Supporting information for:

Green fluorescent genetically encoded calcium indicator based on calmodulin/M13-peptide from fungi

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S1 Supporting information

Figure A. Alignment of the amino acid sequences for CaMs and peptides from CaM-dependent kinases and calcineurins found in different species.

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Table A. Brightness and contrasts for FGCaMP and other ratiometric green and yellow GECIs.

Table B. K_d values and fluorescence contrasts for 402- and 493-forms of FGCaMP and its mutants.

Table C. List of primers.

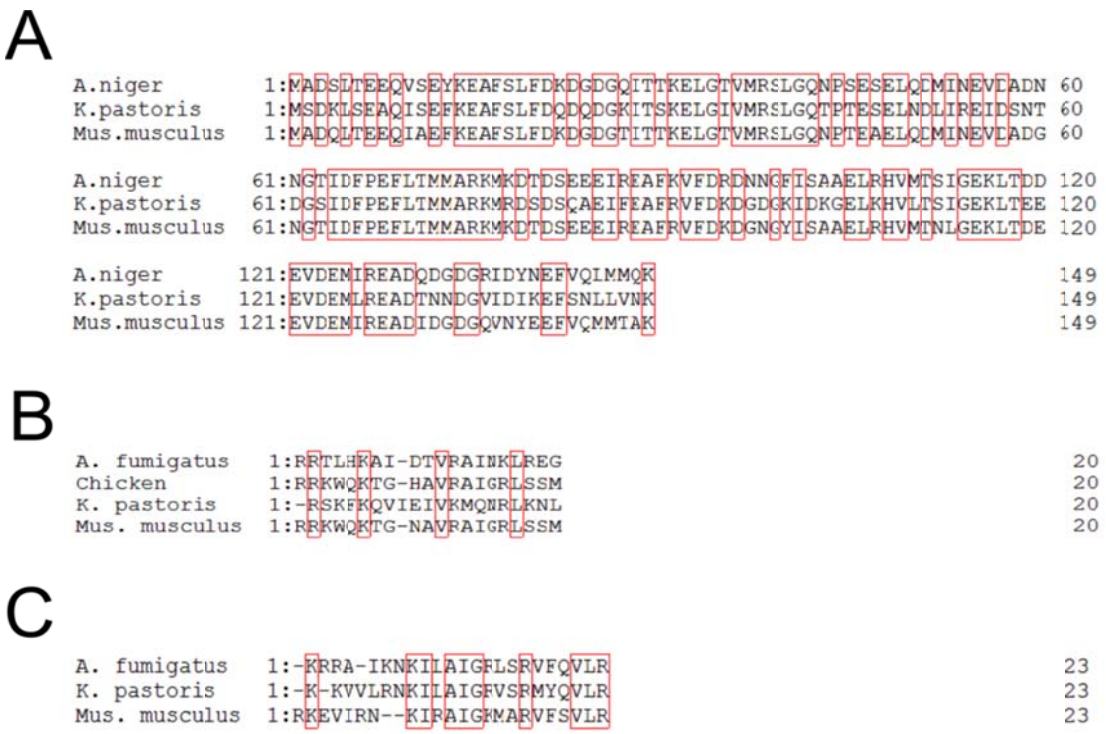


Figure A. Alignment of the amino acid sequences for CaMs and peptides from CaM-dependent kinases and calcineurins found in different species. (A) Alignment of the amino acid sequences for CaMs from *A. niger*, *K. pastoris*, and *M. musculus*. (B) Alignment of the amino acid sequences for M13-like peptides from CaM-dependent kinases from *A. fumigatus*, chicken, *K. pastoris*, and *M. musculus*. (C) Alignment of the amino acid sequences for calcineurins from *A. fumigatus*, *K. pastoris*, and *M. musculus*. Identical residues are selected with red boxes.

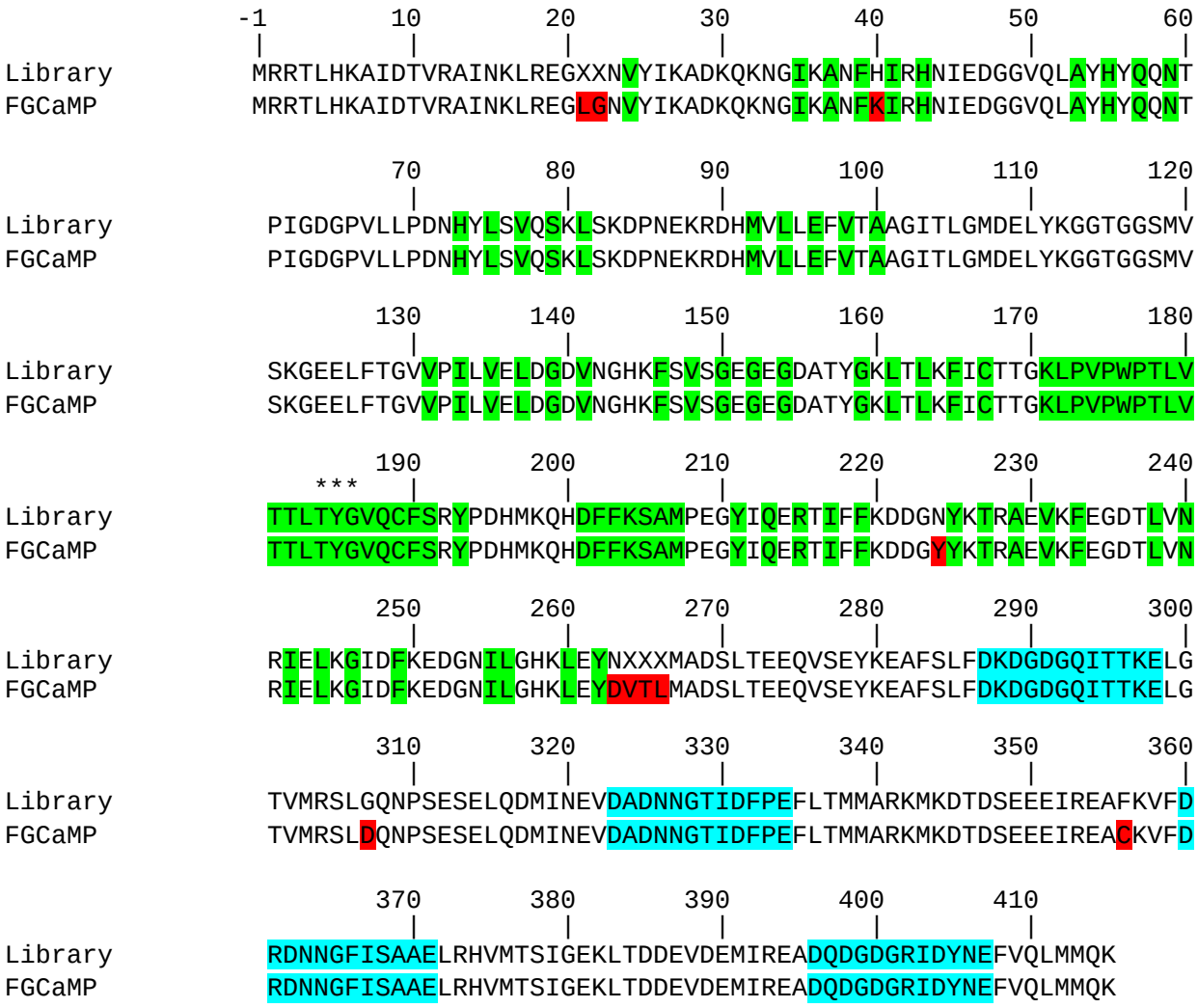


Figure B. Alignment of the amino acid sequences for original library and FGCaMP calcium indicator. Alignment numbering follows that of FGCaMP. Residues from fluorescent part buried in β-can are highlighted with green. Residues that are forming chromophore are marked with asterisks. EF1-EF4 hands responsible for calcium binding are highlighted with cyan color. Mutations in final FGCaMP protein are highlighted with red.

