Supporting Information

"Development and Application of a Near-infrared Fluorescence Probe for Oxidative Stress Based on Differential Reactivity of Linked Cyanine Dyes"

(Journal of the American Chemical Society)

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Experimental procedure

Materials

General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., Sigma-Aldrich Japan, Dojindo, or Acros Organics, and were used without further purification. Xanthine oxidase from bovine milk was purchased from Sigma-Aldrich Japan. Superoxide dismutase from bovine erythrocytes was purchased from MP Biomedicals, LLC. Apocynin was purchased from Merck, Ltd. (Former Calbiochem). Zymosan from *Saccharomyces cerevisiae* was purchased from Invitrogen Corp.

Instruments

^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300 instrument (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR) or a JEOL JNM-LA400 instrument (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR); δ values are in ppm relative to TMS. Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuTOF (ESI). HPLC purification and analyses were performed on a reverse-phase column (GL Sciences (Tokyo, Japan), Inertsil ODS-3 30 mm × 250 mm for purification and Inertsil ODS-3 4.6 mm × 250 mm for analyses) fitted on a Jasco PU-1587 system for purification and a Jasco PU-980 system for analyses. Elution was done with eluent A (0.1 mM acetic acid/triethylamine buffer (pH 7.4)) and eluent B (CH_3CN with 20% H_2O).

UV-visible spectroscopy and fluorometric analysis

Instruments

UV-visible spectra were obtained on a spectrometer (UV-1600, Shimadzu, Japan). Fluorescence spectroscopic studies were performed on a fluorescence spectrophotometer (F4500, Hitachi, Japan). The excitation slit widths, the emission slit widths and the photomultiplier voltage were 2.5 nm, 5.0 nm and 700 V for Cy5, and 10 nm, 10 nm, and 400 V for Cy7, respectively. Dyes were dissolved in DMF as stock solutions. The stock solutions of dyes were diluted in aqueous buffers to the required concentration for measurement. The final concentrations of dyes are given in figure legends.

Relative fluorescence quantum yields

Relative fluorescence quantum yields (ϕ_{fl}) were obtained by comparing the area under the emission spectrum of the test samples with that of a solution of cresyl violet in methanol ($\phi_{fl} = 0.54$) [SR1] or indocyanine green in DMSO ($\phi_{fl} = 0.13$) [SR2].

pH profile and autoxidation of FOSCY-1

Aqueous solutions of 0.1 M Na_3PO_4, Na_2HPO_4, NaH_2PO_4 and H_3PO_4 were prepared. By mixing two of them at certain ratios, 0.1 M sodium phosphate buffers of various pH values were prepared, and pH value of each buffer was measured. FOSCY-1 was diluted to 1 μM in buffers for measurement. Autoxidation was induced by light-

irradiation with a BPS-X500B (Bunkokeiki). A solution of 1 μM FOSCY-1 in 0.1 M sodium phosphate buffer at pH 7.4 containing 0.1% DMF as a cosolvent was prepared and light-irradiated (645 nm, 1.0 mW/cm^2).

Analysis of ROS reactivity

Absorption or fluorescence spectra were obtained after addition of ROS under the conditions described below.

Preparation of stock solutions or generation of ROS

(a) H_2O_2

H_2O_2 was diluted appropriately in water. The concentration of H_2O_2 was determined based on the molar extinction coefficient at 240 nm (43.6 M^{-1} cm^{-1}).[SR3] Then, a H_2O_2 stock solution in water was prepared. To a solution of dye in 0.1 M sodium phosphate buffer at pH 7.4 containing 0.1% DMF as a cosolvent, H_2O_2 solution (final 10 mM) was added at room temperature and the spectrum was measured 30 min later.

(b) Generation of $^{\bullet}OH$

$Fe(ClO_4)_2$ was dissolved in water. To a solution of dye and H_2O_2 in 0.1 M sodium phosphate buffer at pH 7.4 containing 0.1% DMF as a cosolvent, the $Fe(ClO_4)_2$ solution was added at room temperature. Then, $^{\bullet}OH$ was generated from Fe^{2+} and H_2O_2 (Fenton reaction). The final concentration of H_2O_2 was 1 mM and that of $Fe(ClO_4)_2$ was as indicated in each figure.

(c) $ONOO^-$

NaONOO solution was diluted appropriately in 0.1 M NaOHaq.. The concentration of $ONOO^-$ was determined based on the molar extinction coefficient at 302 nm (1670 M^{-1} cm^{-1}).[SR4] Then, a $ONOO^-$ stock solution in 0.1 M NaOHaq. was prepared. To a solution of dye in 0.1 M sodium phosphate buffer at pH 7.4 containing 0.1% DMF as a cosolvent, $ONOO^-$ solution was added at room temperature. The final concentration of $ONOO^-$ was as indicated in each figure.

(d) ^-OCl

NaOCl solution was diluted appropriately in 0.1 M NaOHaq.. The concentration of $^{-}$OCl was determined based on the molar extinction coefficient at 292 nm (350 M^{-1} cm^{-1}).[SR3] Then, a $^{-}$OCl stock solution in 0.1 M NaOHaq. was prepared. To a solution of dye in 0.1 M sodium phosphate buffer at pH 7.4 containing 0.1% DMF as a cosolvent, $^{-}$OCl solution was added at room temperature. The final concentration of $^{-}$OCl was as indicated in each figure.

(e) Generation of $O_2^{\bullet-}$

Xanthine oxidase (XO) was dissolved in 0.1 M sodium phosphate buffer at pH 7.4. Xanthine was dissolved in DMF. To a solution of dye in 0.1 M sodium phosphate buffer at pH 7.4 containing 0.1% DMF as a cosolvent, XO solution (final 4 mU/mL) and xanthine solution (final 33 μM, containing 6.7% DMF as a cosolvent) were added at room temperature. Spectra were obtained at the times indicated in each figure after the addition of xanthine.

