

# **cNTnC and fYTnC2, Genetically Encoded Green Calcium Indicators based on Troponin C from Fast Animals**

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**Supplementary Tables and Figures.**

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**Supplementary Methods.**

**Table S1.** Data collection and refinement statistics. \* Highest resolution range is shown in parentheses.

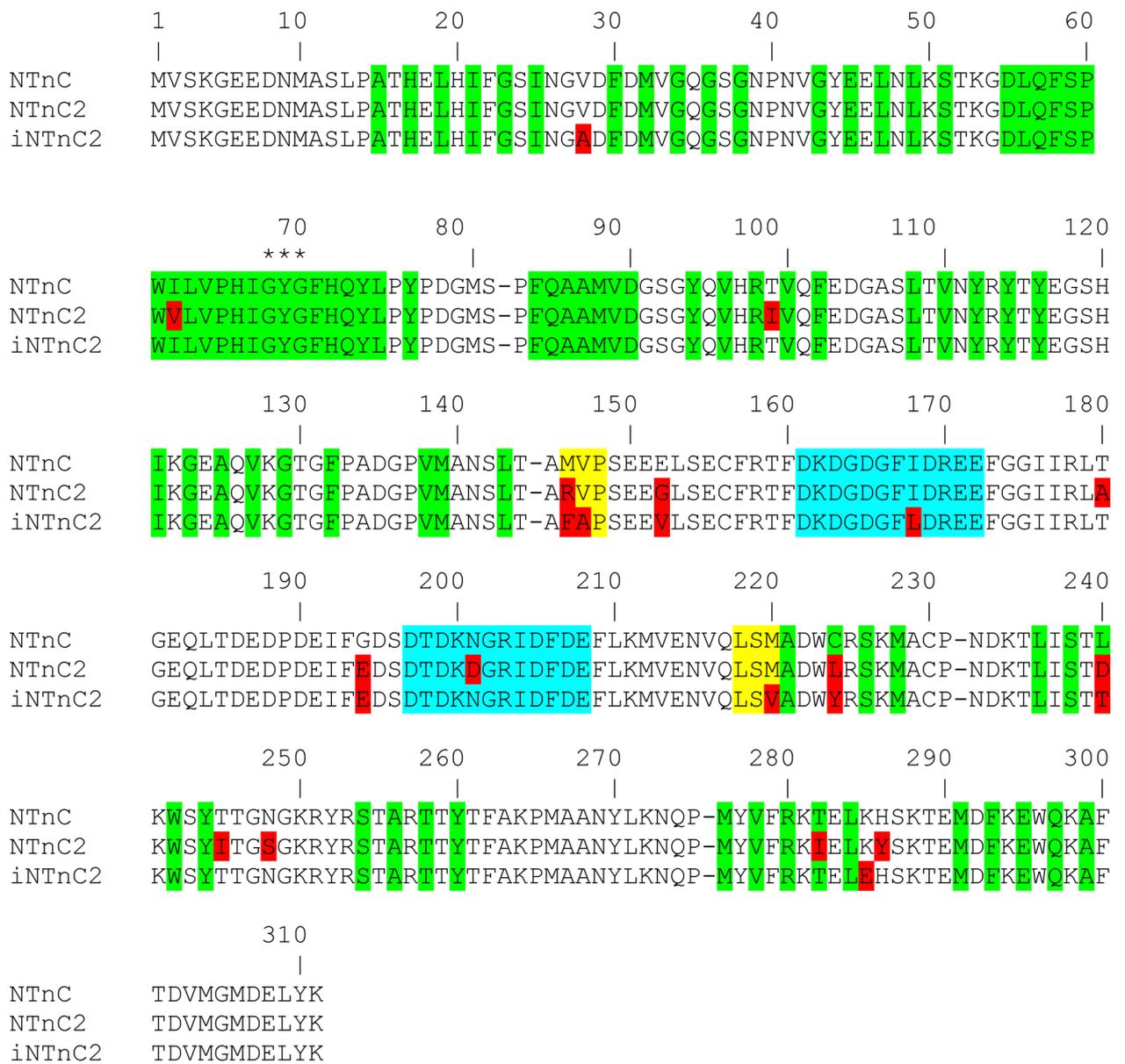
PDB ID	5MWC
<b>Data collection</b>	
Diffraction source	ESRF (ID30A-3)
Detector	Eiger 4M
Wavelength (Å)	0.9677
Crystal-to-detector distance (mm)	170
Rotation range per image (°)	0.15
Total rotation range (°)	120
Space group	P4 <sub>1</sub> 2 <sub>1</sub> 2
Cell size	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	82.10, 82.10, 157.47
$\alpha$ , $\beta$ , $\gamma$ (°)	90, 90, 90
Resolution (Å)	46.73-2.45 (2.50-2.45)*
R <sub>merge</sub> (%)	6.2 (97.3)
$\langle I \rangle / \langle \sigma(I) \rangle$	23.9 (2.3)
Occupancy	99.7 (100)
Redundancy	8.9 (9.4)
CC <sub>1/2</sub>	99.9 (73.3)
<b>Refinement</b>	
Reflections used in refinement	19409
R <sub>work</sub> / R <sub>free</sub>	23.8 / 31.0
Number of non-hydrogen atoms	
Protein	4073
Ligands/ions	36
Solvent	46
Average B-factor	63.2
Protein	63.6
Ligands/ions	44.1
Solvent	46.3
R.m.s deviations	
Bond length (Å)	0.01
Angles (°)	1.86
Ramachandran plot	
Most favored (%)	95.9
Allowed (%)	2.9
Outliers (%)	1.2
MolProbity score	2.38

**Table S2.** *In vitro* properties of fYTnC2 compared to YTnC. <sup>a</sup> Data from [1]. Data marked with asterisk (\*) were determined in this paper. <sup>b</sup> Quantum yields (QYs) were determined at pH 7.20. EGFP (QY=0.60 [2]) was used as the reference standard. <sup>c</sup> The extinction coefficients ( $\epsilon$ ) were determined by alkaline denaturation. <sup>d</sup> Brightness was calculated as a product of the quantum yield and extinction coefficient and normalized to the brightness of EGFP, which has an extinction coefficient of 56,000 M<sup>-1</sup>·cm<sup>-1</sup> and a quantum yield of 0.6 [2]. <sup>e</sup> The Hill coefficient is shown in brackets. <sup>f</sup> EGFP had a maturation half-time of 14 min. <sup>g</sup> Half-time to bleaching up to 50%. One-photon photobleaching was performed under a mercury lamp with drops in oil. Standard deviations are shown. EGFP had a photobleaching half-time of 305 ± 38 s.

