Genetically encoded bioluminescent voltage indicator for multi-purpose use in wide range of bioimaging

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Supplementary Information

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Supplementary Methods

Gene construction of fGEVIs. To construct CS-CDF-Mermaid2-PRE and CS-CDF-VSFP-BF1.2-PRE, we used the In-Fusion HD Cloning Kit (Clontech), following the manufacturer's protocol. To construct CS-CDF-ArclightQ239-PRE and CS-CDF-QuasAr2-PRE, the cDNAs of fGEVIs (ArclightQ239 [Addgene: Plasmid #36856] and QuasAr2 [Addgene: Plasmid #51694]) were amplified by PCR using a sense primer containing a *Bgl*II site and Kozak sequence, and a reverse primer containing a *Xho*I site and a stop codon. Then each of them was subcloned between the *Bam*HI and *Xho*I sites of CS-CDF-Mermaid2-PRE. All of the constructs were verified by DNA sequencing and the primers are listed in **Supplementary Table 1 and 2**.

Investigation of furimazine toxicity. HEK293T cells were cultured on collagen-coated 35-mm glass-bottom dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Just before irradiation, the medium was replaced with phenol red-free DMEM/F12 supplemented with 10% FBS and 1 μM SYTOX AADvanced dead cell stain (Molecular Probes). The cells were continuously irradiated by excitation light for 5 minutes on Eclipse Ti-E inverted microscope (Nikon) equipped with a 10×, NA 0.5, Plan Fluor objective lens (Nikon), a FF01-472/30-25 excitation filter and a FF502-Di01 dichroic mirror (Semrock). The cells were maintained at 37°C by using a stage-top incubator (Tokai Hit). The illumination power was measured above the objective using a power meter (Thorlab). Then irradiated cells were subsequently observed using DIC and fluorescence of SYTOX for the following 12 hours at 10 minutes interval. The fluorescence of SYTOX was measured with a FF01-472/30-35 excitation filter, a FF502-Di01 dichroic mirror and a FF01-641/75 emission filter

(Semrock). For treatment with furimazine, the medium was replaced with phenol red-free DMEM/F12 supplemented with 10% FBS, 1 μM SYTOX AADvanced dead cell stain and 50 μM furimazine. Then the cells were observed in the identical microscope set-up for the following 12 hours at 10 minutes interval.

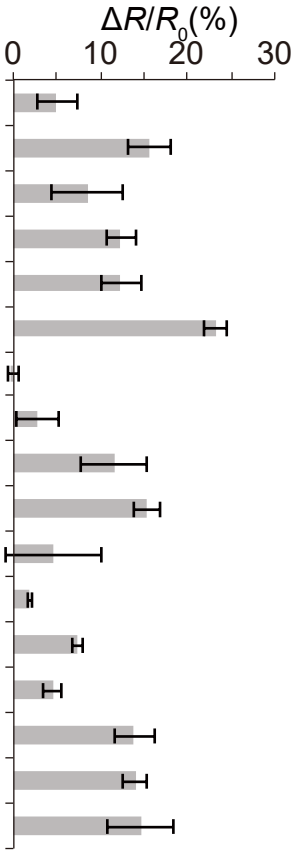
Voltage imaging using fGEVIs. We performed voltage imaging using an Eclipse Ti-E inverted microscope with a 40×, NA 1.30, Plan Fluor oil-immersion objective (Nikon) and an iXon Ultra EMCCD camera (Andor Technology). Instead of a 40×, NA 1.30, Plan Fluor oil-immersion objective, a 20×, NA 0.7, Plan Fluor objective (Nikon) was used for voltage imaging in aggregates of hiPSC-CMs. For Mermaid2, a FF01-438/24-25 excitation filter, a FF458-Di02-25×36 dichroic mirror (Semrock) and same setup of W-VIEW GEMINI A12801-01 (Hamamatsu) as for LOTUS-V, were used. For VSFP BF1.2, a FF01-500/24-25 excitation filter, a FF520-Di02-25×36 dichroic mirror (Semrock) and W-VIEW GEMINI A12801-01 (Hamamatsu) equipped with a FF580-FDi01-25×36 dichroic mirror, FF01-542/27-25 and FF01-624/40-25 emission filters (Semrock) were used. For ArclightQ239, a FF02-472/30-25 excitation filter, a FF495-Di03-25×36 dichroic mirror and a FF01-520/35-25 emission filter (Semrock) were used. For FlicR1.0, a FF01-562/40-25 excitation filter, a FF593-Di03-25×36 dichroic mirror and a FF01-624/40-25 emission filter (Semrock) were used. For QuasAr2, we constructed a homemade, total internal reflection fluorescence (TIRF) microscope based on an IX71 inverted microscope (Olympus) with a 60×, NA 1.40, Plan Apo oil-immersion objective (Olympus), a 638 nm red diode laser (Coherent), an Evolve512 EMCCD camera (Photometrics) and Di01-R405/488/561/635-25×36 dichroic mirror with a FF01-692/40-25 emission filter (Semrock).