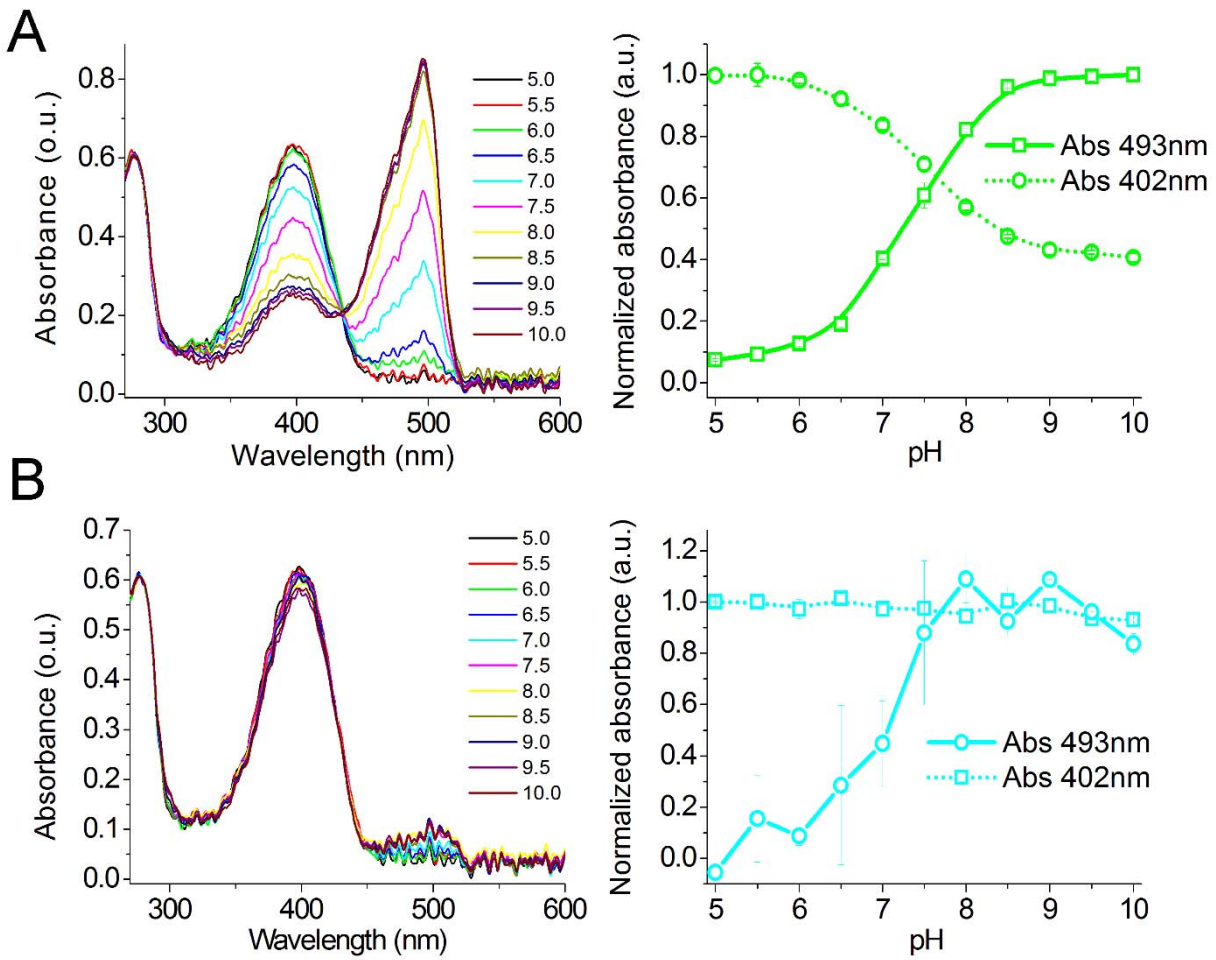


Figure C. pH dependence of absorbance for the purified FGCaMP indicator. (A, B) Absorbance spectra (left) and the normalized absorbance at 402 and 493nm (right) for FGCaMP in Ca^{2+}-bound (A) and Ca^{2+}-free (B) states in the range of pH values of 5-10. Error bars represent the standard deviation.

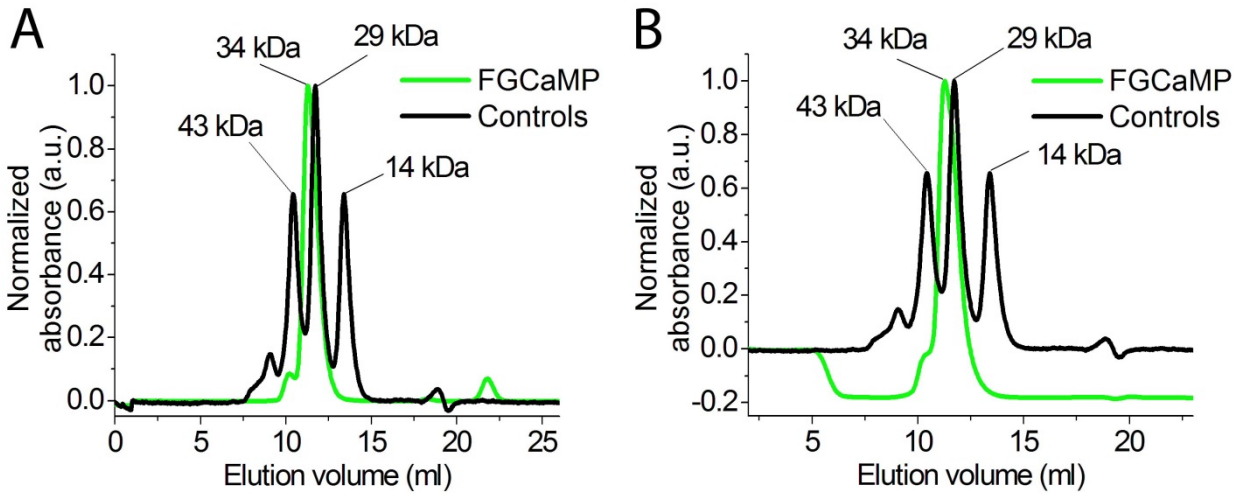


Figure D. Size-exclusion chromatography for FGCaMP protein. Fast protein liquid chromatography of FGCaMP in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl buffer supplemented with 2 mM EDTA (A) or in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl buffer supplemented with 1 mM $CaCl_2$ (B).