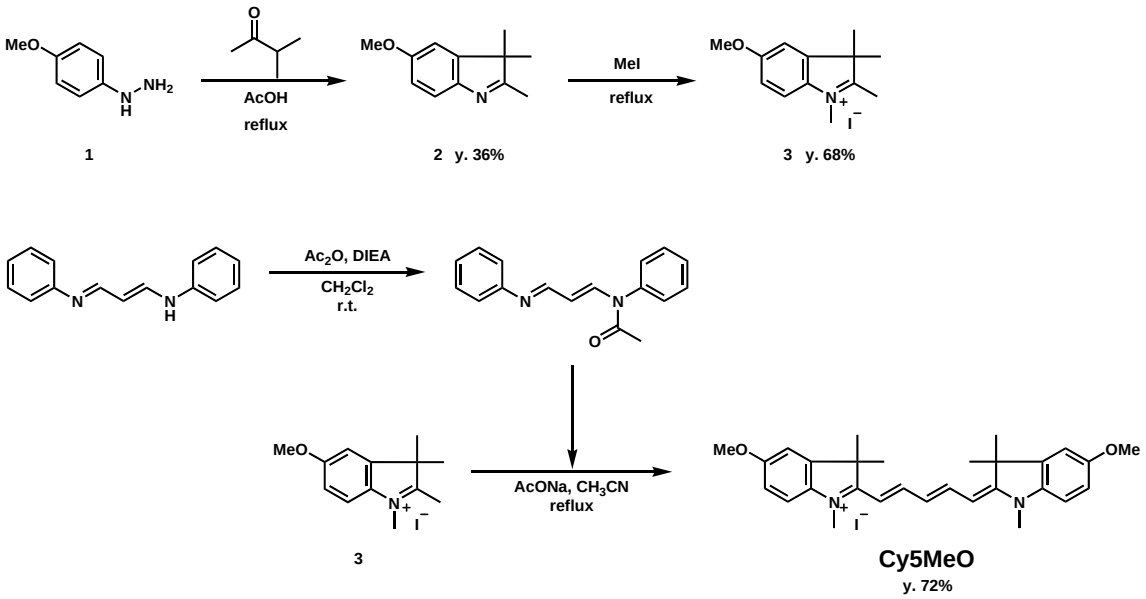
(f) $^1O_2$

EP-1 (3-(1,4-dihydro-1,4-epidioxy-1-naphthyl)propionic acid) was dissolved in DMF.[SR5] To a solution of dye in D_2O containing 0.1% DMF as a cosolvent, EP-1 solution (final 0.2 mM, contaning 0.3% DMF as a cosolvent) was added at 37 °C. Spectra were obtained at the times indicated in each figure after the addition of EP-1.

Cyclic voltammerty

Cyclic voltammetry was performed on an ALS 630 B electrochemical analyzer (BAS Inc., Japan). A three-electrode arrangement in a single cell was used for the measurements: a Pt wire was used as the auxiliary electrode, a Pt electrode and a GC electrode as the working electrode, and a Ag/AgCl (sat. NaCl) electrode as the reference electrode. Sample solutions contained 1 mM of each sample and 0.1 M sodium phosphate as a supporting electrolyte.

Synthesis and characterization



Scheme 1. Synthesis of Cy5MeO.

5-Methoxy-2,3,3-trimethylindolenine (2)

4-Methoxyphenylhydrazine hydrochloride (**1**) (6.0 g, 34 mmol) and 3-methyl-2-butanone (4 mL, 37 mmol) were dissolved in acetic acid (30 mL). The solution was refluxed for 12 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added dichloromethane, and the whole was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na_2SO_4, filtered, and evaporated to afford **2** as a brown oil (2.3 g, yield 36%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.30 (d, 1H, J = 8.4 Hz), 7.03 (d, 1H, J = 2.6 Hz), 6.79 (dd, 1H, J = 8.4, 2.6 Hz), 3.75 (s, 3H), 2.15 (s, 3H), 1.21 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 185.4, 157.4, 147.4, 146.7, 119.3, 112.2, 108.2, 55.4, 53.3, 22.6, 14.8. HRMS (ESI$^+$): Calcd for $[M+H]^+$, 190.12319, Found, 190.11894 (–4.25 mmu).

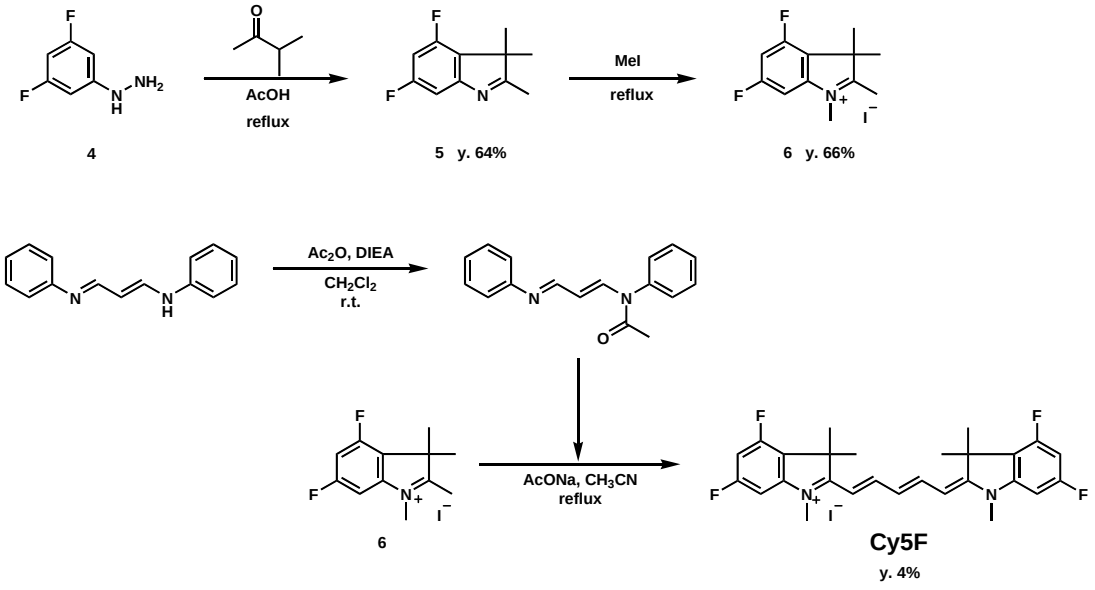
5-Methoxy-1,2,3,3-tetramethylindolenium iodide (3)

Compound **2** (2.3 g, 12 mmol) was dissolved in methyl iodide (20 ml). The solution was refluxed for 15 hours, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with *n*-hexane and dried under reduced pressure to afford **3** as a brown solid (2.7 g, yield 68%). ^{1}H NMR

(300 MHz, DMSO-d_6): δ 7.80 (d, 1H, J = 8.8 Hz), 7.47 (d, 1H, J = 2.4 Hz), 7.11 (dd, 1H, J = 8.8, 2.4 Hz), 3.95 (s, 3H), 3.84 (s, 3H), 2.72 (s, 3H), 1.52 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 193.1, 160.6, 143.7, 135.4, 116.1, 114.2, 109.2, 56.2, 53.8, 35.0, 21.8, 14.2. HRMS (ESI$^+$): Calcd for $[M–I]^+$, 204.13884, Found, 204.13386 (–4.98 mmu).

5,5'-Dimethoxy-1,1',3,3,3',3'-hexamethylindodicarbocyanine iodide (Cy5MeO)

Malonaldehyde dianilide hydrochloride (0.8 g, 3.1 mmol) was dissolved in a mixture of dichloromethane (12 mL) and DIEA (1 mL). To the mixture was added dropwise a mixture of acetic anhydride (0.4 mL) and dichloromethane (3 mL), followed by stirring at room temperature for 6 hours. The reaction mixture was added dropwise to a refluxing acetonitrile solution (20 mL) containing **3** (800 mg, 2.4 mmol) and sodium acetate (0.4 g, 4.9 mmol). Reflux was continued for 18 hours, then the reaction mixture was allowed to cool to room temperature, and the resulting solid was removed by filtration. The filtrate was evaporated, and the crude product was purified by recrystallization from 2-propanol to afford **Cy5MeO** as green crystals (495 mg, yield 72%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.09 (t, 2H, J = 12.9 Hz), 7.02-6.87 (m, 6H), 6.75 (t, 1H, J = 12.9 Hz), 6.22 (d, 2H, J = 12.9 Hz), 3.85 (s, 6H), 3.66 (s, 6H), 1.73 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.4, 158.1, 152.5, 142.7, 136.5, 125.7, 113.2, 110.9, 109.1, 103.4, 56.0, 32.6, 28.1, 25.4. HRMS (ESI$^+$): Calcd for $[M–I]^+$, 443.26985, Found, 443.26870 (–1.16 mmu). Anal. Calcd for $C_{29}H_{35}IN_2O_2$•2-PrOH: C, 60.95; H, 6.87; N, 4.44. Found: C, 60.55; H, 6.68; N, 4.49.