Voltage imaging using Di-8-ANEPPS in hiPSC-CMs. Before imaging, hiPSC-CMs were loaded with 40 μM Di-8-ANEPPS (Invitrogen) in Reprocardio assay medium (ReproCell) for 10 min. Imaging was conducted using an Eclipse Ti-E inverted microscope with a 20×, NA 0.7, Plan Fluor objective (Nikon). A FF01-438/24-25 excitation filter, a FF458-Di02-25×36 dichroic mirror (Semrock) were used for fluorescence excitation. Fluorescence was split by W-VIEW GEMINI A12801-01 (Hamamatsu) equipped with a FF562-Di03-25×36 dichroic mirror, and passed thorough FF01-537/26-25 and FF01-593/LP-25 emission filters (Semrock).

Supplementary Video 1 | An overlaid movie of bright field and averaged $\Delta R/R_0$ of LOTUS-V upon the contraction of hiPSC-CMs (n = 23 sessions).

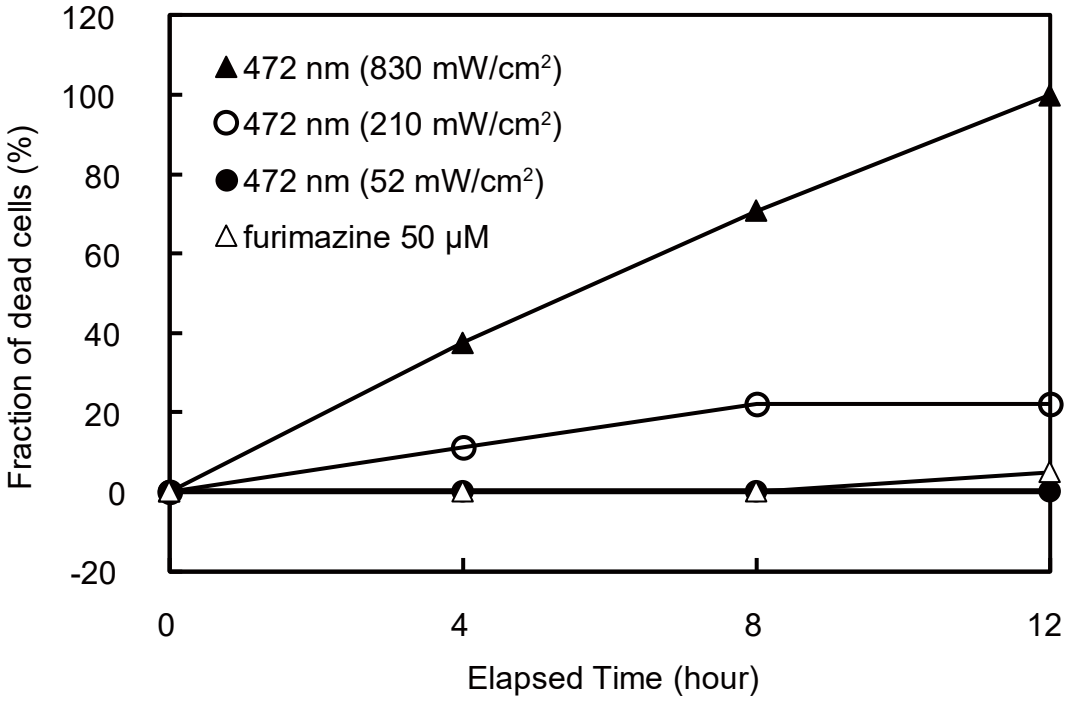
N-VSD(1-**X**)	**A**	C-VSD((**X**+1)-**Y**)	**B**