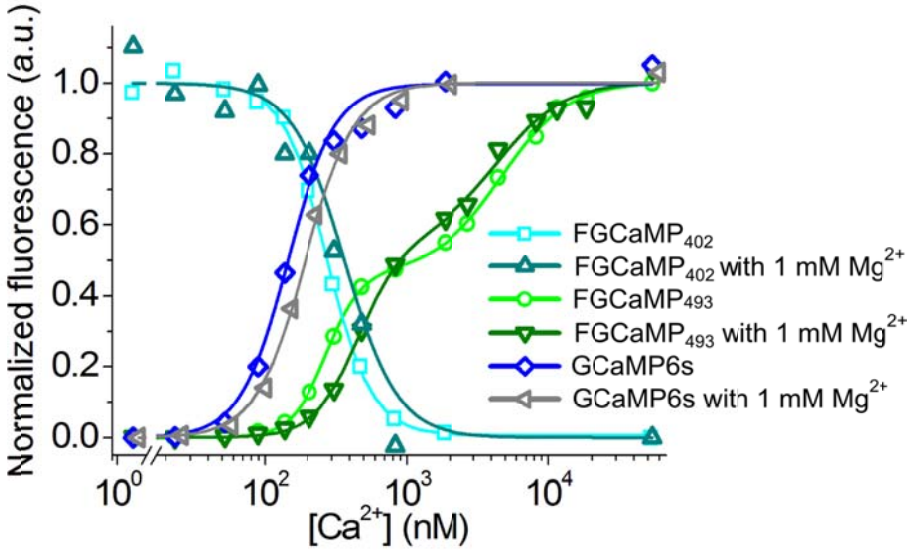


Figure E. Ca^{2+} titration curves for FGCaMP and GCaMP6s in the absence or presence of 1 mM $MgCl_2$. Ca^{2+} titration curves for 402- and 493-forms of FGCaMP were acquired using 402 and 490 nm excitation light, respectively. Fluorescence changes were normalized to maximal and minimal values. Experimental data were fitted to Hill equation or double Hill equation $y = V_1*x^{n1}/(K_{d1}{}^{n1}+x^{n1}) + V_2*x^{n2}/(K_{d2}{}^{n2}+x^{n2})$, respectively.

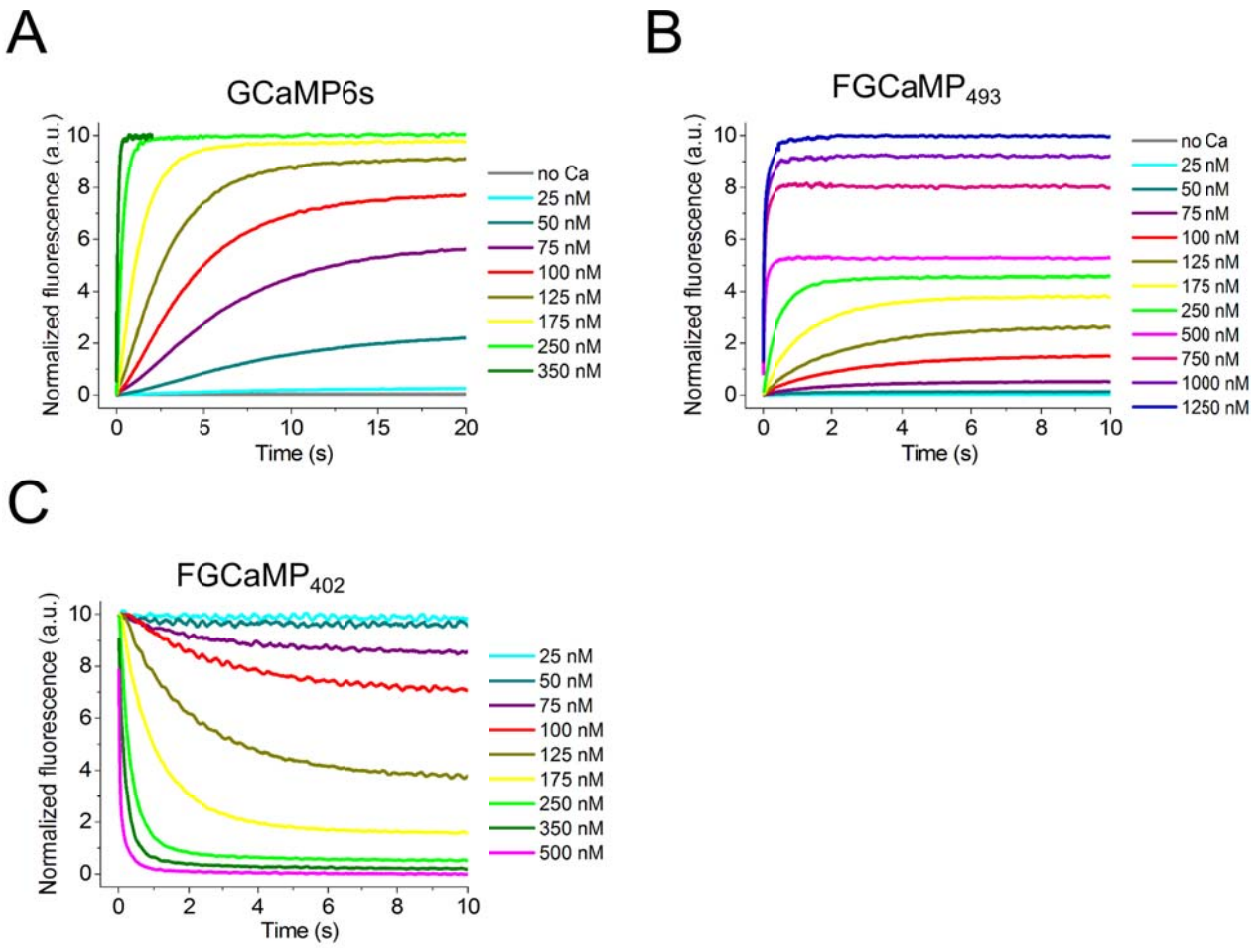


Figure F. Kinetic curves obtained from Ca^{2+}-association experiments. Fluorescence changes are normalized to F_0 of 0 and F_{max} of 10 for GCaMP6s (A) and FGCaMP using excitation at 500 nm (B) and to F_0 of 10 and F_{min} of 0 for FGCaMP using excitation at 402 nm (C). Ca^{2+} concentrations in the mixing chamber are specified in the figure legends.