Properties			Proteins			
			fYTnC2		YTnC <sup>a</sup>	
			apo	sat	apo	sat
<b>Absorption maximum (nm)</b>			414	494 (406)	413	495 (405)
<b>Excitation maximum (nm)</b>			416 (500)	498 (418)	412, 501	502 (413)
<b>Emission maximum (nm)</b>			516	518	514	516 (516)
<b>Quantum yield <sup>b</sup></b>			0.08±0.01 (0.09±0.02)	0.69±0.01 (0.24±0.02)	0.012	0.19 (0.03)
<b><math>\epsilon</math> (mM<sup>-1</sup> cm<sup>-1</sup>) <sup>c</sup></b>			98.1±9.4	46.6±2.8 (39.5±2.4)	28±2	29±3 (20±2)
<b>Brightness vs EGFP (%) <sup>d</sup></b>			23	96 (28)	1	17 (2)
<b><math>\Delta F/F</math></b>	<b>Purified protein</b>	<b>0 mM Mg<sup>2+</sup></b>	18±1		10.6±0.4	
		<b>1 mM Mg<sup>2+</sup></b>	17±1		2.9±0.2	
	<b>HeLa cells</b>		4.0±1.5		2.0±0.4	
<b>pKa</b>			9.1±0.1	6.4±0.1	5.2±0.1, 8.2±0.1	6.3±0.1
<b>K<sub>d</sub> (nM) <sup>e</sup></b>	<b>0 mM Mg<sup>2+</sup></b>		477±14 [n=2.6±0.2]		223±10 [n=1.4±0.1]	
	<b>1 mM Mg<sup>2+</sup></b>		709±38 [n=2.0±0.2]		410±19 [n=1.7±0.2]	
<b>Maturation half-time (min) <sup>f</sup></b>			4.6		16	
<b>Photobleaching half-time (s) <sup>g</sup></b>			194±57		193±36*	

**Table S3.** List of primers.

<b>Primer</b>	<b>Primer sequence (5'-3')</b>	<b>Comment</b>
<b>Neon-BglII-2</b>	gacAGATCTATGGTGAGCAAGGGCGAG	Use for mutagenesis of NTnC-derived mutants
<b>Neon-EcoRI-r2-r</b>	tcgaattcttactgtacagctcgtccatg	
<b>Fw-LSSmOrange-BglII</b>	GTACGGCTCCAGGGCCTTCATCAAGC ACCCCGCCGATATC	Use for mutagenesis of YTnC2-derived mutants
<b>Rv-GFP-EcoRI</b>	GATATCGGCGGGGTGCTTGATGAAGG CCCTGGAGCCGTAC	
<b>Fw-M146NNS</b>	GATGGCCCTGCGCCTGAAGGACGGC GGCCGCTACCTGGC	Use for mutagenesis of NTnC
<b>Rv-M146NNS</b>	GCCAGGTAGCGGCCCGCTCCTTCAG GCGCAGGGCCATC	
<b>Rv-C224NNS</b>	GGCGGCCGCTACCTGGCAGACGTCA GGACCACCTACAAG	
<b>Fw-L240NNS</b>	CTTGTAGGTGGTCCTGACGTCTGCCA GGTAGCGGCCGCC	
<b>YTnC-NES3-r</b>	CAAGGCCAAGAAGCCCGTGCAGATG CCCGGCGCCTACAAC	Use for cloning of NTnC- and YTnC2-derived mutants into pAAV-CAG-NES plasmid



**Figure S1.** Alignment of amino acid sequences for NTnC2, iNTnC2 and NTnC indicators. Amino acids internal to the  $\beta$ -can are marked by green color, amino acids of linkers are marked by yellow color, mutations in NTnC2 and iNTnC2 are highlighted by red color. Chromophore is marked by asterisks.

Acinonyx jubatus	1:SEEE	LAEC	FRIF	DRNAD	GYIDA	EELAE	IFKAS	GEHV	TDDE	IESIM	KDGD	KNND	GRID	FDE	60	
Calypte anna	1:SEEE	LANC	FRIF	DRNAD	GFIDA	EELAE	ILRAT	GEQV	TEED	IEDM	KDSD	KNND	GRID	FDE	60	
Crotalus adamanteus	1:SEEE	LAEC	FRIF	DRNAD	GFLDA	EELVE	IFRMS	GEAV	SEEE	IQEL	MRDG	KNND	GRID	FDE	60	
Falco peregrinus	1:SEEE	LANC	FRIF	DRNAD	GFIDIE	EELGE	ILRAT	GEHV	TEED	IEDM	KDSD	KNND	GRID	FDE	60	
Harpegnathos saltator	1:MQEE	LKEA	FRMY	DREG	NGYIT	TATL	KEIL	AALD	DKLT	SSDL	DGII	AEID	TDGS	GTVD	FDE	
Myotis lucifugus	1:SEEE	LAEC	FRIF	DRNAD	GYIDA	EELTE	IFRAS	GEHV	TEEE	IESIM	KDGD	KNND	GRID	FDE	60	
Toadfish	1:SEEE	SECF	FRIF	DKD	GNGF	IDREE	FGDI	IRLT	GEQL	TDEP	DEIF	GDSD	TDKN	GRID	FDE	60
Acinonyx jubatus	61:FL	KM	ME	GV											69	
Calypte anna	61:FL	KM	ME	GV											69	
Crotalus adamanteus	61:FL	KM	ME	GV											69	
Falco peregrinus	61:FL	KM	ME	GV											69	
Harpegnathos saltator	61:FM	EM	MT	GE	-										68	
Myotis lucifugus	61:FL	KM	ME	GV											69	
Toadfish	61:FL	KM	V	ENV											69	

**Figure S2.** Alignment of truncated troponins C from fast animals. Identical amino acid residues are marked by red frames.