Name	X	Y	A	B
69NL-VSD-249mNG	69	249	NanoLuc	mNeon
69NL-VSD-249VN	69	249	NanoLuc	Venus
84NL-VSD-249mNG	84	249	NanoLuc	mNeon
84NL-VSD-249VN	84	249	NanoLuc	Venus
103NL-VSD-249mNG	103	249	NanoLuc	mNeon
103NL-VSD-249VN (LOTUS-V)	103	249	NanoLuc	Venus
103NL-VSD(D129R)-VN (LOTUS-V(D129R))	103	249	NanoLuc	Venus
103NL-VSD-249VN(cp50)	103	249	NanoLuc	cp50Venus
103NL-VSD-249VN(cp157)	103	249	NanoLuc	cp157Venus
103NL-VSD-249VN(cp173)	103	249	NanoLuc	cp173Venus
103NL-VSD-249VN(cp195)	103	249	NanoLuc	cp195Venus
103NL-VSD-249VN(cp229)	103	249	NanoLuc	cp229Venus
103NL-VSD-243VN	103	243	NanoLuc	Venus
103NL-VSD-239VN	103	239	NanoLuc	Venus
103VN-VSD-249NL	103	249	Venus	NanoLuc
103VN-VSD-243NL	103	243	Venus	NanoLuc
103VN-VSD-239NL	103	239	Venus	NanoLuc



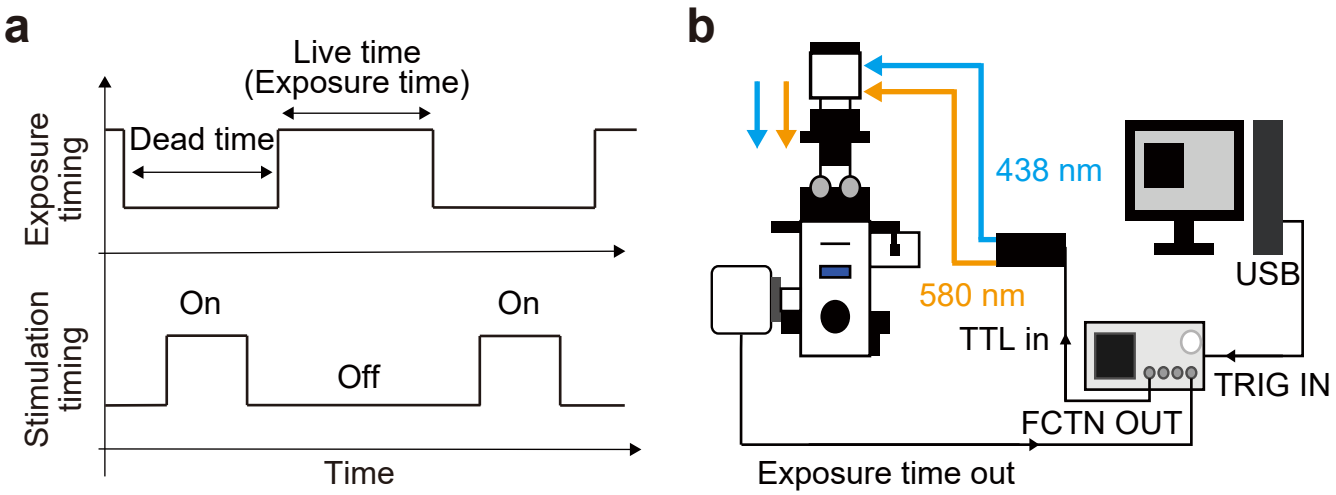
Supplementary Figure 1 | Characterization of bGEVIs with KCl stimulation

Multiple combinations of insertion (**X**), linkage sites (**Y**), FRET donors and acceptors (**A** or **B**) tested in our experiments. The dynamic range of the ratio change ($\Delta R/R_0$) in each construct to the KCl stimulation is shown in right panel. Error bars indicate mean ± s.e.m.



