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Supplementary Materials for

Bright and sensitive red voltage indicators for imaging action potentials in brain slices and pancreatic islets

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This PDF file includes:

Supplementary Figures

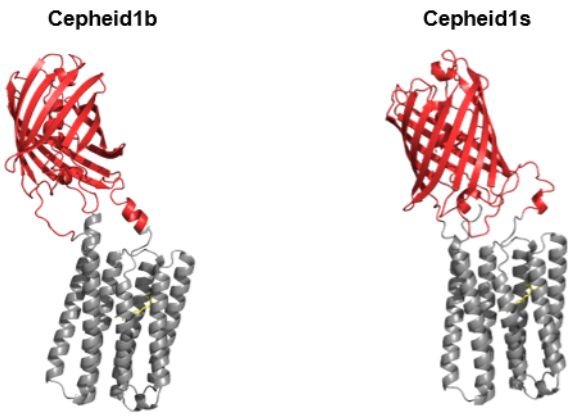

Fig. S1. Predicted tertiary structures of Cepheid1b and Cepheid1s by AlphaFold2. Retinal and RFP fluorophore are added by manual alignment with crystallography data in RCSB PDB.

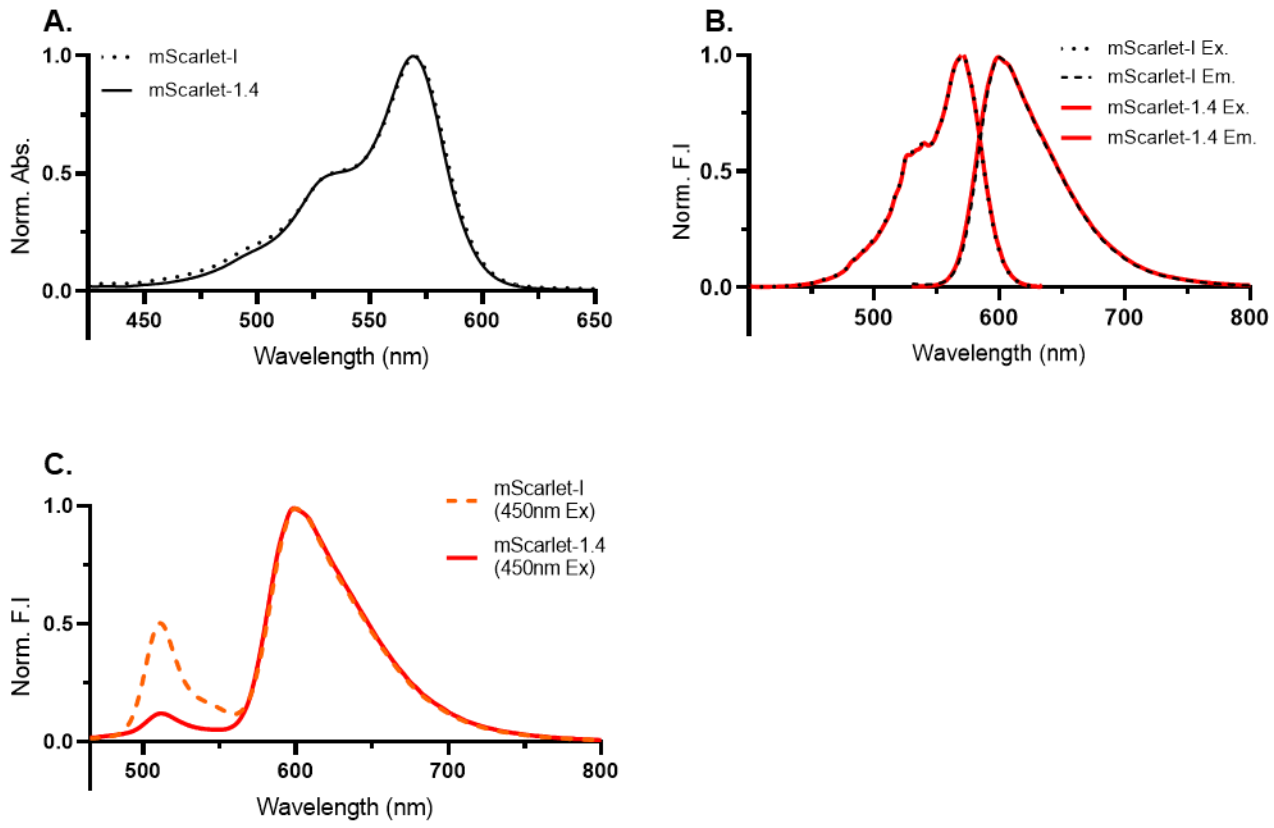

Fig. S2. Spectra of mScarlet-I1.4 and mScarlet-I. (A) Normalized absorption spectra. **(B)** Normalized excitation and emission spectra (right). **(C)** Normalized emission spectra of mScarlet-I and mScarlet-I1.4 excited with 450 nm (10 nm bandwidth) light.

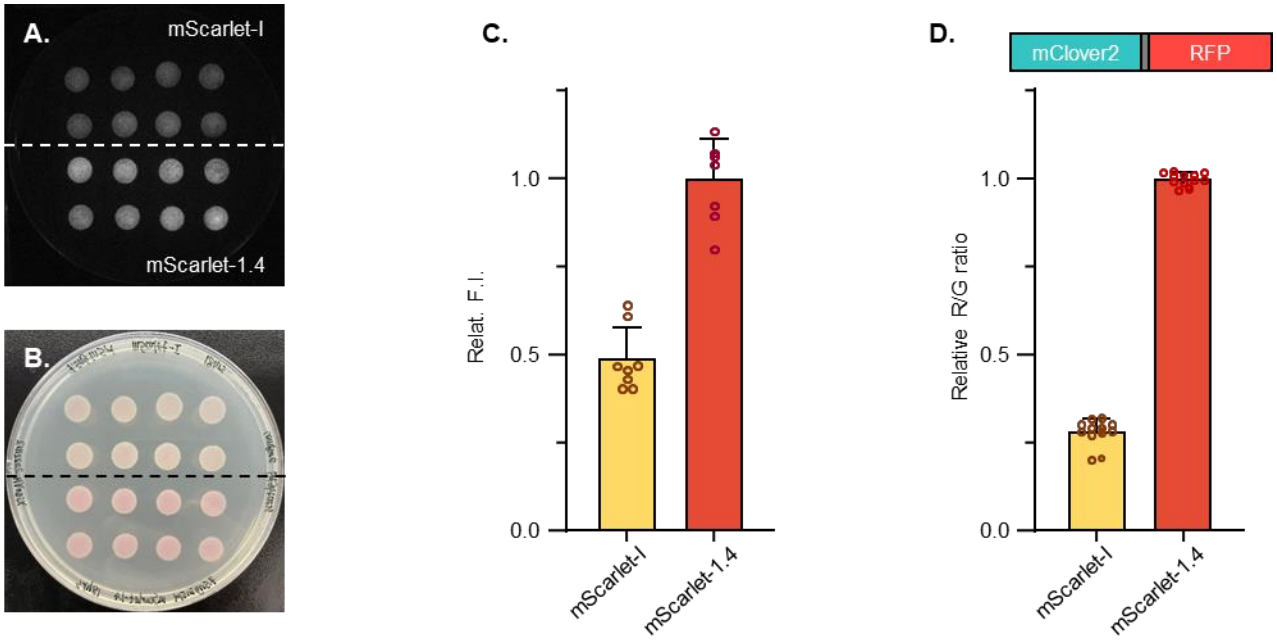

Fig. S3. Brightness comparison of mScarlet-I1.4 to mScarlet-I in E.coli. (A) Red channel image of E.coli transfected with the same amount of pNCs_mScarlet-I or pNCs_mScarlet-I1.4 plasmids and cultured under 34 °C for 12 hr. **(B)** LED illuminated brightfield image of the same petri dish in **A**. **(C)** Relative red channel fluorescent intensity of E.coli, mScarlet-I1.4 is about 1 fold brighter than mScarlet-I, **(D)** Clover2 were fused to the N terminal of mScarlet-I and mScarlet-I1.4 with the same flexible linker, both of these fused FPs were cloned into pNCs vector and introduced into E.coli, after 12 hr culture under 34 °C in solid LB plate bacterial colonies were suspended in PBS and measured with microplate reader (Tecan M1000pro).

