Electronic Supplementary Material (ESI) for ChemComm.

†Electronic Supplementary Information (ESI)

Ratiometric and photoconvertible fluorescent protein-based voltage indicator prototypes

A. S. Abdelfattah,[a] V. Rancic,[b] B. Rawal,[b] K. Ballanyi[b] and R. E. Campbell*[a]

[a]Department of Chemistry, University of Alberta, Edmonton, Alberta, T6G 2G2 Canada

[b]Department of Physiology, University of Alberta, Edmonton, Alberta, T6G 2H7 Canada

*To whom correspondence should be addressed. E-mail:

robert.e.campbell@ualberta.ca

Experimental procedures

Molecular biology to construct tandem dimer voltage indicator variants and FlicGR variants. Polymerase chain reaction (PCR) amplification was used to construct the DNA linker libraries for tdFlicR and FlicGR variants. Synthetic oligonucleotides (Integrated DNA Technologies) were used as primers for amplification and Pfu polymerase (Thermo Fisher Scientific) was used to maintain high fidelity DNA replication.

For tdFlicR variants, overlap PCR was used to link CiVSD to the 3' end of FlicR1. In the case of tdFlicR-ASAP variants, overlap PCR was used to link ASAP1 (pcDNA3.1/Puro-CAG-ASAP1 was a gift from Michael Lin (Addgene plasmid # 52519))[1] to the 3' end of FlicR1. Two codons at the junction were encoded as NNK to fully randomize those positions in all linker libraries. For FlicGR variants, DNA encoding the first 239 amino acids from CiVSD (VSD239) was generated by PCR amplification of CiVSD domain from the voltage sensor FlicR1.[2] DNA encoding the cpmMaple variant

was generated by PCR amplification of the gene encoding GR-GECO1.1. Overlap PCR was used to join the two genes together. This design was expected to maximize the chance of coupling voltage-induced conformational changes to a change in the mMaple chromophore environment. The cpmMaple barrel had been engineered to be sensitive to conformational changes relayed through a Ca^{2+} binding domain.[3]

Random mutagenesis was performed with error-prone PCR amplification using Taq polymerase (New England Biolabs) in the presence of $MnCl_2$ (0.1 mM), 200 μM dATP, 200 μM dGTP, 1000 μM dTTP, and 1000 μM dCTP. Randomization of targeted codons was performed with QuikChange Lightning kits (Agilent Technologies). Staggered extension process (StEP) recombination using low fidelity Taq polymerase (New England Biolabs) was used to construct libraries in directed evolution rounds that had more than one FlicGR template (winners from previous round). StEP PCR was performed in the presence of 0.1 mM $MnCl_2$, 200 μM dATP, 200 μM dGTP, 1000 μM dTTP, and 1000 μM dCTP.

Restriction endonucleases (Thermo Fisher Scientific) were used to digest PCR products and expression vectors. Agarose gel electrophoresis was used to purify DNA products from PCR and restriction digestion reactions. The DNA was extracted from the gels using the GeneJET gel extraction kit (Thermo Fisher Scientific). Ligations were performed using T4 DNA ligase (Thermo Fisher Scientific).

Plasmids for *E. coli* and mammalian cell expression. We used a plasmid we previously constructed (pcDuEx0.5) as the vector for expression of the constructs in *E. coli* and HeLa and HEK 293 cells.[2] The gene was inserted between *BamH*I and *Xba*I restriction sites. For expression of variants in neurons, genes were cloned from plasmid

pcDuEx0.5 into the *BamH*I and *Hind*III sites of pAAV2 vector (Addgene: 61249).[4] Expression was controlled using human synapsin I promoter to preferentially express in neurons. A 3' Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) sequence was used to enhance expression.

Screening libraries in *E. coli*. Gene libraries were used to transform electro-competent *E. coli* strain DH10B (Invitrogen) by electroporation (10 kV/cm, ~ 5ms pulse) (MicroPulser™ Electroporator, BIO-RAD). *E. coli* cells were then plated and cultured at 37 °C on LB agar plates supplemented with ampicillin (400 μg/ml) to obtain 500-1000 colonies per plate. Colonies were then imaged using a custom imaging setup described previously.[5]

To screen for tdFlicR variant brightness, plate images were acquired using a 560/40 nm excitation filter and 630/60 nm emission filter to screen for brightness in the red channel. In the case with green/red ratiometric voltage indicator constructs, images were acquired in the green (470/40 nm excitation filter and 525/50 nm emission filter) and red (560/40 nm excitation filter and 630/60 nm emission filter) channels. For each linker library, ~5,000 colonies (10 plates) were screened. Colonies with the top 0.01% fluorescence brightness were manually picked and cultured in 4 ml LB media supplemented with ampicillin (100 μg/ml). Plasmids were then extracted using GeneJET plasmid miniprep kit (Thermo Fisher Scientific).

For tdFlicR-ASAP libraries we first compared the amino acid sequence of GgVSD and CiVSD to analyze the S1/S4 interface to determine if they would be likely to form a heterodimer analogous to the CiVSD homodimer. Alignment of the two VSD sequences revealed high similarity in sequence identity for the S1 and S4 α-helices (Fig. S3). In

addition, the residues that were not identical represented highly conservative substitutions (Fig. S3). Inspection of the CiVSD homodimer S1/S4 interface (PDB ID: 4G80)[6] revealed 14 residues from the S1 and S4 α-helices pointing towards the dimer interface. Nine residues are identical between CiVSD and GgVSD and five residues were conservative substitutions, namely Leu127Ile, Val220Thr, Leu224Val, Val227Ile, and Ala231Ile relative to the CiVSD sequence. We concluded that fusing GgVSD as part of our tandem dimer construct would very likely result in formation of a heterodimer between CiVSD and GgVSD.

To screen for FlicGR mutant brightness, libraries of genes encoding potential photoconvertible variants were inserted in pcDuEx0.5 vector and used to transform *E. coli*. After overnight growth on agar plates, , the green fluorescence of the colonies was imaged using a 470/40 nm excitation filter and 525/50 nm emission filter. The colonies were then photoconverted for 10-20 min using a custom built illumination source composed of six 9 × 11 arrays of 405 nm light emitting diodes (LED) (OptoDiode Corporation, Newbury Park, CA). The red fluorescence of the colonies was then imaged using a 560/40 nm excitation filter and 630/60 nm emission filter and the photoconversion efficiency was assessed on a colony-by-colony basis (Fig. S5). In a typical round of directed evolution, we used error-prone PCR to create a large library based on our most promising template(s), and then screened the library using the workflow represented in Fig S5. For each round of random mutagenesis, ~10,000 colonies (10-20 plates) were screened. For each library generated by targeted codon randomization, ~3 times more colonies than the expected randomization library size were screened. Colonies that exhibited bright green fluorescence before

photoconversion and bright red fluorescence following photoconversion, were manually picked and cultured in 4 ml LB media supplemented with ampicillin (100 µg/ml). Plasmids were isolated from overnight cultures using GeneJET plasmid miniprep kit (Thermo Fisher Scientific).

