

Supplementary Information

Orthogonal fluorescent chemogenetic reporters for multicolor imaging

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Sequences

The sequence of CFAST is in bold

DNA Sequence of greenFAST

atggagcatgttgcctttggcagtgaggacatcgagaacactctggcctaaaatggacgacgaacaact
ggatgggttggcctttggcgcaattcagctcgatggtgacgggaatatcctgcagtacaatgctgctg
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ggaacggattctcccaggtttaccgcaaattcaaggaaggcgtagcgtcagggaatctgaacacat
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Protein sequence of green FAST

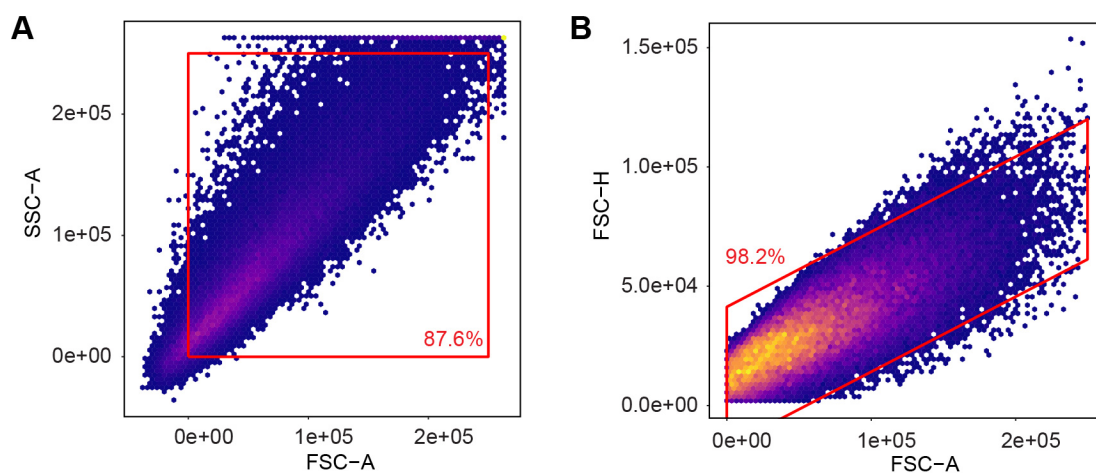
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GTDSPEFYRKFKEGVASGNLNTMFEWMIPTSRGPTKVKVHMKKAL**SGDSYWVFVKRV**

DNA sequence of redFAST

atggagcatgttgcctttggcagtgaggacatcgagaacactctggcctaaaatggacgacggacaact
ggatgggttggccttaggcgcaattcagctcgatggtgacgggaatatcctgcagtacaatgctgctc
agggagacatcacaggcgcagatcccaaacaggtgattgggaagaacttcttcaaggatggtgcacct
ggaacggattctcccaggtttaccgcaaattcaaggtaggcgtagcgtcagggaatctgaacacat
gttcgaatggatgataccgacaacaggggaccaaccaaggtcaaggtgcacatgaagaagccctt
ccggtgacagctattgggtctttgtgaaacgggtg

Protein sequence of redFAST

MEHVAFGSEDIENTLAKMDDGQLDGLALGAIQLDGDGNIQYNAAQGDITGADPKQVIGKNFFKDVAP
GTDSPEFYGKFKVGVASGNLNTMFEWMIPTNRGPTKVKVHMKKAL**SGDSYWVFVKRV**



Supplementary Figure 1. Gating strategy. The graphs show the gating strategy used during the cytometry analysis presented on Figure 1e,g to select non-debris cells (A) and cell singlets (B). 130,000 events were analyzed.