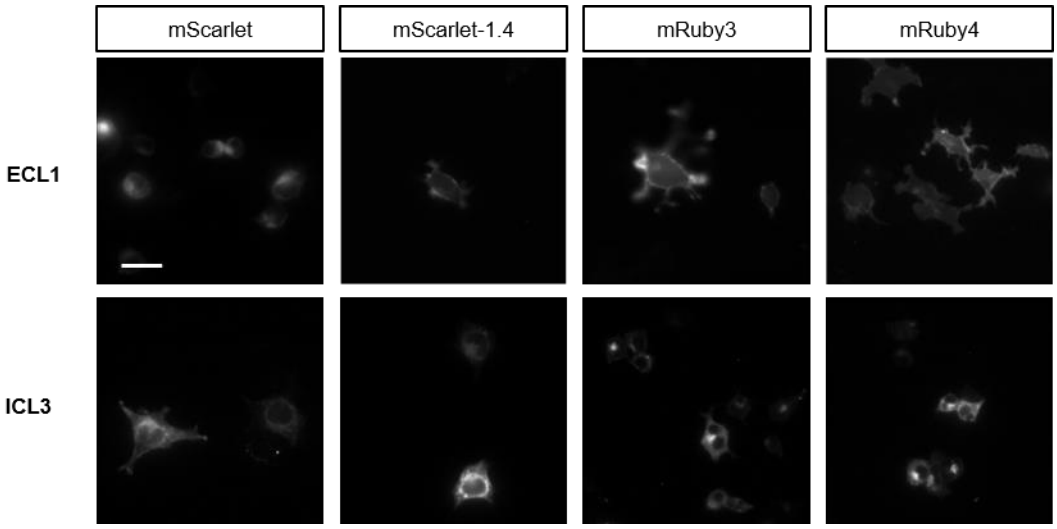

Fig. S4. Epifluorescence images showing expression and trafficking of extracellular loop1 (ECL1) inserted variants. Scale bar = 20 μm.

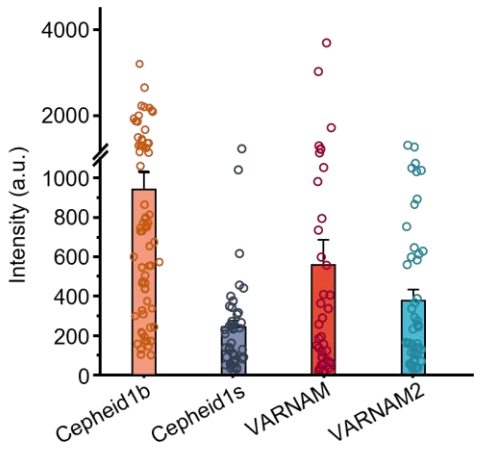

Fig. S5. Brightness of Cepheid indicators and VARNAM series. Each data point indicates the average photon count number in one cell, under wide field epifluorescence imaging with 561 nm laser.

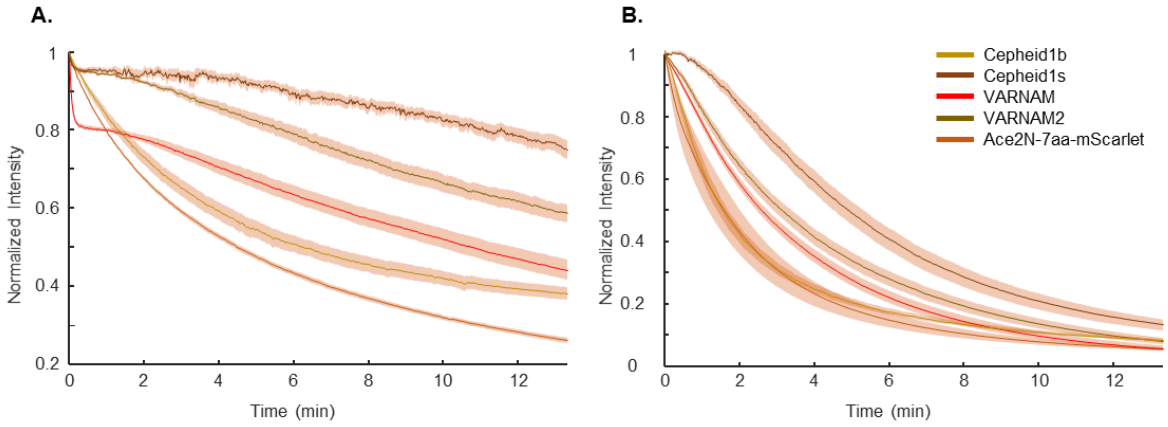

Fig. S6. Photostability comparison of Cepheid1 and other red GEVIs. (A) 1.59 W/cm^2 561 nm laser illumination (n = 7, 7, 6, 6, 5 cells). **(B)** 7.95 W/cm^2 561 nm laser illumination (n = 7, 7, 7, 6, 5 cells).

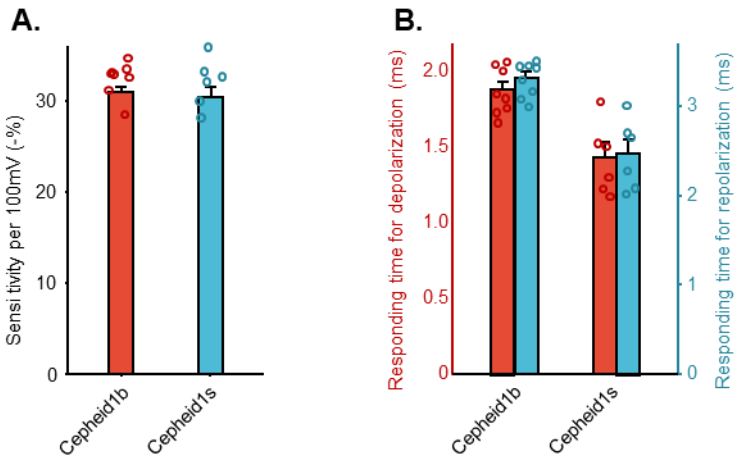

Fig. S7. Characteristics of Cepheid1 voltage response sensitivity and kinetics in HEK293T cells. (A) Sensitivity of Cepheid1b and Cepheid1s to 100 mV (-70 mV to +30 mV). **(B)** Kinetics of Cepheid1b and Cepheid1s to depolarization (-70 mV to +30 mV) and to repolarization (+30 mV to -70 mV).