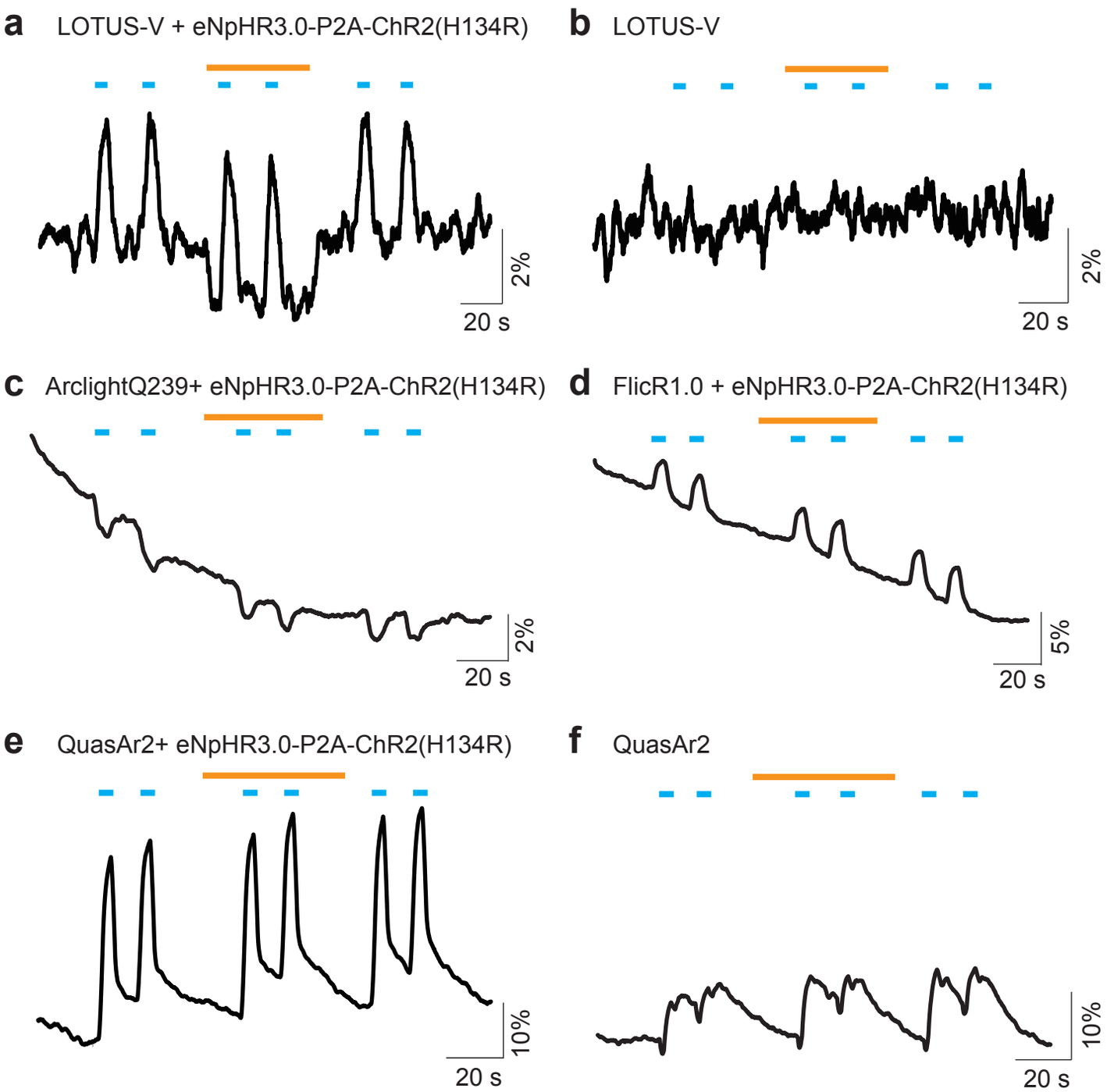
Supplementary Figure 2 | Investigation of Furimazine toxicity

HEK293T cells were either irradiated by the excitation light with varied intensity, or treated with 50 μM furimazine. Each data point was collected from 60 cells.