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fNTnC_library 1:MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDLOFSP 60
aNTnC_library 1:MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDLOFSP 60
cNTnC_library 1:MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDLOFSP 60
CrNTnC_library 1:MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDLOFSP 60
hNTnC_library 1:MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDLOFSP 60
mNTnC_library 1:MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDLOFSP 60
*****

fNTnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
aNTnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
cNTnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
CrNTnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
hNTnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
mNTnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
*****

fNTnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELANCFRIFDRNADGFI DAEELGEILRAT 180
aNTnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELAEFCRIFDRNADGYI DAEELAEIFKAS 180
cNTnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELANCFRIFDRNADGFI DAEELAEILRAT 180
CrNTnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELAEFCRIFDRNADGFL DAEELVEIFRMS 180
hNTnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX MQEELKEAFRMYDREGNGYI TTATLKEILAAL 180
mNTnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELAEFCRIFDRNADGYI DAEELTEIFRAS 180
*****

fNTnC_library 181:GEHVTEEDIEDLMKDS DKNN DGR ID FDEFLKMMEGVQ XSMADWLRSKMACPNDKTLISTD 240
aNTnC_library 181:GEHVTDDEIESLMKDGDKNN DGR ID FDEFLKMMEGVQ XSMADWLRSKMACPNDKTLISTD 240
cNTnC_library 181:GEQVTEEDIEDMMKDS DKNN DGR ID FDEFLKMMEGVQ XSMADWLRSKMACPNDKTLISTD 240
CrNTnC_library 181:GEAVSEEEIQELMRDGDKNNDGR ID FDEFLKMMEGVQ XSMADWLRSKMACPNDKTLISTD 240
hNTnC_library 181:DDKLTSSDLGIIAEIDT DGS GTV D FDEFMEMMTGE -XSMADWLRSKMACPNDKTLISTD 239
mNTnC_library 181:GEHVTEEEIESLMKDGDKNN DGR ID FDEFLKMMEGVQ XSMADWLRSKMACPNDKTLISTD 240
*****

fNTnC_library 241:KWSYITGSGKRYRSTARTT YTFDKPMAANYLKNQPT YVFRKIELKYSKTEMDFKEWQKAF 300
aNTnC_library 241:KWSYITGSGKRYRSTARTT YTFDKPMAANYLKNQPT YVFRKIELKYSKTEMDFKEWQKAF 300
cNTnC_library 241:KWSYITGSGKRYRSTARTT YTFDKPMAANYLKNQPT YVFRKIELKYSKTEMDFKEWQKAF 300
CrNTnC_library 241:KWSYITGSGKRYRSTARTT YTFDKPMAANYLKNQPT YVFRKIELKYSKTEMDFKEWQKAF 300
hNTnC_library 240:KWSYITGSGKRYRSTARTT YTFDKPMAANYLKNQPT YVFRKIELKYSKTEMDFKEWQKAF 299
mNTnC_library 241:KWSYITGSGKRYRSTARTT YTFDKPMAANYLKNQPT YVFRKIELKYSKTEMDFKEWQKAF 300
*****

fNTnC_library 301:TDVMGMDELYK 311
aNTnC_library 301:TDVMGMDELYK 311
cNTnC_library 301:TDVMGMDELYK 311
CrNTnC_library 301:TDVMGMDELYK 311
hNTnC_library 300:TDVMGMDELYK 310
mNTnC_library 301:TDVMGMDELYK 311
*****

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**Figure S3.** Alignment of amino acid sequences of libraries of GECIs containing truncated troponins C of various fast animals and a derivative of the mNeonGreen fluorescent protein with a *trans*-chromophore. f — *Peregrine falcon*, a — *Acinonyx jubatus* cheetah, c — *Calypte anna* hummingbird, m — *Myotis lucifugus* bat, Cr — *Crotalus adamanteus* rattlesnake, h — *Harpegnathos saltator* ant. Amino acids forming the chromophore are shown in green, linker amino acids in yellow, and amino acids in contact with calcium ions in blue.

```

aNTnCcis_lib 1:MVSKGEEENMASLPATHELHIFGSINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
cNTnCcis_lib 1:MVSKGEEENMASLPATHELHIFGSINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
fNTnCcis_lib 1:MVSKGEEENMASLPATHELHIFGSINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
*****

aNTnCcis_lib 61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
cNTnCcis_lib 61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
fNTnCcis_lib 61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
*****

aNTnCcis_lib 121:IKGEAQVEGTGFPADGLVMTNSLTA XXX SEEELAEFRIF DRNADGY IDAEELAEIFKAS 180
cNTnCcis_lib 121:IKGEAQVEGTGFPADGLVMTNSLTA XXX SEEELANCFRIF DRNADGF IDAEELAEILRAT 180
fNTnCcis_lib 121:IKGEAQVEGTGFPADGLVMTNSLTA XXX SEEELANCFRIF DRNADGF IDIEELGEILRAT 180
*****.*****.***.***.***.***.

aNTnCcis_lib 181:GEHVTDDEIESLMKDG DKNN DGR ID FDE FLKMMEGVQ XXX ADWCVSKKTYPNDKTIVSTL 240
cNTnCcis_lib 181:GEQVTEEDIEDMMKDS DKNN DGR ID FDE FLKMMEGVQ XXX ADWCVSKKTYPNDKTIVSTL 240
fNTnCcis_lib 181:EEHVTEEDIEDLMKDS DKNN DGR ID FDE FLKMMEGVQ XXX ADWCVSKKTYPNDKTIVSTL 240
.***.***.***.***.*****

aNTnCcis_lib 241:KWAFFITDNGKRYRSTARTTYTFAPMAANYLKNQPMYVFRKTELKHSKTELNFKEWQKAF 300
cNTnCcis_lib 241:KWAFFITDNGKRYRSTARTTYTFAPMAANYLKNQPMYVFRKTELKHSKTELNFKEWQKAF 300
fNTnCcis_lib 241:KWAFFITDNGKRYRSTARTTYTFAPMAANYLKNQPMYVFRKTELKHSKTELNFKEWQKAF 300
*****

aNTnCcis_lib 301:TDVMGMDELYK 311
cNTnCcis_lib 301:TDVMGMDELYK 311
fNTnCcis_lib 301:TDVMGMDELYK 311
*****

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**Figure S4.** Alignment of amino acid sequences of GECIs libraries containing truncated troponins C from various fast animals and a derivative of the mNeonGreen fluorescent protein with a *cis*-chromophore. — *Peregrine falcon* falcon, a — *Acinonyx jubatus* cheetah, c — *Calypte anna* hummingbird. Amino acids forming the chromophore are shown in green, linker amino acids in yellow, and amino acids in contact with calcium ions in blue.