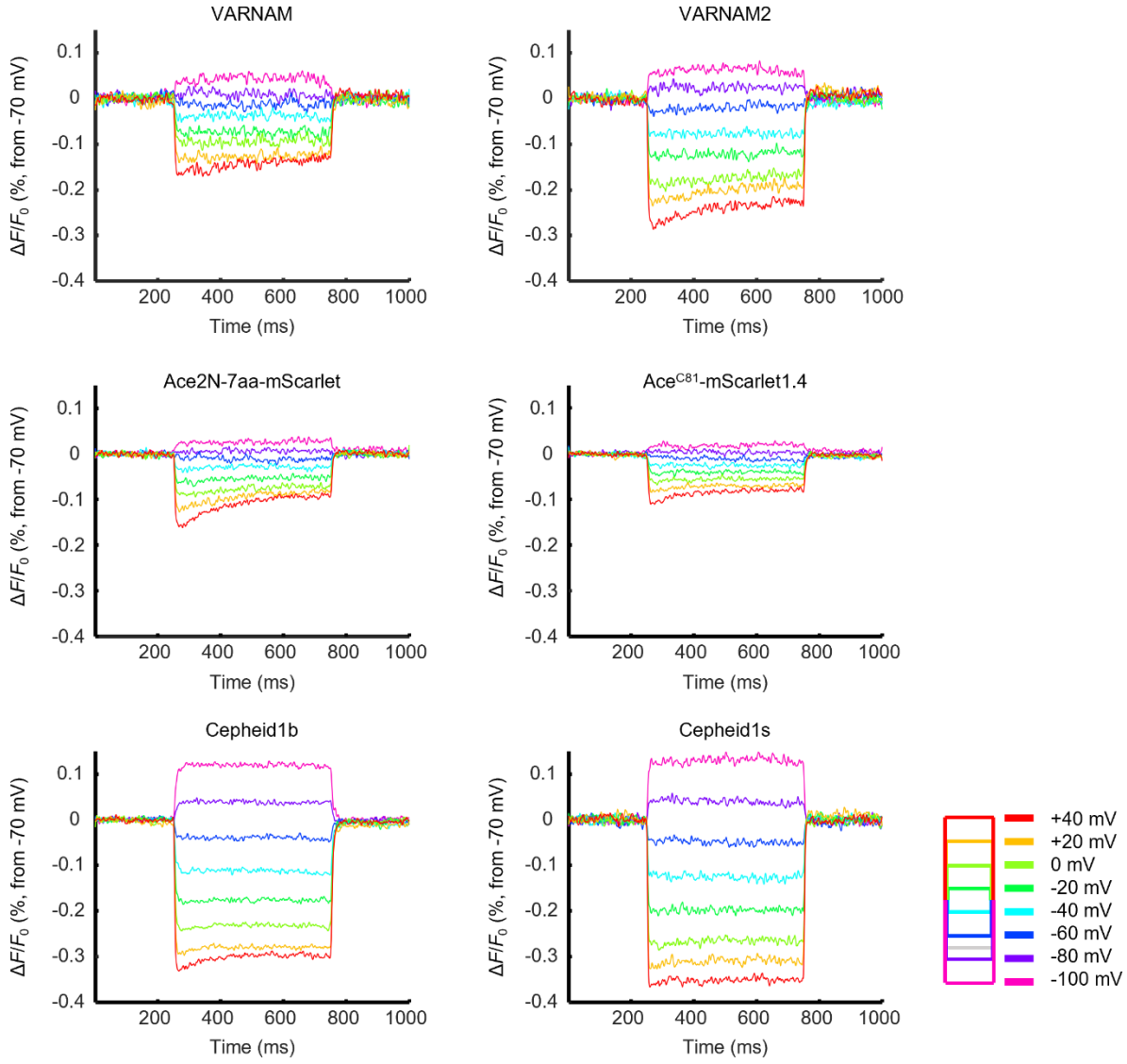

Fig. S8. Representative fluorescence traces of red GEVIs in response to a series of voltage steps. The membrane potential was controlled via whole-cell voltage clamp, and a series of step waveforms were applied from -100 mV to 40 mV in increments of 20 mV). The dynamic range has been normalized to the fluorescence at membrane voltage V_m = 70 mV.

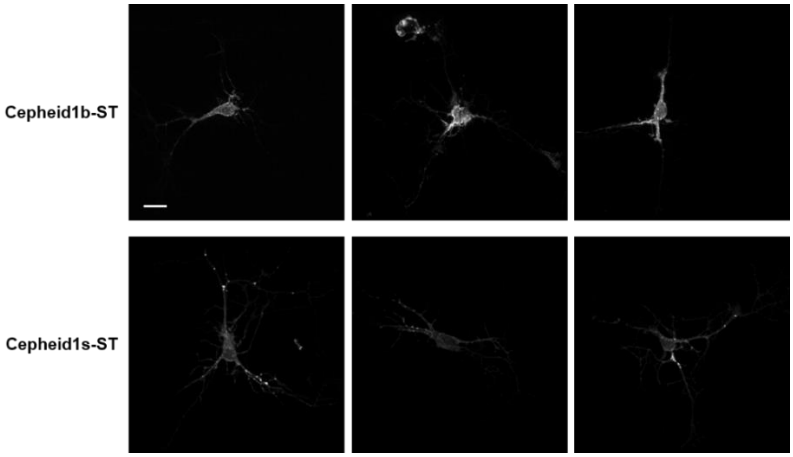

Fig. S9. Localization of Cepheid1b/s-ST in cultured neurons. Images acquired by confocal Z projection. Scale bar = 20 μm.

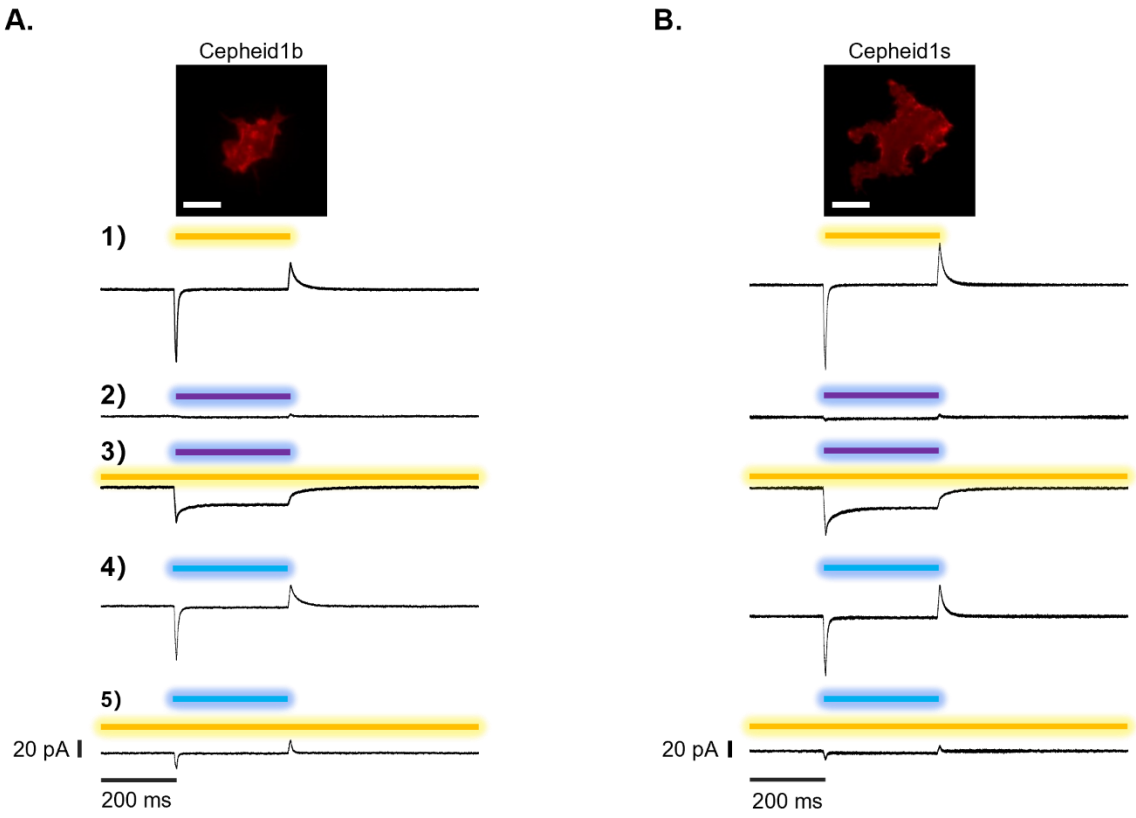

Fig. S10. Photocurrent of Cepheid1b and Cepheid1s. Photocurrent of HEK293T cells expressing Cepheid1b **(A)** and Cepheid1s **(B)** measured in different illumination conditions**.** Top: epifluorescence images of GEVI-expressing HEK293T cells. Bottom: photocurrent elicited by illumination or co-illumination at 561 nm (1.6 to 1.7 W/cm^2, yellow bar), 405 nm (1.6 W/cm^2, purple bar) and 488 nm (3.9 to 4.3 W/cm^2, blue bar). Scale bar = 20 μm.