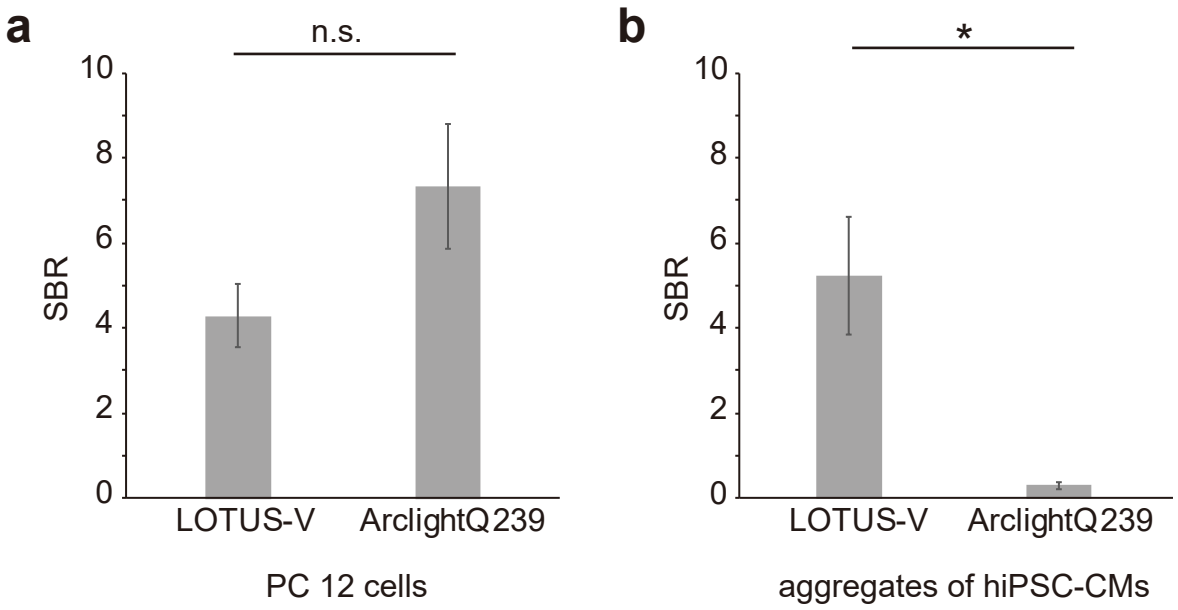
Supplementary Figure 3 | System setup for optogenetic stimulation during dead time

(**a**) Stimulation timing for dead time imaging. The stimulation light for ChR2(H134R) or eNpHR3.0 was irradiated during camera's dead time. (**b**) Setup for dead time imaging to activate ChR2(H134R) and eNpHR3.0. The exposure time-out signals for triggering the multifunctional generator were transferred from an EMCCD camera iXon Ultra (Andor) thorough 50 Ω BNC cables. Pulse signals generated from a multifunctional generator were transferred to an LED-based light source LightEngine (Lumencore) via TTL input to turn on/off the blue light. A Windows PC was used to record the image data and to control the on/off timing of the stimulation light. Arrowheads indicate the signal flow. 438/24 nm and 580/27 nm light were irradiated from above the culture dish. Irradiation from above the dish was applied by replacing the halogen lamp for the transmitted light source with the LightEngine liquid light guide, connected using a homemade adaptor.



