Supplementary information

Orange/far-red hybrid voltage indicators with reduced phototoxicity enable reliable long-term imaging in neurons and cardiomyocytes

Shuzhang Liu[1,#], Jing Ling[2,#], Peng Chen[3], Chang Cao[1], Luxin Peng[1], Yuan Zhang[4], Guangshen Ji[5], Yingna Guo[5], Peng R. Chen[1,2], Peng Zou[1,2,6,7,*] and Zhixing Chen[2,3,4,*]

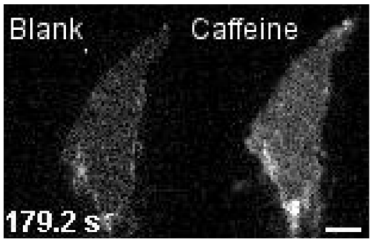
1. College of Chemistry and Molecular Engineering, Synthetic and Functional Biomolecules Center, Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of the Ministry of Education, PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing 100871, China
2. Peking-Tsinghua Center for Life Science, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China
3. PKU-Nanjing Institute of Translational Medicine, Nanjing 211800, China
4. College of Future Technology, Institute of Molecular Medicine, National Biomedical Imaging Center, Beijing Key Laboratory of Cardiometabolic Molecular Medicine, Peking University, Beijing 100871, China
5. State Key Laboratory of Membrane Biology, College of Life Sciences, Peking University, Beijing 100871, China
6. PKU-IDG/McGovern Institute for Brain Research, Beijing 100871, China
7. Chinese Institute for Brain Research, Beijing 102206, China

[#] These authors contributed equally to this work.

* E-mail: zhixingchen@pku.edu.cn (Z.C.); zoupeng@pku.edu.cn (P.Z.)

Table of Contents

Supplementary Video



Video S1. Voltage imaging of HVI-COT-Cy3 in cardiomyocytes

The cardiomyocytes expressing HVI-COT-Cy3 were imaged for 3 min at 100 Hz (18,000 frames) before (left) and after (right) 50 μM caffeine treatment. Scale bar = 10 μm.

Supplementary Tables

Table S1. Photophysical properties of the fluorophores used in this study

Fluorophore name	λ_{ex} (nm)	λ_{em} (nm)	Φ ($^1O_2$)
Cy3	560 [a]	574 [a]	0.0036
COT-Cy3	561 [a]	574 [a]	0.0023
Cy5	649 [a]	667 [a]	0.00041
COT-Cy5	650 [a]	667 [a]	0.00021

[a] Tetrazine dyes were reacted with equimolar 4-TCO and their spectra were measured in Tyrode's buffer.

Table S2. Photobleaching half-life of HVIs and Voltron2

Voltage indicators	Excitation wavelength (nm)	Illumination intensity ($W \cdot cm^{-2}$)	Emission filter (nm)	Photobleaching half-life $t_{1/2}$ (s) [a]
HVI-Cy3	532	8.0	585 / 65	1341 ± 102, n = 13
HVI-COT-Cy3	532	8.0	585 / 65	3210 ± 349, n = 19
$Voltron2_{525}$	532	8.0	585 / 65	73 ± 12, n = 33
HVI-Cy5	637	2.0	700 / 75	78 ± 9, n = 12
HVI-COT-Cy5	637	2.0	700 / 75	434 ± 97, n = 13

[a] measured in formaldehyde-fixed HEK293T cells expressing HVI-Cyanine or Voltron2-JF dye to reduce motion and depolarization-induced artifacts. "±" represents s.d.

Table S3. Voltage sensitivities of HVIs and Voltron2

Voltage indicators	$\Delta F/F_0$ per AP (%) [a]	SNR [a]	$\Delta F/F_0$ per 100 mV (%) [b]
HVI-Cy3	-24.4 ± 0.2, n = 5	52.4 ± 5.0	-39.1± 0.8, n = 9 [1]
HVI-COT-Cy3	-33.4 ± 0.8, n = 6	78.0 ± 9.6	-52.0 ± 1.1, n = 6
$Voltron2_{525}$	-11.8 ± 1.6, n = 5	41.2 ± 7.9	-19.0 ± 0.8, n = 5
HVI-Cy5	-13.5 ± 1.0, n = 5	42.6 ± 8.7	-19.6 ± 0.8, n = 7 [1]
HVI-COT-Cy5	-10.1 ± 0.3, n = 6	27.4 ± 3.5	-16.0 ± 0.8, n = 5
$Voltron2_{635}$	n.d.	n.d.	-5.8 ± 0.9, n = 6

[a] measured in cultured rat neurons at 484 Hz under the illumination of 532 nm (5.0 $W \cdot cm^{-2}$) and 637 nm (1.5 $W \cdot cm^{-2}$), respectively.

[b] measured in HEK293T cells at 1058 Hz under the illumination of 532 nm (5.0 $W \cdot cm^{-2}$) and 637 nm (1.5 $W \cdot cm^{-2}$), respectively.

"±" represents s.e.m.; n.d., not determined.

Table S4. Imaging apparatus for fluorescent imaging

Indicators	Fluorophores	Laser excitation wavelength	Emission filter
HVI-Cy3	Cy3	532 nm	585 / 65 nm
HVI-COT-Cy3	COT-Cy3	532 nm	585 / 65 nm
$Voltron2_{525}$	JF525	532 nm	585 / 65 nm
HVI-Cy5	Cy5	637 nm	700 / 75 nm
HVI-COT-Cy5	COT-Cy5	637 nm	700 / 75 nm
$Voltron2_{635}$	JF635	637 nm	700 / 75 nm
GCaMP6s	cpEGFP	488 nm	525 / 50 nm

Dichroic mirror: Chroma ZT405/488/561/640rpc and ZT405/488/532/642rpc

Table S5. List of reagents used in this study

Reagent	Vendor	Catalog Number
DNA extraction kit	TIANGEN	DP118-02
Dulbecco's Modified Eagle's medium (DMEM)	Gibco	C11995500BT
Fetal Bovine Serum (FBS)	Gibco	100099044
Trypsin-EDTA (0.25%)	Gibco	25200056
Neurobasal™ Medium	Gibco	21103049
B-27™ Supplement	Gibco	17504044
GlutaMAX™ Supplement	Gibco	35050061
5-Bromo-2′-deoxyuridine (BrdU)	Sigma	B5002
Collagenase Type II	Worthington	WBC-LS004202
Pancreatin	Sigma	P3292
Penicillin-streptomycin	Beyotime	C0222
Tyrode’s Salts Solution	macgene	CC018
Adenosine 5’-triphosphate disodium (ATP·Na_2)	Amresco	0220
Guanosine-5'-triphosphate disodium (GTP·Na_2)	Yuanye	56001-37-7
Magnesium acetate	Sinopharm	30110518
Matrigel® Matrix	Corning	356234
poly-D-lysine	Sigma	P7280-5X5M
Laminin Mouse Protein	Gibco	23017015
Opti-MEM™ Medium	Gibco	31985062
Lipofectamine™ 2000 Reagent	Invitrogen	11668019
Lipofectamine™ LTX & PLUS™	Invitrogen	15338100
Formaldehyde solution	Aladdin	F111934
Imidazole	Aladdin	I108707-100g
IPTG	Inalco	1758-400
Ni-NTA agarose	Qiagen	30210
HEPES	Amresco	0511
EGTA	Sigma	03777-10G
2-APB	Abcam	ab120124
Gabazine	Abcam	ab120042
NBQX	Abcam	ab120045
D-AP5 (APV)	Abcam	ab120003
Cyclothiazide	Abcam	ab120061
Caffeine	Merck	205548
Cy3 Tetrazine	Click Chemistry Tools	1204-5
Me-tetrazine-Disulfo-Cyanine5	Confluore	BCE-29

Supplementary Figures

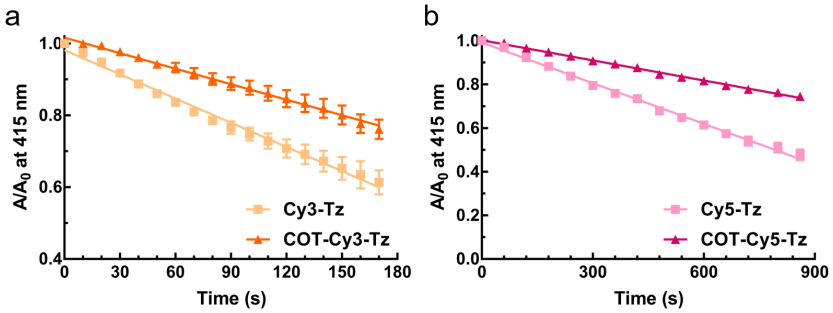


Figure S1. Singlet oxygen generation yield of cyanine dyes

Plots of the relative absorbance change of DPBF versus illumination time in (a) Cy3-Tz and COT-Cy3-Tz, (b) Cy5-Tz and COT-Cy5-Tz. Error bars represent s.e.m., n = 4 wells.

