angles. Energy profiles were calculated independently for dihedrals $\chi_1 - \chi_5$ starting from energy minimized planar conformation of **N871b** by rotating corresponding dihedral within range $-180^\circ - 180^\circ$ with step of 2° . Dashed lines indicate regions where energy disturbed by nonbonded interactions. These regions were not taken into account during development of dihedral parameters for MD simulation of **N871b**.

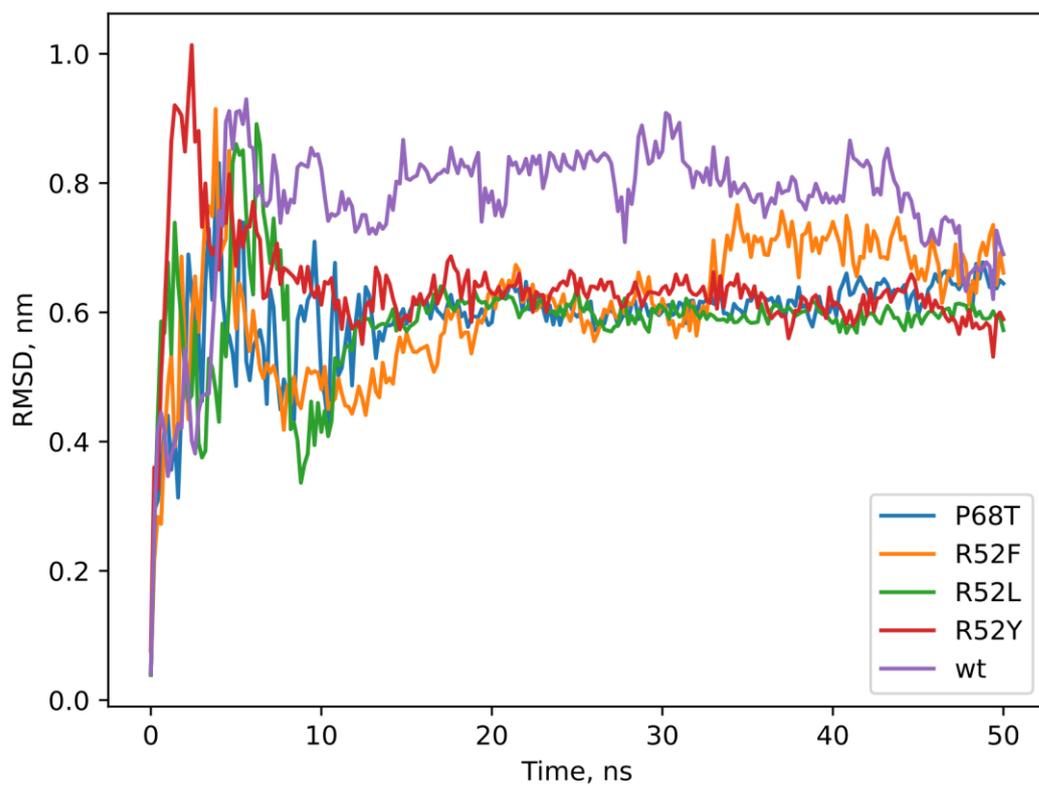


Figure S24. RMSD of all atoms of protein-**N871b** complexes from starting (energy minimized) structure during equilibration stage of MD simulation. Result of MD simulation of FAST (model 1 in PDB ID 7AVA, wt) and its 4 mutants are shown.