Supplementary Figure 4 | Voltage imaging using various GEVIs with optogenetic stimulation

(**a,b**) Optical response of LOTUS-V in PC12 cells coexpressing eNpHR3.0 and ChR2(H134R) (**a**), or without optical control tools (**b**) upon light irradiation. (**c,d**) Optical response of ArclightQ239 (**c**) and FlicR1.0 (**d**) in PC12 cells coexpressing both eNpHR3.0 and ChR2(H134R). The excitation light for ArclightQ239 (472 nm, 1.01 mW/cm^2) and FlicR1.0 (562 nm, 499 mW/cm^2) was applied from the bottom of an imaging dish. (**e,f**) Optical response of QuasAr2 in PC12 cells coexpressing eNpHR3.0 and ChR2(H134R) (**e**), or without optical control tools (**f**) upon light irradiation. The signal of QuasAr2 increased even in the absence of ChR2(H134R) and eNpHR3.0, suggesting its photochromic property. The excitation light for QuasAr2 (631 nm, 60 W/cm^2) was applied from the bottom of an imaging dish. Blue and orange bars indicate the durations of blue (438 nm, 25.5 mW/cm^2) and orange (580 nm, 47 mW/cm^2) light irradiation, respectively. The signals processed by the moving average (window length of 20 frames) is shown.

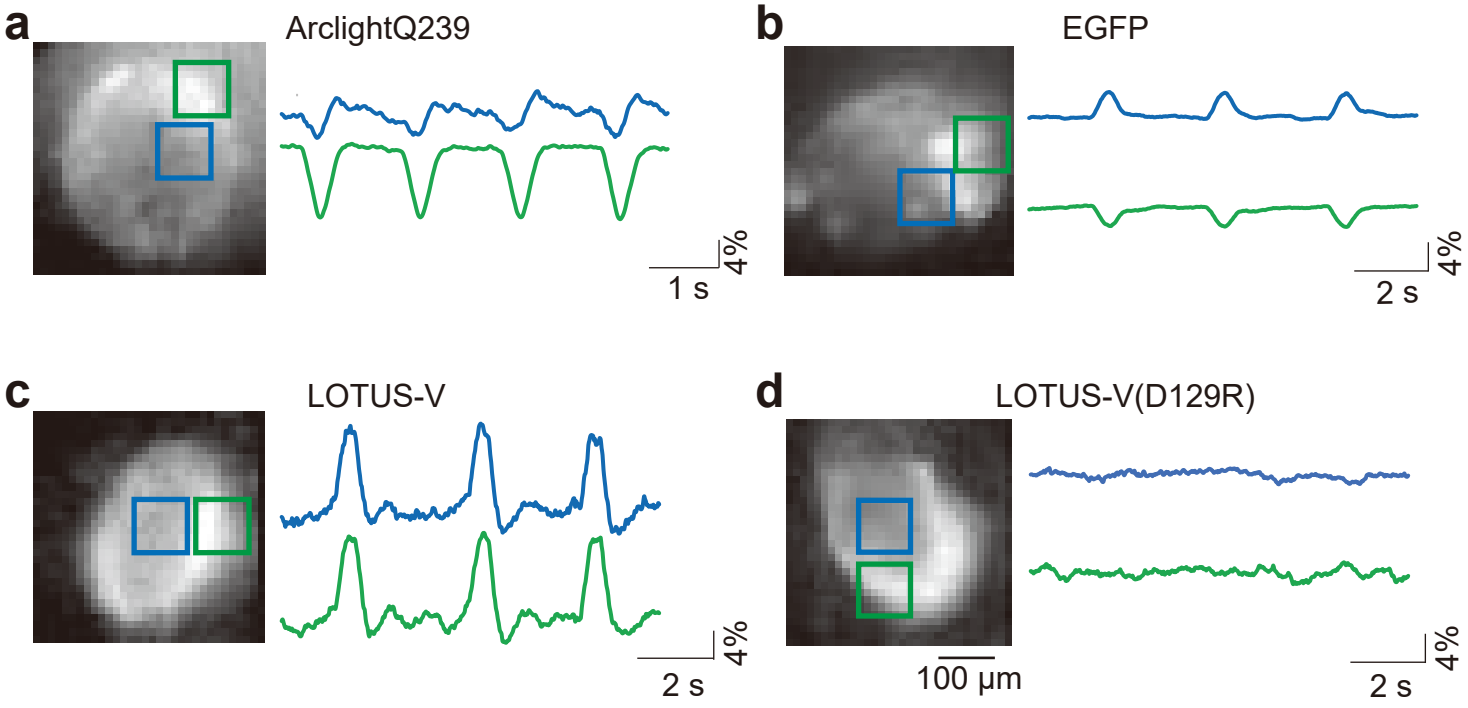


Supplementary Figure 5 | Signal to background ratio in PC12 cells and aggregates of hiPSC-CMs