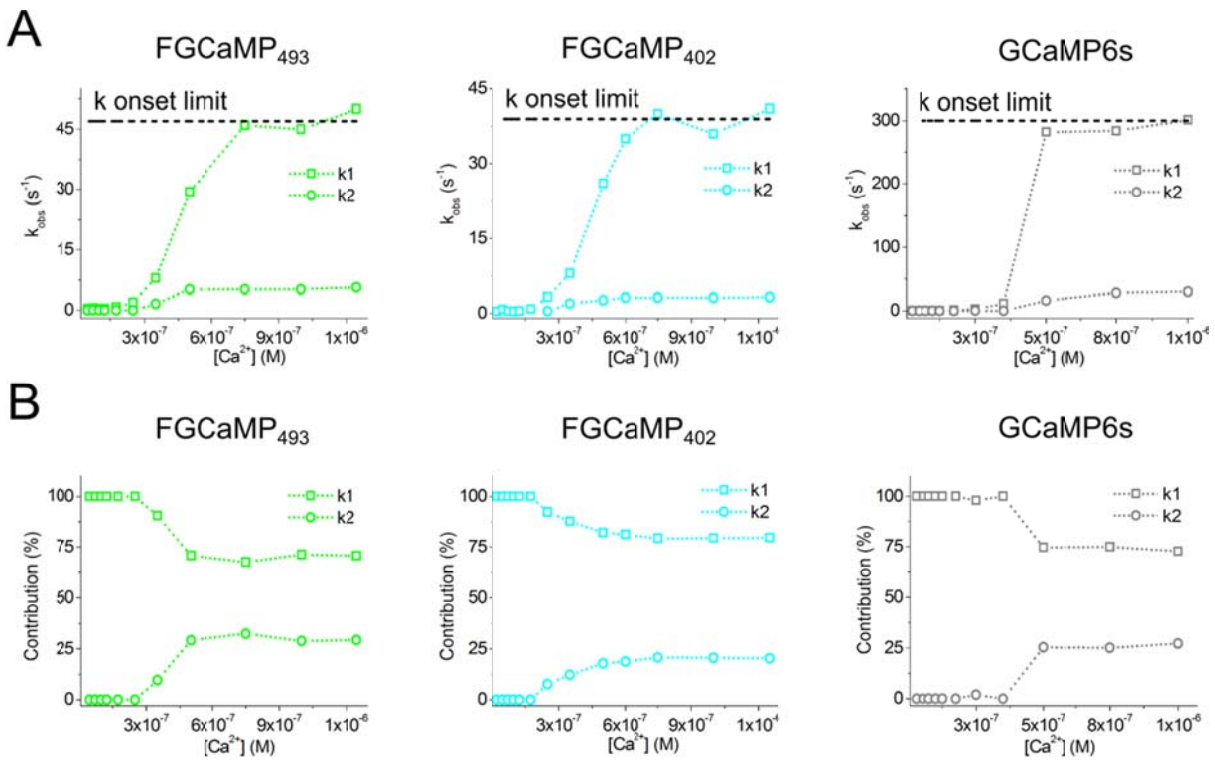


Figure G. Detailed analysis of the onset kinetics for Ca^{2+}-binding. (A) Observed association rate constants (k1, k2) obtained by fitting the kinetic curves to double exponentials. (B) Relative contributions of the k1 and k2 exponentials. Onset rate limit values (k^{onset} limit) correspond to saturation levels of the observed association rate constants (at > 600-800 nM Ca^{2+} concentrations).

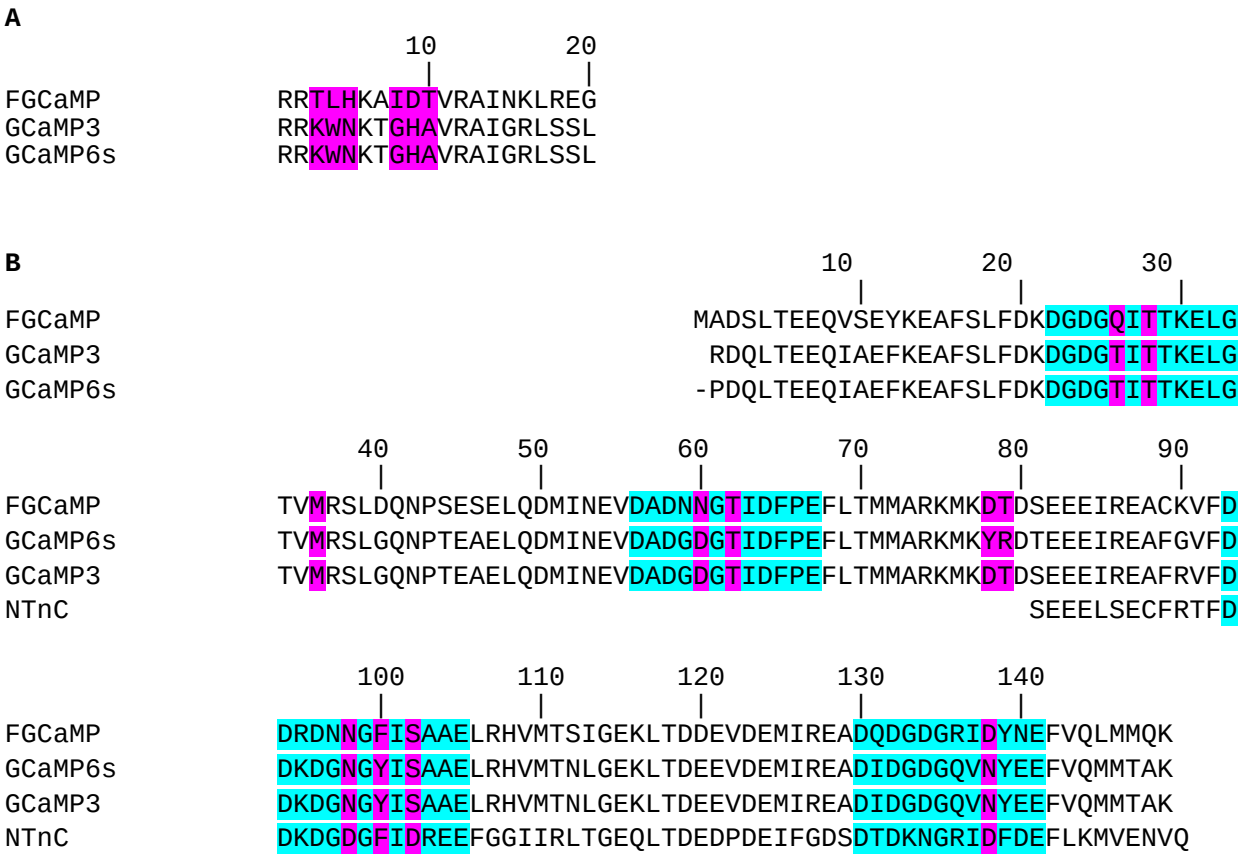


Figure H. Alignment of the amino acid sequences of M13-like peptides and CaMs from FGCaMP and the other calcium indicators. (A) Alignment of peptides from CaM-dependent kinase for FGCaMP, GCaMP3, and GCaMP6s indicators. Mutated randomized residues are highlighted with magenta. (B) Alignment of CaMs from FGCaMP, GCaMP3, GCaMP6s, and NTnC. Four EF1-EF4 hands responsible for calcium-binding are highlighted with cyan, mutated residues are highlighted with magenta. Alignment numbering follows that for GCaMP6s.