Supplementary Table 1. Clones isolated from the green selection

Clones	Number of appearances	Mutations	K_D for HMBR (μM)	K_D for HBR-3,5DOM (μM)
1	7	G21E, P68T, G77R	0.09	16.2
2	1	F62L, P68S, T70K, Y76F, K80N	0.05	7.2
3	1	P68T, T70K	n.d.	n.d.
4	1	S8R, F62L, P68H, T70P, N87D	n.d.	n.d.
6	4	P68T, F75L, E93D	0.13	13.0
7	1	G35S, D36G, S72T, E93D, V107M	0.08	3.3
12	1	P68T, T70R	n.d.	n.d.
21	2	Q41R, E93D, V107M	0.10	4.4
24	1	P68T, T70K, E93V, G115S	n.d.	n.d.

n.d. not determined

Supplementary Table 2. Clones isolated from the red selection

Clones	Number of appearances	Mutations	K_D for HMBR (μM)	K_D for HBR-3,5DOM (μM)
1	3	K17R, D19G, F28L, A30T, E46Q, K60R	0.92	0.51
2	7	A30V, R52S, K60R, V83A, K111R, S117C, Y118F	1.60	0.6
4	2	G21R, F28L, E46Q	0.98	0.78
5	1	F28L, E46Q, S117R	1.47	0.88
6	1	L33F, Q41H, E46Q, K111N	1.72	1.0
7	1	R52A, K80M, S99I	1.45	1.0
10	6	R52A, E81V, S99N	1.75	1.0
17	1	D20H, F28I, E46Q	1.1	0.84

Supplementary Table 3. Rationally designed clones

Plasmid	Mutations	K_D for HMBR (μM)	K_D for HBR-3,5DOM (μM)
302	green clone 6 V107M	0.05	16.4
303	green clone 21 P68T	0.13	15.6
304	green clone 21 P68T T70K	0.10	7.4
305	green clone 21 V122I	0.55	25.5
306	red clone 7 I99N	1.33	0.7
307	red clone 10 F28L	9.4	6.4
308	red clone 10 F28L E46Q	12	1.2

Supplementary Table 4. Average fluorescence lifetime determination of FAST:fluorogen complexes

protein	fluorogen	monoexponential fit	biexponential fit	
		τ (ns)	τ_1 (ns)	τ_2 (ns)
iFAST	HMBR	1.50 ± 0.02	1.7 ± 0.02	0.7 ± 0.07
iFAST	HBR-3,5DOM	2.62 ± 0.06	2.77 ± 0.05	0.5 ± 0.2
greenFAST	HMBR	1.11 ± 0.01	1.18 ± 0.09	0.4 ± 0.4
greenFAST	5:10*	1.10 ± 0.01	1.22 ± 0.09	0.6 ± 0.4
redFAST	HBR-3,5DOM	2.42 ± 0.03	2.48 ± 0.07	0.4 ± 0.5
redFAST	5:10*	2.39 ± 0.05	2.46 ± 0.03	0.13 ± 0.09

* in presence of 5 μ M HMBR + 10 μ M HBR-3,5DOM

See Supplementary Tables 5-10 for individual fit results.

Supplementary Table 5. Fluorescence lifetime determination of iFAST:HMBR.

	monoexponential fit			biexponential fit				
	A	τ (ns)	χ^2	A1	τ_1 (ns)	A2	τ_2 (ns)	χ^2
Cell1	10578.46	1.475	4.472	7092.61	1.716	4934.4	0.729	0.973
Cell2	14741.84	1.509	4.826	10437.04	1.718	6104.15	0.743	0.987
Cell3	13313.04	1.471	5.762	8398.85	1.742	6707.23	0.772	1.029
Cell4	4073.04	1.509	1.935	3063.62	1.689	1523.5	0.679	0.975
Cell5	7365.25	1.519	2.77	5432.43	1.711	2841.34	0.709	0.976
Cell6	14109.8	1.485	4.934	9950.92	1.695	5976.06	0.72	0.994
Cell7	3942.55	1.511	1.9	3024.85	1.682	1433.01	0.65	0.961
Cell8	4792.98	1.512	2.012	3518.86	1.703	1838.22	0.725	0.922
Cell9	7427.48	1.513	2.724	5139.95	1.731	3103.13	0.794	1.01
Cell10	11558.12	1.468	5.301	7410.5	1.732	5775.46	0.747	1.069
Cell11	4528.78	1.504	1.969	3358.88	1.689	1723.41	0.7	0.911
Cell12	5223.34	1.479	2.603	3818.87	1.676	2211.79	0.643	0.924
Cell13	11762.86	1.498	4.163	8864.49	1.677	4537.6	0.645	0.961
Cell14	6579.2	1.487	3.014	4726.88	1.693	2805.27	0.67	0.934
Cell15	8212.45	1.515	2.748	6425.35	1.673	2820.98	0.645	0.988
Cell16	5028.68	1.508	2.009	3516.45	1.717	2039.22	0.798	0.954
Cell17	6203.44	1.492	2.981	4239.95	1.726	2806.52	0.729	1.026
Cell18	4904.13	1.494	2.69	3928.75	1.642	1899.4	0.492	1.131
Cell19	9044.41	1.478	3.958	5851.78	1.736	4399.14	0.763	0.911
Cell20	12247.06	1.486	4.79	8476.43	1.707	5404.3	0.725	1.055