```

aTnCtrns      1:MVSKGEEDNMAASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDQLQFSP 60
aTnC_library  1:MVSKGEEDNMAASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDQLQFSP 60
cNtnCtrns     1:MVSKGEEDNMAASLPATHELHIFGSINGVDFDMVGI GSGNPNVGYEELNLKSTKGDQLQFSP 60
cNtnC_library 1:MVSKGEEDNMAASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDQLQFSP 60
fNtnCtrns     1:MVSKGEEDNMAASLPATHELHIFGSINGVDFDMVGQSGNPNVGYEELNLKSTKGDQLQFSP 60
fNtnC_library 1:MVSKGEEDNMAASLPATHELHIFGSINGVDFDMVGQSGGNPNVGYEELNLKSTKGDQLQFSP 60
*****.*****.*****.*****

aTnCtrns      61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
aTnC_library  61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
cNtnCtrns     61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
cNtnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
fNtnCtrns     61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
fNtnC_library 61:WVLVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRIVQFEDGASLTVDYRYTYEGSH 120
*****.*****

aTnCtrns      121:IKGEAQVKGTGFPADGPMANSLTARVQ SEEELAECFCIFDGNADGYIDAEEMAEIFKAS 180
aTnC_library  121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELAECFRIFDRNADGYIDAEELAEIFKAS 180
cNtnCtrns     121:IKGEAQVKGTGFPADGPMANSLTARVY SEVELANCFRIFDRNADGFIDAEELAEILRIT 180
cNtnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELANCFRIFDRNADGFIDAEELAEILRAT 180
fNtnCtrns     121:IKGEAQVKGTGFPADGPMANSLTARVY SEEELANSFRICDRNADGFIDIEELGEILRAT 180
fNtnC_library 121:IKGEAQVKGTGFPADGPMANSLTARVX SEEELANCFRIFDRNADGFIDIEELGEILRAT 180
*****.*****

aTnCtrns      181:GEHVTDEIEDLSMKDGDKNNDGRIDFDEF LKMMEGVQMSMANWLRSKMACPNDKTLLIST 240
aTnC_library  181:GEHVTDEIEDLSMKDGDKNNDGRIDFDEF LKMMEGVQXSMADWLRSKMACPNDKTLLIST 240
cNtnCtrns     181:GEQVTEEDIEDMMKDS DKNNDGRIDFDEF LKMTIEGVQLMVDWLRSKMACPNDKTLLIST 240
cNtnC_library 181:GEQVTEEDIEDMMKDS DKNNDGRIDFDEF LKMMEGVQXSMADWLRSKMACPNDKTLLIST 240
fNtnCtrns     181:ERVTEEDIEDLMKDS DKNNDGRIDFDEF LKMMEGAQLSMADWLRSKMACPNDKTLLIST 240
fNtnC_library 181:GEHVTDEIEDLMKDS DKNNDGRIDFDEF LKMMEGVQXSMADWLRSKMACPNDKTLLIST 240
.* **...*.*****.*****

aTnCtrns      241:KWSYITGSGKRYRSTARTTYTFDKPMAANYLKNQPTYVFRKIELKYSKTEMDFKEWQKAF 300
aTnC_library  241:KWSYITGSGKRYRSTARTTYTFDKPMAANYLKNQPTYVFRKIELKYSKTEMDFKEWQKAF 300
cNtnCtrns     241:KWSYITGSGNRYRATARTTYTFDKPMAANYLKNQPTYVSRKIELKYFKTEMDFIEWQKAF 300
cNtnC_library 241:KWSYITGSGKRYRSTARTTYTFDKPMAANYLKNQPTYVFRKIELKYSKTEMDFKEWQKAF 300
fNtnCtrns     241:KWSYITGSGKRYRITARTTYTFDKPMAANYLKNQPTYVFRKIELKYSKTEMDFKEWQKAF 300
fNtnC_library 241:KWSYITGSGKRYRSTARTTYTFDKPMAANYLKNQPTYVFRKIELKYSKTEMDFKEWQKAF 300
*****.***.*****.*****