Cell culture. HeLa cells (ATCC, CCL-2) or HEK 293A cells (CRL-11268, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (supplemented with 10% fetal bovine serum (FBS) (Sigma), 2 mM GlutaMax (Invitrogen), penicillin-G potassium salt (50 units/ml), and streptomycin sulfate (50 µg/ml)) and incubated for 48 h at 37 °C, 5% CO_2. Cells were split and cultured on collagen-coated 35 mm glass bottom dishes (Matsunami) to ~50% confluency. Transfection was performed by incubating HeLa cells or HEK 293 cells with a mixture of 1 µg of plasmid DNA and 2 µL of Turbofect (Thermo Fisher Scientific) for 2 hours following the manufacturer's instructions. Imaging was performed 24-48 hours after transfection.

Induced transmembrane voltage (ITV) screening. Both tandem dimer voltage indicator variants and FlicGR variants were co-expressed with the inward rectifier potassium channel, Kir2.1 (pGEMTEZ-Kir2.1 was a gift from Richard Axel & Joseph Gogos & C. Ron Yu (Addgene plasmid # 32641))[7] in HeLa cells. In the case of red tdFlicR variants, ArcLight Q239 (ArcLight-Q239 was a gift from Vincent Pieribone (Addgene plasmid # 36856))[8] was used as an internal reference. Expression of Kir2.1 in HeLa cells helped maintain the resting potential around -60 mV, which is appropriate for screening neuronal voltage indicators. A uniform electric field of ~50 V/cm was applied across the cell culture to create an ITV using a custom-built field stimulation system. A pulse generator (PG 58A, Gould Advance Ltd.) was used to apply a 10 ms square-wave

pulse at ~0.5 Hz. An amplifier (6824A 40V/25A HP/Agilent) was used to obtain a pulse amplitude of 25 V. Two parallel platinum electrodes (0.5 cm apart) were used to deliver the pulses to the cell culture. Fluorescence was imaged (in both the green and red channels) at a frame rate of 100 Hz for 10 seconds during multiple electric field pulses.

Imaging ITV in HeLa cells. Imaging was carried out in HEPES (25 mM) buffered Hanks' Balanced Salt Solution (HBSS). An inverted fluorescence microscope (Eclipse Ti-E, Nikon) equipped with a 200 W metal halide lamp (PRIOR Lumen) and a 60× oil objective was used to image HeLa cells. Images were acquired at 100 Hz with 4×4 binning using a 16-bit QuantEM 512SC electron-multiplying CCD camera (Photometrics). A FITC/Cy2 filter set (470/40 nm (excitation), 525/50 nm (emission), and a 495LP dichroic mirror (set number 49002, Chroma)) was used to image ArcLight Q239 and FlicGR variants before photoconversion. The same filter set was used to image the green channel of tdFlicR-ASAP variants. A TRITC/Cy3 filter set (545/30 nm (excitation), 620/60 nm (emission), and a 570LP dichroic mirror (set number 49005, Chroma)) was used to image the red channel of tdFlicR, tdFlicR-ASAP, and the red form of FlicGR variants. The NIS Elements Advanced Research software (Nikon) was used to control the microscope and camera. The raw fluorescence traces of both the red and green channels were extracted from identical regions of interest in cells and exported into a customized Microsoft Excel spreadsheet. Background subtraction, photobleaching corrections, and calculations of average $\Delta F/F_{min}$, were performed in Excel (Microsoft). In the case of tdFlicR variants, the average $\Delta F/F_{min}$ of tdFlicR signals were compared to those of ArcLight signals from the same cells, and the ratio of $\Delta F/F_{min}$ of FlicR over ArcLight was reported. The best variant in terms of maximum mean ratio in

each library was identified and the isolated plasmid DNA was submitted for DNA sequencing.

Tandem dimer voltage indicators and FlicGR libraries tested for ITV. For tdFlicR linker libraries, truncation of 0, 40 or 50 residues from the second CiVSD did not result in variants that were bright enough to report induced transmembrane voltage (ITV) in HeLa cells. Truncation of more than 60 residues from the second CiVSD resulted in constructs with sufficient fluorescence to enable high-speed imaging and report ITV in the HeLa cell screen. In libraries with 60, 70, 80, 90, 100 or 110 residues truncated, the amino acids Ala, Arg, Gly, Lys and His tended to be selected for at the two randomized positions at the fusion site between cpmApple and the second CiVSD. For these libraries (i.e., 60, 70, 80, 90, 100 or 110 residues truncated), we found that that all variants exhibited similar brightness and voltage sensitivity. Linker libraries in which more than 110 residues were truncated exhibited an almost complete loss of fluorescence, possibly due to poor folding efficiency of the second CiVSD domain. We further applied our screening strategy to additional truncation libraries (truncations in two residue increments from Δ100 to Δ116 with randomization of the two residues at the junction) however were unable to identify variants that surpassed tdFlicR Δ110AR's performance.

For the tdFlicR-ASAP library, inspection of the GgVSD amino acid sequence reveals that its flexible N-terminus is 59 residues shorter than CiVSD (Fig. S3) so we chose to fuse the full length GgVSD because, as described above, truncation of 60 residues from the N-terminus of CiVSD in tdFlicR constructs resulted in variants with bright fluorescence and voltage sensitivity in mammalian cells.

For FlicGR libraries, fluorescence response to stimulation was recorded in the green channel (before photoconversion) and the red channel (after photoconversion). Variants that exhibited the highest voltage sensitivity in both channels were used as gene templates for the next round of library creation and screening. A mixture of the 3 to 6 variants with the brightest fluorescence, most efficient photoconversion, expected pattern of membrane localization, and largest responses to changes in membrane potential, would be typically used as the template for the next round of library creation by random mutagenesis. This procedure was carried out six times to reach FlicGR1.

Fluorescence imaging and electrophysiology of HEK 293 cells. All imaging and electrophysiology measurements were performed in Tyrode buffer (120 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, NaH_2PO_4, 26 mM NaHCO, 310 mM glucose). The pH of the solution was adjusted to 7.4 by ongoing gassing with carbogen mixture 95% O_2 and 5% CO_2. Filamented glass micropipettes (Harvard Apparatus) were pulled to a tip resistance of 4–6 MΩ (Puller model: PP-830, Narishige), and filled with internal solution containing 140 mM K-Gluconate, 1 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM BAPTA, 10 mM HEPES, 1 mM ATP-Na_2, (pH 7.3), adjusted to 295 mOsm with sucrose. Patch clamp recording and current injections were performed with a single electrode clamp amplifier (SEC-05L, NPI Electronic Tamm, Germany). The membrane potential was varied from -70 mV to +30 mV (50 ms spikes at 10 Hz). The signals were digitalized (A/D converter, PowerLab 8/3, ADInstruments) and sampled at 5 kHz using LabChart7 software (AD Instruments) and Pulse (HEKA, Lambrecht, Germany).