(a,b) Signal to background ratio (SBR) of LOTUS-V and ArclightQ239 in PC12 cells (4.29 ± 0.74 and 7.32 ± 1.45, respectively; n = 5 cells, p = 0.143, two-tailed Wilcoxon rank sum test) (**a**) or aggregates of hiPSC-CMs (5.23 ± 1.39 and 0.31 ± 0.09, respectively; n = 5 aggregates, p = 0.036, two-tailed Wilcoxon rank sum test) (**b**). For fair comparison with ArclightQ239, the SBR of Venus signal from LOTUS-V was regarded as the SBR of LOTUS-V. Error bars indicate mean ± s.e.m.

Supplementary Figure 6 | Long-term imaging using ArclightQ239 in aggregates of hiPSC-CMs

Time course of $\Delta F/F_0$ of ArclightQ239 at 0 min and 60 min. Images were taken at 20 ms/frame. Excitation light (472 nm, 2.84 mW/cm^2) was applied continuously for 60 min. The ratio change processed by the moving average (window length of 20 frames) is shown in all graphs.



Supplementary Figure 7 | Evaluation of a motion artefact in an aggregate of hiPSC-CMs

(**a-d**) Time course of the signal changes of ArclightQ239 (**a**), EGFP (**b**), LOTUS-V (**c**), LOTUS-V(D129R) (**d**) in the local places of an aggregates of hiPSC-CMs. Blue and green lines indicate the signal changes in each ROI. Images were taken at 20 ms/frame. The ratio change processed by the moving average (window length of 20 frames) is shown in all graphs.