aTnCtrns      301:TDVMGMDELYK 311
aTnC_library  301:TDVMGMDELYK 311
cNtnCtrns     301:TDV VGMDELYK 311
cNtnC_library 301:TDVMGMDELYK 311
fNtnCtrns     301:TD VGMDELYK 311
fNtnC_library 301:TDVMGMDELYK 311
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**Figure S5.** Alignment of amino acid sequences for developed indicators with *trans*-chromophore and libraries of calcium indicators containing truncated troponins C of various fast animals and a derivative of mNeonGreen fluorescent protein with *trans*-chromophore. f — *Peregrine falcon* falcon, a — *Acinonyx jubatus* cheetah, c — *Calypte anna* hummingbird. Linker amino acids are in red, chromophore-forming amino acids are in green, amino acids in contact with calcium ions are in blue, and amino acid substitutions in the resulting mutants compared to the original libraries are in pink, amino acids of linkers in the resulting mutants after overlap mutagenesis are in yellow.

```

aNTnCcis      1:MVSKGEEENMASLPATHELHIFGSIINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
aNTnCcis_lib  1:MVSKGEEENMASLPATHELHIFGSIINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
cNTnCcis      1:MVSKGEEENMASLPATHELHIFGSIINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
cNTnCcis_lib  1:MVSKGEEENMASLPATHELHIFGSIINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
fNTnCcis      1:MVSKGEEENMASLPATHELHIFGSIINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
fNTnCcis_lib  1:MVSKGEEENMASLPATHELHIFGSIINGIDFDMVGQGTGNPNPDGYEELNLKSTMGDLQFSP 60
                *****.*****.*****

aNTnCcis      61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGA LTVNRYRYTEGSH 120
aNTnCcis_lib  61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
cNTnCcis      61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
cNTnCcis_lib  61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
fNTnCcis      61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
fNTnCcis_lib  61:WILVPHI GYG FHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNRYRYTEGSH 120
                *****.***.***.*****

aNTnCcis      121:IKGEAQVEGTGFPADG P VMTNSLTAKIF SEEELAECS IFDCNADGY IDAEELAEIFKAS 180
aNTnCcis_lib  121:IKGEAQVEGTGFPADGLVMTNSLTAXXX SEEELAECSFRIFDRNADGY IDAEELAEIFKAS 180
cNTnCcis      121:IKGEAQVEGTGFPADG P VMTNSLTAKIY SEEELAECSFRIFDRNADGFI DAEELAEILRAT 180
cNTnCcis_lib  121:IKGEAQVEGTGFPADGLVMTNSLTAXXX SEEELANCFRIFDRNADGFI DAEELAEILRAT 180
fNTnCcis      121:IVGEAQVEGTGFPADG P VMTNSLTAKIST SEEELANCFRIFDRNADGFI DIEELGEILRAT 180
fNTnCcis_lib  121:IKGEAQVEGTGFPADGLVMTNSLTAXXX SEEELANCFRIFDRNADGFI DIEELGEILRAT 180
                *.***** **.****** *****.*.*.*****.***.***.*.*..

aNTnCcis      181:GEHVTDEIESLMKDG DKNDGRIDFDE FLKMMEGVQ VVPT DWCVSKKTYPNDKTIVSTL 240
aNTnCcis_lib  181:GEHVTDEIESLMKDG DKNDGRIDFDE FLKMMEGVQ XXXADWCVSKKTYPNDKTIVSTL 240
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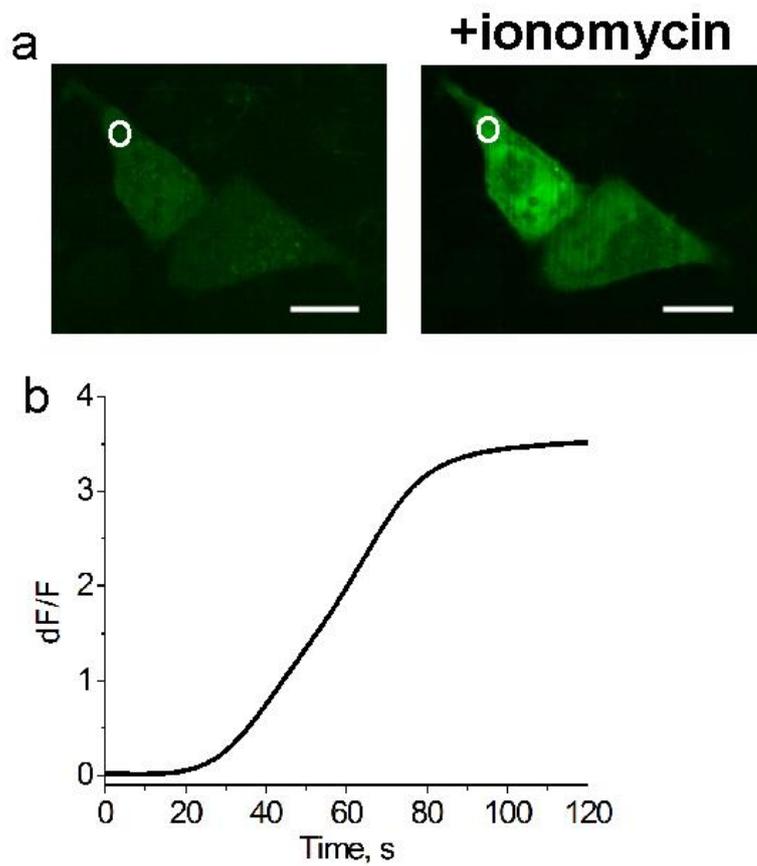
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                **.*...*****.*****.*****.*****

aNTnCcis      301:TDVMGMDELYK 311
aNTnCcis_lib  301:TDVMGMDELYK 311
cNTnCcis      301:TDVMGMDELYK 311
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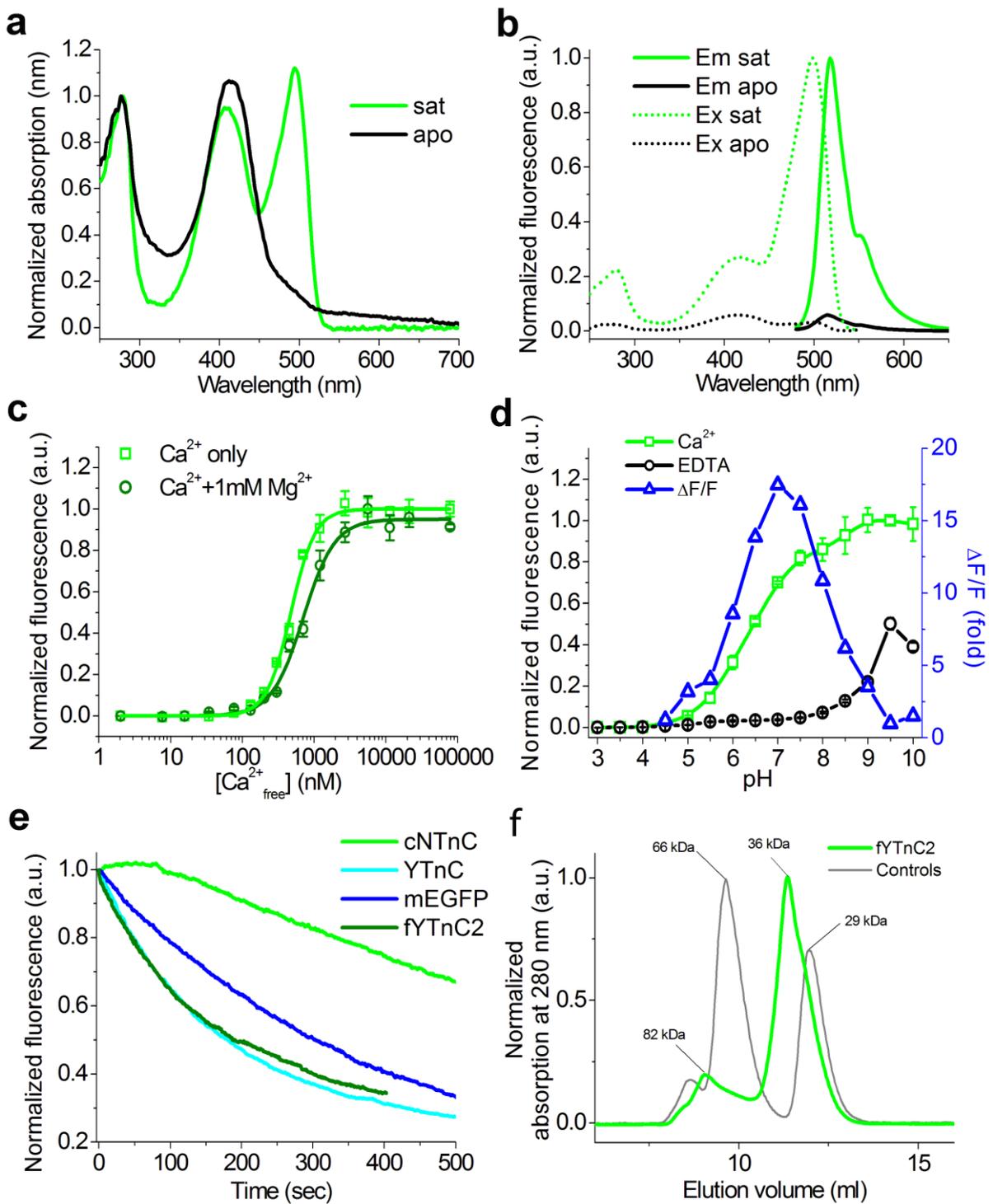
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**Figure S6.** Alignment of the amino acid sequences of the developed indicators with a cis chromophore and libraries of calcium indicators containing truncated troponins C from various fast animals and a derivative of the mNeonGreen fluorescent protein with a cis chromophore. f — *Peregrine falcon*, a — *Acinonyx jubatus* cheetah, c — *Calypte anna* hummingbird. Chromophore-forming amino acids are in green, linker amino acids are in yellow, amino acids in contact with calcium ions are in blue, and amino acid substitutions in the resulting mutants compared to the original libraries are in pink.

