

Supplementary Table 1. Amino acid positions and mutations identified from the screen.

amino acid positions identified from residue map stage	wild-type sequence	target mutation stage	target checking stage
3	Y	-	-
18	T	-	-
19	N	-	-
47	G	-	-
60	A	-	-
62	F	-	-
71	A	S	S
74	T	-	-
75	W	-	-
77	S	-	-
84	I	LV	LV
88	A	LSTV	not found
90	E	ADFNPQT	ADNPQ
91	M	FILST	FL
93	K	-	-
94	V	LT	L
95	I	-	-
98	F	AMRV	MV
99	F	AEMRTV	AERTV
100	F	not found	-
102	F	GLNPST	LNPST
103	K	AGHIPRTW	GPTW
111	A	CGHLS	CGLS
112	T	DGNPRSW	GNS
114	H	AGV	GV
115	R	PS	PS
117	Q	LPY	LPY
123	E	A	A
132	L	ACG	C
133	I	CLP	not found
134	H	KR	KR
136	S	-	-
151	G	-	-
173	V	-	-
181	G	-	-
185	G	-	-
187	N	not found	-

188	T	L V	L
202	T	A L R S	not found
205	K	A E P Q R S T V	A V
206	G	not found	-
210	Q	L V	L V
214	G	-	-
246	T	-	-
250	T	I Q	I Q
251	I	-	-
269	V	-	-
272	H	-	-
273	E	-	-

In order to prioritize residues to screen, we selected amino acid positions from the residue map stage with the highest improvement (A88, E90, M91, F98, F99, F102, K103, T112, H114, R115, Q117, H134, N187, T188, T202, K205, G206, Q210, T250; normalized mean > 1.5 from **Supp. Fig. 1b**) as well as those that were near the 1.5-fold changed residues (A71, I84, V94, F100, A111, E123, L132) to screen for target mutations. ‘-’ indicates that the amino acid position has not been selected according to this criteria. ‘not found’ indicates amino acid positions that have been screened but mutations with statistically significant improvement have not been identified.

Supplementary Table 2. Layout of site-saturation libraries screened in the residue map stage.

Empty					
Water					
Wild-type	Library 1	Library 2	Library 3	...	Library 11
Wild-type	Library 1	Library 2	Library 3		Library 11
Wild-type	Library 1	Library 2	Library 3		Library 11
Wild-type	Library 1	Library 2	Library 3		Library 11
Water					
Empty					

Supplementary Table 3. Layout of mutants screened in the target mutation stage.

Water	Water							Water	Empty
	Wild-type	Mutant 1	Mutant 2		Mutant 6	Mutant 7	Mutant 8		
	Mutant 8	Mutant 7	Mutant 6	...	Mutant 2	Mutant 1	Wild-type		
	Wild-type	Mutant 9	Mutant 10		Mutant 14	Mutant 15	Mutant 16		
	Mutant 16	Mutant 15	Mutant 14	...	Mutant 10	Mutant 9	Wild-type		
	Wild-type	Mutant 17	Mutant 18		Mutant 22	Mutant 23	Mutant 24		
	Mutant 24	Mutant 23	Mutant 22	...	Mutant 18	Mutant 17	Wild-type		
	Water								

Supplementary Table 4. Layout of mutants screened in the target checking stage.

Water	Water							Water	Empty
	Wild-type	Mutant 1	Mutant 2		Mutant 6	Mutant 7	Mutant 8		
	Mutant 8	Mutant 7	Mutant 6	...	Mutant 2	Mutant 1	Wild-type		
	Wild-type	Mutant 1	Mutant 2		Mutant 6	Mutant 7	Mutant 8		
	Mutant 8	Mutant 7	Mutant 6	...	Mutant 2	Mutant 1	Wild-type		
	Water								
	Empty								
	Empty								

Supplementary Table 5. Composition of intracellular and extracellular solutions used to assess the ion selectivity of channelrhodopsins.

Solution	[Na] (mM)	[K] (mM)	[Ca] (mM)	[H] (mM)	pH	Other (mM)
Intracellular	0	140	0	5.10E-05	7.40	5 EGTA, 2 MgCl ₂ , 10 HEPES
145 mM NaCl	145	5	1	5.10E-05	7.40	10 HEPES, 5 glucose, 2 MgCl ₂
145 mM KCl	0	145	1	5.10E-05	7.40	10 HEPES, 5 glucose, 2 MgCl ₂
90 mM CaCl ₂	0	5	90	5.10E-05	7.40	10 HEPES, 5 glucose, 2 MgCl ₂
135 mM NMDG	5	5	1	5.10E-04	6.40	135 NMDG, 10 HEPES, 5 glucose, 2 MgCl ₂