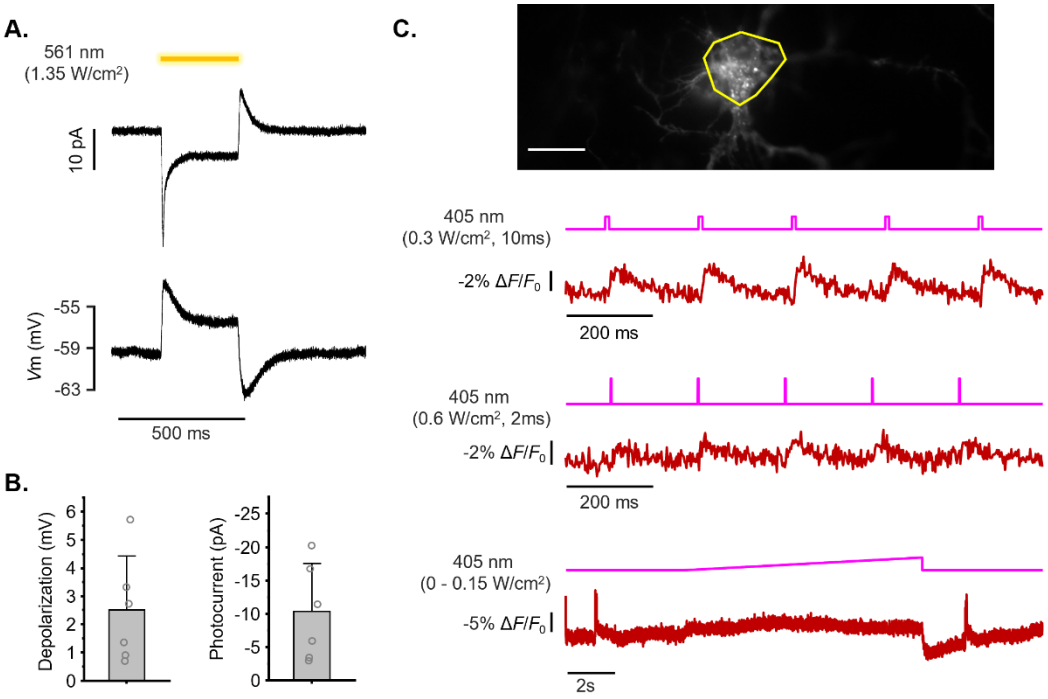

Fig. S11. Optical crosstalk in all-optical electrophysiology experiments. (A-B) Neuron expressing Cepheid1b-ST-P2A-CheRiff was illuminated with 561 nm laser at imaging intensity, while photocurrent and depolarization were recorded via whole-cell patch clamp. Example trail-averaged traces **(A)** and statistics **(B)** are shown. **(C)** Top, example of a neuron expressing Cepheid1s-ST; yellow circle is the ROI, and scale bar is 20 μm. Below, red fluorescence artifact caused by 405 nm illumination.

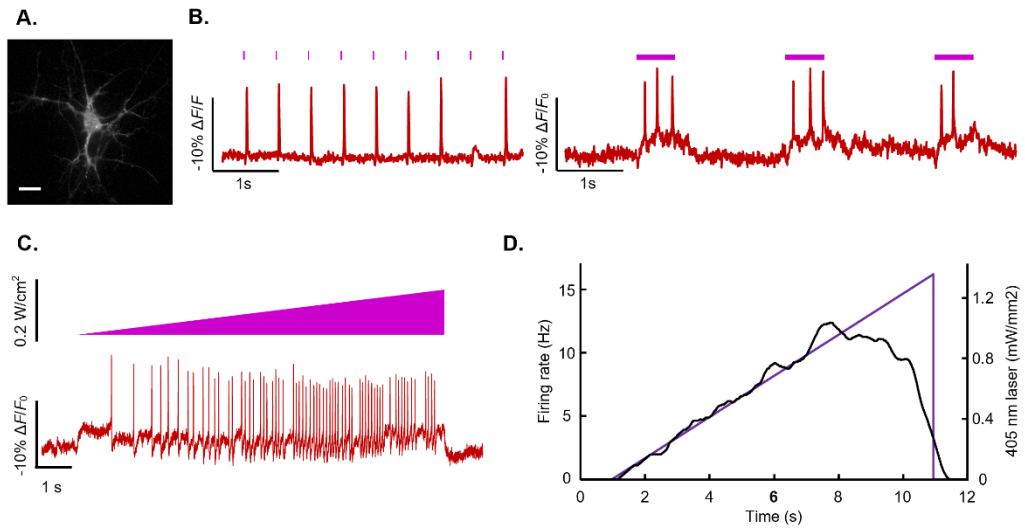

Fig. S12. All-optical electrophysiology in cultured neurons. (A) Epifluorescence images showing expression of Cepheid1s-ST in a neuron transfected with Cepheid1s-ST-P2A-CheRiff (scale bar = 20 μm). **(B)** APs triggered by 405 nm pulse illumination, recorded by Cepheid1s-ST under wide field illumination with 561 nm laser. **(C)** APs triggered by 405 nm ramp illumination and recorded by Cepheid1s-ST. **(D)** Firing rate in the trial in **(C)** aligned with stimulation intensity.