Scheme 2. Synthesis of Cy5F.

4,6-Difluoro-2,3,3-trimethylindolenine (5)

3,5-Difluorophenylhydrazine hydrochloride (**4**) (5.0 g, 28 mmol) and 3-methyl-2-butanone (5 mL, 46 mmol) were dissolved in acetic acid (30 mL). The solution was refluxed for 15 hours, then the reaction mixture was allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added dichloromethane, and the whole was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na_2SO_4, filtered, and evaporated to afford **5** as a brown oil (3.5 g, yield 64%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.23 (dd, 1H, J = 7.9, 2.4 Hz), 7.10 (td, 1H, J = 10.1, 2.4 Hz), 2.20 (s, 3H), 1.24 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 188.6, 161.9, 161.7, 158.6, 158.5, 153.8, 153.6, 150.8, 150.7, 150.6, 150.5, 150.4, 150.2, 136.3, 136.2, 136.1, 136.0, 106.0, 105.9, 105.7, 105.6, 103.0, 102.7, 102.6, 102.4, 54.8, 22.1, 15.1. HRMS (ESI$^+$): Calcd for $[M+H]^+$, 196.09378, Found, 196.09221 (–1.57 mmu).

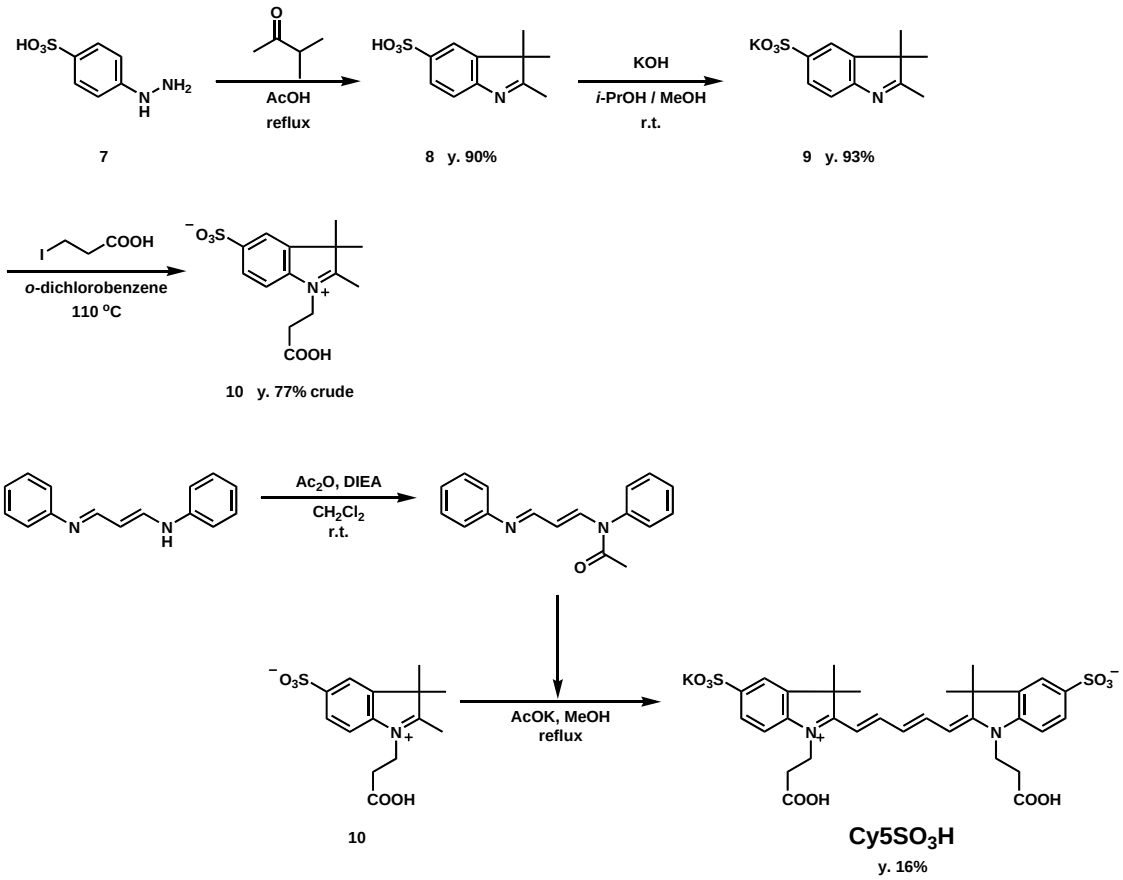
4,6-Difluoro-1,2,3,3-tetramethylindolenium iodide (6)

Compound **5** (2.7 g, 14 mmol) was dissolved in methyl iodide (20 ml). The solution was refluxed for 12 hours, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with *n*-hexane and dried under reduced pressure to afford **6** as a brown-pink solid (3.1 g, yield 66%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.77 (d, 1H, J = 7.3 Hz), 7.61 (t, 1H, J = 10.6 Hz), 4.05 (s, 3H), 2.80 (s, 3H), 1.57 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 197.0, 164.0, 163.9, 160.7, 160.6, 151.6, 151.5, 148.3, 148.1, 146.5, 146.3, 125.7, 125.6, 108.1, 108.0, 107.7, 107.6, 105.5, 105.2, 105.1, 104.8, 55.3, 38.1, 21.5, 14.5. HRMS (ESI$^+$): Calcd for $[M–I]^+$, 210.10943, Found, 210.10463 (–4.80 mmu).

4,4',6,6'-Tetrafluoro-1,1',3,3,3',3'-hexamethylindodicarbocyanine iodide (Cy5F)

Malonaldehyde dianilide hydrochloride (1.1 g, 4.1 mmol) was dissolved in a mixture of dichloromethane (12 mL) and DIEA (1.3 mL). To the mixture was added dropwise a mixture of acetic anhydride (0.6 mL) and dichloromethane (3 mL), followed by stirring at room temperature for 3 hours. The reaction mixture was added dropwise to a refluxing acetonitrile solution (20 mL) containing **6** (1.1 g, 3.3 mmol) and sodium acetate (0.6 g, 7.3 mmol). Reflux was continued for 20 hours, then the reaction mixture was allowed to cool to room

temperature, and the resulting solid was removed by filtration. The filtrate was evaporated, and the crude product was purified by recrystallization from 2-propanol to afford **Cy5F** as green crystals (40 mg, yield 4%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.63 (t, 2H, J = 13.7 Hz), 6.91-6.79 (m, 5H), 6.19 (d, 2H, J = 13.7 Hz), 3.84 (s, 6H), 1.85 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.2, 161.4, 161.3, 158.9, 158.8, 155.4, 149.4, 149.3, 146.9, 146.8, 145.8, 145.7, 127.7, 125.9, 125.8, 106.6, 106.5, 106.4, 106.3, 104.8, 104.6, 104.5, 104.3, 104.0, 50.8, 34.8, 34.7, 28.3. HRMS (ESI^+): Calcd for $[M-I]^+$, 455.21104, Found, 455.21406 (+3.02 mmu). Anal. Calcd for $C_{27}H_{27}F_4IN_2 \cdot 0.5H_2O$: C, 54.83; H, 4.77; N, 4.74. Found: C, 55.06; H, 4.67; N, 4.70.