Widefield imaging was performed on an upright FV1000 confocal microscope (OlympusCanada, Markham, ON, Canada) equipped with software (FluoView1000,

Olympus Canada), a 20× XLUMPlanF1 water immersion objective (numerical aperture (NA) = 1.00), and connected to a 200 W xenon arc lamp (Lambda XL, Sutter Instruments). Images were acquired at 100 Hz with 2×2 binning using a CMOS digital camera (Hamamatsu Orca-Flash2.8; Hamamatsu Photonics). Dual band excitation and barrier filter sets (475/10 nm (excitation), 570/10 nm (excitation), 530/30 nm (emission), and 625/50 nm (emission)) and a polychromatic mirror having two bandpass transmission regions (510-555 nm and 585-65 nm bandpass) along with a beam splitter (DV2 Multichannel Imaging System, Photometrics) was used to spectrally separate the red and green emission channels. The two emission channels were projected simultaneously on the fast CMOS camera.

Culturing dissociated rat hippocampal neurons. Dissociated E18 Sprague Dawley hippocampal cells in Hibernate® EB Complete Media were purchased from BrainBits LLC. The cells were grown on 35 mm glass bottom dish (In Vitro Scientific) coated with poly-D-lysine (EMD Millipore, A-003-E) containing 2 ml of NbActiv4 (BrainBits LLC) supplemented with 2% FBS, penicillin-G potassium salt (25 units/ml), and streptomycin sulfate (25 µg/ml). Half of the culture media was replaced every 3 days. Neuronal cells were transfected on day 8 using Lipofectamine 2000 (Life Technologies) following the manufacturer instructions. Briefly, 1-2 µg of plasmid DNA and 4 µL of Lipofectamine 2000 (Life Technologies) were added to 100 µl of NbActive4 medium to make the transfection medium. This medium was then incubated at room temperature for 10-15 minutes. Half of the culture medium (1 ml) from each neuron dish was taken out and combined together with an equal volume of fresh NbActiv4 medium (supplemented with 2% FBS, penicillin-G potassium salt (25 units/ml), and streptomycin sulfate (25 µg/ml))

to make a 1:1 mixture and incubated at 37 °C and 5% CO_2. 1 ml of fresh conditioned (at 37 °C and 5% CO_2) NbActiv4 medium was then added to each neuron dish. The transfection medium was then added and the neuron dishes were incubated for 2-3 hours at 37 °C in a CO_2 incubator. The media was then replaced using the conditioned 1:1 medium prepared previously. The cells were incubated for 48-72 hours at 37 °C in a CO_2 incubator before imaging.

Imaging primary neuron cultures. Imaging was carried out in HEPES (25 mM) buffered HBSS. Widefield imaging was performed on an inverted Nikon Eclipse Ti-E microscope equipped with a 200 W metal halide lamp (PRIOR Lumen), 60× oil objectives (NA = 1.4, Nikon), and a 16-bit QuantEM 512SC electron-multiplying CCD camera (Photometrics). A TRITC/Cy3 filter set (545/30 nm (excitation), 620/60 nm (emission), and a 570LP dichroic mirror, set number 49005, Chroma) was used to image tdFlicR variants. A dual band excitation and barrier filter sets (475/10 nm (excitation), 570/10 nm (excitation), 530/30 nm (emission) and 625/50 nm (emission)) and a polychromatic mirror having two bandpass transmission regions (510-555 nm and 585-65 nm bandpass) along with a beam splitter (DV2 Multichannel Imaging System, Photometrics) was used to image tdFlicR-VK-ASAP simultaneously in both the green and red channels. A FITC/Cy2 filter set (470/40 nm (excitation), 525/50 nm (emission), and a 495LP dichroic mirror (set number 49002, Chroma)) was used to image FlicGR variants in the green channel before photoconversion. A TRITC/Cy3 filter set (545/30 nm (excitation), 620/60 nm (emission), and a 570LP dichroic mirror, set number 49005, Chroma) was used to image FlicGR in the red channel after

photoconversion. For time-lapse imaging, neurons were imaged at 100 Hz imaging frequency with 4×4 binning.

Fluorescence spectra of voltage indicators in live cells. To record the fluorescence spectra in live cells, HeLa cells were transfected and imaged using a laser scanning microscope (Zeiss LSM 880) 24-48 hours post transfection. The fluorescence emission spectrum was recorded in Lambda mode with a 3 nm resolution using the spectral imaging channels. The resulting spectra are shown in Fig. S8. Lambda stacks were acquired using a 63× oil objective (NA = 1.4, Zeiss). The dichroic mirrors used were: MBS 561 for imaging tdFlicR, MBS 458/561 for imaging tdFlicR-VK-ASAP, MBS 488 for imaging the green state of FlicGR1, and MBS 561 for imaging the red state of FlicGR1. The unmixing tool of the ZEN software (Zeiss) was used to analyze the data and obtain the emission spectra.

Cartoon representation of indicators. Schematic representations of tdFlicR and tdFlicR-ASAP indicators shown in Fig. S1F and Fig. 2A respectively are prepared using PyMol.[9] tdFlicR is shown by placing cpmApple between two CiVSDs. Model of tdFlicR is represented by the crystal structures of CiVSD (PDB ID 4G80),[6] cpmApple (PDB ID 4I2Y).[10] tdFlicR-ASAP is composed of CiVSD linked to cpmApple linked to GgVSD which has a circularly permutated superfolder GFP[11] inserted between S3 and S4 α-helices. Model of tdFlicR-ASAP is represented by the crystal structures of CiVSD (PDB ID 4G80),[6] cpmApple (PDB ID 4I2Y),[10] and cpGFP (PDB ID 3EVR).[12]

Acknowledgement of plasmid sources. We thank Michael Lin for the gift of pcDNA3.1/Puro-CAG-ASAP1 (Addgene plasmid # 52519), Richard Axel and Joseph

Gogos and C. Ron Yu for the gift of pGEMTEZ- Kir2.1 (Addgene plasmid # 32641), and Vincent Pieribone for the gift of ArcLight-Q239 (Addgene plasmid # 36856).