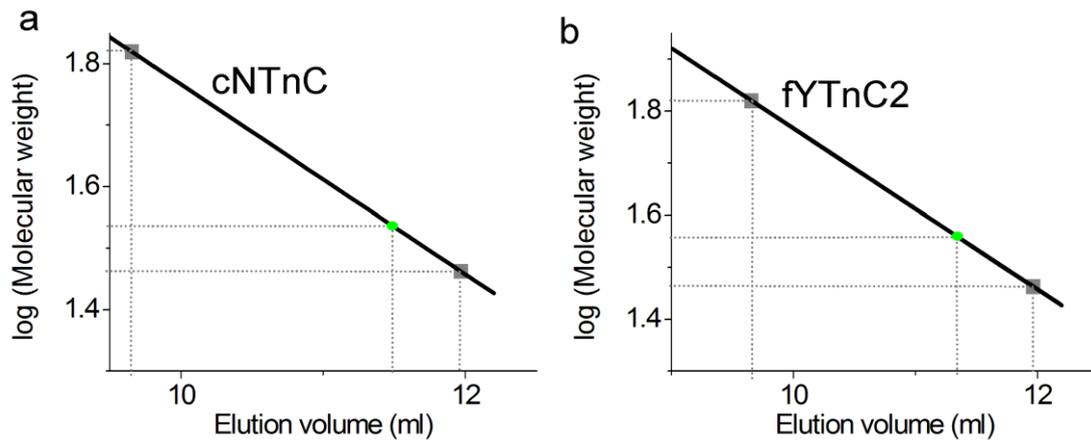




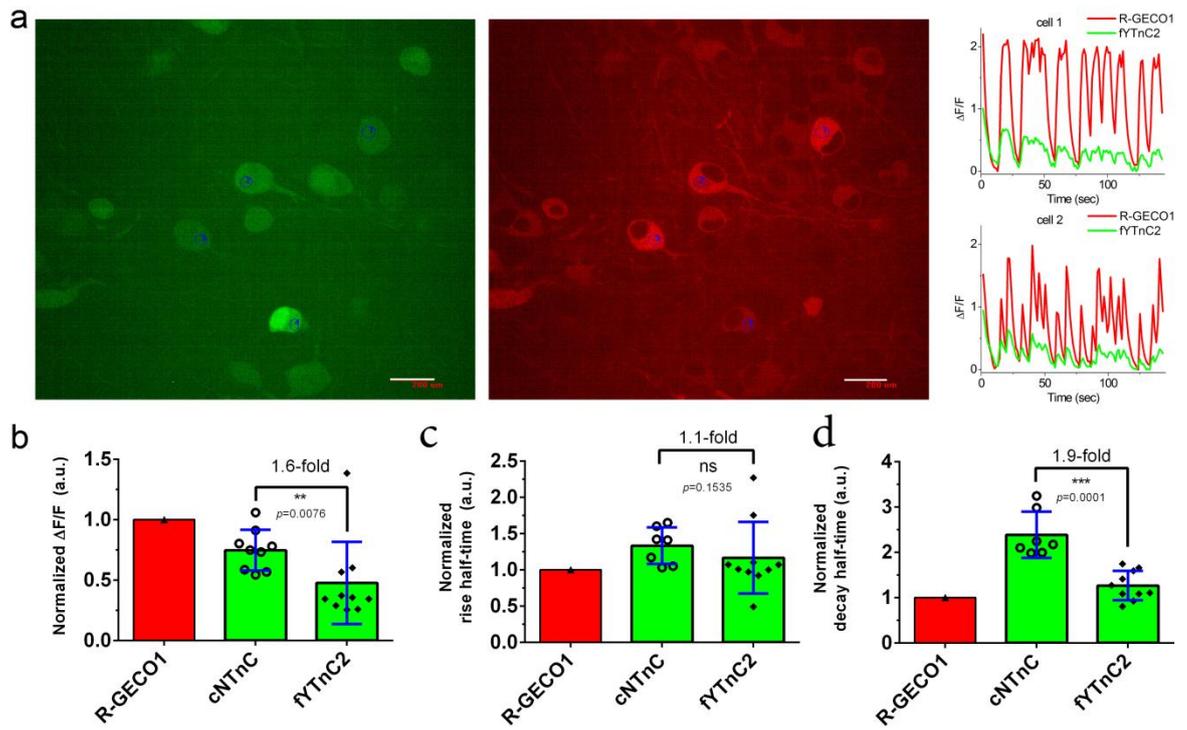
**Figure S8.** Response of fYTnC2 to ionomycin-induced increase of  $\text{Ca}^{2+}$  concentration in HeLa Kyoto cells. Top, confocal images of HeLa cells expressing fYTnC2 in green fluorescence channel (with 488 nm excitation) before and after ionomycin addition. Bottom, the graph shows the changes in green fluorescence as a result of the addition of 5  $\mu\text{M}$  ionomycin. The graph illustrates changes in green fluorescence in the areas indicated with white circles. Scale bars, 100  $\mu\text{m}$ .



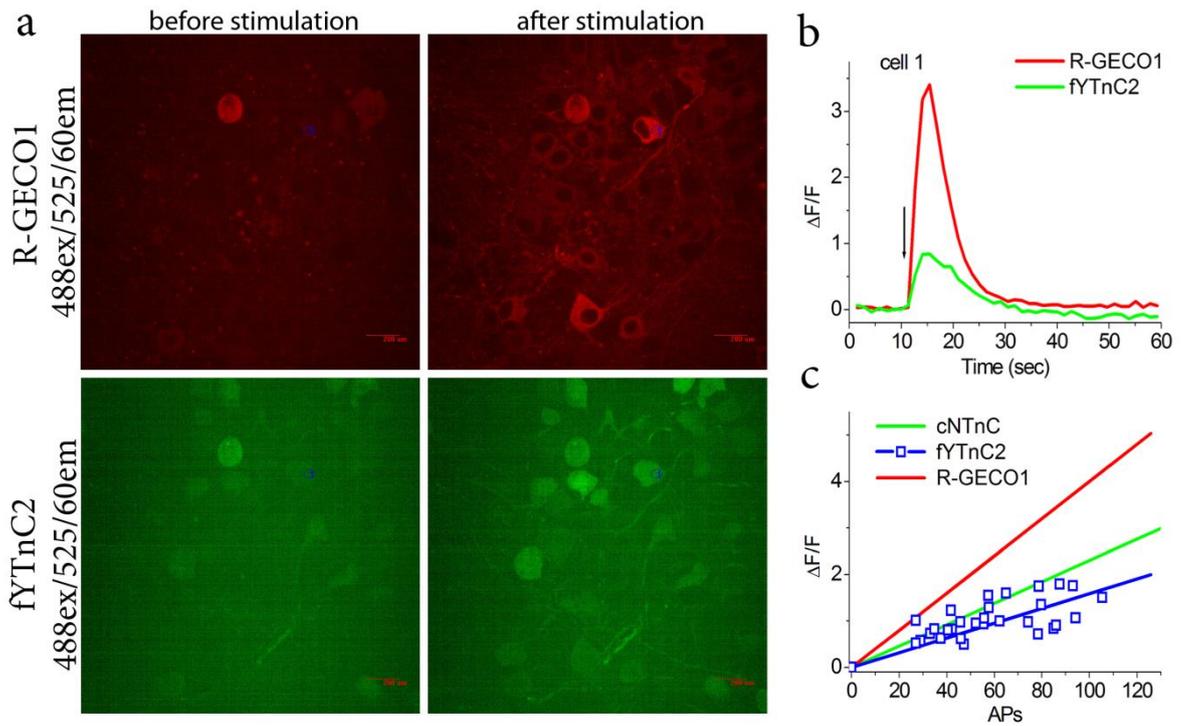
**Figure S9.** Properties fYTnC2 in vitro. (a) Absorbance spectra of fYTnC2 in Ca<sup>2+</sup>-free (apo) and Ca<sup>2+</sup>-bound (sat) states. (b) Excitation and emission spectra of fYTnC2 in Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-bound states. (c) Ca<sup>2+</sup> titration curves for fYTnC2 in the absence or presence of 1 mM MgCl<sub>2</sub>. (d) Intensity and dynamic range of fYTnC2 as a function of pH. The ΔF/F fluorescence response (fold) at each pH value was determined as the ratio of fYTnC2 fluorescence intensity in the absence of Ca<sup>2+</sup> to that in the presence of Ca<sup>2+</sup>. (e) Photobleaching curves for fYTnC2, cNTnC and YTnC in the Ca<sup>2+</sup>-bound states and for mEGFP. (f) Fast protein liquid chromatography of fYTnC2. fYTnC2 was eluted in 20 mM Tris-HCl (pH 7.80) and 200 mM NaCl buffer. The molecular weight of fYTnC2 was calculated from a linear regression of the dependence of the logarithm of the control molecular weights vs. elution volume (**Figure S7**). Error represents the standard error of the estimate for the average of three records.



**Figure S10.** Fast protein liquid chromatography of cNTnC and fYTnC2 protein. cNTnC (a) and fYTnC2 (b) were eluted in 20 mM Tris-HCl (pH 7.80) and 200 mM NaCl buffer supplemented with 5 mM CaCl<sub>2</sub> as shown in Figures 8f and S6f. The molecular weight of cNTnC and fYTnC2 were calculated from the dependence of logarithm of control molecular weights vs. elution volume.



**Figure S11.** Spontaneous neuronal activity of fYTnC2 co-expressed with R-GECO1 in neuronal cultures. (a) Left, confocal images of green and red fluorescence with excitation at 488 and 561 nm of HeLa cells co-expressing fYTnC2 with R-GECO1. Scale bars, 50  $\mu\text{m}$ . Right, the graphs show the changes in green and red fluorescence in two cells in the areas indicated with blue circles. (b) The  $dF/F$  responses for cNTnC and fYTnC2 were calculated according to the  $dF/F$  response of R-GECO1 in the same cell. (c,d) The rise (c) and decay (d) half-times for cNTnC and fYTnC2. (b–d) Error bars are the standard deviations across 7–10 cells. Ns, not significant,  $p>0.05$ . \*\*,  $p$ -value is from 0.001 to 0.01. \*\*\*,  $p$ -value is from 0.0001 to 0.001.



**Figure S12.** Comparison of the responses of green fYTnC2 and red R-GECO1 indicators to the external field stimulation of neurons co-expressing the GECIs in dissociated neuronal cultures. Neuronal cultures co-expressing the NES-fYTnC2 and NES-R-GECO1 indicators were imaged and stimulated on DIV 21–22th. Neuronal cultures were transduced on DIV fourth with a mixture of rAAVs carrying NES-fYTnC2 and NES-R-GECO1. (a) Confocal images of neuronal culture co-expressing the NES-cNTnC and NES-R-GECO1 indicators before (left) and after (right) electrical stimulation. Scale bar, 50  $\mu\text{m}$ . (b) The graph illustrates  $\Delta F/F$  changes in the green and red fluorescence of the fYTnC2 and R-GECO1 indicators in