Scheme 3. Synthesis of $Cy5SO_3H$.

2,3,3-trimethylindolenine-5-sulfonic acid (8)

Hydrazinobenzenesulfonic acid (**7**) (12.9 g, 67 mmol) and 3-methyl-2-butanone (7 mL, 67 mmol) were dissolved in acetic acid (30 mL). The solution was refluxed for 14 hours, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with diethyl ether and dried under reduced pressure to afford **8** as a pink solid (18.0 g, yield 90%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.77 (d, 1H, J = 1.3

Hz), 7.64 (dd, 1H, J = 8.0, 1.3 Hz), 7.45 (d, 1H, J = 8.0 Hz), 2.40 (s, 3H), 1.33 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 193.2, 148.3, 146.2, 144.2, 125.7, 119.9, 117.3, 53.7, 22.3, 15.2. HRMS (ESI$^+$): m/z calcd for $[M+H]^+$, 240.06944; found, 240.06907 (–0.36 mmu).

Potassium salt of 2,3,3-trimethylindolenine-5-sulfonate (9)

Compound **8** (18.0 g, 59 mmol) was dissolved in methanol (20 mL). To this solution was added 2-propanol saturated with potassium hydroxide (300 mL), followed by stirring. The resulting yellow solid was collected by filtration. The solid was washed with 2-propanol, and dried under reduced pressure to afford **9** (15.2 g, yield 93%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.62 (d, 1H, J = 1.7 Hz), 7.54 (dd, 1H, J = 7.9, 1.7 Hz), 7.32 (d, 1H, J = 7.9 Hz), 2.20 (s, 3H), 1.23 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 188.8, 153.6, 145.2, 145.1, 125.1, 119.1, 118.1, 53.2, 22.5, 15.1. HRMS (ESI$^+$): m/z calcd for $[M+H]^+$, 278.02532; found, 278.02719 (+1.87mmu).

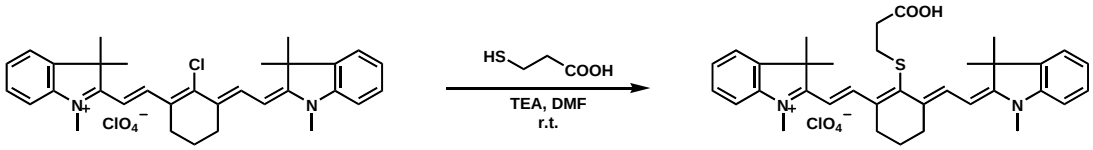
1-(β-carboxyethyl)-2,3,3-trimethylindolenium-5-sulfonate (10)

Compound **9** (30.5 g, 0.11 mol) and 3-iodopropionic acid (25.0 g 0.13 mol) were suspended in *o*-dichlorobenzene (150 mL). The suspension was stirred at 110 °C for 19 hours, then allowed to cool to room temperature, and the supernatant was removed. The residue was washed with 2-propanol and diethyl ether, then dried under reduced pressure to afford **10** as a reddish solid (26.5 g, yield 77% crude). The crude **10** was used without further purification in the next reaction. HRMS (ESI$^+$): m/z calcd for $[M+H]^+$, 350.04645; found, 350.04381 (–2.64 mmu).

Potassium salt of 1,1'-bis-(β-carboxyethyl)-3,3,3',3'-tetramethylindodicarbocyanine-5,5'-disulfonate (Cy5SO$_3$H)

Malonaldehyde dianilide hydrochloride (2.5 g, 9.8 mmol) was dissolved in a mixture of dichloromethane (15 mL) and DIEA (1.5 mL). To the mixture was added dropwise a mixture of acetic anhydride (1.5 mL) and dichloromethane (5 mL), followed by stirring at room temperature for 4 hours. The reaction mixture was added dropwise to a refluxing methanol solution (20 mL) containing **10** (6.8 g, 22 mmol) and potassium acetate (1.0 g, 10 mmol). Reflux was continued for 10 hours, then the mixture was allowed to cool to room temperature. The resulting solid was collected by filtration and washed with 2-propanol and diethylether. The crude product was

purified by reversed-phase ODS chromatography (H_2O / methanol, 5:1) to afford **Cy5SO$_3$H** as a gold powder (1.1 g, yield 16%). ^{1}H NMR (300 MHz, D_2O): δ 7.92 (t, 2H, *J* = 13.0 Hz), 7.69-7.64 (m, 4H), 7.21 (d, 2H, *J* = 8.6 Hz), 6.49 (t, 1H, *J* = 13.0 Hz), 6.22 (d, 2H, *J* = 13.0 Hz), 4.16 (t, 4H, *J* = 7.2 Hz), 2.49 (t, 4H, *J* = 7.2 Hz), 1.54 (s, 12H). ^{13}C NMR (100 MHz, D_2O): δ 179.9, 175.4, 156.1, 145.6, 143.3, 140.4, 127.8, 127.5, 121.2, 112.5, 105.8, 50.5, 42.8, 36.3, 28.2. HRMS (ESI^-): m/z calcd for $[M–K]^-$, 657.15766; found, 657.15571 (–1.95 mmu).

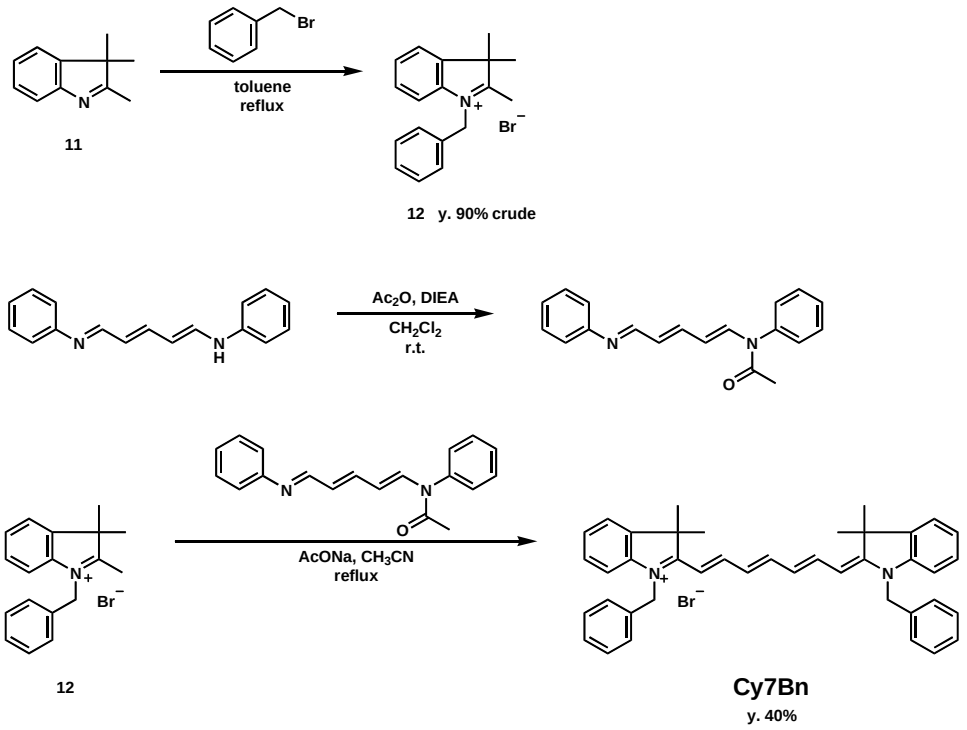


IR786 **IR786S**
y. 89%

Scheme 4. Synthesis of IR786S.