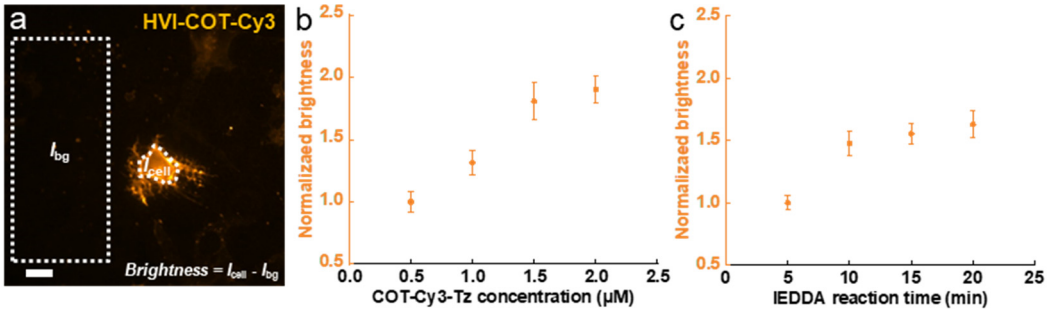


Figure S2. Optimization of COT-Cy3-Tz labeling in HEK293T cells

HVI-expressing HEK293T cells were labeled with 100 μM 4-TCO by 5 μM LplA for 30 min. Then they were labeled with COT-Cy3-Tz at a series of concentrations and incubation times. (a) Representative wide-field imaging of HVI-COT-Cy3 in HEK293T cell. Scale bar = 20 μm. (b) IEDDA labeling with 0.5 to 2 μM COT-Cy3-Tz for 15 min. Whole-cell fluorescence intensities were normalized to 0.5 μM. (c) IEDDA labeling with 1.5 μM COT-Cy3-Tz for 5 to 20 min. Fluorescence intensities were normalized to 5 min. Error bars represent s.e.m. n = 16-32 cells.

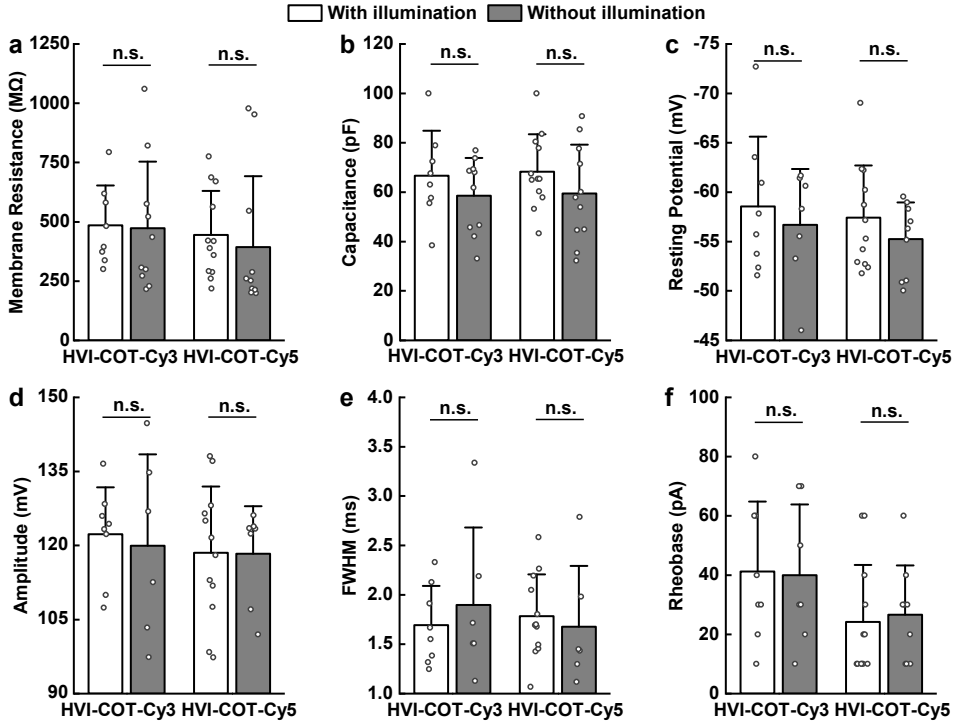


Figure S3. Electrophysiological properties of cultured neurons expressing HVI-COT-Cy3 (or HVI-COT-Cy5) with and without illumination

"HVI-COT-Cy3 w/o illumination" group: neurons labeled with COT-Cy3-Tz were kept for 30 min at room temperature. "HVI-COT-Cy3 with illumination" group: neurons were illuminated with a Xenon lamp equipped with a 480-540 nm filter at ~1.5 $W \cdot cm^{-2}$ for 30 min. "HVI-COT-Cy5 w/o illumination" group: neurons labeled with COT-Cy5-Tz were kept for 15 min at room temperature. "HVI-COT-Cy5 with illumination" group: neurons were illuminated with a Xenon lamp equipped with a 600-660 nm filter at ~0.8 $W \cdot cm^{-2}$ for 15 min. Membrane resistance (a) and membrane capacitance (b) were recorded in whole-cell voltage-clamp mode. Resting potential (c), amplitude (d), and the full width at half maximum (FWHM, e) of action potentials (APs) were obtained in current-clamp mode (~200 pA current injection for 10 ms). For rheobase measurements (f), neurons were stimulated by 1-s stepwise current injection in 10 pA increments for 10 cycles. Statistical significance was determined by Mann-Whitney U-test. Error bars represent s.d.

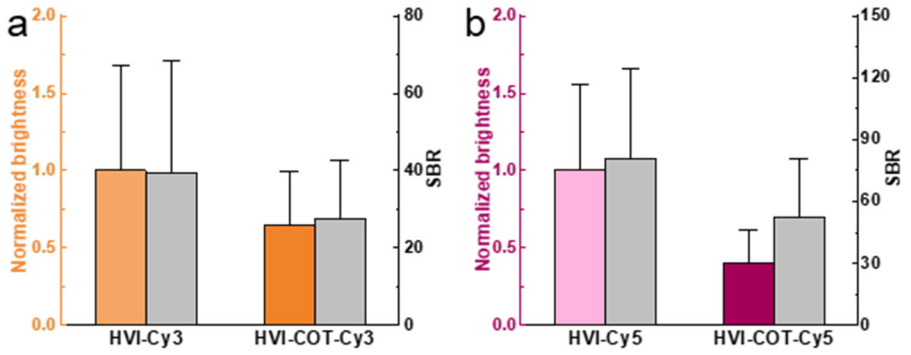


Figure S4. Relative brightness and labeling signal-to-background ratio (SBR) comparison in cultured neurons

(a) Neurons expressing HVI-COT-Cy3 (n = 36 cells) or HVI-Cy3 (n = 43 cells). (b) Neurons expressing HVI-COT-Cy5 (n = 39 cells) or HVI-Cy5 (n = 40 cells). Error bars represent s.d.