Supplementary Table 6. Fluorescence lifetime determination of greenFAST:HMBR

	monoexponential fit			biexponential fit				
	A	τ (ns)	χ^2	A1	τ_1 (ns)	A2	τ_2 (ns)	χ^2
Cell1	27370.09	1.113	2.057	26939.49	1.121	26696.94	0.033	1.664
Cell2	14098.96	1.128	1.395	11391.21	1.198	3150.07	0.726	1.092
Cell3	17045.11	1.112	1.706	16735.95	1.121	14765.24	0.038	1.406
Cell4	9503.22	1.132	1.227	5862.89	1.259	4022.13	0.876	1.022
Cell5	13197.7	1.129	1.293	12972.02	1.141	1302.8	0.163	1.15
Cell6	13836.96	1.129	1.337	11161.69	1.205	3318.65	0.729	0.999
Cell7	18519.36	1.112	1.369	7338.28	1.306	11600.95	0.948	0.996
Cell8	21487.22	1.115	1.544	21224.2	1.122	2826.47	0.117	1.333
Cell9	16927.76	1.11	1.393	16522.99	1.115	3091.94	0.08	1.248
Cell10	26328.43	1.086	2.526	17419.74	1.216	10552.16	0.736	1.068
Cell11	17946.31	1.103	1.725	17647.59	1.11	18925.53	0.031	1.485
Cell12	4859.09	1.121	1.133	4710.63	1.129	11053.38	0.014	1.036
Cell13	6036.19	1.122	1.128	1799.51	1.417	4442.82	0.954	0.888
Cell14	9214.84	1.078	1.508	9018.14	1.088	7124.8	0.044	1.309
Cell15	4349.51	1.107	0.9	3387.54	1.19	1165.22	0.741	0.791

Supplementary Table 7. Fluorescence lifetime determination of greenFAST in presence of both fluorogens (5 μ M HMBR + 10 μ M HBR-3,5DOM)

	monoexponential fit			biexponential fit				
	A	τ (ns)	χ^2	A1	τ_1 (ns)	A2	τ_2 (ns)	χ^2
Cell1	11173.58	1.078	1.303	7705.49	1.194	4133.85	0.725	0.768
Cell2	6930.13	1.105	1.248	6203.97	1.156	1177.52	0.504	0.987
Cell3	18878.47	1.107	1.7	18509.18	1.113	33085.25	0.019	1.472
Cell4	5446.24	1.107	0.847	5236.14	1.112	72432.55	0.003	0.804
Cell5	10142.73	1.076	1.76	9783.5	1.093	6448.44	0.064	1.369
Cell6	16119.46	1.104	1.577	8451.9	1.274	8452.32	0.859	0.958
Cell7	15506.13	1.113	1.43	15037.19	1.116	9696.21	0.02	1.355
Cell8	16499.81	1.089	1.733	9112.22	1.252	8263.47	0.828	0.967
Cell9	5101.52	1.124	1.029	5019.57	1.135	582.18	0.14	0.975
Cell10	7193.56	1.103	1.143	3666.89	1.287	3891.32	0.855	0.841
Cell11	15727.38	1.112	1.359	7656.04	1.286	8733.3	0.902	0.933
Cell12	24572.01	1.097	1.928	13650.78	1.255	12148.35	0.838	0.971
Cell13	12991.7	1.106	1.388	4386.7	1.353	9150.58	0.944	0.978
Cell14	12118.07	1.109	1.379	3783.49	1.383	8864.49	0.947	0.94
Cell15	13471.46	1.095	1.386	8461.83	1.227	5686.63	0.808	0.898