Name of Primer	Sequence (5' to 3')
F-*Hind* III-VSD_1	AGTCAAGCTTGCCACCATGGAGGGATTC
F-*EcoR* I-GVG-VSD_70	GCAGAATTCGGCGTGGGCGAGGAACGAATAGATATACC
F-*EcoR* I-GVG-VSD_85	GCAGAATTCGGCGTGGGCGAGAATGAACATGGAG
F-*EcoR* I-GVG-VSD_104	GCAGAATTCGGCGTGGGCCGCGTCCAGTTTCGTGTCC
R-VSD_69-*BamH* I	ATTGGATCCTGTCTTGTCTCTGG
R-VSD_84-*BamH* I	ATTGGATCCCCCCACCATAGACCTG
R-VSD_103-*BamH* I	ATTGGATCCCCTACACCAGTAGTAG
R-VSD_239-*Xho* I	CATCTCGAGTTGTTGATGGGAATAAAATATTC
R-VSD_243-*Xho* I	CATCTCGAGTGAAGCCTTCATTTGTTGATG
R-VSD_249-*Xho* I	CATCTCGAGTGATATTGTTCTTCTGCTTGAAGCC
F-*BamH* I-NLuc_1	ATTGGATCCGATGGTCTTCACACTCG
F-*Xho* I-NLuc_1	CATCTCGAGATGGTCTTCACACTCGAAG
R-NLuc_171-*EcoR* I	GCAGAATTCCGCCAGAATGCGTTCG
R-NLuc_171-x-*Not* I	ATTGCGGCCGCTTACGCCAGAATGCGTTCG
F-*Xho* I-gfp_1	TATCTCGAGATGGTGAGCAAGGGCGAGG
F-*BamH* I-gfp_1	TTGGATCCGATGGTGAGCAAGGGCGAGGAG
F-*Xho* I-gfp_50	TAATCTCGAGATGACCGGCAAGCTGCCC
F-*Xho* I-gfp_157	TAATCTCGAGATGCAGAAGAACGGCATCAA
F-*Xho* I-gfp_173	TAATCTCGAGATGGACGGCGGCGTGCAG
F-*Xho* I-gfp_195	TAATCTCGAGATGCTGCCCGACAACCACTA
F-*Xho* I-gfp_229	TAATCTCGAGATGATCACTCTCGGCATGG
R-Venus_49-x-*NotI*	ATTGCGGCCGCTTAGGTGCAGATCAGCTTCAGGG
R-Venus_156-x-*Not* I	ATTGCGGCCGCTTACTTGTCGGCGGTGATATAGA
R-Venus_172-x-*Not* I	ATTGCGGCCGCTTACTCGATGTTGTGGCGGATCT
R-Venus_194-x-*Not* I	ATTGCGGCCGCTTACAGCACGGGGCCGTCGCCGA
R-Venus_228-x-*Not* I	ATTGCGGCCGCTTACCCGGCGGCGGTCACGAACT
R-gfp_239-x-*Not* I	ATTGCGGCCGCTTACTTGTACAGCTCGTCCATG
R-gfp_239-*EcoR* I	GCAGAATTCCTTGTACAGCTCGTCCATGCC
F-*EcoR* I-P2A-1	AATTCGGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGG
F-P2A-*Not* I-22	CTGGAGACGTGGAGGAGAACCCTGGACCTTGC
R-P2A-*Not* I1	CTCCAGCCTGCTTCAGCAGGCTGAAGTTAGTAGCTCCGCTTCCG
R-*EcoR* I-P2A-22	GGCCGCAAGGTCCAGGGTTCTCCTCCACGT
F-*Hind* III-eNpHR_1	AGTCAAGCTTGCCACCATGACAGAGAC
R-ERex_7-*EcoR* I	GCAGAATTCCACCTCGTTCTCGTAGCAGAAC
F-*Not* I-ChR2_1	ATTGCGGCCGCATGGACTATGGCGGCG
R-ChR2_309-x-*Xba* I	AGTCTCTAGATTATGGCACGGCTCCGGCCT
F-LOTUS-V_428 Xho1 del	GAAGAACAATATCACTGGAGATGGTGAGCAAGGG
F-*Bgl* II-VSD_1 kozak	GACTAGATCTGCCACCATGGAGGGATTC
R-*Xho* I-x-gfp_238	CATCTCGAGTTACTTGTACAGCTCGTCC
F-VSD_1	TGTCGTGAACACGCTGGATCCGCCACCATGGAGGG
R-gfp_238	GACGCGGCCCACGCCGAATTCCTTGTACAGCTCGTC
R-x-gfp238	CGATAAGCTTGATCCCTCGAGTTACTTGTACAGCTC
F-LOTUS-V_D129R	GTCTTCCTAATTTTCTTGCGCATCATCCTCATGATC
F-gfp Y66G	GACCACCCTGGGCggCGGCCTGCAGTG

Supplementary Table 1 | List of primers for gene construction (LOTUS-V and eNpHR3.0-P2A-ChR2(H134R))

Name of Primer	Sequence (5' to 3')
F-*Hind* Ⅲ-VSD_1	AGTCAAGCTTGCCACCATGGAGGGATTC
F-*Bam* HI-VSD	TGTCGTGAACACGCTGGATCCGCCACCATGGAGGG
R-gfp_238-*Eco*RI	GACGCGGCCCACGCCGAATTCCTTGTACAGCTCGTC
F-gfp_1	GGCGTGGGCCGCGTCCAGTTTCGTGTCCGA
R-gfp_238-x-*Xho* I	CGATAAGCTTGATCCCTCGAGTTACTTGTACAGCTC
F-*Bam* HI-Gorgi ex_1	TGTCGTGAACACGCTGGATCCGCCACCATGAGGAGC
R-SEpH_238-x-*Xho* I	CATCTCGAGTCATTTGTATAGTTCATCCATG
F-*Bgl* Ⅱ-koz-QuasAr2_1	GACTAGATCTGCCACCATGGGGACCTGGATG
R-mOrange2_237-x-*Xho* I	CATCTCGAGTTATTCATTCTCATAACAAAATCC

Supplementary Table 2 | List of primers for gene construction (Mermaid2, VSFP BF1.2, ArclightQ239 and QuasAr2)