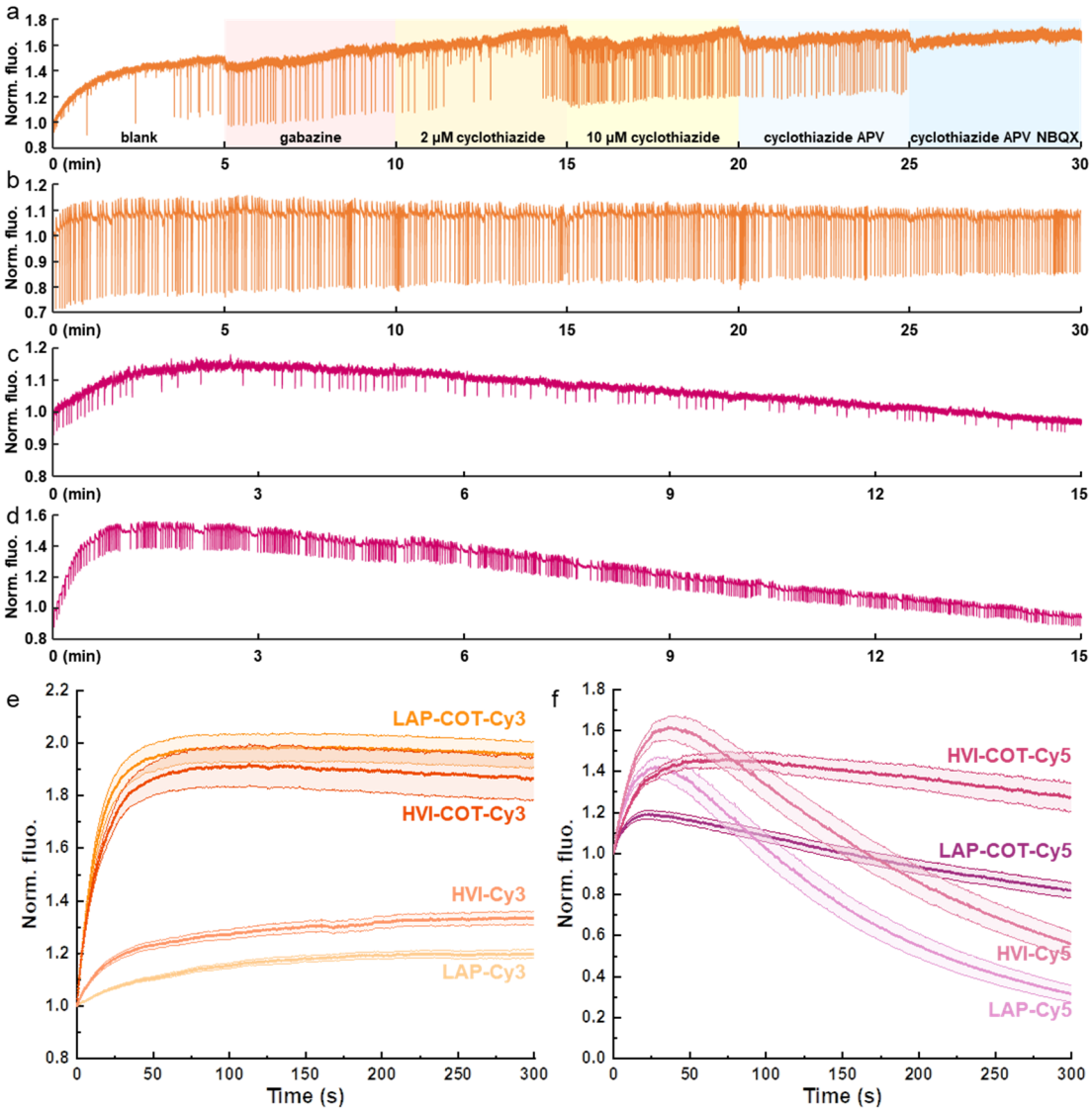


Figure S5. Long-term imaging of HVI-COT-Cy3 and HVI-COT-Cy5 without photobleaching correction (a) Raw trace of 30-min imaging of HVI-COT-Cy3 in neuron (corresponding Fig 2d). (b) Raw trace of 30-min imaging of HVI-COT-Cy3 in cardiomyocyte (corresponding Fig 4b). (c) Raw trace of 15-min imaging of HVI-COT-Cy5 in neuron (corresponding Fig 3b). (d) Raw trace of 15-min imaging of HVI-COT-Cy5 in cardiomyocyte (corresponding Fig 5b). (e-f) HEK293T cells expressing HVI or displaying LAP on cell surface have been labeled with COT-Cy3 / Cy3 / COT-Cy5 / Cy5, and been illuminated by 1.5 $W \cdot cm^{-2}$ 532 nm laser (e) or 0.8 $W \cdot cm^{-2}$ 637 nm laser (f) for 5 min. n = 11~31 cells. Error bars represent s.e.m. The illumination-induced brightening is an intrinsic property of these cyanine fluorophores, which has been investigated previously in mitochondrial imaging and speculated as self-dequenching process [2].

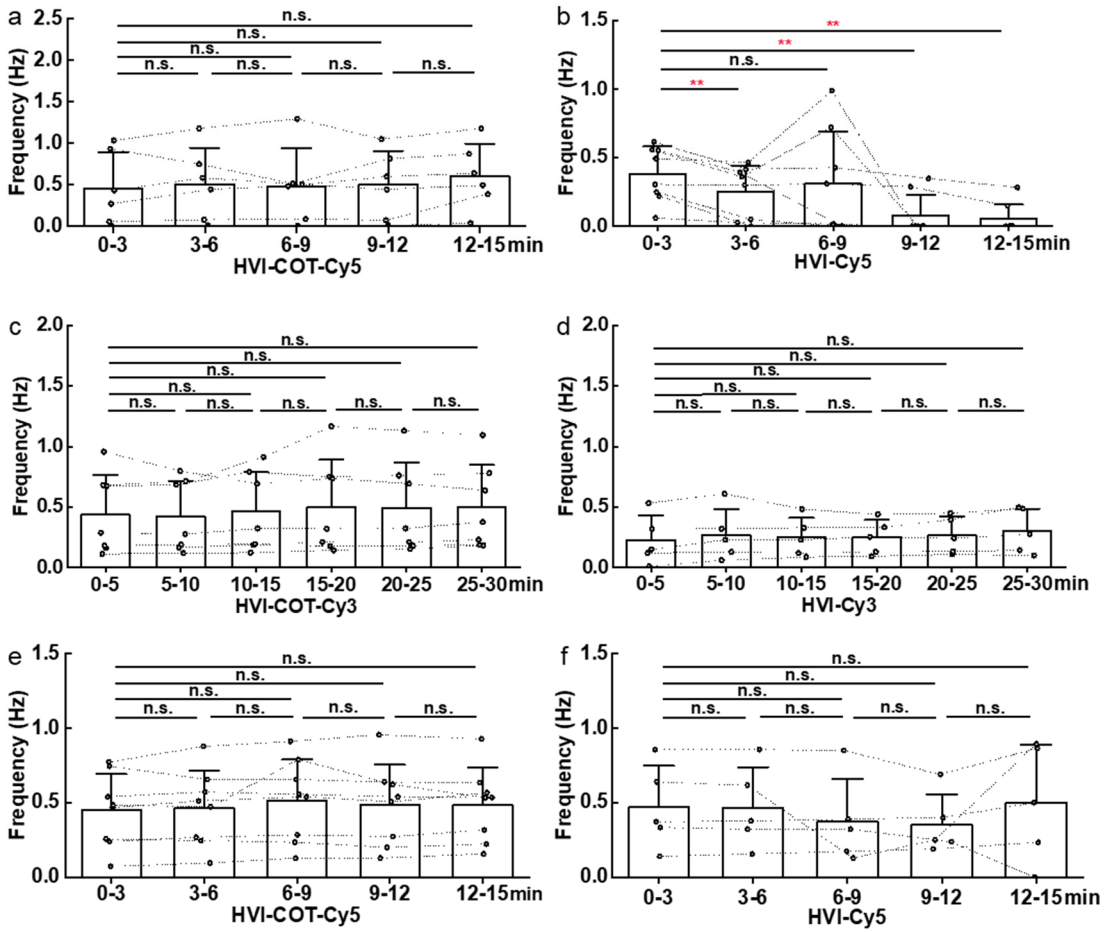


Figure S6. Statistics of AP burst frequency in cultured neurons or cardiomyocytes

(a-b) Neurons expressing HVI-COT-Cy5 (a, n = 6 cells) or HVI-Cy5 (b, n = 8 cells) during each round of recording. (c-d) Cardiomyocytes expressing HVI-COT-Cy3 (c, n = 7 cells) or HVI-Cy3 (d, n = 5 cells) during each round of recording. (e-f) Cardiomyocytes expressing HVI-COT-Cy5 (e, n = 8 cells) or HVI-Cy5 (f, n = 5 cells) during each round of recording. Statistical significances are determined by One-Way Repeated Measures ANOVA (* $p < 0.05$, ** $p < 0.01$; n.s., not significant). Error bars represent s.d.

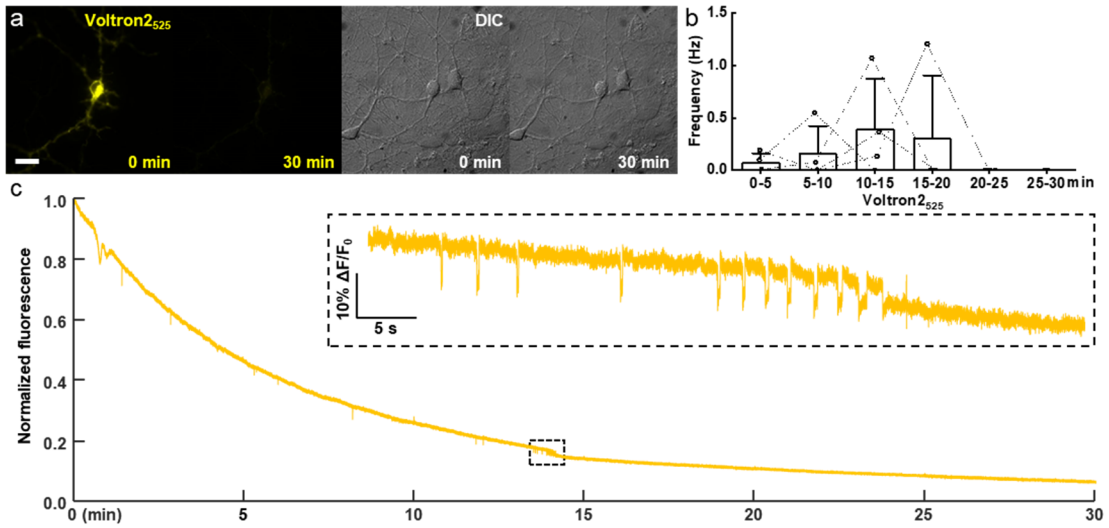


Figure S7. Long-term voltage imaging of Voltron2$_{525}$ in neurons

(a) Wide-field imaging of neuron expressing Voltron2$_{525}$ before/after 30-min illumination, scale bar = 20 μm. (b) Statistics of AP frequency in cultured neurons during six rounds of 5-min voltage imaging (n = 4 cells). (c) Representative normalized fluorescent signal of Voltron2$_{525}$ showcasing photobleaching and spontaneous spikes. A zoomed-in signal in the box was presented on the top, corresponding to phototoxicity-induced depolarization events. The illumination intensity of the 532 nm laser was 1.5 W·cm^{-2}, and the camera frame rate was 400 Hz.