Supplementary Table 8. Fluorescence lifetime determination of iFAST:HBR-3,5DOM

	monoexponential fit			biexponential fit				
	A	τ (ns)	χ^2	A1	τ_1 (ns)	A2	τ_2 (ns)	χ^2
Cell1	1965.42	2.586	1.947	1736.53	2.77	704.13	0.457	1.078
Cell2	2639.56	2.642	1.782	2412.05	2.778	662.7	0.496	1.172
Cell3	2201.31	2.596	1.918	1950.74	2.777	723.07	0.49	1.072
Cell4	4469.52	2.609	2.381	4010.16	2.768	1259.21	0.525	1.049
Cell5	4425.4	2.678	1.637	3846.89	2.872	930.57	1.001	1.022
Cell6	2653.59	2.611	2.162	2373.2	2.776	926.74	0.425	1.077
Cell7	7151.08	2.595	3.627	6395.55	2.761	2237.1	0.484	1.083
Cell8	6423.93	2.612	3.102	5758.78	2.773	1854.14	0.517	1.081
Cell9	2456.01	2.448	3.813	2017.73	2.743	1425.48	0.434	1.078
Cell10	1910.88	2.676	1.237	1880.57	2.681	1928.43	0.026	1.13
Cell11	2501.35	2.645	1.416	2325.02	2.757	532.52	0.477	1.02
Cell12	1688.3	2.666	1.257	1532.78	2.813	346.11	0.671	0.988
Cell13	1996.75	2.677	1.237	1837.91	2.803	374.38	0.635	0.969
Cell14	1747.61	2.678	1.242	1631.1	2.789	316.94	0.537	1.029
Cell15	2607.02	2.647	1.495	2558.18	2.668	1694.42	0.072	1.304

Supplementary Table 9. Fluorescence lifetime determination of redFAST:HBR-3,5DOM

	monoexponential fit			biexponential fit				
	A	τ (ns)	χ^2	A1	τ_1 (ns)	A2	τ_2 (ns)	χ^2
Cell1	1814.86	2.396	1.208	1713.03	2.478	331.12	0.418	0.997
Cell2	2414.63	2.455	1.072	2281.37	2.53	265.37	0.76	0.97
Cell3	2063.17	2.431	1.132	2042.45	2.437	2392.74	0.032	1.059
Cell4	2957.77	2.445	1.167	2919.34	2.458	1086.28	0.055	1.097
Cell5	828.31	2.347	1.208	768.06	2.446	399.47	0.19	0.887
Cell6	891.13	2.45	0.898	744.72	2.625	183.3	1.391	0.853
Cell7	765.46	2.41	1.038	787.37	2.413	-1518.57	0.031	1.021
Cell8	979.48	2.439	0.918	911.8	2.531	124.54	0.824	0.867
Cell9	515.49	2.374	0.896	515.73	2.374	-155.36	0	0.898
Cell10	1863.2	2.455	1.026	1522.06	2.652	425.65	1.38	0.911
Cell11	3337.64	2.454	1.179	3300.5	2.458	3716.89	0.012	1.075
Cell12	2921.11	2.445	1.135	2891.01	2.449	2692.52	0.015	1.081
Cell13	1938.98	2.415	1.227	1817.72	2.507	374.3	0.442	0.967
Cell14	1436.13	2.424	1.01	1412.72	2.437	699.3	0.04	0.958
Cell15	2346.33	2.451	0.989	2344.62	2.452	1060.38	0	0.991
Cell16	1432.24	2.416	1.124	1325.28	2.524	264.85	0.557	0.939
Cell17	1479.55	2.41	1.053	1457.19	2.431	811.07	0.056	0.965