Supplementary Figures

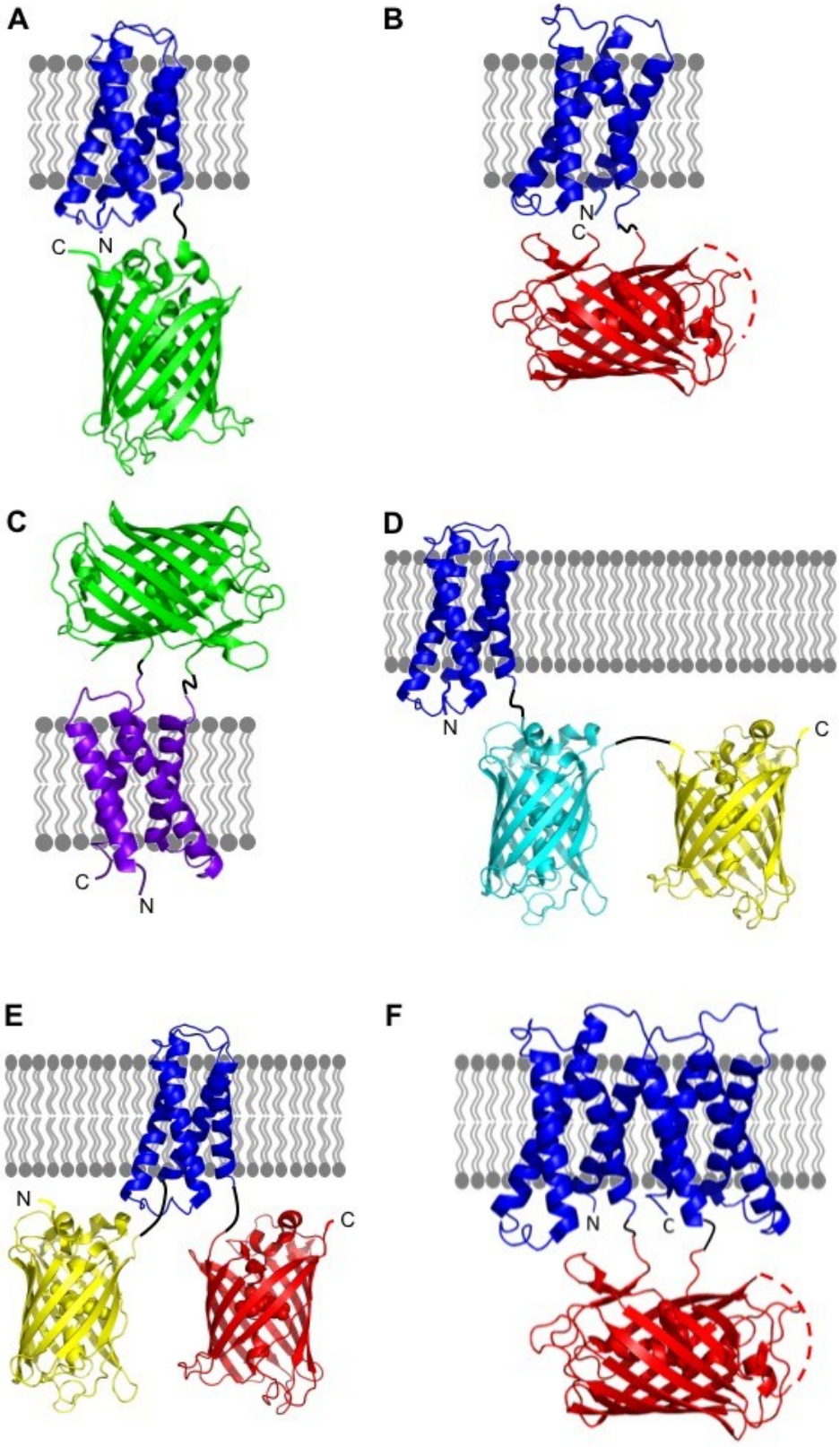

Fig. S1. Schematic representations of voltage indicator designs. (A) A single FP is fused to S4 of the CiVSD.[8,13] (B) A cpFP is fused to S4 of the CiVSD.[2,14,15] (C) A cpFP is fused between the S3 and S4 helices of GgVSD. In this design the FP barrel is outside the cell.[1] (D) A FRET pair is fused to S4 of CiVSD.[13,16–18] (E) A FRET pair is fused to S1 and S4 and flank the CiVSD.[19] (F) Representation of tdFlicR topology. Cartoon representations were prepared using PyMol.[9]

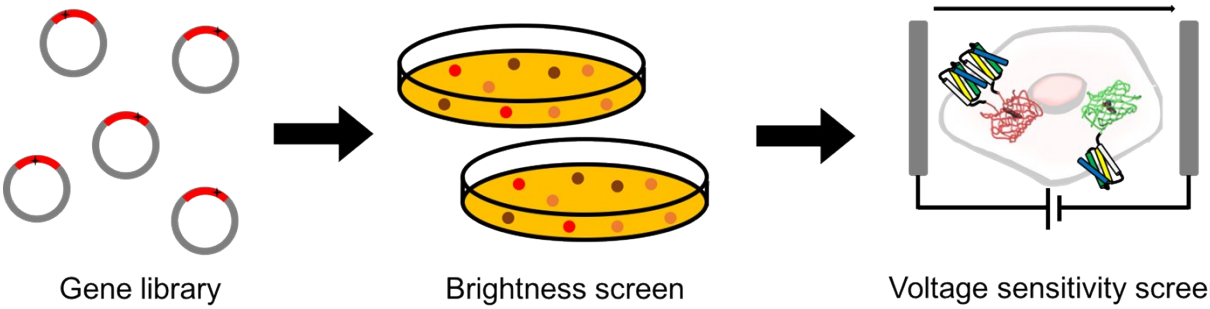

Fig. S2. Schematic representation of screening method used to select tdFlicR variants. First a gene library was constructed and used to transform *E. Coli*. Colonies were screened and the brightest ones were selected to test their voltage sensitivity in mammalian cells using ITV as described in Experimental procedures.

.