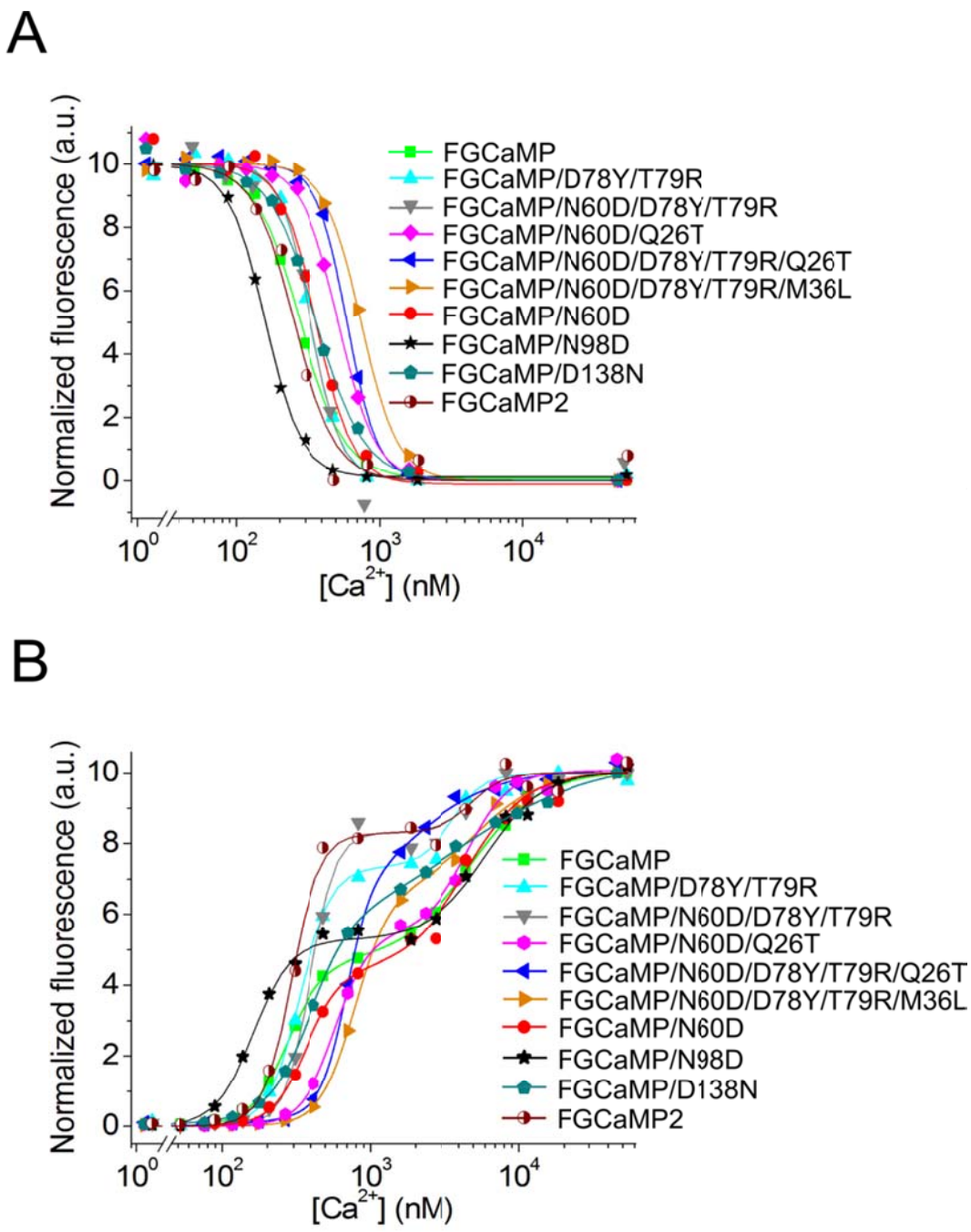
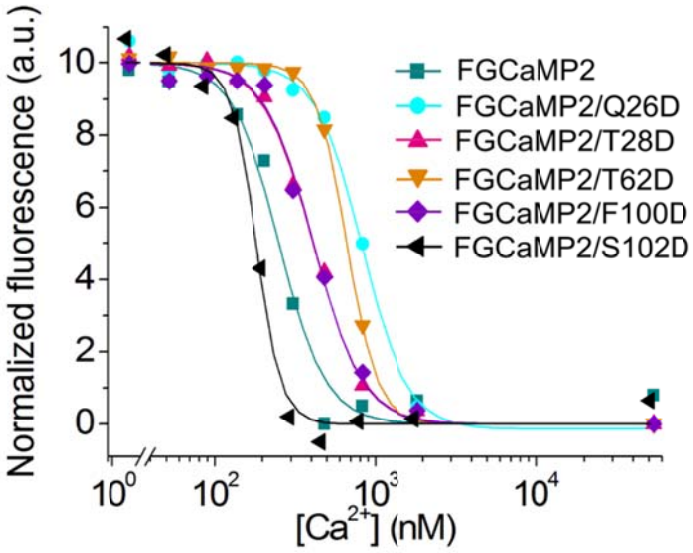


Figure I. Ca^{2+} titration curves for FGCaMP mutants. (A, B) Ca^{2+} titration curves for 402- (A) and 493-forms (B) of FGCaMP mutants using excitation at 402 and 490 nm, respectively. FGCaMP2 corresponds to FGCaMP/N60D/D78Y/T79R/N98D. Alignment numbering follows that for CaM from GCaMP6s. Fluorescence is normalized to maximal and minimal values. SD corresponds to 20%.

A



B

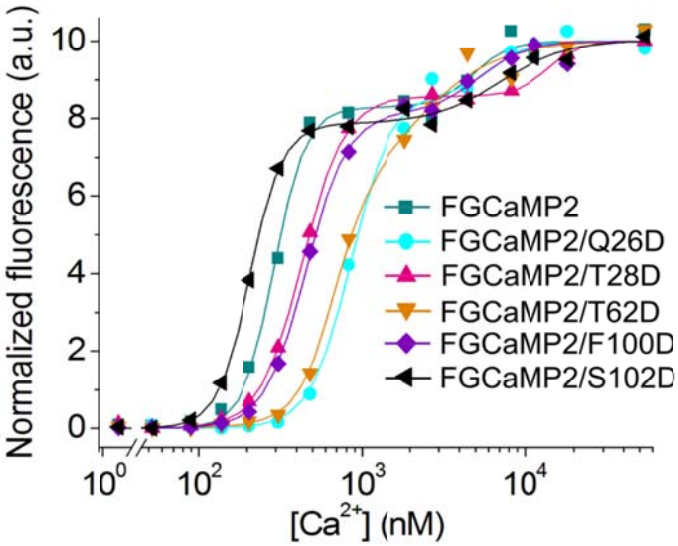


Figure J. Ca^{2+} titration curves for FGCaMP2 mutants with mutations in CaM part. (A, B) Ca^{2+} titration curves for 402- (A) and 493-forms (B) of FGCaMP2 mutants using 402 and 490 nm excitation, respectively. FGCaMP2 corresponds to FGCaMP/N60D/D78Y/T79R/N98D. Alignment numbering follows that for CaM from GCaMP6s. Fluorescence is normalized to maximal and minimal values. SD corresponds to 20%.