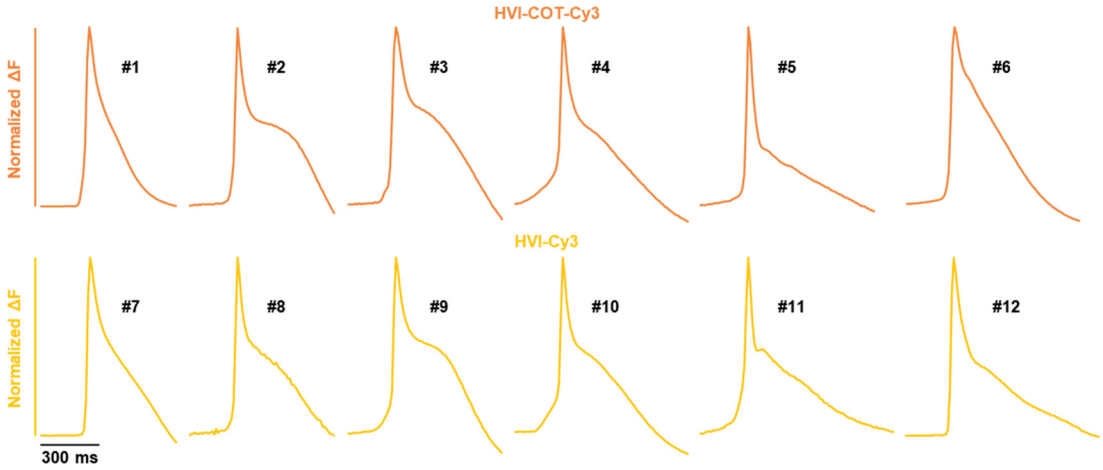


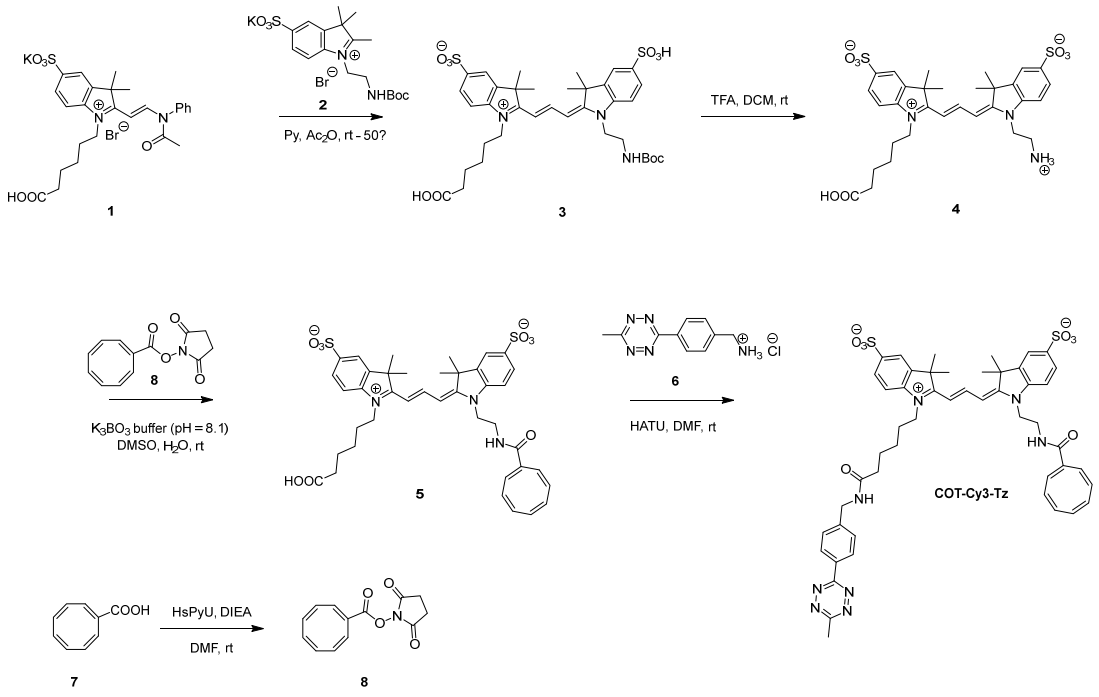
Figure S8. Variation of AP forms from cultured cardiomyocytes

Representative normalized average AP traces from individual cardiomyocytes expressing HVI-COT-Cy3 (#1 ~ #6) or HVI-Cy3 (#7 ~ #12) without drug treatment.

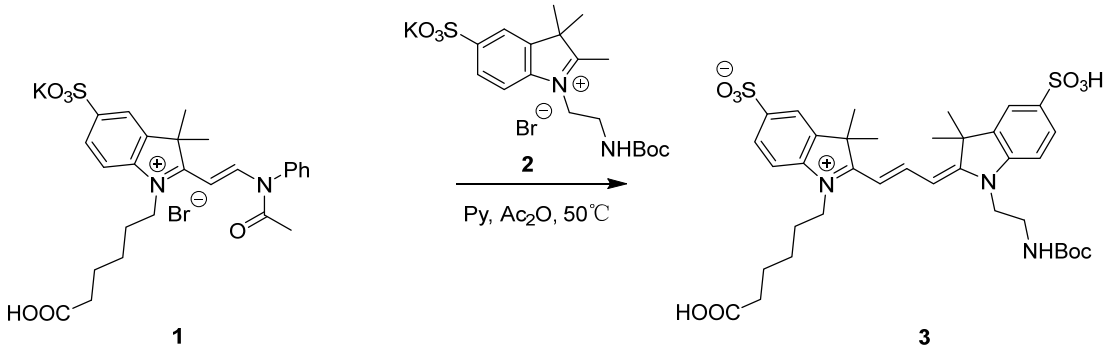
Supplementary Methods

Chemical synthesis

Synthesis of compound **COT-Cy3-Tz**



Synthesis of 2-((E)-3-((E)-1-(2-((tert-butoxycarbonyl)amino)ethyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)prop-1-en-1-yl)-1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (**3**)



Compounds **1** and **2** were prepared following previously published procedures [3]. To a solution of compound **1** (50 mg, 0.081 mmol) and compound **2** (41 mg, 0.081 mmol) in pyridine (0.80 mL) was added acetic anhydride (0.80 mL) at room temperature. The mixture was stirred at 50℃ and stirred for 5 h. The reaction mixture was then concentrated and dried in a vacuum oven. Then the mixture was redissolved in a minimal volume of DMF and then diluted to 3 mL with ddH_2O. Purification of the residue by reversed-HPLC (ACN/H_2O, mobile phase in a gradient of 3-50% acetonitrile) provided the compound **3** (6.5 mg, 11%) as a red solid.

ESI-HRMS: m/z calculated for $C_{36}H_{48}N_3O_{10}S_2$ $[M]^+$ 746.28 found 746.59.

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.59 (t, J = 13.4 Hz, 1H), 7.99 - 7.86 (m, 4H), 7.42 (d, J = 8.2 Hz, 2H), 6.59 (d, J = 13.4 Hz, 1H), 6.49 (d, J = 13.4 Hz, 1H), 4.28 (t, J = 6.4 Hz, 2H), 4.18 (t, J = 7.0 Hz, 2H), 3.57 - 3.52 (m, 2H), 2.33 (t, J = 6.4 Hz, 2H), 1.90 - 1.84 (m, 2H), 1.84 - 1.78 (m, 12H), 1.74 - 1.67 (m, 2H), 1.56 - 1.48 (m, 2H), 1.23 (s, 9H).