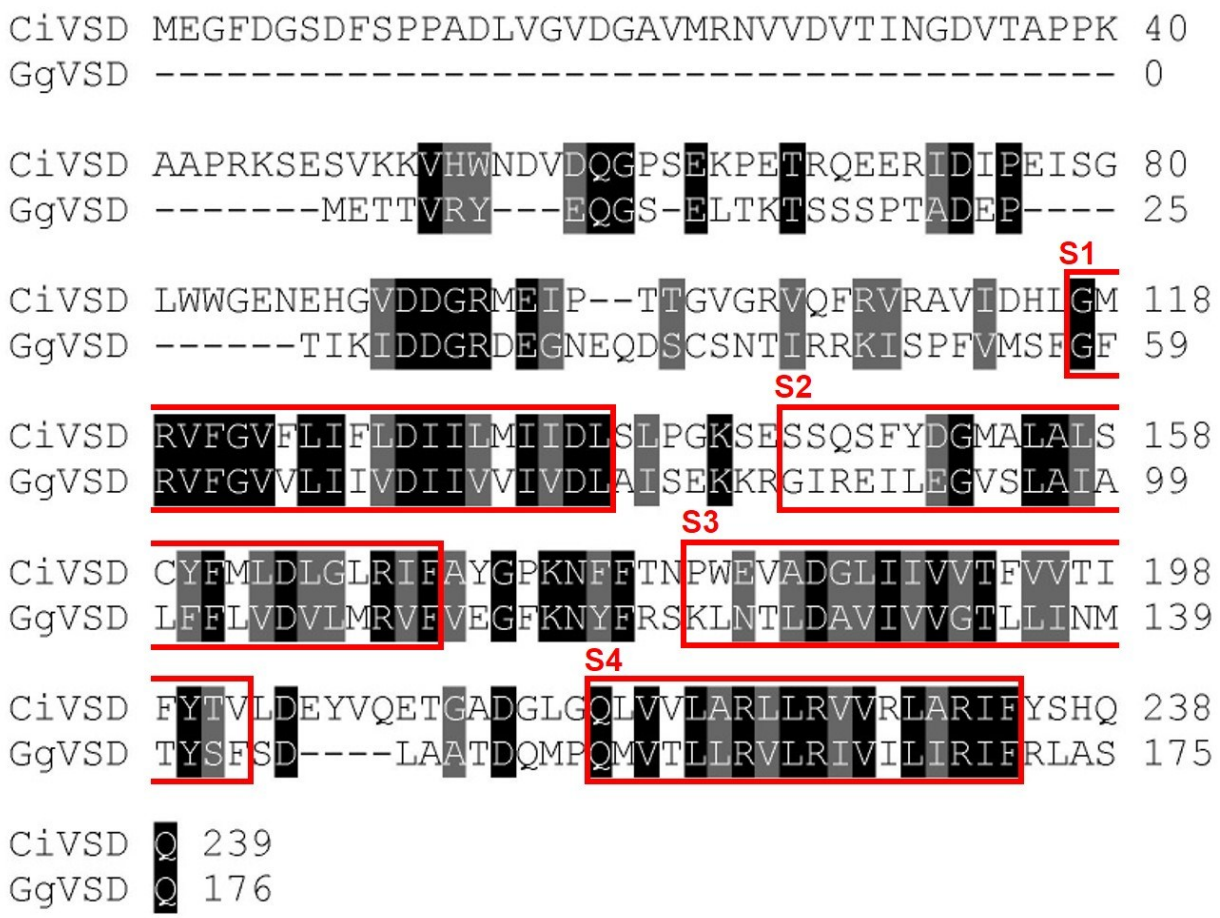

Fig. S3. Sequence alignment of CiVSD (as present in FlicR1) and GgVSD (as present in ASAP1). The high level of sequence identity between S1 and S4 indicates that CiVSD and GgVSD are likely to form a heterodimer. Conserved amino acids are highlighted in black and similar amino acids are shaded in grey. Dashes indicate gaps in the alignment. S1-S4 α-helices are labeled, highlighted in red and boxed. Amino acid sequences aligned using Clustal Omega.[20,21]

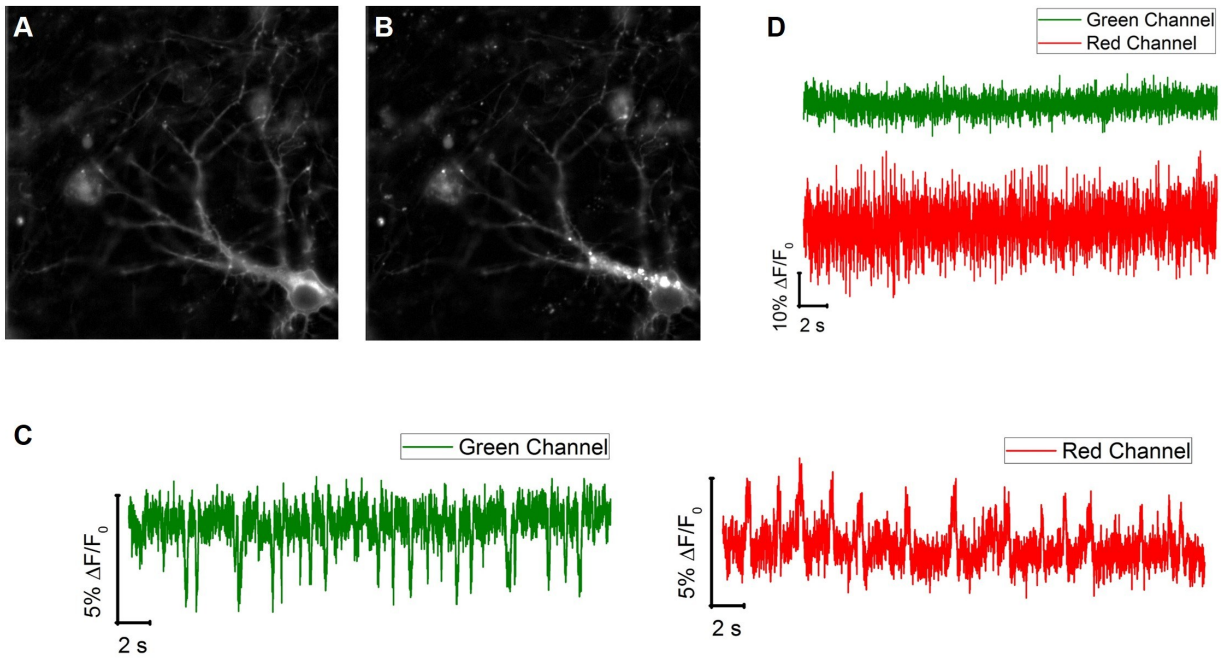

Fig. S4. tdFlicR-VK-ASAP imaging in neurons. (A-B) Image of cultured hippocampal neuron expressing tdFlicR-VK-ASAP in the green channel (A) and red channel (B). Note that the fluorescence puncta are only present in the red channel (B). (C) Consecutive (i.e., not simultaneous) two-color fluorescence imaging of spontaneous activity waveforms in rat hippocampal neuron culture with tdFlicR-VK-ASAP indicator showing successful reporting in the green channel (left) and red channel (right). (D) Simultaneous two-color fluorescence imaging of spontaneous activity in rat hippocampal neuron culture with tdFlicR-VK-ASAP indicator. The fluorescence intensity was low and true signals could not be distinguished from noise.