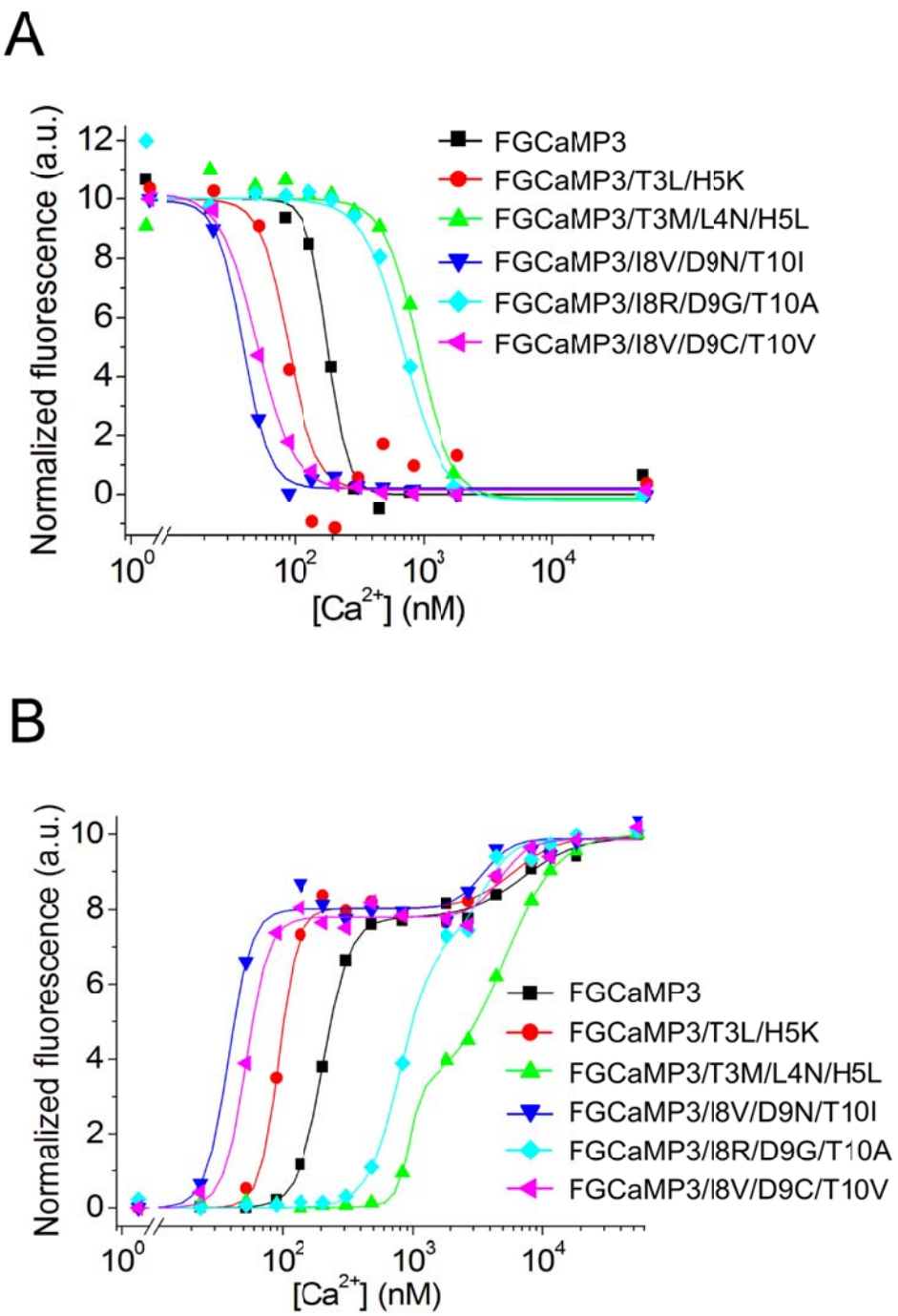


Figure K. Ca^{2+} titration curves for FGCaMP3 variants with mutations in the M13-like peptide. (A, B) Ca^{2+} titration curves for 402- (A) and 493-forms (B) of **F**GCaMP3 mutants using 402 and 490 nm excitation, respectively. FGCaM**P**3 corresponds to FGCaMP/N60D/D78Y/T79R/N98D/S102D. Alignment numbering follows that for CaM or M13 peptide from GCaMP6s. Fluorescence is normalized to minimal and maximal fluorescence values. SD corresponds to 20%.

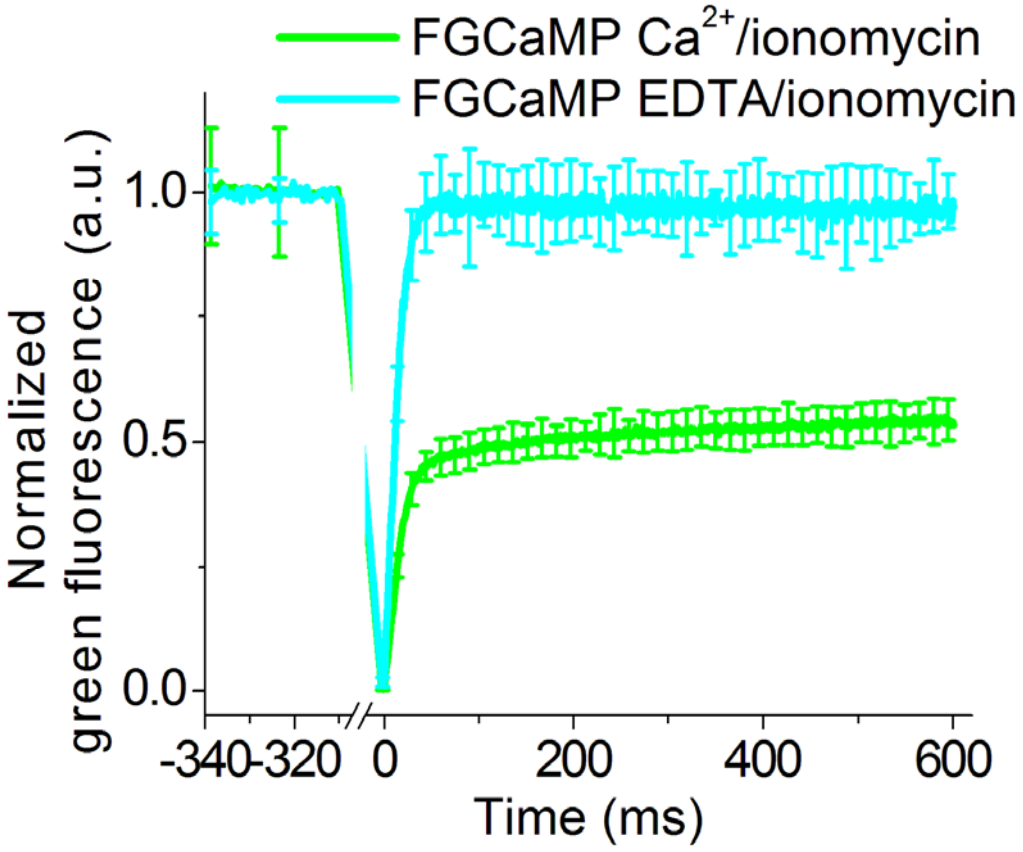


Figure L. Reversible changes in the mobile fraction of the FGCaMP indicator at the transition from high to low Ca^{2+} concentrations in HeLa Kyoto cells studied by FRAP experiments. The graphs illustrate FRAP induced changes in green fluorescence of FGCaMP in response to the addition of 2 mM $CaCl_2$/5 µM ionomycin (high Ca^{2+} concentrations) followed by washing and addition of 1 mM EDTA/5 µM ionomycin (low Ca^{2+} concentrations). Error bars are standard deviations shown for each 20^{th} dot on plots.

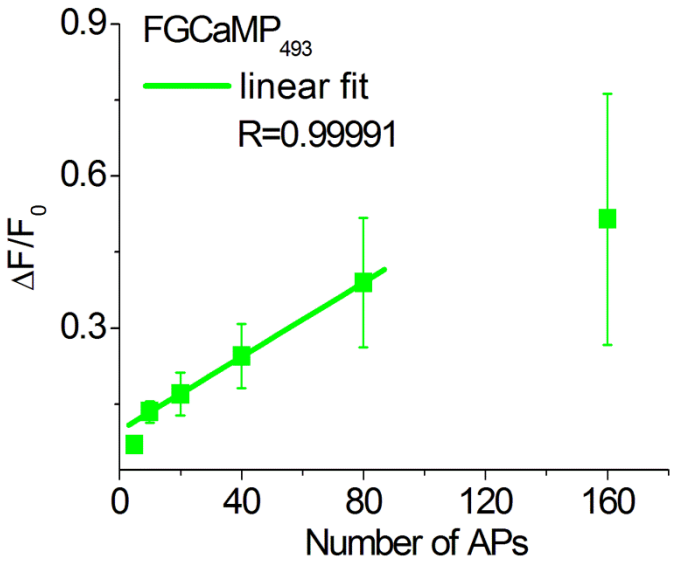


Figure M. Fluorescence changes in response to the external electric field in cultured neurons expressing FGCaMP indicator. Linear region is fitted to a linear equation. APs were induced by electrical pulses of 1 ms, 5 V, and 87 Hz.