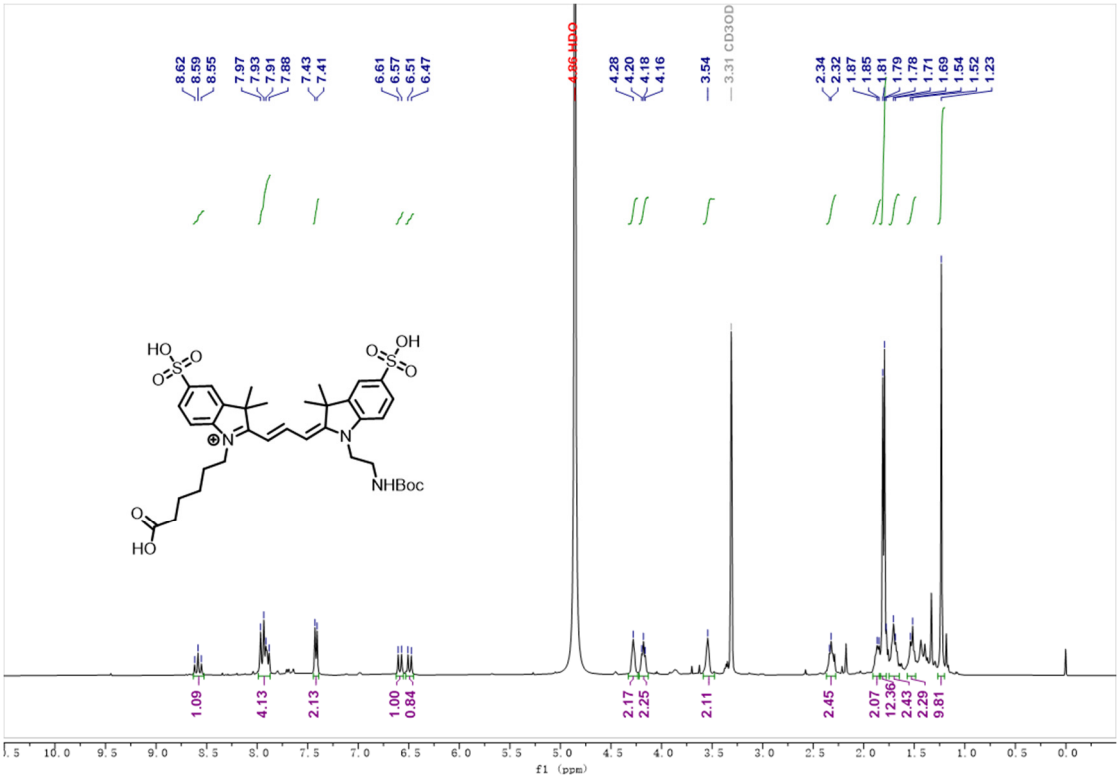
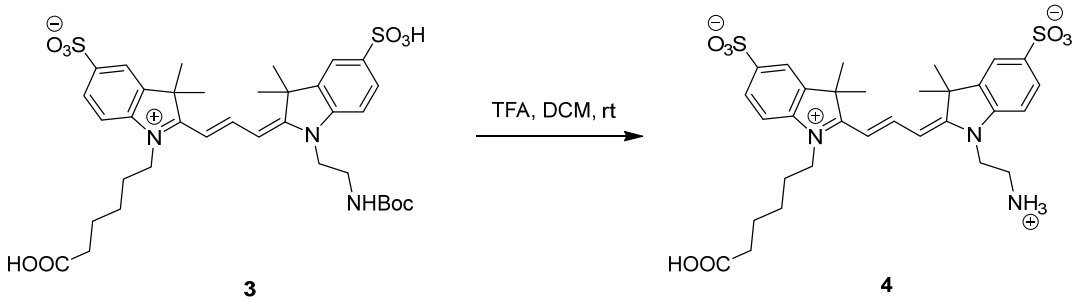


Figure S9. ^{1}H-NMR spectrum of compound **3**

Synthesis of 2-((E)-3-((E)-1-(2-ammonioethyl)-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)prop-1-en-1-yl)-1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (**4**)

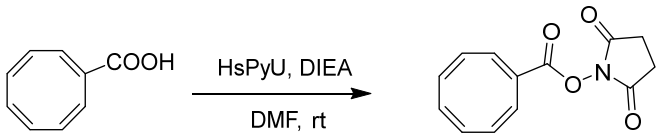


3 **4**

To a mixture of compound **3** (6.5 mg, 8.7 μmol) in DCM (0.40 mL) was added TFA (0.10 mL) at room temperature. The mixture was stirred for 2 h. The reaction was monitored by TLC until completion. The crude compound **4** was concentrated *in vacuo* and used directly in the next step.

ESI-HRMS: m/z calculated for $C_{31}H_{40}N_3O_8S_2$ $[M]^+$ 646.23 found 646.26.

Synthesis of 2,5-dioxopyrrolidin-1-yl (1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxylate (**8**)

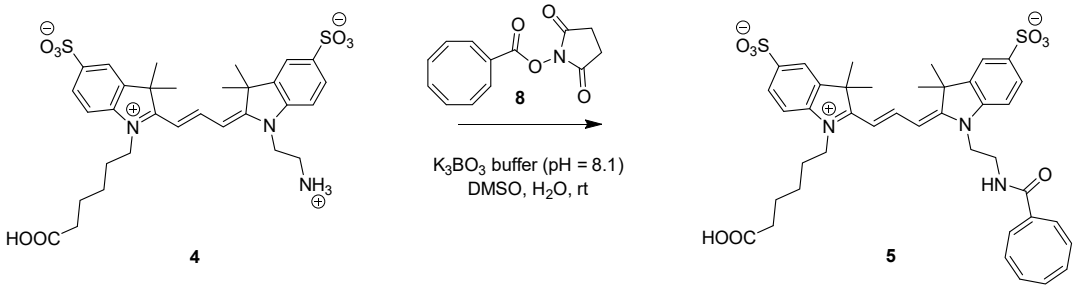


7 **8**

To a solution of compound **7** (15 mg, 0.10 mmol) in DMF (0.30 mL) were added HsPyu (49.4 mg, 0.12 mmol) and DIEA (38.7 mg, 0.30 mmol) in turn at room temperature. The mixture was stirred for 6h. Then the mixture was concentrated under reduced pressure to afford the residue, which was purified by prep-TLC (DCM) to afford the compound **8** (22.0 mg, 90%) as a yellow solid.

The NMR is in accordance with published result [3].

Synthesis of 1-(5-carboxypentyl)-2-((E)-3-((E)-1-(2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (**5**)



To a solution of compound **4** in DMSO (0.35 mL) and H_2O (0.53 mL) were added compound 8 (7.4 mg, 30 μmol) and K_3BO_3 buffer (pH 8.1, 0.14 mL) in turn at room temperature. After addition, the mixture was stirred at this temperature for 1 h. The reaction was monitored by LCMS until completion. The reaction mixture was purified by reversed-HPLC (ACN/H_2O, mobile phase in a gradient of 3-50% acetonitrile) to afford compound **5** (5.0 mg, 6.1μmol, 70%) as a red solid.

ESI-HRMS: m/z calculated for $C_{40}H_{46}N_3O_9S_2$ $[M]^+$ 776.27 found 776.48.

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.56 (d, J = 13.2 Hz, 1H), 7.99 - 7.82 (m, 4H), 7.44 - 7.37 (m, 2H), 6.63 - 6.55 (m, 2H), 6.48 (d, J = 13.4 Hz, 1H), 5.96 - 5.64 (m, 6H), 4.38 (t, J = 6.0 Hz, 2H), 4.17 (t, J = 7.5 Hz, 2H), 3.78 - 3.71 (m, 2H), 2.22 (t, J = 7.2 Hz, 2H), 1.90 - 1.82 (m, 2H), 1.79 (s, 12H), 1.74 - 1.68 (m, 2H), 1.54 - 1.51 (m, 2H).

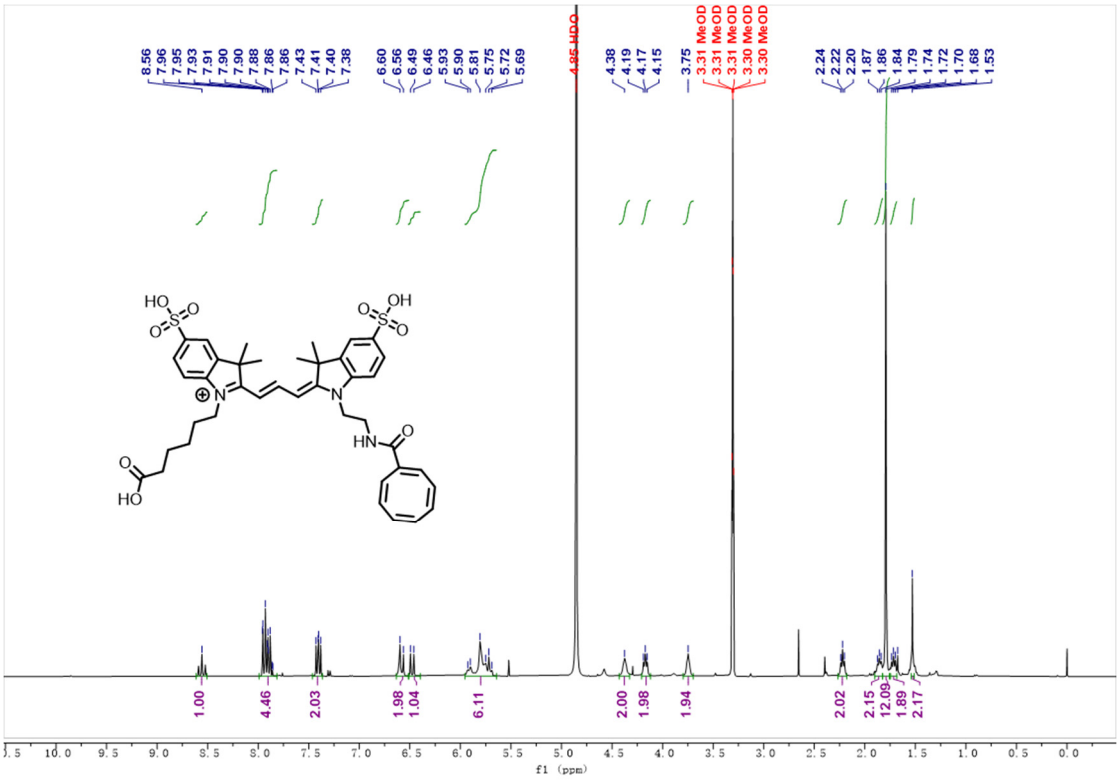
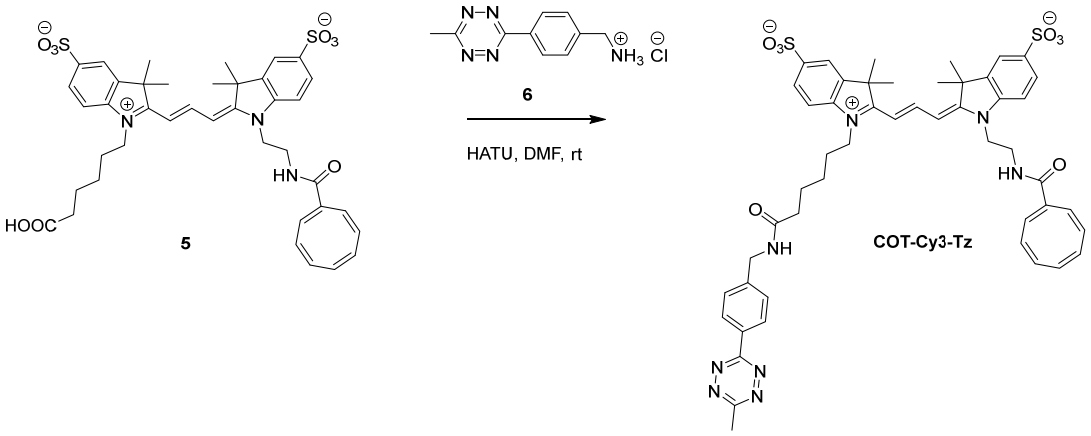