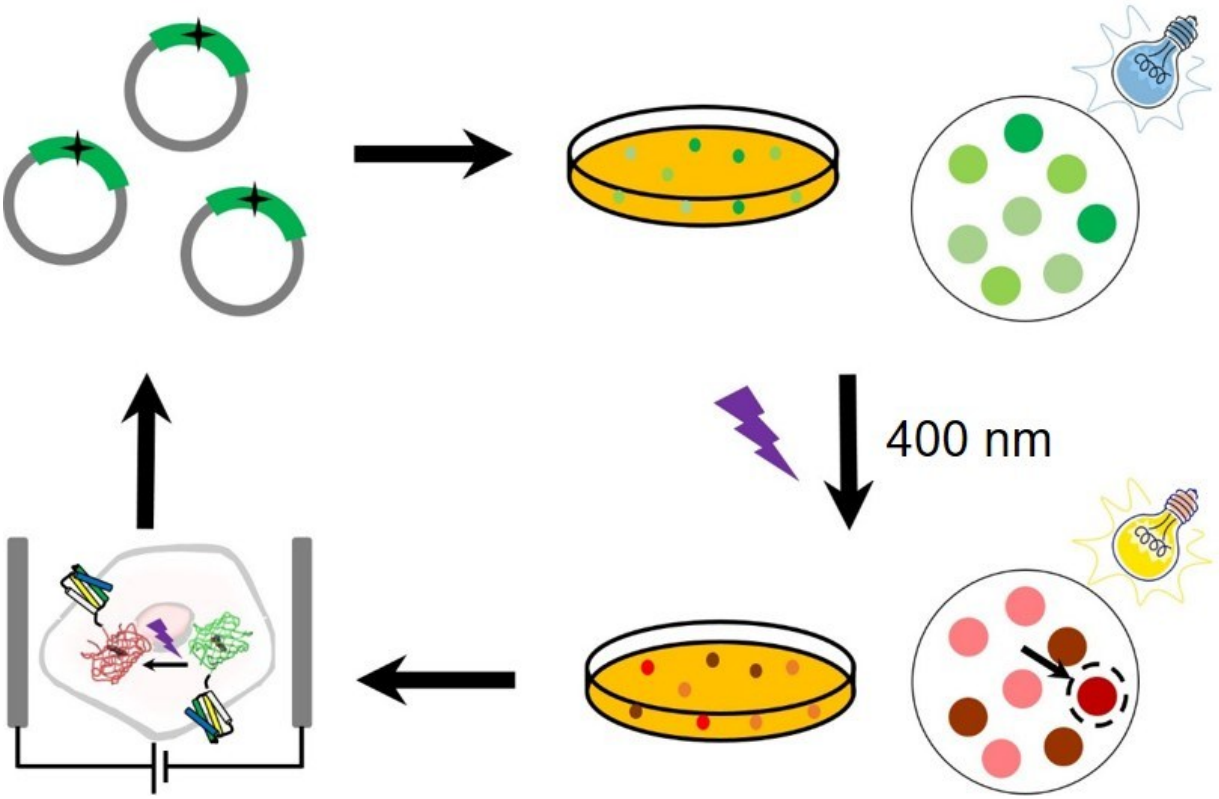

Fig. S5. Schematic representation of directed evolution strategy used to evolve FlicGR. First, libraries of DNA encoding indicator genes were used to transform *E. coli* and cultured on agar plates. Second, *E. coli* colonies expressing FlicGR were illuminated with blue light and green fluorescence was imaged. Subsequently, *E. coli* colonies were subjected to ~400 nm light for photoconversion. The same colonies were then illuminated with yellow light and red fluorescence was imaged. Colonies with efficient photoconversion and/or bright red fluorescence were picked and screened for voltage sensitivity in mammalian cells. Voltage sensitivity of FlicGR variants was then tested via ITV stimulation in HeLa cells. The process was repeated for several rounds.

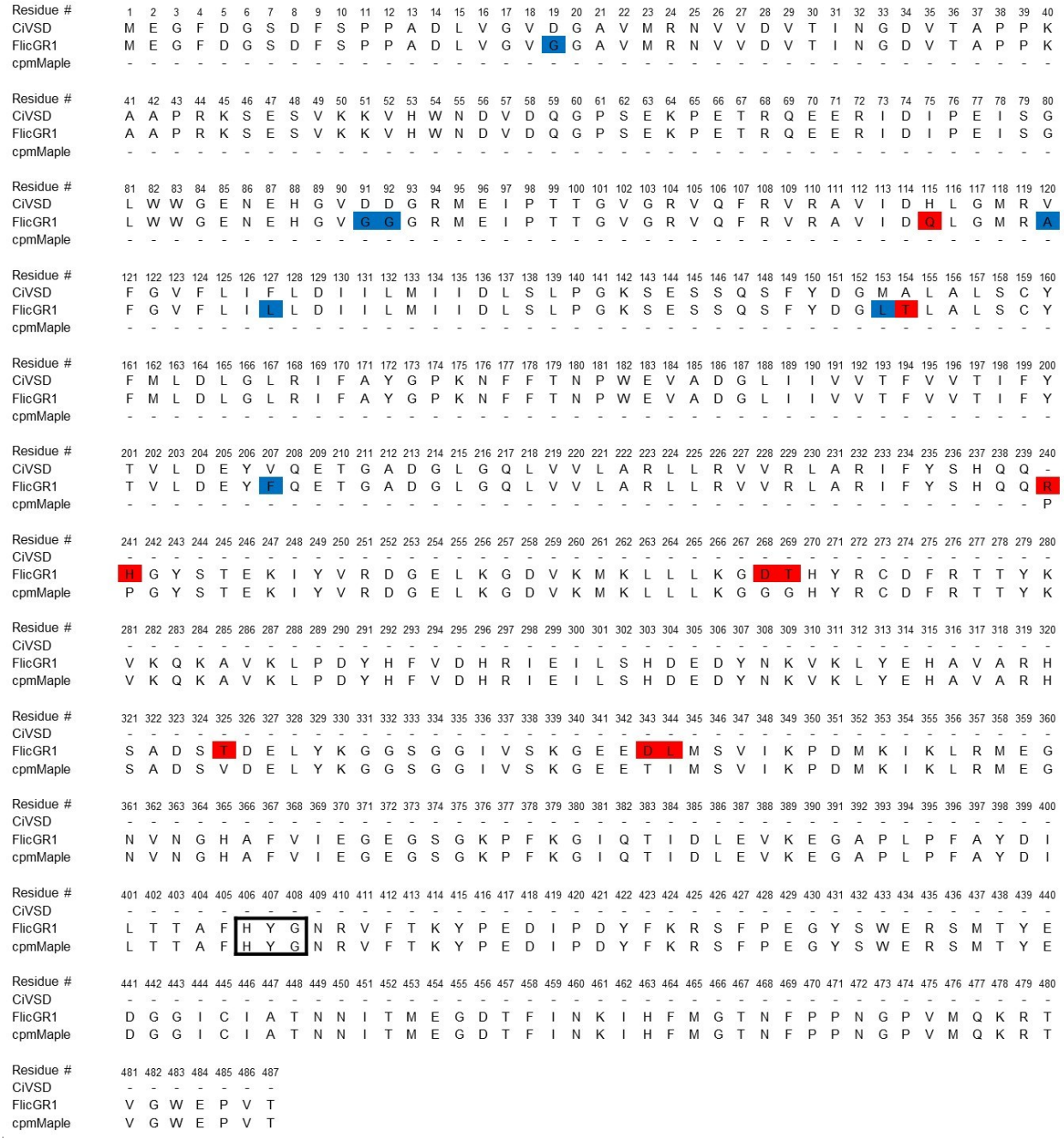

Fig. S6. Sequence alignment of FlicGR1. Alignment of FlicGR1 gene with the CiVSD domain (top) and cpmMaple from GR-GECO1.1 (bottom). Blue highlighted residues are amino acid mutations in the CiVSD domain carried forward from our work on FlicR1.[2] Residues highlighted in red are mutations with respect to the starting template. Residues HYG in the box correspond to the mMaple chromophore.