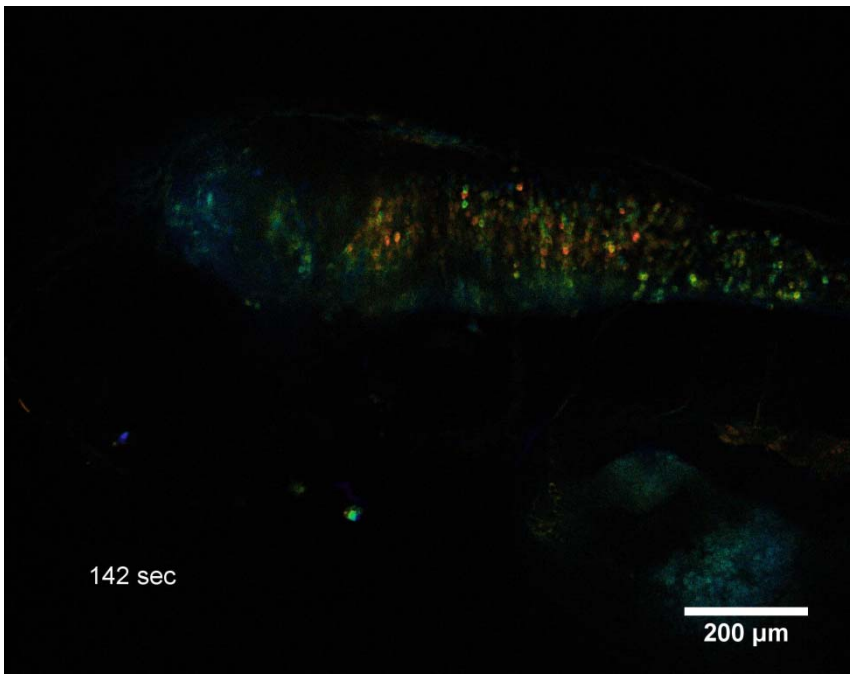


Figure N. Confocal ratiometric calcium imaging with FGCaMP during 4-AP induced neuronal activity in paralyzed larvae embedded in ultra-low-melting agarose gel. Example of frame from S1 Video is shown.

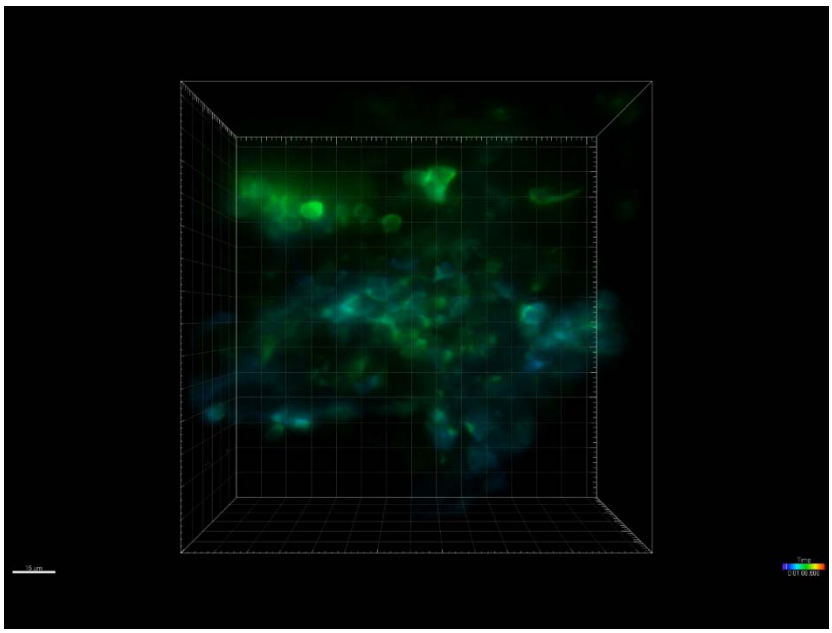


Figure O. Light sheet ratiometric calcium imaging with FGCaMP during 4-AP induced neuronal activity in paralyzed larvae embedded in ultra-low-melting agarose gel. Example of frame from S2 Video is shown.

Table A. Brightness and contrasts for FGCaMP and other ratiometric green and yellow GECIs.

Protein		Brightness	Fluorescence contrast (fold)	Fluorescence Ratio (fold)
FGCaMP	**402-form^{apo}**	26	6.9±0.5	101
	493-form^{sat}	49	14.7±0.6	
GEX-GECO	**390-form^{apo}**	6.7[a]	1.1±0.1	18
	482-form^{sat}	1[a]	16.1±0.4	
Ratiometric-pericam [b]	**418-form^{apo}**	7.2	ND	10
	494-form^{sat}	1.9	ND	
Y-GECO [c]	**523-form^{apo}**	30	15	200
	413-form^{sat}	1.2	18	

[a] ref [1]. [b] ref [2]. [c] ref [3]

Table B. K_d values and fluorescence contrasts for 402- and 493-forms of FGCaMP and its mutants.

Protein	402-form		493-form			
	K_{d1} (nM)	Contrast (fold)	K_{d1} (nM)	K_{d2} (μM)	Contrast K_{d1} (fold)	Contrast K_{d2} (fold)
FGCaMP	276±9	5.1±0.8	273±7	4.7±0.2	9.3±0.6	1.7±0.1
FGCaMP/N60D	370±30	5.5±0.8	370±40	4.4±0.6	9.2±0.6	1.8±0.1
FGCaMP/N98D	159±3	5.8±0.9	161±8	6.0±0.5	8.9±0.5	1.7±0.2
FGCaMP/D138N	364±13	5.4±0.8	390±9	5.7±2.4	9.9±0.3	1.6±0.1
FGCaMP/N60D/Q26T	517±24	3.2±0.5	575±32	4.7±0.2	10.1±0.1	1.8±0.1
FGCaMP/D78Y/T79R	330±8	3.7±0.6	333±8	3.6±0.3	10.0±0.4	1.3±0.1
FGCaMP/N60D/D78Y/T79R	332±20	3.4±0.5	390±20	4.9±1.1	10.7±0.3	1.2±0.1
FGCaMP/N60D/D78Y/T79R/Q26T	600±15	3.4±0.5	693±17	3.2±1.9	11.7±0.3	1.2±0.1
FGCaMP/N60D/D78Y/T79R/M36L	743±23	3.4±0.5	788±47	4.6±1.0	13.0±0.6	1.3±0.1
FGCaMP2 [a]	259±11	3.3±0.5	292±10	4.8±1.1	11.3±0.4	1.2±0.1
FGCaMP2/Q26D	824±34	4.3±0.6	857±33	6.4±1.5	10.8±2	1.2±0.1
FGCaMP2/T28D	412±13	4.6±0.7	433±4	14±1	13±1	1.1±0.1
FGCaMP2/T62D	665±10	3.7±0.6	669±60	2.1±0.8	9.4±1.5	1.4±0.2
FGCaMP2/F100D	415±19	3.7±0.6	455±16	5.3±1.1	10.2±0.1	1.2±0.1
FGCaMP3 [a]	183±8	3.0±0.5	208±4	7±1	10.9±0.2	1.3±0.1
FGCaMP3/T3L/H5K	90±20	2.3±0.4	93±2	6±2	6.7±0.3	1.2±0.1
FGCaMP3/T3M/L4N/H5L	910±80	3.3±0.5	900±300	5.1±0.3	4.9±0.3	2.2±0.1
FGCaMP3/I8V/D9N/T10I	40±2	2.7±0.4	39±3	3.5±0.7	6.3±0.1	1.3±0.1
FGCaMP3/I8R/D9G/T10A	700±70	3.0±0.5	790±50	3.6±0.5	4.6±0.3	1.2±0.1
FGCaMP3/I8V/D9C/T10V	51±2	4.6±0.7	52±2	4.8±0.7	7.6±0.4	1.2±0.1

[a] FGCaMP2 and FGCaMP3 corresponds to FGCaMP/N60D/D78Y/T79R/N98D and FGCaMP2/S102D, respectively.