Figure S10. ^{1}H-NMR spectrum of compound **5**

Synthesis of 2-((E)-3-((E)-1-(2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-1-(6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)-3H-indol-1-ium-5-sulfonate (**COT-Cy3-Tz**)



To a mixture of compound **5** (5.0 mg, 6.1 μmol), HATU (2.9 mg, 7.7 μmol) and TEA (2.3 mg, 23 μmol) in DMF (0.2 mL) was added compound **6** (3.0 mg, 13 μmol) at room temperature. After addition, the mixture was stirred at this temperature for 2 h. Then the mixture was concentrated under reduced pressure to afford a residue, which was purified by reversed-HPLC (ACN/0.05% HCl in water, mobile phase in a gradient of 5-50% acetonitrile) to afford the compound (2.3 mg, 2.4 μmol, 39%) as a red solid.

ESI-HRMS: m/z calculated for $C_{50}H_{55}N_8O_8S_2$ $[M]^+$ 959.36 found 959.39.

^{1}H NMR (400 MHz, CD_3CN and D_2O) δ 8.47 - 8.38 (m, 3H), 7.86 (d, *J* = 6.3 Hz, 2H), 7.80 (d, *J* = 8.3 Hz, 2H), 7.51 (d, *J* = 8.3 Hz, 2H), 7.32 - 7.22 (m, 2H), 6.62 (s, 1H), 6.35 - 6.27 (m, 2H), 5.92 – 5.57 (m, 6H), 4.42 (s, 2H), 4.24 - 4.13(m, 2H), 4.01 (t, *J* = 8.3 Hz, 2H), 3.66-3.54 (m, 2H), 2.97 (s, 3H), 2.26 (t, *J* = 7.4 Hz, 2H), 1.82 - 1.74 (m, 2H), 1.73 - 1.65 (m, 14H), 1.51 - 1.41 (m, 2H).

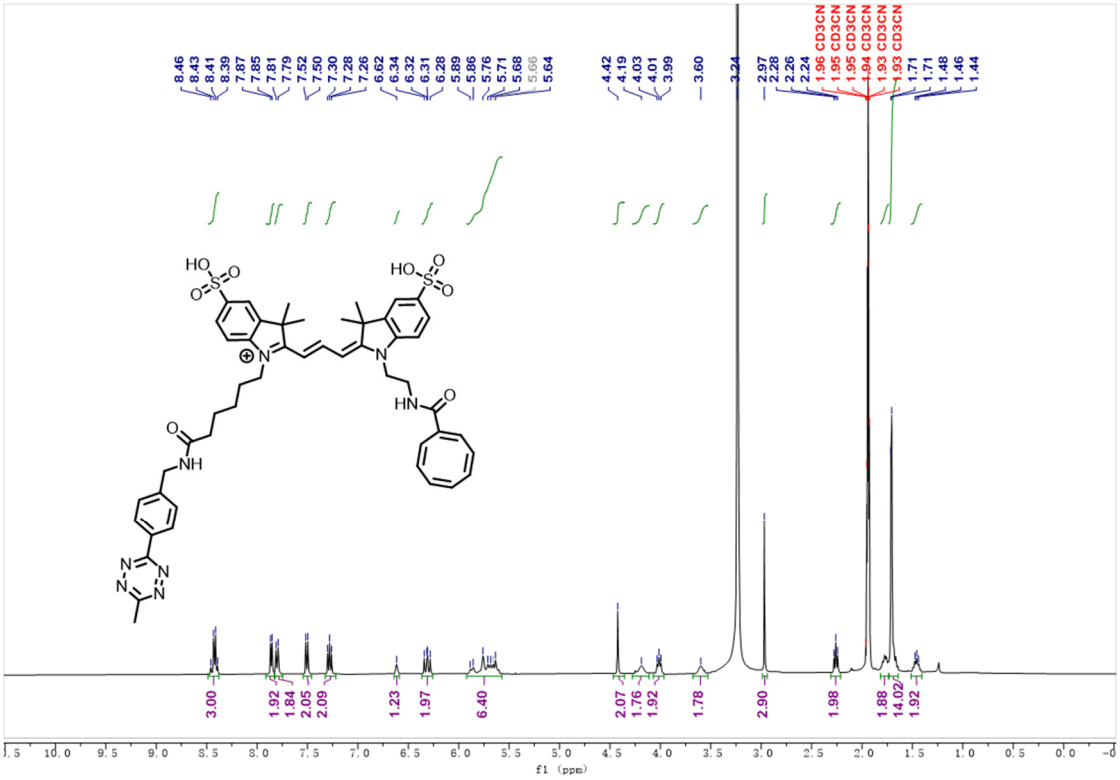
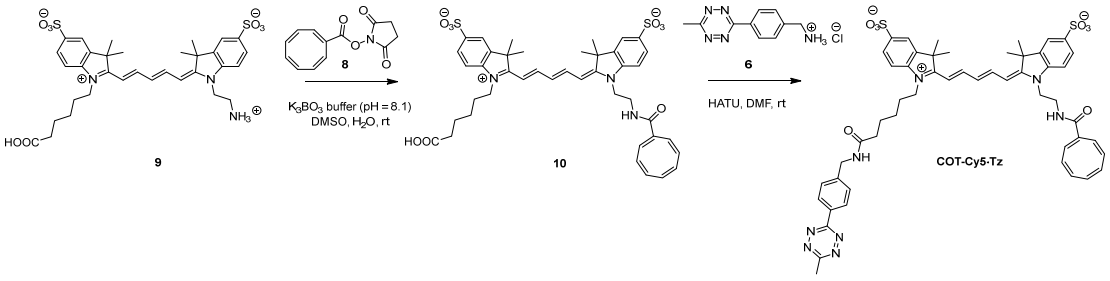
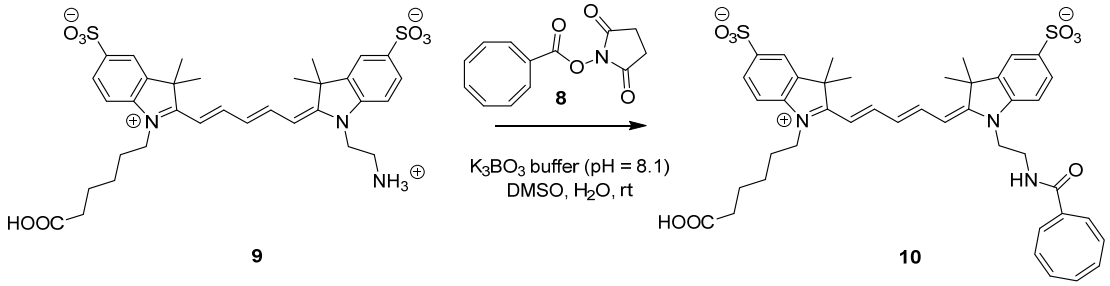


Figure S11. ^{1}H-NMR spectrum of **COT-Cy3-Tz**

Synthesis of compound **COT-Cy5-Tz**



Synthesis of 1-(5-carboxypentyl)-2-((1E,3E)-5-((E)-1-(2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (**10**)



The compound **9** was prepared following previously published procedures [4]. To a solution of compound **9** (4.5 mg, 6.7 μmol) in DMSO (0.53 mL) and H_2O (0.79 mL) were added compound **8** (5.4 mg, 22 μmol) and K_3BO_3 buffer (pH 8.1, 0.21 mL) in turn at room temperature. After addition, the mixture was stirred at this temperature for 1 h. The reaction was monitored by LCMS until completion. The reaction mixture was purified by reversed-HPLC (ACN/H_2O, mobile phase in a gradient of 3-50% acetonitrile) to afford the compound **10** (3.8 mg, 4.5 μmol, 67%) as a blue solid.

ESI-HRMS: m/z calculated for $C_{42}H_{48}N_3O_9S_2$ $[M]^+$ 802.28 found 802.49.