Supplementary Table 10. Fluorescence lifetime determination of redFAST in presence of both fluorogens (5 μ M HMBR + 10 μ M HBR-3,5DOM)

	monoexponential fit			biexponential fit				
	A	τ (ns)	χ^2	A1	τ_1 (ns)	A2	τ_2 (ns)	χ^2
Cell1	1428.92	2.262	3.688	1254.12	2.423	2067.49	0.119	1.04
Cell2	3816.95	2.378	2.664	3596.76	2.445	2731.71	0.108	1.057
Cell3	3905.82	2.434	1.486	3719.87	2.504	678.7	0.388	1.08
Cell4	7004.25	2.43	1.511	6923.67	2.444	9689.39	0.028	1.192
Cell5	6466.97	2.429	2.127	6160.42	2.496	1506.33	0.284	1.172
Cell6	2483.86	2.443	1.648	2365.88	2.507	1096.33	0.147	1.049
Cell7	6634.34	2.43	1.489	6571.84	2.441	8457.66	0.029	1.265
Cell8	3026.95	2.38	1.853	2868.4	2.449	1241.79	0.171	1.088
Cell9	3683.57	2.398	1.544	3551.47	2.445	1203.28	0.151	1.038
Cell10	4610.46	2.434	1.447	4561.21	2.446	6194.79	0.029	1.23
Cell11	4329.77	2.341	3.864	4045.72	2.426	3852.1	0.104	1.171
Cell12	4008.91	2.274	8.293	3577.52	2.406	6120.45	0.101	1.323
Cell13	13575.71	2.405	7.021	12842.39	2.463	10664.73	0.094	1.375
Cell14	5577.29	2.402	5.794	5189.37	2.489	6302.6	0.09	1.215
Cell15	5242.42	2.368	5.231	4875	2.46	4898.98	0.113	1.203

Supplementary Table 11. Sequence of oligonucleotides used in this study

Name	Sequence
ag175	gcagcggcggaggggatccatggagcatgttcctttggc
ag182	ggatccccctccgcccgtgccgcctcctccggagacctgttgagattcgtcgg
ag184	ggatccccctccgcccgtgccgcctcctccgattctccagtttagaagctccacatc
ag189	gtggtgctcgagctattactacacccgtttataaagaccaatagc
ag195	gcctgtgatgtctccctgagcagcattgtac
ag196	tacaatgctgctcaggagacatcacaggc
ag216	ttcgtagctagcatggagcatgttcctttg
ag217	ttgtcggatcccaccgtttcacaaagac
ag224	atggctagcgaacacctgtatttcaggggcatggagcatgttcctttggc
ag311	aaagcttattctgaagaggactgtaataggcggccgactctagatcataatc
ag313	ctcacctgtcctgccgagaaagtatcca
ag314	tggatactttctcggcaggagcaaggtag
ag321	gccctgaaaatacaggtttcgctagc
ag322	taatagctcgagcaccaccaccac
ag347	taataggcggccgactctag
ag354	gtggtggtgctcgcagctattacacccgtttcacaagaccaatag
ag356	gtcctctcagaaataagctttgttcggatcccaccgtttcacaagaccaatag
ag357	ccggactcagatctgccaccatggagcatgttcctttggcag
ag358	ggtggcagatctgagtcggtag
ag420	ccaaccaaggtcaagatgcacatgaagaaag
ag421	ctttctcatgtgcatcttgacctgtgtg
ag422	caaggatgtgcaactggaacggattctc
ag423	gagaatccgttcagttgcaacatccttg
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ag426	gaatggatgataccgacaaacaggggaccaaccaag
ag427	ctgggtggccccctgtttgtcggatcatccattc
ag428	gatgggtggccttaggcgaattcagctc
ag429	gagctgaattgcgctaaggccaaccatc
ag472	ctagagtcgcccgcctattaggaaagggcttctcatgtcac
ag491	ggaggcggatctgccaccatggagcatgttcctttggcag
ag492	catggtggcagatccgcctc
ag527	gccaaaggcaacatgctccatgaattccaagtcctctcagaataagctttgttc
ag528	atggagcatgttcctttg
ag530	caccgtttcacaaagacc
ag532	gtcgaagcaggctggagacgtggaggagaaccctggacctatggagcatgttcctttg
ag533	gggtctttgtgaaacgggtgggatccatcacactggcgg
ag534	ctagagtcgcccgcctattacagcgccttctccgttttc
ag550	tgctgaagcaggctggagacgtggaggagaaccctggacctgtgagcaagggcgaggagg
ag554	catcaagtccaagggcaaggactccgcccggcggctccatggagcatgttcctttggc
ag555	gagtccttgccctggactgatg
ag598	tacagcatgctgccgagc
ag599	cctgctcagcaggctgaagtagtagctccgctcctatagtgctcctgatcctgggctg
ag675	gctcggcagcatgctgtacacccgtttcacaagacc
ag677	ctaccggactcagatctgccaccatggcggtggcacttgatcaagaagttcagatcca
ag678	ctccatgaccggtggatccccctcctctggagatggactcgaactcttgatcaagtc
ag679	ggaggggatccaccggtatggagcatgttcctttg
ag795	ctaccggactcagatctgccaccatgggtcccggcaagaagaag
ag796	ccaaaggcaacatgctccatgtctggttaatcacactcatggtg
Kan-F	gcatcaaccaaacctgtattcattcgtg
Kan-R	cacgaatgaataacggtttggtgatgc