2-[4'-(*β*-Carboxyethylthio)-7'-(1'',3'',3''-trimethylindolenine)-3',5'-trimethyleneheptatrien-1-yl]-1,3,3-trimethylindolenium perchlorate (IR786S)

To a solution of IR786 perchlorate (1.5 g, 2.6 mmol) in DMF (10 mL) were added 3-mercaptopropionic acid (265 μL, 3.0 mmol) and triethylamine (425 μL, 3.0 mmol). The mixture was stirred at room temperature for 20 hours, then dichloromethane was added, and the whole was washed with brine. The organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by recrystallization from 2-propanol to afford **IR786S** as dark red crystals (1.5 g, yield 89%). ^{1}H NMR (300 MHz, CD_3OD): δ 8.87 (d, 2H, *J* = 14.3 Hz), 7.50-7.25 (m, 8H), 6.26 (d, 2H, *J* = 14.3 Hz), 3.64 (s, 6H), 3.06 (t, 2H, *J* = 6.8 Hz), 2.68 (t, 4H, *J* = 6.1 Hz), 2.59 (t, 2H, *J* = 6.8 Hz), 1.93 (m, 2H), 1.75 (s, 12H). ^{13}C NMR (100 MHz, CD_3OD): δ 174.9, 174.6, 157.8, 147.1, 144.5, 142.4, 134.8, 129.8, 126.2, 123.3, 111.8, 102.2, 50.4, 35.7, 33.9, 31.6, 28.2, 27.2, 22.2. HRMS (ESI$^+$): m/z calcd for $[M–ClO_4]^+$, 553.28887; found, 553.28932 (+0.45 mmu). Anal. Calcd for $C_{35}H_{41}ClN_2O_6S•1.5H_2O$: C, 61.80; H, 6.52; N, 4.12. Found: C, 62.15; H, 6.20; N, 4.08.



Scheme 5. Synthesis of Cy7Bn.

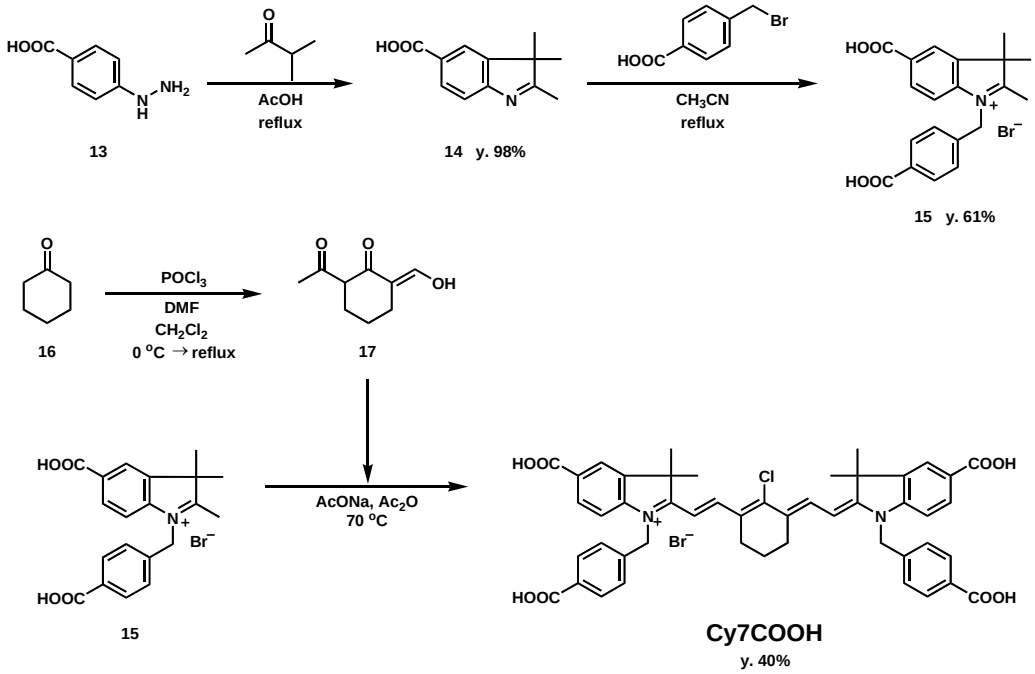
1-Benzyl-2,3,3-trimethylindolenium bromide (12)

2,3,3-Trimethylindolenine (**11**) (1 ml, 6.2 mmol) and benzyl bromide (0.8 ml, 6.7 mmol) were suspended in toluene (20 mL). The reaction mixture was refluxed for 18 hours, then allowed to cool to room temperature, and the resulting solid was collected by filtration. The solid was washed with *n*-hexane and dried under reduced pressure to afford **12** as a red solid (1.8 g, yield 90% crude). The crude **12** was used without further purification in the next reaction. HRMS (ESI^+): Calcd for $[M–Br]^+$, 250.15957 Found, 250.15803 (–1.54 mmu).

1,1'-Dibenzyl-3,3,3',3'-tetramethylindotricarbocyanine bromide (Cy7Bn)

Glutaconaldehydedianil hydrochloride (0.4 g, 1.4 mmol) was dissolved in a mixture of dichloromethane (5 mL) and DIEA (0.5 mL). To the mixture was added dropwise a mixture of acetic anhydride (0.25 mL) and dichloromethane (5 mL), followed by stirring at room temperature for 4 hours. The reaction mixture was added dropwise to a refluxing acetonitrile solution (20 mL) containing **12** (0.2 g, 0.6 mmol) and sodium acetate (0.5 g, 6.1 mmol), and reflux was continued for 4 hours. The reaction mixture was allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added dichloromethane, and the whole was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried

over Na_2SO_4, filtered, and evaporated. The crude product was purified by semi-preparative HPLC. Purification was performed until HPLC analysis showed a single peak of the product, affording **Cy7Bn** as a green powder (78 mg, yield 40%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.93 (t, 2H, *J* = 13.2 Hz), 7.57 (t, 1H, *J* = 12.7 Hz), 7.51 (d, 2H, *J* = 7.3 Hz), 7.38-7.18 (m, 16H), 6.44 (t, 2H, *J* = 12.7 Hz), 6.27 (d, 2H, *J* = 13.2 Hz), 5.23 (s, 4H), 1.66 (s, 12H). ^{13}C NMR (100 MHz, CD_3OD): δ 173.8, 158.4, 153.5, 143.9, 142.3, 136.0, 130.2, 130.0, 129.1, 129.0, 127.5, 126.2, 123.5, 112.0, 105.5, 50.5, 28.1, 23.5. HRMS (ESI^+): Calcd for $[M–Br]^+$, 561.32697, Found, 561.32259 (–4.39 mmu).