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.31 (t, J = 13.2 Hz, 2H), 7.94 - 7.83 (m, 4H), 7.38 - 7.30 (m, 2H), 6.71 - 6.59 (m, J = 12.4 Hz, 2H), 6.40 - 6.32 (m, 2H), 5.96 - 5.70 (m, 6H), 4.31 (t, J = 6.0 Hz, 2H), 4.17 - 4.07 (t, J = 7.4 Hz, 2H), 3.76 - 3.64 (m, 2H), 2.25 - 2.14 (m, 2H), 1.87 - 1.80 (m, 2H), 1.78 - 1.72 (m, 12H), 1.55 - 1.46 (m, 2H), 1.37 - 1.26 (m, H).

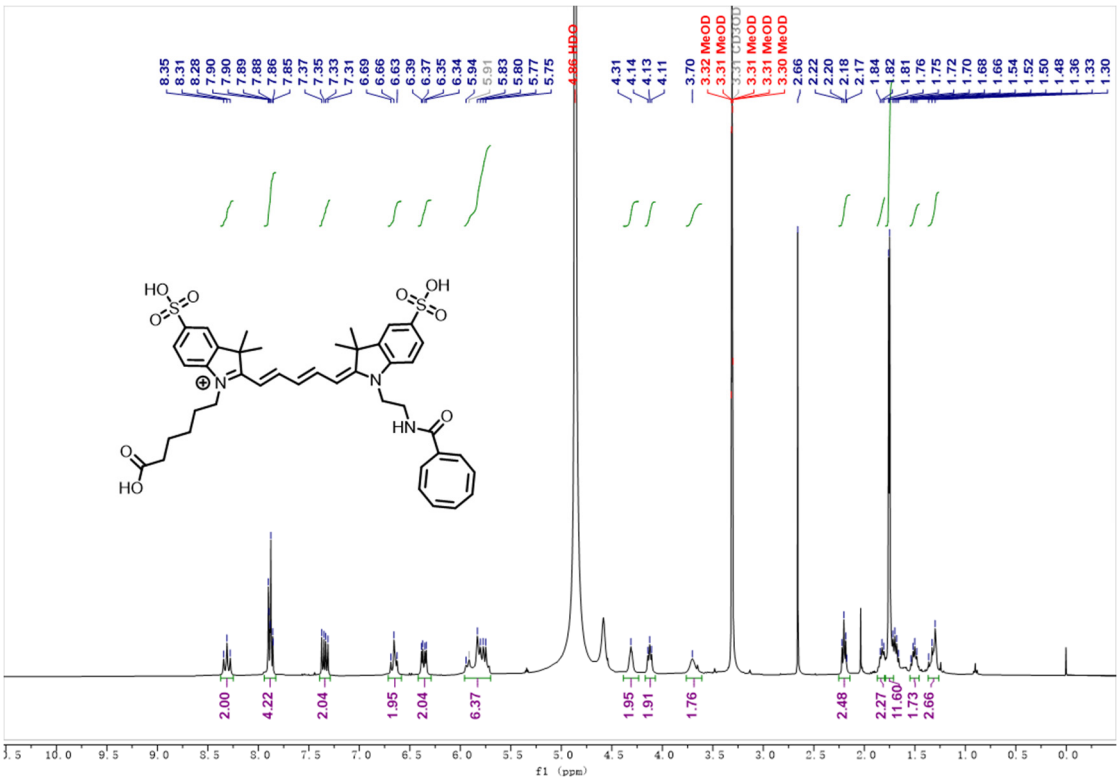
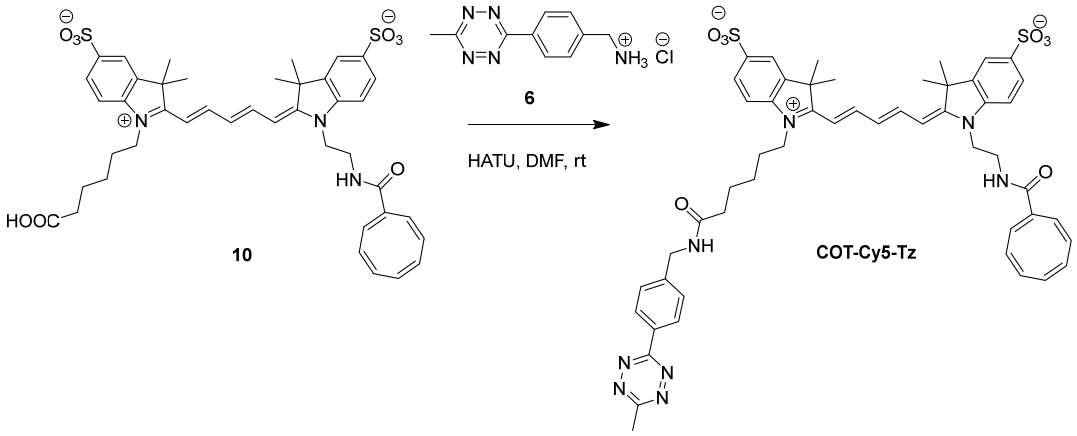


Figure S12. ^{1}H-NMR spectrum of compound **10**

Synthesis of 2-((1E,3E)-5-((E)-1-(2-((1E,3Z,5Z,7Z)-cycloocta-1,3,5,7-tetraene-1-carboxamido)ethyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)-3H-indol-1-ium-5-sulfonate, potassium salt (**COT-Cy5-Tz**)



To a mixture of compound **10** (1.7 mg, 2.1 μmol), HATU (1.2 mg, 3.3 μmol) and TEA (0.77 mg, 7.6 μmol) in DMF (0.15 mL) was added compound **6** (1.0 mg, 4.3 μmol) at room temperature. After addition, the mixture was stirred at this temperature for about 2 h. Then the mixture was concentrated under reduced pressure to afford a residue, which was purified by reversed-HPLC (ACN/0.05% HCl in water, mobile phase in a gradient of 5-50% acetonitrile) to afford the compound (0.86 mg, 0.84 μmol, 40%) as a blue solid.

ESI-HRMS: m/z calculated for $C_{42}H_{48}N_3O_9S_2$ $[M]^+$ 985.37 found 985.60.

^{1}H NMR (400 MHz, CD_3CN and D_2O) δ 8.53 (d, J = 8.0 Hz, 2H), 8.25 - 8.13 (m, 2H), 8.03 - 7.94 (m, 4H), 7.69 (d, J = 8.1 Hz, 2H), 7.50 - 7.42 (m, 2H), 6.75 (s, 1H), 6.59 (d, J = 12.0 Hz, 1H), 6.34 (d, J = 13.1 Hz, 1H), 6.26 (d, J = 13.7 Hz, 1H), 6.07 - 5.72 (m, 6H), 4.60 (s, 2H), 4.46 - 4.25 (m, 2H), 4.17 (t, J = 6.9 Hz, 2H), 3.95 - 3.63 (m, 2H), 2.99 (s, 3H), 2.45 (t, J = 7.1 Hz, 2H), 1.97 - 1.89 (m, 2H), 1.85 - 1.72 (m, 14H), 1.57 - 1.47 (m, 2H).

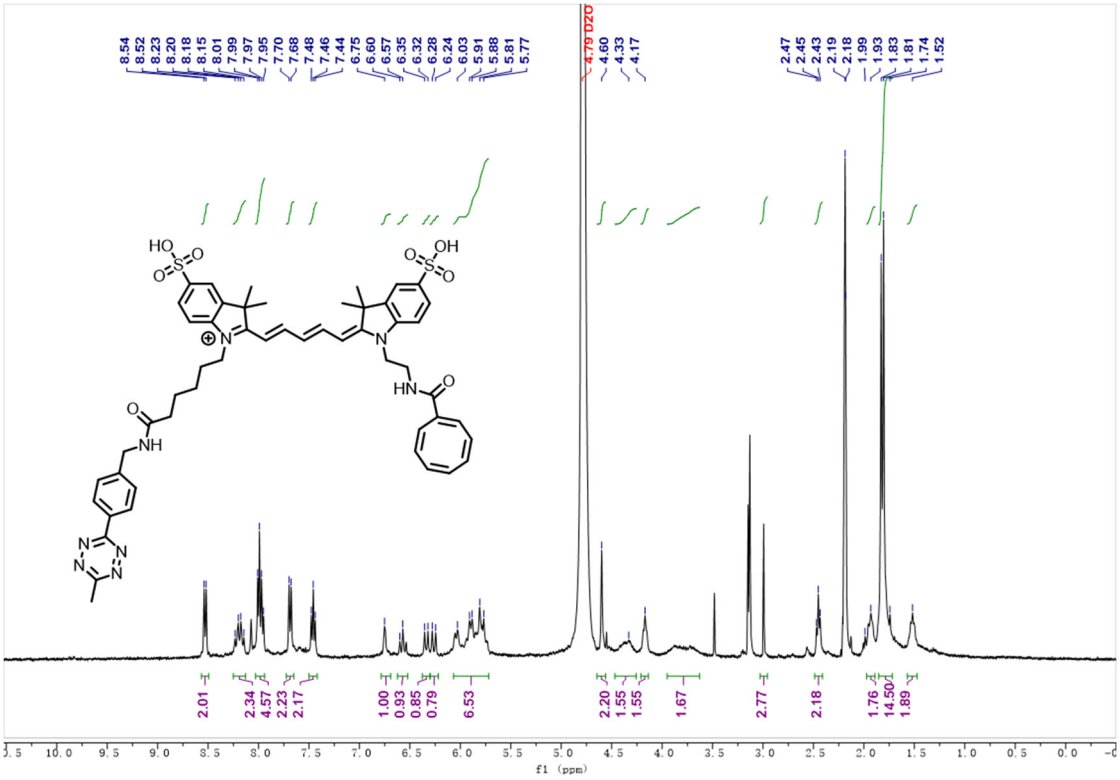


Figure S13. ^{1}H-NMR spectrum of **COT-Cy5-Tz**

General procedures on synthetic chemistry

Unless otherwise stated, all commercially available materials were purchased at the highest commercial quality and used without further purification. Anhydrous dichloromethane (DCM), N, N-dimethylformamide (DMF), and tetrahydrofuran (THF) were purchased from MREDA (China) or Innochem (China). All reactions were carried out under a nitrogen atmosphere with dry solvents, unless otherwise mentioned.