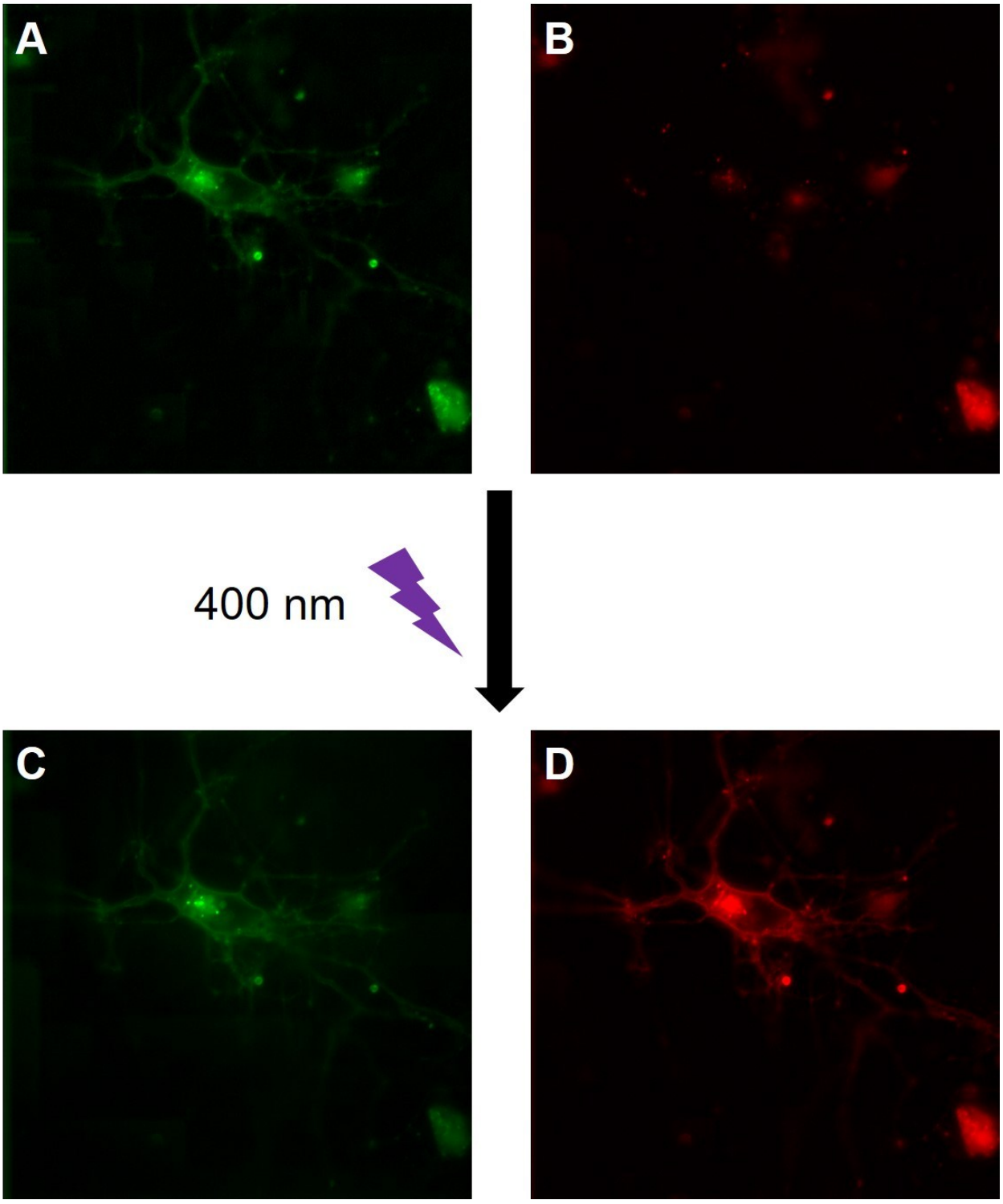

Fig. S7. FlicGR1 imaging in neurons. (A-B) Image of a cultured hippocampal neuron expressing FlicGR1 in the green channel (A) and red channel (B) before photoconversion. (C-D) Image of cultured hippocampal neuron expressing FlicGR1 in the green channel (C) and red channel (D) after photoconversion.