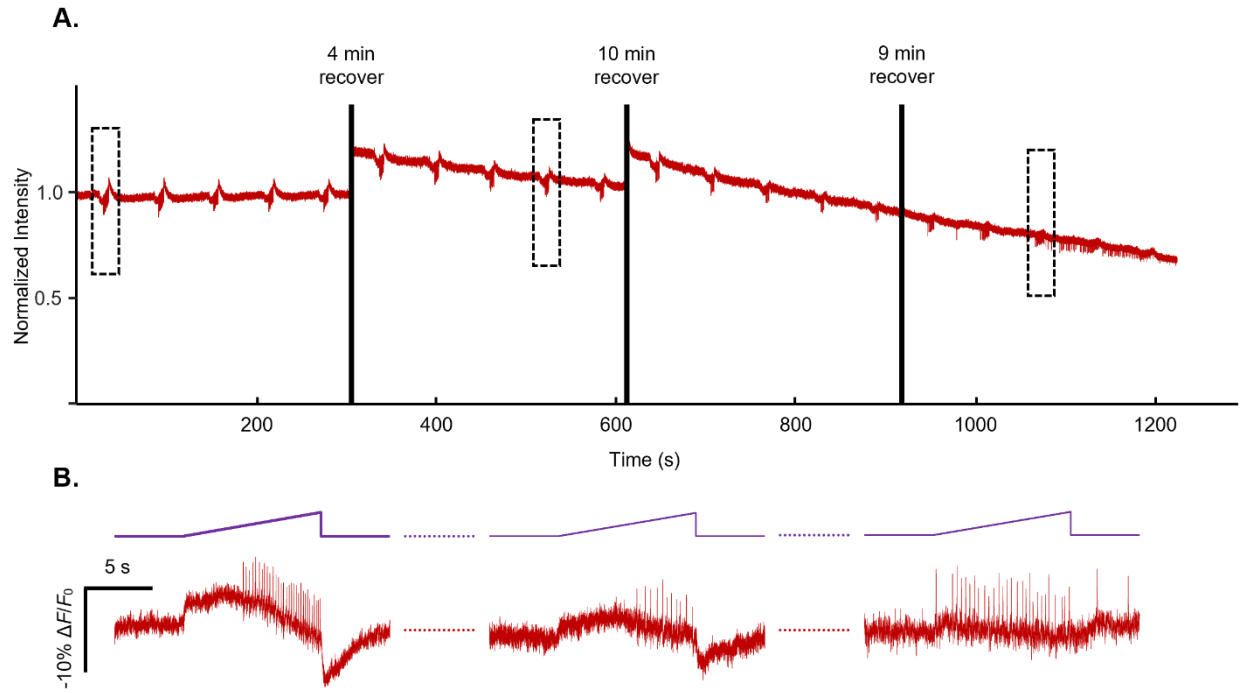

Fig. S13. long term optical recording under photo-activation of CheRiff. (A) Photobleaching curve of the long-term imaging with Cepheid1s-ST. 10 s-long, ramp-shaped stimulation is conducted every 60s. The cultured neuron was recovered for 3-10 min in dark after a 300 s imaging. **(B)** Below, zoomed-in traces (red) of selected stimulation sessions in the dotted box in **(A)**, in alignment with optogenetic stimulation intensity patterns (magenta).

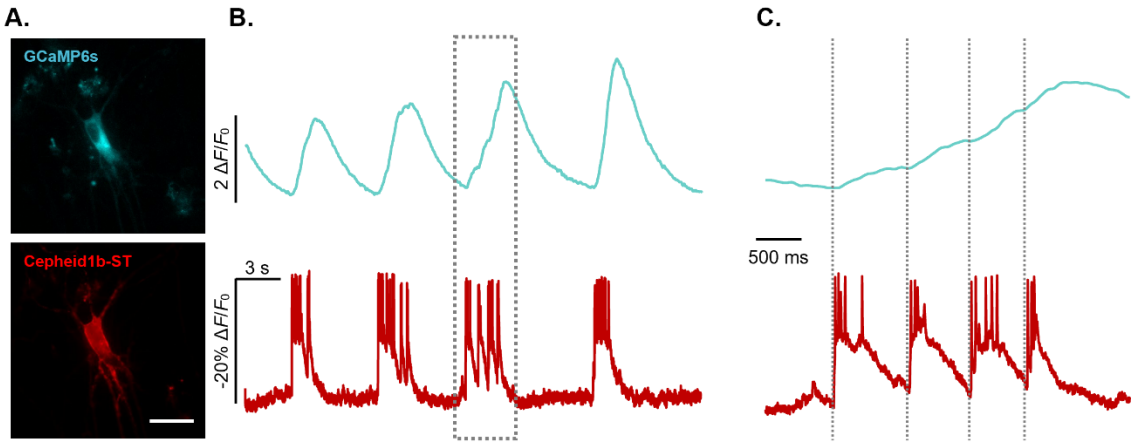

Fig. S14. Dual-color imaging using GCaMP6s and Cepheid1b-ST. (A) Epifluorescence image of a neuron expressing GCaMP6s and Cepheid1b-ST. Scale bar, 20 μm. **(B)** Calcium and voltage activity in the same neuron. **(C)** A zoomed in view of fluorescence traces in the dashed gray box in **(B)**.

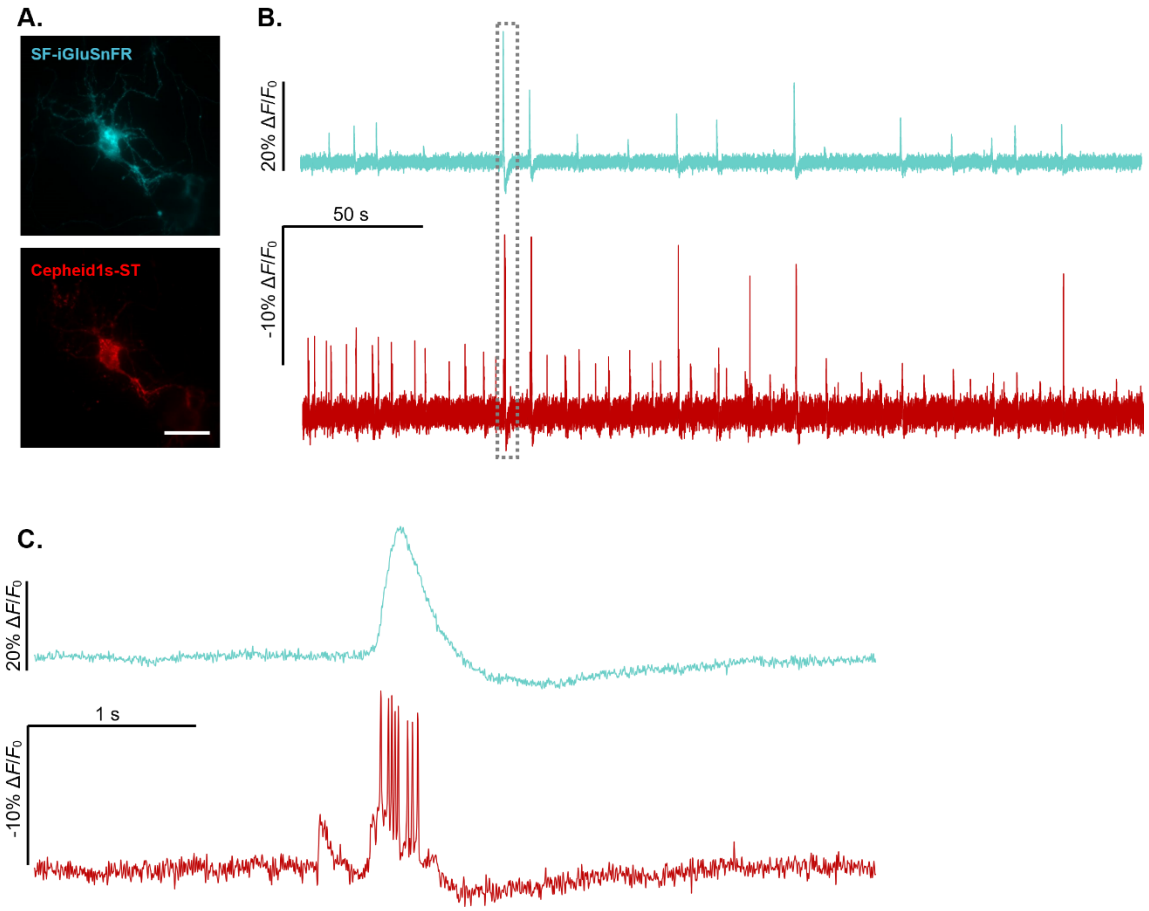

Fig. S15. Long-term optical recording of glutamate events and electrophysiology. (A) Epifluorescence image of a neuron expressing SF-iGluSnFR and Cepheid1b-ST. Scale bar, 20 μm. **(B)** Glutamine and voltage activity in the same neuron. **(C)** A zoomed in view of fluorescence traces in the dashed gray box in **(B)**.