Reactions were monitored by Thin Layer Chromatography on plates (GF254) (Yantai Chemicals) using UV light as visualizing agent or by LC/MS (4.6 mm × 150 mm 5 μm C18 column; 2 μL injection; 5-100% CH_3CN/H_2O, linear gradient, with constant 0.1 % v/v formic acid additive; 6-10 min run; 0.6 mL/min flow; ESI; positive or negative ion mode; UV detection with ACQUITY PDA). If not specially mentioned, flash column chromatography uses silica gel (200-300 mesh, Tsingtao Haiyang Chemicals). Preparative HPLC separations were performed using Teledyne Isco EZ Prep UV-Vis and a RediSep Prep C18 column (100 Å, 5 μm, 20 × 150 mm).

NMR spectra were recorded on Brüker Advance 400 (^{1}H 400 MHz, ^{13}C 101 MHz) and are calibrated using residual undeuterated solvent (Chloroform-d at 7.26 ppm ^{1}H NMR, 77.16 ppm ^{13}C NMR; CD_3OD at 3.31 ppm ^{1}H NMR, 49.00 ppm ^{13}C NMR; DMSO-d_6 at 2.50 ppm ^{1}H NMR, 39.52 ppm ^{13}C NMR). Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = triplet of doublets, m = multiplet, br = broad), coupling constant (Hz), integration. Data for ^{13}C NMR are reported by chemical shift (δ ppm). High-resolution mass spectrometric data were obtained using Waters Acquity I class UPLC synapt G2-SI using ESI (electrospray ionization).

Measurement of ROS generation ability of fluorophores

1,3-Diphenylisobenzofuran (DPBF) was applied to evaluate the ROS ($^1O_2$) generation ability of fluorophores. The mixed solution containing 0.8 μM and 1μM COT-Cy3/5-Tz (pre-reacted with equimolar 4-TCO) was illuminated with an LED lamp (520-530 nm, 0.050 $W \cdot cm^{-2}$; 620-630 nm, 0.0125 $W \cdot cm^{-2}$). At regular time points, the change of the UV–Vis absorption spectrum of DPBF was observed at 415 nm, resulting in a linear plot of the absorbance intensity versus time, and the quantum yield of $^1O_2$ was determined by comparing the slope for the samples with that obtained for the standards [5].

HEK293T cell culture and transfection

Human embryonic kidney (HEK) 293T cells were incubated in Dulbecco's modified eagle medium (DMEM, Gibco™) supplemented with 10% v/v fetal bovine serum (FBS, Gibco™) in an incubator at 37 °C with 5% CO_2. For experiments, HEK293T cells were seeded in a 24-well plate. At 70-90% confluency, cells in each well were transfected by using 500 ng plasmid and 1 μL Lipofectamine™ 2000 Reagent mixed in Opti-MEM™ Medium for 4 h. After that, cells were digested by Trypsin-EDTA (0.25%, Gibco™), reseeded on Matrigel® Matrix-coated 14-mm glass coverslips, and then cultured in cell medium for 24 h before labeling with fluorophore.

Neonatal rat neuron culture and transfection

For primary rat hippocampus neuron culture, 14-mm glass coverslips were pre-coated with poly-D-lysine (Sigma) solution and Laminin Mouse Protein (Gibco™) solution at 37 °C within 2 days. After that, the coverslips were washed twice with ddH_2O and let dry at room temperature. To isolate neurons, neonatal Sprague-Dawley rats' heads were cut off with scissors. Then, the brain was isolated from the skull and put into the ice-chilled dissection solution (DMEM with high glucose and penicillin-streptomycin antibiotics). Next, the hippocampi were separated from brains under a dissection scope, cut into small pieces, and incubated with Trypsin-EDTA (0.25%) for 15 min at 37°C. Thereafter, the trypsin was gently replaced with DMEM containing 10% FBS. After being repeatedly pipetted for 1 min and incubated on ice for 5 min, the tissue fragments were sedimented at the bottom of the centrifuge tube. The supernatant was collected and diluted with neural culture medium (Neurobasal™ medium supplemented with B-27™ supplement, GlutaMAX™ supplement, and penicillin-streptomycin) to a final cell density of 6×10^4 cells/mL. 1 mL cell suspension was added to each 24-well containing one coverslip. Every four days, half of the neural culture medium was replaced with fresh medium.

For the transfection experiments, cultured neurons were transfected at DIV8 (8 days *in vitro*). Neurons for each 24-well were treated with a mixture of 0.5-1 μg plasmids and 1 μL Lipofectamine™ LTX for 45 min. After 4-6 days, the transfected neurons were labeled and imaged.

Neonatal rat cardiomyocytes culture and transfection

Neonatal rat cardiomyocytes were isolated from Sprague-Dawley rats born within 24 h. The

whole hearts were isolated, minced and rinsed in Tyrode's buffer. And then, six cycles of digestion using collagenase type II (0.08% w/v, Worthington) and pancreatin (0.1% w/v, Sigma) were performed for each cycle about 6 min at 37 °C. At the end of each cycle, the suspension was centrifuged for 5 min at 100 g and the supernatant was collected, pooled and resuspended in cardiomyocytes culture medium (DMEM containing 10% v/v FBS, 100 μM BrdU and penicillin-streptomycin solution). Isolated cells were pre-plated for 2 h on culture flasks in a humidified incubator at 37 °C with 5% CO_2 to separate cardiomyocytes and fibroblasts. Isolated cardiomyocytes were resuspended in cardiomyocytes culture medium. The density of cardiomyocytes was adjusted to 1.5×10^5 cells/mL. After neonatal rat cardiomyocytes had been cultured for 36-48 h, the medium was rinsed for further experiments.

Cardiomyocytes were transfected on DIV1-2, and cells were switched from the culture medium into Opti-MEM™ for more than 30 min in the incubator. Thereafter, cells were transfected with 1.5 μg plasmids using 1.5 μL PLUS™ reagent and 1.5 μL Lipofectamine™ LTX reagent mixed in Opti-MEM™ Medium. After 12 h, the transfection medium was changed to the culture medium, and the transfected cardiomyocytes were incubated for 2-4 days before experiments.

Data analysis

Both electrical data and fluorescence images were analyzed by the custom MATLAB software (MathWorks, version R2018b). For each cell, fluorescence intensities were taken from the mean pixel values of a manually created ROI around the soma. After camera bias removal (100 and 400 for 1-by-1 and 2-by-2 binning, respectively), the raw optical traces of spontaneous electrical activities averaged from ROIs were corrected with background subtraction and photobleaching removal. The trend lines were generated by a smooth function using a 2.5-s (999 consecutive data points) moving average to the background-subtracted data. The photobleaching of the traces was removed by normalizing to the corresponding trend lines. Statistical analyses were performed using Excel (Microsoft Excel 2019) and Origin (version 2019b).

Data availability statement

Data presented in this study are provided in Supplementary Tables.

Code availability statement

MATLAB code can be downloaded from GitHub at https://github.com/PKUCHEMZouLab/HVI.

References

[1] Liu, S.; Lin, C.; Xu, Y.; Luo, H.; Peng, L.; Zeng, X. et al. A far-red hybrid voltage indicator enabled by bioorthogonal engineering of rhodopsin on live neurons. *Nat Chem* **2021**, *13*, 472-479.

[2] Perry, S. W.; Norman, J. P.; Barbieri, J.; Brown, E. B.; Gelbard, H. A. Mitochondrial membrane potential probes and the proton gradient: A practical usage guide. *Biotechniques* **2011**, *50*, 98-115.

[3] Pati, A. K.; El Bakouri, O.; Jockusch, S.; Zhou, Z.; Altman, R. B.; Fitzgerald, G. A. et al. Tuning the baird aromatic triplet-state energy of cyclooctatetraene to maximize the self-healing mechanism in organic fluorophores. *Proc Natl Acad Sci USA* **2020**, *117*, 24305-24315.

[4] Zheng, Q.; Jockusch, S.; Zhou, Z.; Altman, R. B.; Zhao, H.; Asher, W. et al. Electronic tuning of self-healing fluorophores for live-cell and single-molecule imaging. *Chem Sci* **2017**, *8*, 755-762.

[5] Redmond, R. W.; Gamlin, J. N. A compilation of singlet oxygen yields from biologically relevant molecules. *Photochem Photobiol* **1999**, *70*, 391-475.