Alignment numbering follows that for CaM or M13 peptide from GCaMP6s.

Table C. List of primers.

Primer	Primer sequence (5'-3')
CaMA-rv	GTATATTTCATTGCTCCCCACCCTTTCCGC
CaMN-BglII	CGAGATCTATGGCCGACTCTCTGACCGAAGAGCAAG
CaMN-HindIII reverse	CCAAGCTTTTATTTTTGCATCATGAGCTGGACGAACTCGTTG
CaMK-BglII	CGAGATCTATGTCCGATAAATTGTCCGAAGCCCAAATTTC
CaMK-HindIII reverse	CCAAGCTTCTATTTATTAACTAGGAGATTGGAAAATTCCTTG
M13Fc-BglII forward	CAGAGATCTAAAAGACGGGCGATCAAGAACAAG
M13Fc reverse	CCGCAACACCTGGAAGACACGCGAGAGACGTCCGATGGCAAGAATCTTGTTC TTGATCGCCCG
M13Fc-cpEGFP forward	GTCTTCCAGGTGTTGCGGNNSNNSAACGTCTATATCAAGGCC
M13Fc-cpEGFP reverse	GGCCTTGATATAGACGTTSNNSNNCCGCAACACCTGGAAGAC
cpEGFP-CaMF forward	CACAAGCTGGAGTACAACNNSNNSNNSATGGCCGACTCTCTGACCGAAGAGC AAG
cpEGFP-CaMF reverse	CTTGCTCTTCGGTCAGAGAGTCGGCCATSNNSNNSNNGTTGTACTCCAGCTTG TG
M13Fk-BglII forward	CAGAGATCTCGTCGAACGCTGCACAAAGCC
M13Fk reverse	CCCTCGCGGAGCTTGTTGATGGCGCGGACGGTATCGATGGCTTTGTGCAGCG TTCG
M13Fk-cpEGFP forward	CAACAAGCTCCGCGAGGGANNSNNSAACGTCTATATCAAGGCC
M13Fk-cpEGFP reverse	GGCCTTGATATAGACGTTSNNSNNTCCCTCGCGGAGCTTGTTG
M13KPc-BglII forward	CAGAGATCTAAGAAAGTGGTACTCAGAAATAAG
M13KPc reverse	GGAGAACTTGATACATCCTGGATACTCTTCCAATTGCCAGAATCTTATTTCTGA GTACCACTTTC
M13KPc-cpEGFP forward	GGATGTATCAAGTTCTCCGTNNSNNSAACGTCTATATCAAGGCC
M13KPc-cpEGFP reverse	GGCCTTGATATAGACGTTSNNSNNACGGAGAACTTGATACATCC
cpEGFP-CaMKP forward	CACAAGCTGGAGTACAACNNSNNSNNSATGTCCGATAAATTGTCCGAAGCCCA AATTTC
cpEGFP-CaMKP reverse	GAAATTTGGGCTTCGGACAATTTATCGGACATSNNSNNSNNGTTGTACTCCAG CTTGTG
M13KPk-BglII forward	CAGAGATCTAGATCCAAGTTCAAGCAAG
M13KPk reverse	GTTCTTCAATCTGTTTTGCATCTTGACAATCTCGATGACTTGCTTGAACTTGGA TC
M13KPk-cpEGFP forward	GCAAAACAGATTGAAGAACCTCNNSNNSAACGTCTATATCAAGGCC
M13KPk-cpEGFP reverse	GGCCTTGATATAGACGTTSNNSNNGAGGTTCTTCAATCTGTTTTGC
FCaMP-M36L forward	CAAGGAGCTCGGCACTGTGCTGCGCTCCCTTGACCAGAAC
FCaMP-M36L reverse	GTTCTGGTCAAGGGAGCGCAGCACAGTGCCGAGCTCCTTG
FCaMP-N60D forward	GAGGTTGACGCTGACAACGACGGAACGATCGACTTCC
FCaMP-N60D	GGAAGTCGATCGTTCCGTCGTTGTCAGCGTCAACCTC

reverse	
FCaMP-78YR forward	GATGGCTCGTAAGATGAAGTACAGAGACACCGAGGAGGAAATCCGCG
FCaMP-78YR reverse	CGCGGATTTCCTCCTCGGTGTCTCTGTACTTCATCTTACGAGCCATC
FCaMP-Q26T forward	CAAGGATGGCGATGGCACGATCACCACCAAGGAGCTC
FCaMP-Q26T reverse	GAGCTCCTTGGTGGTGATCGTGCCATCGCCATCCTTG
FCaMP-N98D forward	GTCTTCGACCGCGACAACGATGGTTTTATCTCCGCCGCG
FCaMP-N98D reverse	CGCGGCGGAGATAAAACCATCGTTGTCGCGGTCGAAGAC
FCaMP-D138N reverse	GAGCTGGACGAACTCGTTGTAGTTGATGCGGCCATCACCGTC
FC-102D forward	GACAACGATGGTTTTATCGACGCCGCGGAGCTGCGCCAC
FC-102D reverse	GTGGCGCAGCTCCGCGGCGTCGATAAAACCATCGTTGTC
FC-BglII-M13NNS1 forward	GGCAGATCTATGCGTAGCCGTCGANNSNNSNNSAAAGCCATCGATACCGTC
FC-BglII-M13NNS2 forward	GGCAGATCTATGCGTAGCCGTCGAACGCTGCACAAAGCCNNSNNSNNSGTCC GCGCCATCAACAAG
G-FCaMP-BamHI-NheI forward	TGGGGATCCGGCTAGGCCACCATGCGTCGAACGCTGCACAAAGCC
GEX-GECO-BglII forward	CAGAGATCTATGGTCGACTCATCACGTC
GEX-GECO-EcoRI reverse	CAGGAATTCTCACTTCGCTGTCATCATTTG

References

1. Zhao Y, Araki S, Wu J, Teramoto T, Chang YF, Nakano M, et al. (2011) An expanded palette of genetically encoded Ca(2) indicators. Science 333: 1888-1891.
2. Nagai T, Sawano A, Park ES, Miyawaki A (2001) Circularly permuted green fluorescent proteins engineered to sense Ca2+. Proceedings of the National Academy of Sciences of the United States of America 98: 3197-3202.
3. Zhao Y, Abdelfattah AS, Ruangkittisakul A, Ballanyi K, Campbell RE, Harrison DJ (2014) Microfluidic cell sorter-aided directed evolution of a protein-based calcium ion indicator with an inverted fluorescent response. Integrative biology : quantitative biosciences from nano to macro 6: 714-725.