Supplementary Table 12. Table of plasmids used in this study

Plasmid code	Expression host	open reading frame
pAG261	<i>E. coli</i>	green clone 1 = greenFAST
pAG262	<i>E. coli</i>	green clone 2
pAG263	<i>E. coli</i>	green clone 6
pAG264	<i>E. coli</i>	green clone 7
pAG265	<i>E. coli</i>	green clone 21
pAG270	<i>E. coli</i>	red clone 1
pAG271	<i>E. coli</i>	red clone 2
pAG272	<i>E. coli</i>	red clone 4
pAG273	<i>E. coli</i>	red clone 5
pAG274	<i>E. coli</i>	red clone 6
pAG275	<i>E. coli</i>	red clone 7
pAG276	<i>E. coli</i>	red clone 10
pAG277	<i>E. coli</i>	red clone 17
pAG302	<i>E. coli</i>	green clone 6 V107M
pAG303	<i>E. coli</i>	green clone 21 P68T
pAG304	<i>E. coli</i>	green clone 21 P68T T70K
pAG305	<i>E. coli</i>	green clone 21 V122I
pAG306	<i>E. coli</i>	red clone 7 I99N
pAG307	<i>E. coli</i>	red clone 10 F28L
pAG308	<i>E. coli</i>	red clone 10 F28L E46Q = redFAST
pAG361	Mammalian	lyn11-greenFAST
pAG362	Yeast	redFAST
pAG364	Mammalian	greenFAST
pAG365	Mammalian	redFAST
pAG369	Mammalian	lyn11-redFAST
pAG372	Mammalian	mito-greenFAST
pAG373	Mammalian	mito-redFAST
pAG374	Mammalian	H2B-greenFAST
pAG375	Mammalian	H2B-redFAST
pAG460	Mammalian	FRB-N-greenFAST
pAG461	Mammalian	FRB-N-redFAST
pAG462	Mammalian	FKBP-N-greenFAST
pAG463	Mammalian	FKBP-N-redFAST
pAG469	Mammalian	LifeAct-redFAST
pAG477	Mammalian	redFAST-Cdt(30-120)-P2A-greenFAST-Gem(1-120)
pAG551	Mammalian	MAP4-greenFAST
pAG552	Mammalian	MAP4-redFAST
pAG646	Yeast	green clone 6 V107M
pAG647	Yeast	green clone 21 P68T
#1113	Vertebrate	pT2iC6-LifeAct-greenFAST
#1135	Vertebrate	redFAST-zGem(1-100)-P2A-greenFAST-zCdt1(1-190)