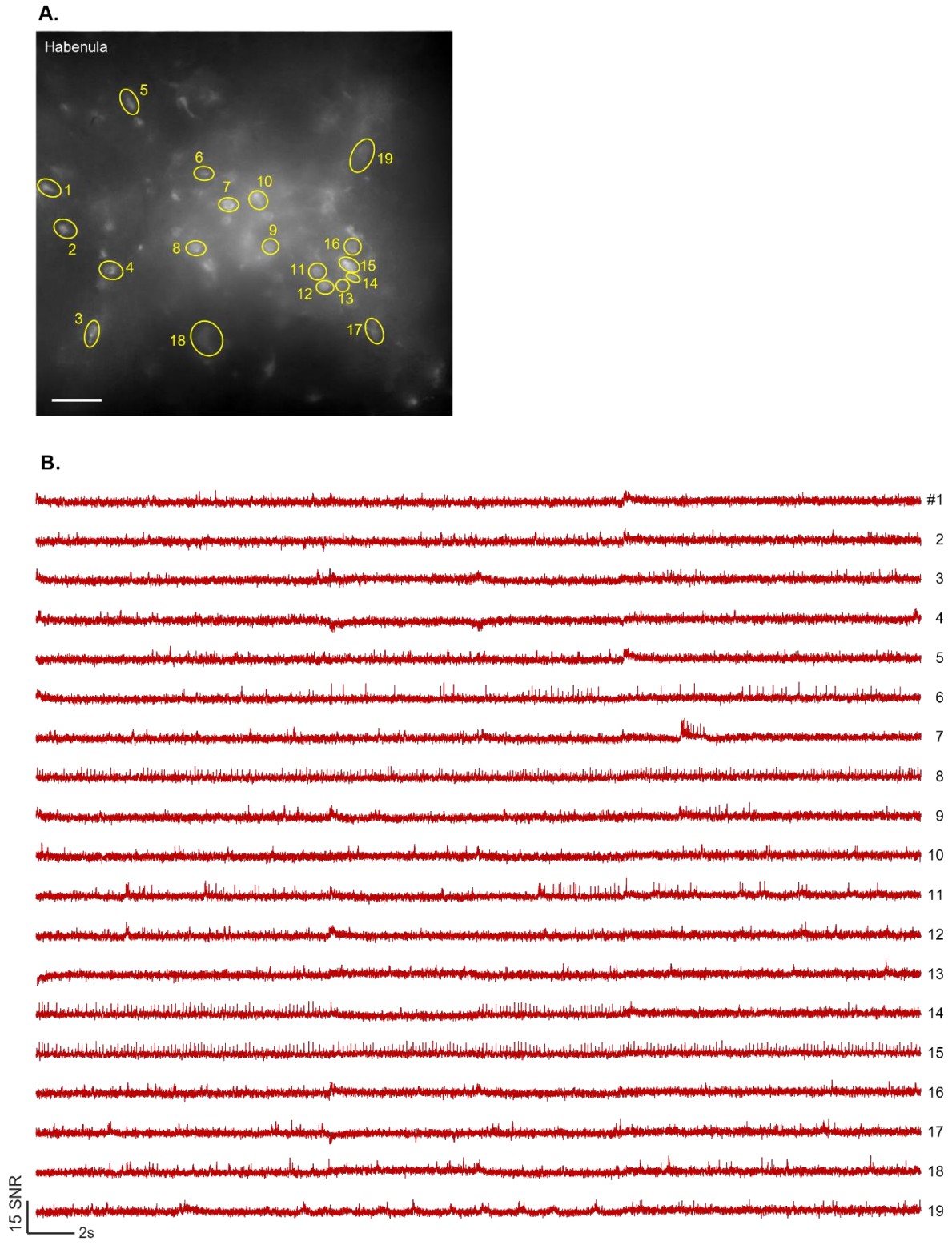

Fig. S16. Imaging of neuronal firing in a large field of view of habenula with Cepheid1b-ST. (A) Epifluorescence images of habenula, scale bar = 20 μm. **(B)** Voltage imaging of spontaneous neural activity in habenula.

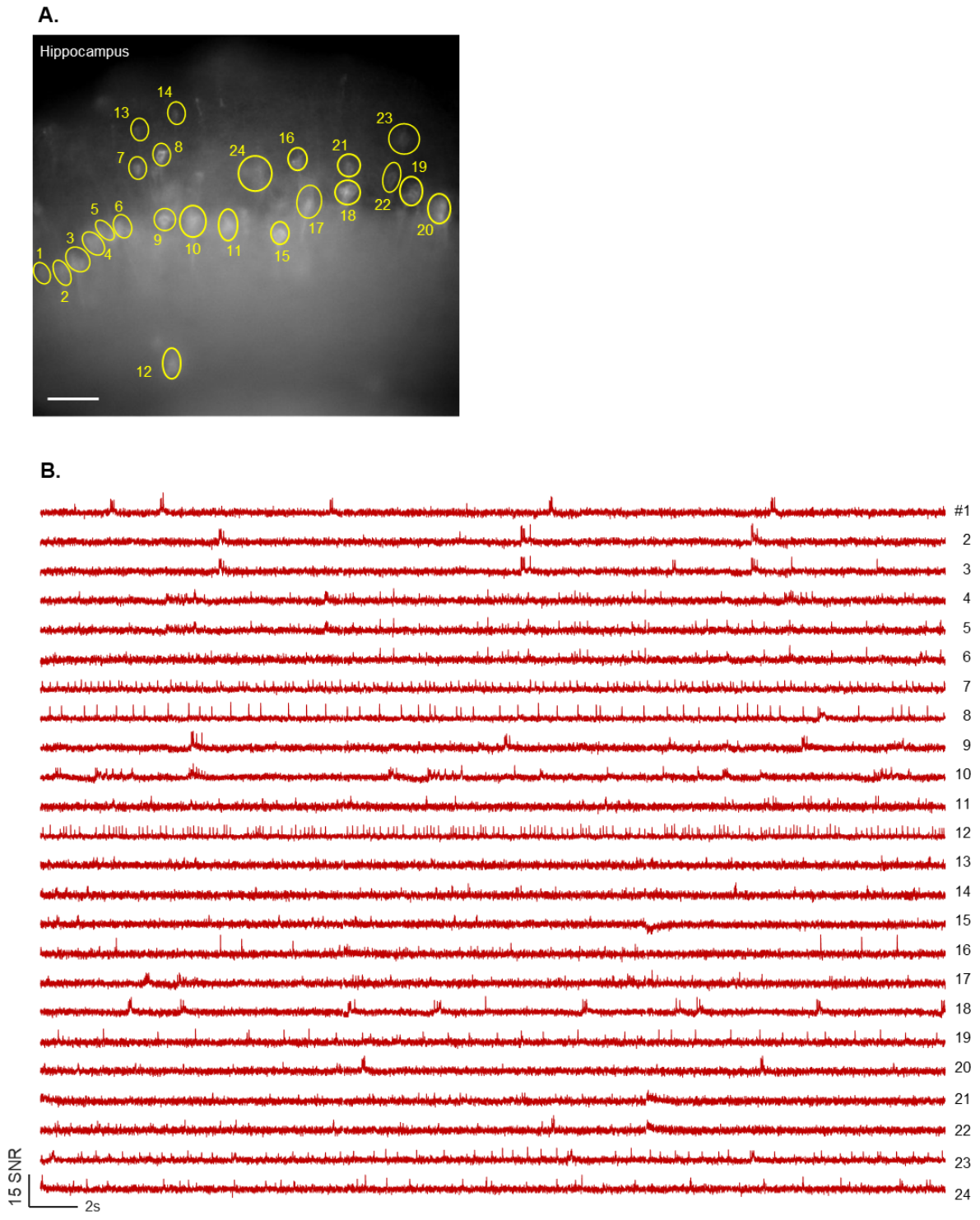

Fig. S17. Imaging of neuronal firing in a large field of view of hippocampus with Cepheid1b-ST. (A) Epifluorescence images of hippocampus, scale bar = 20 μm. **(B)** Voltage imaging of spontaneous neural activity in hippocampus.