response to an electrical field stimulation. The changes on the graph correspond to the area indicated on panel a as a white circle. (c) The dependence of  $\Delta F/F$  responses for the fYTnC2 indicator vs. the number of action potentials (APs). A number of APs was determined according to the  $\Delta F/F$  response of the R-GECO1 indicator (0.04 per 1 AP) co-expressed in the same cell and assuming that response's linearity in the examined AP range (linear fitting for fYTnC2 had R2 value of 0.68211). The dependences of  $\Delta F/F$  responses on APs for cNTnC and R-GECO1 were added for comparison.

## Supplementary Methods.

### *Preparative protein purification.*

For preparative protein purification for X-ray crystallography, the bacterial cells expressing the NTnC protein with a His-tag and Tobacco Etch Virus (TEV) protease cleavage site were harvested by centrifugation for 20 min at 5,000×rpm and 4 °C using Avanti J-E centrifuge (Beckman Coulter, USA). The pellet was further resuspended in 40 mM Tris(tris(hydroxymethyl)aminomethane)-HCl buffer, pH 7.5 supplemented with 400 mM NaCl, 10 mM Imidazole, 0.2% Triton X-100, and 1 mM PMSF (phenylmethylsulfonyl fluoride) (7 ml per 1 g of the cells), and disrupted by sonication (2 sec pulse-6-sec pause, 45% amplitude, for total time of 5 minutes). The crude cell extract was centrifuged for 30 min at 28,000×g and 4 °C using Avanti J-E centrifuge (Beckman Coulter, USA). The supernatant was applied to a 5 ml Ni-NTA Superflow column (Qiagen, Hilden, Germany) equilibrated with the binding buffer (40 mM Tris-HCl, pH 7.5, containing 400 mM NaCl, 10 mM imidazole, and 0.1% (v/v) Triton X-100). The column was further washed using the same binding buffer without Triton X-100 followed by second washing using binding buffer in the absence of Triton X-100, supplemented with 40 mM imidazole. Protein elution was performed using the same binding buffer without Triton X-100 and supplemented with 300 mM imidazole. 1mM DTT (dithiothreitol) (final concentration) and 1mM EDTA (ethylenediaminetetraacetic acid) (final concentration) were added to the protein solution and mixed with TEV protease (1 mg per 10 mg of the protein). The final mix was dialyzed for 16 hours in dialysis buffer (40 mM Tris-HCl, pH 7.8, 400 mM NaCl, 5 mM Imidazole, 2 mM BME (2-mercaptoethanol), 1 mM EDTA), at +4 °C (monitoring of the His-tag cleavage was conducted using 12 % SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)). After dialysis, the protein solution was applied to a Ni-NTA Superflow column (Qiagen, EU), equilibrated with dialysis buffer; TEV protease and cleaved His-tag were absorbed by the Ni-NTA Superflow column (Qiagen, EU) and the flow-through was concentrated till 2.3 ml volume using a 10 kDa cutoff centrifugal filter device (Millipore, Burlington, MA, USA). The buffer of the concentrated protein was exchanged for the 20 mM Tris pH 7.5, 5mM CaCl<sub>2</sub>, 5% (v/v) glycerol buffer using PD-10 column (GE Healthcare, Sweden). The concentrated protein was further applied to a 1 ml ResourceQ column (GE Healthcare, Sweden) equilibrated with the same 20 mM Tris pH 7.5, 5mM CaCl<sub>2</sub>, 5% (v/v) glycerol buffer. Recombinant NTnC was eluted using a linear gradient from 0 to 1M NaCl; protein eluted by one peak at 250 mM NaCl. Protein concentration was measured for each of the fractions individually using Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, Saint Louis, USA), BSA (bovine serum albumin) protein standard (P0914-5AMP, Sigma-Aldrich, Saint Louis, USA) solution as standard. Total protein yield was 4.8 mg. The fractions were loaded onto the Superdex 75 10/300 GL column (GE Healthcare, Sweden), in 20 mM Tris-HCl pH 8.0, 5mM CaCl<sub>2</sub>, 250 mM NaCl buffer. In both cases, 15.4-17.5 ml fractions (corresponding to monomer) were collected and concentrated using a 10 kDa cutoff centrifugal filter device (Millipore, Burlington, MA, USA) till 15 mg/ml concentration for the crystallization. Protein purity at each step was monitored using 12% SDS-PAGE. Chromatography was performed using ÄKTA prime plus and ÄKTA explorer 100 systems (GE Healthcare, Sweden).