Scheme 6. Synthesis of Cy7COOH.

2,3,3-Trimethylindolenine-5-carboxylic acid (14)

4-Hydrazinobenzoic acid hydrochloride (**13**) (2 g, 11 mmol) and 3-methyl-2-butanone (2 mL, 19 mmol) were dissolved in acetic acid (20 mL). The solution was refluxed for 12 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added dichloromethane, and the whole was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na_2SO_4, filtered, and evaporated to afford **14** as a brown solid (2.2 g, yield 98%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.16 (dd, 1H, *J* = 8.1 Hz, 1.7 Hz), 8.07 (d, 1H, *J* = 1.7 Hz), 7.69 (d, 1H, *J* = 8.1 Hz),

2.39 (s, 3H), 1.38 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.3, 169.0, 156.3, 145.2, 130.3, 127.5, 122.9, 119.0, 53.7, 22.5, 14.9. HRMS (ESI^+): Calcd for $[M+H]^+$, 204.10245, Found, 204.10440 (+1.95 mmu)

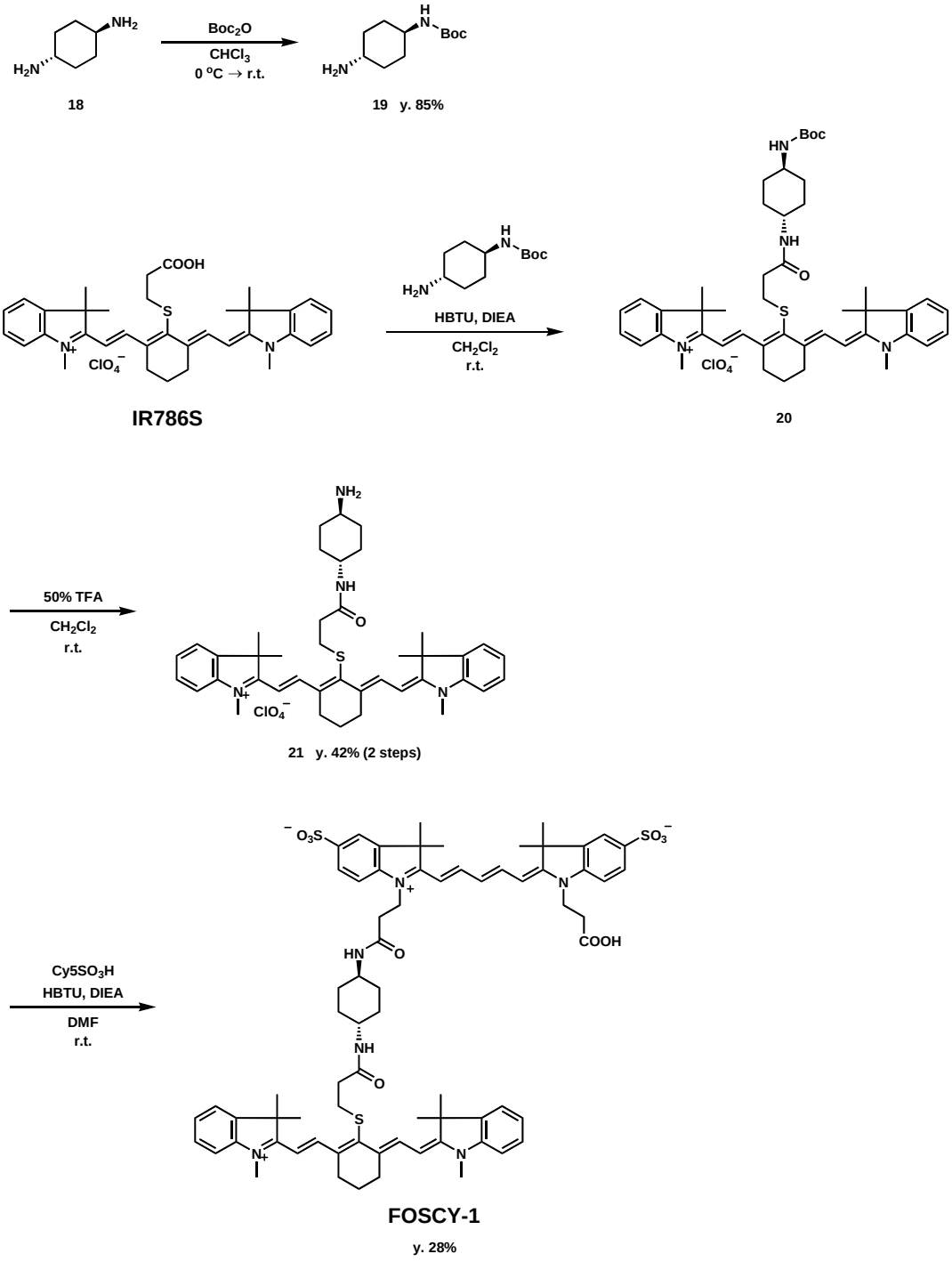
5-Carboxy-1-(*p*-carboxybenzyl)-2,3,3-trimethylindolenium bromide (15)

Compound **14** (1.5 g, 7.4 mmol) and 4-bromomethylbenzoic acid (1.6 g, 7.4 mmol) were dissolved in acetonitrile (10 mL). The solution was refluxed for 12 hours, then allowed to cool to room temperature, and the solvent was removed under reduced pressure. To the resulting residue was added diethyl ether, followed by stirring. The resulting brown solid was collected by filtration. The solid was washed with diethyl ether, and dried under reduced pressure to afford **15** (1.9 g, yield 61%). ^{1}H NMR (300 MHz, CD_3OD): δ 8.41 (s, 1H), 8.22 (dd, 1H, *J* = 8.4 Hz, 1.1 Hz), 8.08 (d, 2H, *J* = 8.6 Hz), 7.84 (d, 1H, *J* = 8.4 Hz), 7.48 (d, 2H, *J* = 8.6 Hz) 5.96 (s, 2H), 3.31 (s, 3H partially overlapped by CD_3OD), 1.73 (s, 6H). ^{13}C NMR (75 MHz, CD_3OD): δ 168.7, 167.9, 145.7, 143.6, 137.4, 132.8, 132.3, 132.2, 131.9, 128.4, 125.8, 117.5, 117.2, 56.6, 52.6, 22.8, 22.6. HRMS (ESI^+): Calcd for $[M-Br]^+$, 338.13923, Found, 338.13772 (−1.52 mmu).