Supplementary Tables

Table S1. FRET efficiency predictions

	R (Å)	κ^2	κ^2/R^6 (10^{-11} Å^{-6})
Ace^{81C}-(C)mScarlet-I1.4	63	0.00026	0.00042
Ace^{81C}-(C)mRuby4	52	0.0045	0.023
Ace^{81C}-(ECL1)mScarlet-I1.4	51	0.26	1.5
Ace^{81C}-(ECL1)mRuby4	48	0.66	5.4
Ace^{81C}-(ICL3)mScarlet-I1.4	54	0.65	2.6
Ace^{81C}-(ICL3)mRuby4	60	0.046	0.10

Table S2. Photophysical properties of mScarlet-I1.4 compared with mScarlet-I

	mScarlet-I	mScarlet-I1.4
Absorption maximum	570 nm	570 nm
Excitation maximum	571 nm	571 nm
Emission maximum	511 nm & 600 nm	512 nm & 600 nm
Green/Red emission ratio	0.502	0.119
Relative brightness in bacteria	0.489 / 0.301	1

Table S3. List of reagents used in this study

Reagent	Vendor	Catalog Number
Phanta® Max Super-Fidelity DNA Polymerase	Vazyme	P505-d2
Lightening Cloning Kit	Biodragon	BDIT0014-100
DNA extraction kit	TIANGEN	DP118-02
Dulbecco's Modified Eagle's medium (DMEM)	Gibco	C11995500BT
Fetal Bovine Serum (FBS)	Gibco	10099141C
Trypsin-EDTA (0.25%)	Gibco	25200056
Neurobasal™ Medium	Gibco	21103049
B-27™ Supplement	Gibco	17504044
GlutaMAX™ Supplement	Gibco	35050061
Penicillin-streptomycin	Beyotime	C0222
Matrigel® Matrix	Corning	356234
poly-D-lysine	Sigma	P7280-5X5M
Laminin Mouse Protein	Gibco	23017015
Opti-MEM® Medium	Gibco	31985062
Lipofectamine® 3000 Reagent	Gibco	L3000008
HEPES	Amresco	0511
EGTA	Sigma	03777-10G
2-APB	Abcam	ab120124
Gabazine	Abcam	ab120042
NBQX	Abcam	ab120045
D-AP5 (APV)	Abcam	ab120003
Creatine Phosphate Sodium	Maklin	C804629
Potassium gluconate	Sigma	G4500
Adenosine 5'-triphosphate Magnesium	Maklin	A922363
HBSS	Gibco	C14175500BT
$NaHCO_3$	Tong Guang	144-55-8
$NaH_2PO_4 \cdot 2H_2O$	Xilong Scientific	13472-35-0
NaCl	Sigma	S3014
Glucose	Sigma	G7021
KCl	Sigma	P9541
$MgCl_2$	Sigma	M2670
$CaCl_2$	Sigma	10043-52-4

Table S4. List of cloning primers used in this study

Construct	Primer sequence (5' to 3')
Ace (N terminus)	Forward: ATGGCTGACGTGGAAACCGAG Reverse: CATTGTCAGGTCCTGGTAGTTCACG
Ace (C terminus)	Forward: AATGGTGAAAGGCAGGTGGTCTAC Reverse: CTTGAAGATAGTCTCATGGGCAATGAGG
mScarlet-I1.4	Forward: TACCAGGACCTGACAATGATGGTGAGCAAGGGCGAGG Reverse: CCTGCCTTTCACCATTCTTGTACAGCTCGTCCATGCCG
mRuby4	Forward: CGTGAACTACCAGGACCTGACAATGCTGATCAAGGAAGAGATGC CCATGAAG Reverse: CGTAGACCACCTGCCTTTCACCATTCTTGTACAGCTCGTCCATGC C
Δ7mOrange2(Y71F)-ER2	Forward: CATCAATGTGGGGGGCAACATGGCCATCATCAAGGAGTTCATG Reverse: AGCTTGATATCGAATTCTCATTACACCTCGTTCTCGTAGCAGAACT TGTA
3xTS	Forward: CCCATGAGACTATCTTCAAGACCGGTGCCGCCGACCG Reverse: GCCCCCCACATTGATGTCAATCTG
Kv2.1 motif	Forward: TGGACGAGCTGTACAAGCAGAGCCAGCCTATCCTGAACAC Reverse: CGATAAGCTTGATATCGAATTCTTACACTTCATTTTCATAGCAGAA GAACCTGG
P2A-CheRiff	Forward: GGCTCCGGAGCCACGAAC Reverse: AGCGTAATCTGGAACATCGTATGGG

Table S5. Spectral properties and imaging apparatus for fluorescent imaging

Indicator	Fluorophore	Excitation max. (nm)	Emission max. (nm)	Laser (nm)	Emission filter (nm)
Cepheid1b	mScarlet-I1.4	570	600	561	630 / 75 (inverted wide-field, confocal) 600 / 50 (upright wide-field, dual-color)
Cepheid1s	mRuby4	558	592	561	630 / 75 (wide-field, confocal) 600/50 (dual-color)
VARNAM(*6*)	mRuby3	558	592	561	630 / 75
VARNAM2(*14*)	mRuby3	558	592	561	630 / 75
Ace2N-7aa-mScarlet(*26*)	mScarlet	569	594	561	630 / 75
AceC81-mScarlet-I1.4	mScarlet-I1.4	570	600	561	630 / 75
GCaMP6s(*18*)	cpEGFP	497 (Ca^{2+}-bound)	515 (Ca^{2+}-bound)	488	525 / 50

Dichroic mirror: Chroma ZT405/488/561/640rpc for confocal imaging and ZT405/488/532/642rpc for inverted wide-field imaging.

ZT561rdc for upright wide-field wide-field imaging. T565LPXR was used to split fluorescence for dual-color imaging.

Table S6. Amino acid sequences of Cepheid1b/s and Cepheid1b/s-ST

Voltage indicator	Amino acid sequence	Feature
Cepheid1b	MADVETETGMIAQWIVFAIMAAAAIAFGVAVHFRP SELKSAYYINIAICTIAATAYYAMAVNYQDLTMMVS KGEAVIKEFMRFKVHMEGSVNGHEFEIEGEGEGR PYEGTQTAKLKVTKGGPLPFSWDILSPQFMYGSR AFTKHPADIPDYYKQSFPEGFTWERVMNFEDGGS VTVTQDTSLEDGTLIYKVKLRGTNFPPDGPVMQKT TMGWEASTERLYPEDGVLKGDIKMALRLKDGGRY IADFKTTYKAKKPVQMPGAYNVDRKLDIVSHNEDY TVVEQYEASVGRHSTGGMDELYKNGERQVVYAR YICWVLTTPLLLLDLIVMTKMGGVMISWVIGADIFMI VFGILGAFEDEHKFKWVYFIAGCVMQAVLTYGMY NATWKDDLKKSPEYHSSYVSLLVFLSILWVFYPVV WAFGSGSGVLSVDNEAILMGILDVLAKPLFGMGC LIAHETIFKTGAADRPVVAVSKAAAASKSRITSEGE YIPLDQIDINVGGKSRITSEGEYIPLDQIDINVGGKS RITSEGEYIPLDQIDINVGGNMAIIKEFMRFKVRMEG SVNGHEFEIEGEGEGRPYEGFQTAKLKVTKGGPL PFAWDILSPHFTFGSKAYVKHPADIPDYFKLSFPE GFKWERVMNYEDGGVVTVTQDSSLQDGEFIYKVK LRGTNFPSDGPVMQKKTMGWEASSERMYPEDGA LKGKIKMRLKLKDGGHYTSEVKTTYKAKKPVQLP GAYIVDIKLDITSHNEDYTIVEQYERAEGRHSTGGM DELYKFCYENEV	**Gray:** Ace^{C81}(1-68)…(69-227) **Red:** mScarlet-I1.4 **Black:** linker **Blue:** transport signal (TS) **Orange:** dark mOrange2 (Δ7mOrange(Y64F)) **Green:** ER-exiting sequences (ER2)