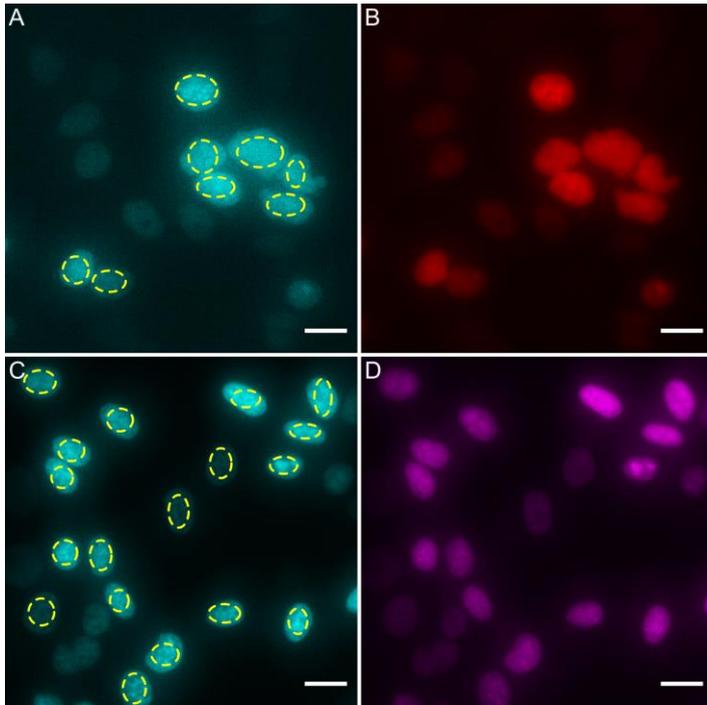


Figure S25. Representative images of HeLa Kyoto nuclei used to evaluate relative brightness of FAST with different chromophores. HeLa Kyoto cells transiently transfected with H2B-TagBFP-FAST/R52L construct with addition of **MCL.1f** (A, B), and **MCL.3a** (C, D) chromophores imaged in Blue channel (A, C), mCherry channel (B), and CY5 channel (D). Scale bars are 20 μm . Yellow dashed circles represent selected ROIs for relative brightness evaluation.

Table S1. Aminoacid sequences of the synthesized mutants. Aminoacids which are extra to the FAST protein sequence are shown in brackets.

mutant	sequence
FAST WT	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
D65K	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFK K VAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
D65R	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFK R VAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
F62L	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKN L FKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
P68K	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDV A KGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
P68R	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDV A RGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
P68T	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDV A TGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
P73S	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDS S EFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
P97T	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMI T TSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
P97T/T98G	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMI TG SRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)

R52A	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG A DPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
R52E	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG E DPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
R52E/D65R	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG E DPKQVIGKNFFK R VAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
R52F	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG F DPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
R52K	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG K DPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
R52L	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG L DPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
R52Y	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG Y DPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
R52D	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITG D DPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
S99E	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPT E RGF TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
S99K	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPT K RGF TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
S99R	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPT R RGF TKVKVHMKKALSGDSYWVFKRV (GGGHHHHHH)
V107I	(M) EHVAFGSEDIEN TLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKNFFKDVAPGTDSPEFYGKFKEGVASGNLNTMFEWMIPTSRGP TKVK I HMKKALSGDSYWVFKRV (GGGHHHHHH)

Table S2. Optical properties of complexes **N871b** with FAST mutants.

Mutant	K_D , μM ^a	ϵ , $\text{M}^{-1}\cdot\text{cm}^{-1}$ ^b	Fluorescence quantum yield (FQY), % ^c	Brightness ($=\epsilon\cdot\text{FQY}$)	Absorbance maxima position, nm	Emission maxima position, nm
FAST WT	0.33±0.01	27000±410	26±1.4	7000±480	553	609
D65K	0.25±0.01	27500±410	26±2.1	7100±680	555	607
D65R	0.34±0.04	27000±410	24±1.2	6450±420	554	609
F62L	0.55±0.08	27500±410	19±0.9	5250±330	552	611
P68K	0.22±0.01	26500±400	18±0.7	4750±260	548	610
P68R	0.31±0.03	27000±410	23±0.4	6100±200	547	609
P68T	0.41±0.03	26500±400	17±1.5	4550±470	527	604
P73S	0.17±0.01	28000±420	20±1.2	5600±420	558	610
P97T	na ^d	na ^d	na ^d	na ^d	na ^d	na ^d
P97T/T98G	na ^d	na ^d	na ^d	na ^d	na ^d	na ^d
R52A	0.79±0.05	28500±430	27±1.0	7700±400	558	607
R52E	1.30±0.07	26000±390	26±0.2	6700±150	556	607
R52E/D65R	1.36±0.22	25000±380	26±1.5	6500±470	556	605
R52F	0.54±0.04	28000±420	23±0.4	6450±210	557	606
R52K	0.42±0.02	29500±440	24±1.2	7100±460	549	605
R52L	0.27±0.01	30000±450	29±1.7	8750±640	556	604
R52Y	0.24±0.02	29000±440	29±1.5	8350±560	558	605
R52D	na ^d	na ^d	na ^d	na ^d	na ^d	na ^d
S99E	0.21±0.01	28500±430	25±1.1	7050±420	556	608
S99K	0.27±0.01	28000±420	25±0.3	7050±190	556	609
S99R	0.24±0.01	28500±430	24±1.2	6800±440	555	608
V107I	0.38±0.05	26500±400	27±3.2	7150±950	555	607