### *Mammalian cell imaging*

Transient transfection of the HeLa Kyoto cells was performed in 24-well format using lipofectamine reagent according to the manufacture's protocol. Cells were cultured using DMEM medium supplemented with 10% FBS, Glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin, at 37 degrees and 5% CO<sub>2</sub>. HeLa cell cultures were imaged 24 h after the transient transfection using a laser spinning-disk

Andor XDi Technology Revolution multi-point confocal system (Andor Technology, UK) equipped with an inverted Nikon Eclipse Ti-E/B microscope (Nikon Instruments, Japan), a 75 W mercury-xenon lamp (Hamamatsu, Japan), a 60× oil immersion objective NA 1.4 (Nikon, Japan), a 16-bit Neo sCMOS camera (Andor Technology, UK), laser module Revolution 600 (Andor Technology, UK), spinning-disk module Yokogawa CSU-W1 (Andor Technology, UK). The green and red fluorescence were acquired using the 488 nm or 561 nm lasers, confocal dichroic mirror 405/488/561/640 and filter wheel, 525/50 or 617/73 emission filters, respectively. During imaging cells were incubated at 37 degrees and 5% CO<sub>2</sub> using a cage incubator (Okolab, Italy).

### *Imaging of neuronal cultures*

Dissociated neuronal cultures were isolated from the C57BL/6 mice at postnatal days 0–1 and were grown on a 24-well cell imaging black plate with a glass bottom; then, the tissue cultures were treated (Eppendorf, Hamburg, Germany) in Neurobasal Medium A (GIBCO, Paisley, Scotland, UK) supplemented with 2% B27 Supplement (GIBCO, Paisley, Scotland, UK), 0.5 mM glutamine (GIBCO, Paisley, Scotland, UK), 50 U/mL penicillin, and 50 g/mL streptomycin (GIBCO, Paisley, Scotland, UK). On the fourth day in vitro, neuronal cultures were transduced with a mixture of rAAV viral particles (DJ serotype) carrying AAV-CAG-NES-R-GECO1 and AAV-CAG-NES-cNTnC, AAV-CAG-NES-aNTnC, AAV-CAG-NES-fNTnC, AAV-CAG-NES-cYTnC, AAV-CAG-NES-aYTnC, or AAV-CAG-NES-fYTnC. The cells were imaged using an Andor XDi Technology Revolution multi-point confocal system on DIV 15 (spontaneous activity at 37°C, 5% carbon dioxide) and 21–22 (electrical field stimulation at r.t.).

Stimulation of neuronal cultures was performed using a self-built electrical system described earlier [1]. In this step, 300 voltage pulses of a 1 ms duration (0.5 ms negative phase, 0.5 ms interphase, and 0.5 ms positive phase) at a 87 Hz frequency with an amplitude of +70 V were applied to the neuronal cultures in 24-well plates through iridium electrodes with a 5 mm gap. Then, 10 μM cyanquinoxaline (6-cyano-7-nitroquinoxaline-2,3-dione) (CNQX) and 100 μM (2R)-amino-5-phosphonovaleric acid (APV or AP5) were added before stimulation to block spontaneous neuronal activity. To quantify the fluorescence intensity, the background noise determined from the adjacent cell-free area was subtracted from mean fluorescence intensity value for the cytosolic sub-region of the cell of the similar area. The rise and decay half-times were calculated as time difference between time point corresponding to the calcium spike maximum and time points at half-maximum on the left and right edges of the spike, respectively.

### **References**

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2. Tsien, R. Y., The green fluorescent protein. *Annu Rev Biochem* **1998**, *67*, 509-44.