Cepheid1s	MADVETETGMIAQWIVFAIMAAAAIAFGVAVHFRP SELKSAYYINIAICTIAATAYYAMAVNYQDLTMLIKE EMPMKVVMTGTVNGHYFKCTGEGEGRPYEGVQT MRIKVIEGGPLPFAFDILATSFMYGSRTFIKYPADIP DFFKQSFPEGFTWERVTRYEDGGVVTVTQDTSLQ DGVLIYNVKVRGENFPSNGPVMQKKTKGWEPNTE MMYPADGGLRGYTDIALKVDGGGHLHCSFVTEYK SKKTVGNIKMPGVHAVDHRLERIEESDNETYVVQR EVAVAKYSNLGGGMDELYKNGERQVVYARYICW VLTTPLLLLDLIVMTKMGGVMISWVIGADIFMIVFGI LGAFEDEHKFKWVYFIAGCVMQAVLTYGMYNAT WKDDLKKSPEYHSSYVSLLVFLSILWVFYPVVWAF GSGSGVLSVDNEAILMGILDVLAKPLFGMGCLIAH ETIFKTGAADRPVVAVSKAAAASKSRITSEGEYIPL DQIDINVGGKSRITSEGEYIPLDQIDINVGGKSRITS EGEYIPLDQIDINVGGNMAIIKEFMRFKVRMEGSVN GHEFEIEGEGEGRPYEGFQTAKLKVTKGGPLPFA WDILSPHFTFGSKAYVKHPADIPDYFKLSFPEGFK WERVMNYEDGGVVTVTQDSSLQDGEFIYKVKLRG TNFPSDGPVMQKKTMGWEASSERMYPEDGALKG KIKMRLKLKDGGHYTSEVKTTYKAKKPVQLPGAYI VDIKLDITSHNEDYTIVEQYERAEGRHSTGGMDEL YKFCYENEV	Gray: Ace^{C81}(1-68)…(69-227) Red: mRuby4 Black: linker Blue: transport signal (TS) Orange: dark mOrange2 (Δ7mOrange(Y64F)) Green: ER-exiting sequences (ER2)
Cepheid1b-ST	MADVETETGMIAQWIVFAIMAAAAIAFGVAVHFRP SELKSAYYINIAICTIAATAYYAMAVNYQDLTMMVS KGEAVIKEFMRFKVHMEGSVNGHEFEIEGEGEGR PYEGTQTAKLKVTKGGPLPFSWDILSPQFMYGSR AFTKHPADIPDYYKQSFPEGFTWERVMNFEDGGS VTVTQDTSLEDGTLIYKVKLRGTNFPPDGPVMQKT TMGWEASTERLYPEDGVLKGDIKMALRLKDGGRY IADFKTTYKAKKPVQMPGAYNVDRKLDIVSHNEDY TVVEQYEASVGRHSTGGMDELYKNGERQVVYAR YICWVLTTPLLLLDLIVMTKMGGVMISWVIGADIFMI VFGILGAFEDEHKFKWVYFIAGCVMQAVLTYGMY NATWKDDLKKSPEYHSSYVSLLVFLSILWVFYPVV WAFGSGSGVLSVDNEAILMGILDVLAKPLFGMGC LIAHETIFKTGAADRPVVAVSKAAAASKSRITSEGE YIPLDQIDINVGGKSRITSEGEYIPLDQIDINVGGKS RITSEGEYIPLDQIDINVGGNMAIIKEFMRFKVRMEG SVNGHEFEIEGEGEGRPYEGFQTAKLKVTKGGPL PFAWDILSPHFTFGSKAYVKHPADIPDYFKLSFPE	Gray: Ace^{C81}(1-68)…(69-227) Red: mScarle-I1.4 Black: linker Blue: transport signal (TS) Orange: dark mOrange2 (Δ7mOrange(Y64F)) Brown: somatic targeting (ST) domain from the voltage-gated potassium channel Kv2.1 Green: ER-exiting sequences (ER2)

	GFKWERVMNYEDGGVVTVTQDSSLQDGEFIYKVK LRGTNFPSDGPVMQKKTMGWEASSERMYPEDGA LKGKIKMRLKLKDGGHYTSEVKTTYKAKKPVQLP GAYIVDIKLDITSHNEDYTIVEQYERAEGRHSTGGM DELYKQSQPILNTKEMAPQSKPPEELEMSSMPSP VAPLPARTEGVIDMRSMSSIDSFISCATDFPEATRF FCYENEV	
Cepheid1s-ST	MADVETETGMIAQWIVFAIMAAAAIAFGVAVHFRP SELKSAYYINIAICTIAATAYYAMAVNYQDLTMLIKE EMPMKVVMTGTVNGHYFKCTGEGEGRPYEGVQT MRIKVIEGGPLPFAFDILATSFMYGSRTFIKYPADIP DFFKQSFPEGFTWERVTRYEDGGVVTVTQDTSLQ DGVLIYNVKVRGENFPSNGPVMQKKTKGWEPNTE MMYPADGGLRGYTDIALKVDGGGHLHCSFVTEYK SKKTVGNIKMPGVHAVDHRLERIEESDNETYVVQR EVAVAKYSNLGGGMDELYKNGERQVVYARYICW VLTTPLLLLDLIVMTKMGGVMISWVIGADIFMIVFGI LGAFEDEHKFKWVYFIAGCVMQAVLTYGMYNAT WKDDLKKSPEYHSSYVSLLVFLSILWVFYPVVWAF GSGSGVLSVDNEAILMGILDVLAKPLFGMGCLIAH ETIFKTGAADRPVVAVSKAAAASKSRITSEGEYIPL DQIDINVGGKSRITSEGEYIPLDQIDINVGGKSRITS EGEYIPLDQIDINVGGNMAIIKEFMRFKVRMEGSVN GHEFEIEGEGEGRPYEGFQTAKLKVTKGGPLPFA WDILSPHFTFGSKAYVKHPADIPDYFKLSFPEGFK WERVMNYEDGGVVTVTQDSSLQDGEFIYKVKLRG TNFPSDGPVMQKKTMGWEASSERMYPEDGALKG KIKMRLKLKDGGHYTSEVKTTYKAKKPVQLPGAYI VDIKLDITSHNEDYTIVEQYERAEGRHSTGGMDEL YKQSQPILNTKEMAPQSKPPEELEMSSMPSPVAP LPARTEGVIDMRSMSSIDSFISCATDFPEATRFFCY ENEV	**Gray:** Ace^{C81}(1-68)…(69-227) **Red:** mRuby4 **Black:** linker **Blue:** transport signal (TS) **Orange:** dark mOrange2 (Δ7mOrange(Y64F)) **Brown:** somatic targeting (ST) domain from the voltage-gated potassium channel Kv2.1 **Green:** ER-exiting sequences (ER2)