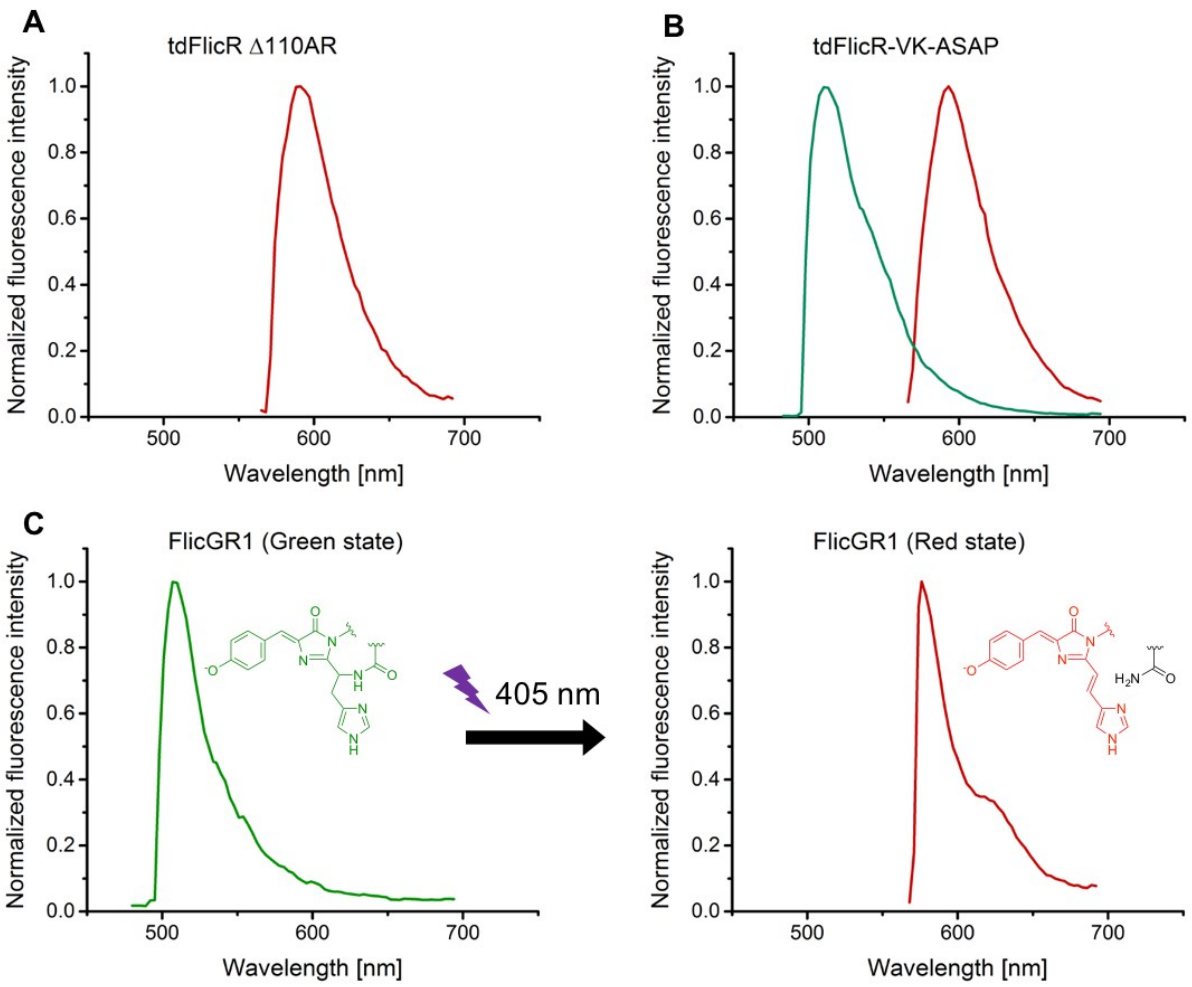

Fig. S8. Fluorescence emission spectra of voltage indicators expressed in Hela cells. (A) Fluorescence emission spectrum of tdFlicR Δ110AR using 561 nm laser excitation. (B) Fluorescence emission spectrum of tdFlicR-VK-ASAP using 458 nm laser excitation (green trace), and using 561 nm laser excitation (red trace). (C) Emission spectra of FlicGR1 before photoconversion (left panel) and after photoconversion (right panel). FlicGR1 expressing cells were photoconverted using blue light illumination (405 nm laser). Left panel: Fluorescence emission spectrum of FlicGR1 using 488 nm laser excitation (green trace) Right panel: Fluorescence emission spectrum of FlicGR1 after photoconversion using 561 nm laser excitation (red trace). Inset: Structure of FlicGR1 chromophore before and after photoconversion.

Supplementary References

1 F. St-Pierre, J. D. Marshall, Y. Yang, Y. Gong, M. J. Schnitzer and M. Z. Lin, *Nat. Neurosci.*, 2014, **17**, 884–889.

2 A. S. Abdelfattah, S. L. Farhi, Y. Zhao, D. Brinks, P. Zou, A. Ruangkittisakul, J. Platisa, V. A. Pieribone, K. Ballanyi, A. E. Cohen and R. E. Campbell, *J. Neurosci.*, 2016, **36**, 2458–2472.

3 H. Hoi, T. Matsuda, T. Nagai and R. E. Campbell, *J. Am. Chem. Soc.*, 2013, **135**, 46–49.

4 J. Wu, L. Liu, T. Matsuda, Y. Zhao, A. Rebane, M. Drobizhev, Y.-F. Chang, S. Araki, Y. Arai, K. March, T. E. Hughes, K. Sagou, T. Miyata, T. Nagai, W.-H. Li and R. E. Campbell, *ACS Chem. Neurosci.*, 2013, **4**, 963–972.

5 Z. Cheng and R. E. Campbell, *Chembiochem*, 2006, **7**, 1147–1150.

6 Q. Li, S. Wanderling, M. Paduch, D. Medovoy, A. Singharoy, R. McGreevy, C. A. Villalba-Galea, R. E. Hulse, B. Roux, K. Schulten, A. Kossiakoff and E. Perozo, *Nat. Struct. Mol. Biol.*, 2014, **21**, 244–252.

7 C. R. Yu, J. Power, G. Barnea, S. O'Donnell, H. E. V Brown, J. Osborne, R. Axel and J. A. Gogos, *Neuron*, 2004, **42**, 553–566.

8 L. Jin, Z. Han, J. Platisa, J. R. A. Wooltorton, L. B. Cohen and V. A. Pieribone, *Neuron*, 2012, **75**, 779–785.

9 L. Schrödinger, *The PyMOL Molecular Graphics System, Version 1.3 r1; Schrödinger, LLC: New York, 2010*.

10 J. Akerboom, N. Carreras Calderón, L. Tian, S. Wabnig, M. Prigge, J. Tolö, A. Gordus, M. B. Orger, K. E. Severi, J. J. Macklin, R. Patel, S. R. Pulver, T. J. Wardill, E. Fischer, C. Schüler, T.-W. Chen, K. S. Sarkisyan, J. S. Marvin, C. I. Bargmann, D. S. Kim, S. Kügler, L. Lagnado, P. Hegemann, A. Gottschalk, E. R. Schreiter and L. L. Looger, *Front. Mol. Neurosci.*, 2013, **6**, 2.

11 J.-D. Pédelacq, S. Cabantous, T. Tran, T. C. Terwilliger and G. S. Waldo, *Nat. Biotechnol.*, 2006, **24**, 79–88.

12 Q. Wang, B. Shui, M. I. Kotlikoff and H. Sondermann, *Structure*, 2008, **16**, 1817–1827.

13 A. Lundby, H. Mutoh, D. Dimitrov, W. Akemann and T. Knöpfel, *PLoS One*, 2008, **3**, e2514.

14 L. Barnett, J. Platisa, M. Popovic, V. A. Pieribone and T. Hughes, *PLoS One*, 2012, **7**, e43454.

15 S. G. Gautam, A. Perron, H. Mutoh and T. Knöpfel, *Front. Neuroeng.*, 2009, **2**, 14.

16 R. Sakai, V. Repunte-Canonigo, C. D. Raj and T. Knöpfel, *Eur. J. Neurosci.*, 2001, **13**, 2314–2318.

17 A. J. Lam, F. St-Pierre, Y. Gong, J. D. Marshall, P. J. Cranfill, M. A. Baird, M. R. McKeown, J. Wiedenmann, M. W. Davidson, M. J. Schnitzer, R. Y. Tsien and M. Z. Lin, *Nat. Methods*, 2012, **9**, 1005–1012.

18 H. Tsutsui, S. Karasawa, Y. Okamura and A. Miyawaki, *Nat. Methods*, 2008, **5**, 683–685.

19 W. Akemann, H. Mutoh, A. Perron, Y. K. Park, Y. Iwamoto and T. Knöpfel, *J. Neurophysiol.*, 2012, **108**, 2323–2337.

20 F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson and D. G. Higgins, *Mol. Syst. Biol.*, 2011, **7**, 539.

21 M. Goujon, H. McWilliam, W. Li, F. Valentin, S. Squizzato, J. Paern and R. Lopez, *Nucleic Acids Res.*, 2010, **38**, W695–W699.