a – represented as mean ± SD (n = 3);

b – represented as result of single measurement ± the precision of the measuring instruments (weighing and pipetting errors);

c – represented as mean ± SD (n = 9, except **R52E** and **R52E/D65R** where n = 3);

d – data was not obtained. due to the poor fluorescence of the complex.

Table S3. NMR parameters of studied FAST/**N871b** complexes

mutant	NMR parameters					
	E46 I ^a	E46 c.s. ^b	Y42 I	Y42 c.s.	W94 I	W94 c.s.
FAST WT	0.20	14.92	0.06	12.69	0.74	12.05
D65K	0.20	14.91	0.04	12.77	0.77	12.01
D65R	0.21	14.91	0 ^c		0.78	12.01
F62L	0.07	14.44	0.05	13.09	0.73	11.85
P68K	0.19	14.88	0.07	12.71	0.81	11.86
P68R	0.22	14.71	0.07	12.75	0.78	11.84
P68T	0.12	14.93	0		0.78	11.78
P73S	0.13	14.98	0.04	12.57	0.75	11.87
P97T	n.a. ^d					
P97T/T98G	0		0		0.36	11.57
R52A	0.06	15.04	0		0.77	12.07
R52E	0		0		0.67	12.18
R52E/D65R	0		0		0.58	12.15
R52F	0.26	15.23	0.38	12.76	0.74	12.17
R52K	0.11	14.86	0.12	13.15/12.68 ^e	0.69	12.05
R52L	0.26	15.03	0.45	12.63	0.76	12.14
R52Y	0.30	15.02	0.38	12.77	0.77	12.08
R52D	0		0		0	
S99E	0.19	14.91	0.06	12.72	0.74	12.07
S99K	0.21	14.91	0.05	12.74	0.77	12.04
S99R	0.19	14.91	0.05	12.75	0.76	12.05
V107I	0		0		0.80	11.7

^aI - integral of the corresponding signal in 1D NMR spectrum, relatively to 1/3 integral of the FAST methyl group signal at -0.66 ppm.

^bChemical shifts of Y42 H η , E46 H ϵ 2, and H ϵ 1 W94 in ppm, measured at 25 °C relative to the signal of TSP at 0.0 ppm.

^c0 - the signal is not observed

^ddata was not obtained, due to the low protein yields and poor fluorescence of the protein in complex with **N871b**.

^eFor R52K two states are observed with 1:1 intensity ratio. See Figure S3.

Table S4. Mutations, found to enhance the activity of FAST, according to the literature.