2-[4'-Chloro-7'-(1''-(*p*-carboxybenzyl)-3'',3''-dimethylindolenine)-3',5'-trimethylenehepta-trien-1-yl]-1-(*p*-carboxybenzyl)-3,3-dimethylindolenium bromide (Cy7COOH)

2-Chloro-1-formyl-3-(hydroxymethylene)-cyclohex-1-ene (**17**) was prepared from anhydrous DMF, phosphorus oxychloride and cyclohexanone (**16**) in dichloromethane according to the literature [SR6], and the crude **17** was used without further purification in the next reaction. Compound **15** (0.5 g, 1.2 mmol), compound **17** (48 mg, 0.3 mmol) and sodium acetate (50 mg, 0.6 mmol) were dissolved in acetic anhydride (15 mL), followed by stirring at 70 °C for 30 minutes under an argon atmosphere. The reaction mixture was allowed to cool to room temperature, and dichloromethane was added. The whole was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by semi-preparative HPLC. Purification was performed until HPLC analysis showed a single peak of the product, affording **Cy7COOH** as a dark red powder (100 mg, yield 40%). ^{1}H NMR (400 MHz, CD_3OD): δ 8.42 (d, 2H, *J* = 14.2 Hz), 8.14 (d, 2H, *J* = 1.5 Hz), 8.03 (dd, 2H, *J* = 8.3 Hz, 1.5 Hz), 7.94 (d, 4H, *J* = 8.3 Hz), 7.33 (d, 2H, *J* = 8.3 Hz), 7.29 (d, 4H, *J* = 8.3 Hz), 6.30 (d, 2H, *J* = 14.2 Hz), 5.48 (s, 4H), 2.51 (t, 4H, *J* = 5.6 Hz), 1.81-1.78 (m, 14H). ^{13}C NMR (100 MHz, CD_3OD): δ 174.1, 173.6, 152.1, 146.0, 145.8, 142.0, 138.5, 138.2, 135.9, 131.7,

131.1, 130.9, 129.5, 127.2, 124.6, 111.5, 103.9, 50.5, 29.1, 28.4, 27.2, 23.4. HRMS (ESI^+): Calcd for $[M-Br]^+$, 811.27862, Found, 811.28195 (+3.33 mmu).



Scheme 7. Synthesis of FOSCY-1.

2-[4'-(*β*-4'''-*trans*-*N*-*tert*-Butoxycarbonylaminocyclohexylcarbamoylethylthio)-7'-(1'',3'',3''-trimethylindolenine)-3',5'-trimethyleneheptatrien-1-yl]-1,3,3-trimethylindolenium perchlorate (20)

N-*tert*-Butoxycarbonyl-*trans*-1,4-cyclohexanediamine (**19**) was prepared from *trans*-1,4-cyclohexanediamine (**18**) and di-*tert*-butyl dicarbonate in chloroform according to the literature.[SR7] **IR786S** (217 mg, 0.33 mmol) and HBTU (173 mg, 0.46 mmol) were dissolved in dichloromethane (10 mL). To this solution were added compound **19** (98 mg, 0.46 mmol) and DIEA (75 μL), followed by stirring at room temperature for 4 hours. Then dichloromethane was added, and the whole was washed with saturated aqueous solution of sodium bicarbonate. The organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude **20** was used without further purification in the next reaction. HRMS (ESI^+): m/z calcd for $[M–ClO_4]^+$, 749.44644; found, 749.44195 (–4.49 mmu).

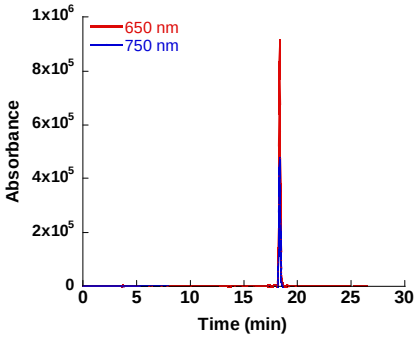
2-[4'-(*β*-4'''-*trans*-Aminocyclohexylcarbamoylethylthio)-7'-(1'',3'',3''-trimethylindolenine)-3',5'-trimethyleneheptatrien-1-yl]-1,3,3-trimethylindolenium perchlorate (21)

Compound **20** was dissolved in a mixture of dichloromethane (10 mL) and trifluoroacetic acid (10 mL). The reaction mixture was stirred at room temperature for 3 hours, then evaporated, and the crude **21** was reprecipitated from 1 drop of methanol and diethylether (about 200 mL). The precipitate was collected by filtration and purified by recrystallization from 2-propanol to afford **21** as dark green crystals (104 mg, yield 42% 2 steps). ^{1}H NMR (300 MHz, CD_3OD): δ 8.87 (d, 2H, *J* = 14.3 Hz), 7.51-7.26 (m, 8H), 6.27 (d, 2H, *J* = 14.3 Hz), 3.65 (s, 6H), 3.61 (m, 1H), 3.05 (t, 2H, *J* = 7.3 Hz), 2.69 (t, 4H, *J* = 5.9 Hz), 2.49 (t, 2H, *J* = 7.3 Hz), 2.03-1.95 (m, 6H), 1.75 (s, 12H), 1.49-1.37 (m, 4H). ^{13}C NMR (100 MHz, CD_3OD): δ 174.5, 172.3, 157.8, 147.1, 144.4, 142.3, 134.8, 129.8, 126.2, 123.3, 111.8, 102.2, 50.4, 50.3, 37.2, 34.3, 32.2, 31.2, 30.5, 30.4, 28.3, 27.2, 22.2. HRMS (ESI^+): m/z calcd for $[M–ClO_4]^+$, 649.39401; found, 649.39725 (+3.24 mmu).

FOSCY-1

Cy5SO$_3$H (233 mg, 0.33 mmol) was dissolved in DMF (10 mL). To the mixture was added dropwise a solution of HBTU (108 mg, 0.28 mmol) in DMF (10 mL), followed by dropwise addition of a solution of **21** (80 mg, 0.11 mmol) and DIEA (25 μL) in DMF (10 mL). The reaction mixture was stirred at room temperature for 9 hours, and evaporated. The resulting solid was purified by semi-preparative HPLC. Purification was performed until

HPLC analysis showed a single peak of the product, affording **FOSCY-1** as a dark red powder (40 mg, yield 28%). ^{1}H NMR (300 MHz, DMF-d_7): δ 8.77 (d, 2H, J = 14.1 Hz), 8.47 (m, 2H), 8.33 (br, 1H), 7.85-7.26 (m, 15H), 6.64-6.53 (m, 2H), 6.37 (m, 3H), 4.42 (br, 4H), 3.78 (s, 6H), 3.04 (t, 2H, J = 7.2 Hz), 2.63 (m, 4H), 2.46 (t, 2H, J = 7.2 Hz), 1.83-1.68 (m, 30H), 1.20-1.07 (m, 4H). ^{13}C NMR (100 MHz, DMF-d_7): δ 179.6, 178.9, 178.4, 177.8, 174.6, 174.5, 168.2, 167.9, 161.0, 160.0, 151.8, 151.6, 150.7, 149.0, 147.8, 147.7, 146.8, 146.5, 146.4, 138.9, 134.2, 131.9, 131.8, 130.5, 128.0, 125.6, 125.5, 116.7, 116.1, 115.8, 110.1, 109.5, 107.2, 54.9, 54.7, 54.6, 53.2, 53.1, 51.6, 51.5, 51.2, 41.5, 38.9, 37.6, 36.7, 32.8, 32.3, 31.6, 26.5, 14.1, 14.0, 13.7. HRMS (ESI$^-$): m/z calcd for [M–H]$^-$, 1287.53328; found, 1287.53710 (+3.83 mmu). HPLC (eluent A/B = 80/20 - 20 min - 0/100; flow rate = 1.0 mL/min): 18.34 min.



Experiments using living cells

Cell culture

HeLa cells were purchased from RIKEN BioResoource Center. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA), supplemented with 10% (v/v) fetal bovine serum (Invitrogen Corp., Carlsbad, CA), penicillin (100 units/mL) -streptomycin (100 μg/mL) liquid (Invitrogen Corp., Carlsbad, CA) in a humidified incubator containing 5% CO_2 in air.