target	name	mutations												
HBR-3OM	iFAST	V107I												
HMBR	V122I	V122I												
HMBR	gFAST	G21E	P68T	G77R										
HMBR	gFAST1	F62L	P68S	T70K	Y76F	K80N								
HMBR	gFAST2	P68T	T70K											
HMBR	gFAST3	S8R	F62L	P68H	T70P	K80N								
HMBR	gFAST4	P68T	F75L	E93D										
HMBR	gFAST5	G35S	D36G	S72T	E93D	V107M								
HMBR	gFAST6	P68T	T70R											
HMBR	gFAST7	Q41R	E93D	V107M										
HMBR	gFAST8	P68T	T70K	E93V	G115S									
HBR-3,5DOM	rFAST	F28L	E46Q	R52A	E81V	S99N								
HBR-3,5DOM	rFAST1	K17R	D19G	F28L	A30T	E46Q	K60R							
HBR-3,5DOM	rFAST2	A30V	R52S	K60R	V83A	K111R	S117C	Y118F						
HBR-3,5DOM	rFAST3	G21R	F28L	E46Q										
HBR-3,5DOM	rFAST4	F28L	E46Q	S117R										
HBR-3,5DOM	rFAST5	L33F	Q41H	E46Q	K111N									
HBR-3,5DOM	rFAST6	R52A	K80M	S99I										
HBR-3,5DOM	rFAST7	R52A	E81V	S99N										
HBR-3,5DOM	rFAST8	D20H	F28I	E46Q										
HBO-3M	AR68	D65V	E93D	M109L	S117R									
HBO-3M	AR611	V83I	M109L											
HBO-3M	AR71	M109L												
HBO-3M	AR72	Q41L	M109L											
HBO-3M	AR710	V83I	T103I											
HBO-3M	AR712	K17R	A30V	E74K	E93V									
HBO-3M	AR714	T50S	E93Q	M95I										
HPAR-3OM	frFAST	F62L	D71V	P73S	E74G	V107I								
HBP-3,5DM	AR53	Q41H	V83L	M95I										
HBP-3,5DM	AR57	Q41K	S72T	V83A	M95I									
HBP-3,5DM	AR512	K17N	Q41K	M95T										
HBP-3,5DM	AR513	Q41L	S117I											
HBP-3,5DM	AR521	V83E	S117R											
HBP-3,5DM	AR62	E93G	M95V	M109L										
HBP-3,5DM	AR71	D20N	E81Q	V83I	M109L									
HBP-3,5DM	AR76	G25R	A84S											
HBP-3,5DM	AR710	Q32K	Y76N											
HBP-3,5DM	BR52	K17N	Q41K	S72T	M95T									
HBP-3,5DM	BR511	K17N	G25E	S72T	V83A	M95T								
HBP-3,5DM	BR519	Q41K	K80M	A84S	N89D	M95T	M109L							
HBP-3,5DM	BR61	K17N	G21E	G25R	A30V	Q41L	S72T	V83A	M95T	S117R				
HBP-3,5DM	BR610	Q41K	S72T	V83A	S117R									
HBP-3,5DM	BR615	N13S	Q41K	S72T	V83A	M95T								
HBP-3,5DM	BR619	K17I	G25R	Q41K	A44T	K60R	V38A	N89D	E93K	M95T	M109L	S117R		

HBP-3,5DM	BR73	K17N	Q41K	D65E	S72T	V83A	K106M	M109L				
HBT-3,5DM	BR611	A16D	Q41K	Y76H	V83A	M95T	M109L	S117R				
HBT-3,5DM	tFAST	G25R	Q41K	S72T	A84S	M95A	M109L	S117R				
HBT-3,5DM	BR617	A27V	Q41K	S72T	A84S	M95A	M109L	S117R				
HBT-3,5DM	BR624	K17N	G21E	G25R	A30V	Q41L	S72T	V83A	M95T	M109L		
HBT-3,5DM	BR72	Q41K	K80R	V83A	M95T	M109L						
HBT-3,5DM	BR74	Q41K	M95T	S117I								
HBO-3,5DOM	BR67	G7D	Q41K	S72T	A84S	M95I	M109L	S117R	R124L			
HBO-3,5DOM	BR73	K17N	Q41K	A84S	M95T	M109L	S117R					
HBO-3,5DOM	BR77	Q41K	D48G	S72T	V83A	M95T	M109L	S117R				
HBO-3,5DOM	BR722	G21R	G25R	Q41K	Q60E	M95T	M109L	A112G				
HMBR	mutant1	F62L	P68C	D71R	P73S							
HMBR	mutant2	D19N	F62L	P68C	D71R	P73S	M95T	S99K				
HMBR	mutant3	F62L	P68E	D71R	P73S	M95T	S99K					