HL60 cells were purchased from RIKEN BioResoource Center. The cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Invitrogen Corp., Carlsbad, CA), supplemented with 10% (v/v) fetal bovine serum (Invitrogen Corp., Carlsbad, CA), penicillin (100 units/mL) -streptomycin (100 μg/mL) liquid (Invitrogen Corp., Carlsbad, CA) in a humidified incubator containing 5% CO_2 in air.

Porcine neutrophils were obtained from *porcine* blood purchased from Tokyo Shibaura Zouki Co. Ltd..[SR8] The blood (2 L) was added to 5% (w/v) EDTA-3Na aq. (0.2 L) and then to 3% (w/v) polyvinylpyrrolidone solution (0.5 L) and mixed gently. The mixture was kept at room temperature for 30-40 minutes and the supernatant was separated from sedimenting erythrocyte aggregates by siphoning. Leucocytes in the supernatant were precipitated by centrifugation at 4 °C, 250 g for 10 minutes. Contaminating erythrocytes in the precipitate were lysed by the addition of 0.2% (w/v) NaCl aq. (50 mL). After 30 seconds, the suspension was made iso-osmolar by the addition of 1.6% (w/v) NaCl aq. (50 mL). The pellet obtained by centrifugation at 4 °C, 250 g for 10 minutes was suspended in Krebs-Ringer phosphate (KRP) buffer (114 mM NaCl, 4.6 mM KCl, 2.4 mM $MgSO_4$, 1.0 mM $CaCl_2$, 15 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 5 mM D-glucose). Polymorphonuclear leucocytes were separated from platelets and mononuclear cells by the Conray-Ficoll method (Lymphoprep™, AXIS-SHIELD PoC AS, Oslo, Norway). The polymorphonuclear leucocytes were suspended in KRP buffer and kept in ice until use.

Fluorometric analysis of HL60 cells

HL60 cells were diluted to 1×10^6 cells/mL in Hanks' balanced salts solution (HBSS) and 3 mL of this suspension of HL60 was poured into a plastic cuvette. FOSCY-1 (final 0.1 μM, DMF 0.1% as a cosolvent) was added. The samples were stirred gently at 37 °C, and SOD (final 60 mU/mL) was added in the case of the PMA-stimulated+SOD sample. Then, the measurement of fluorescence intensity at 668 nm with excitation at 645 nm was started. After 1 minute, PMA (final 1 μg, DMF 0.2% as a cosolvent) or DMF 3 μL (final DMF 0.2% as a cosolvent) was added.

Fluorometric analysis of *porcine* neutrophils

Porcine neutrophils were diluted to 1×10^6 cells/mL in KRP buffer and 3 mL of this suspension of *porcine* neutrophils was poured into a plastic cuvette. FOSCY-1 (final 1 μM, DMF 0.1% as a cosolvent) was added. The samples were stirred gently at 37 °C, and SOD (final 60 mU/mL) or ABH (final 1 mM) were added in the case of the +SOD, +ABH, or +SOD+ABH sample. Then, the measurement of fluorescence intensity at 668 nm with excitation at 645 nm was started. After 1 minute, PMA (final 1 μg, DMF 0.2% as a cosolvent) or DMF 3 μL (final DMF 0.2% as a cosolvent) was added.

Imaging system

The imaging system comprised a confocal laser scanning unit (U-LH100HG, Olympus), an inverted microscope (IX81FVF, Olympus) and a 60 × objective lens (PlanApo 60 × O., Olympus). The excitation wavelength was 633 nm and the emission was filtered using a 660-nm band-pass filter.

Imaging of FOSCY-1-loaded HeLa cells

The culture medium was removed, and the cells were washed with HBSS. FOSCY-1 (final 10 μM, DMF 0.1% as a cosolvent) in HBSS was loaded into HeLa cells plated in a 35-mm PLL-coated glass-bottomed dish (Matsunami, D110110), followed by incubation at room temperature for 30 minutes. Without washing, fluorescence images were acquired.

***In vivo* imaging**

C57Bl/6 mice (CLEA Japan, Inc.) were given an intraperitoneal (i.p.) injection of zymosan derived from *Saccharomyces cerevisiae* (1 mg in 1 ml saline) to induce peritonitis. After 4 hours, the mice were anesthetized by i.p. injection of Nembutal (40~50 mg/kg), and their abdominal fur was removed with an electric shaver. Then, the mice were injected i.p. with FOSCY-1 (100 μM in 0.9 ml saline), and divided into four groups; one group was injected i.p. with PMA (3 μg in 0.3 ml saline, n = 6) 2 minutes after FOSCY-1 injection, a second group was injected with saline (0.3 ml, n = 6), and the third and fourth groups were injected with apocynin (400 μM or 4 mM, n = 3 each), which is a NOX inhibitor, followed by PMA. Images were acquired for 60 minutes by using a MAESTRO™ In Vivo Imaging System (CRI Inc., Woburn, MA, USA), with an excitation filter of 620 nm, an emission filter of 680 nm and bright-field settings. Mean fluorescence intensity of the abdominal region for each mouse was measured with Maestro 2.4.0.

Fluorometric analysis of peritoneal exudate cells (PEC)

C57Bl/6 mice (CLEA Japan, Inc.) were given an i.p. injection of zymosan derived from *Saccharomyces cerevisiae* (1 mg in 1 ml saline) to induce peritonitis. After 4 hours, the mice were sacrificed by ether inhalation. A small incision was made in the abdominal skin, and opened up by pulling the skin apart. Cold PBS (5 mL) was injected into the i.p. cavity and the abdominal region was kneaded, then the PBS suspension containing PEC was collected. PEC were diluted to 1×10^6 cells/mL in PBS and 600 μL of the suspension of PEC was poured into a plastic cuvette. FOSCY-1 (final 16.7 μM, DMF 0.1% as a cosolvent) was added. The samples were stirred gently at 37 °C, and apocynin (final 67 μM, 0.33 mM, or 3.3 mM) was added in the case of the apo 1-3 samples. Then, the measurement of fluorescence intensity at 668 nm with excitation at 645 nm was started. After 1 minute, PMA (final 1 μg, DMF 0.2% as a cosolvent) or DMF 3 μL (final DMF 0.2% as a cosolvent) was added.

(Supporting references)

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Supporting Figure

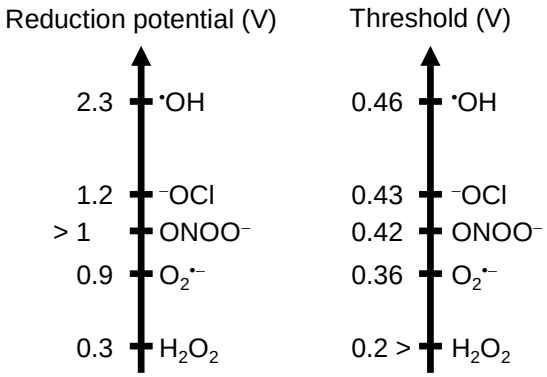

Figure S1. Reduction potentials of ROS and approximate threshold oxidation potentials of reaction with ROS.

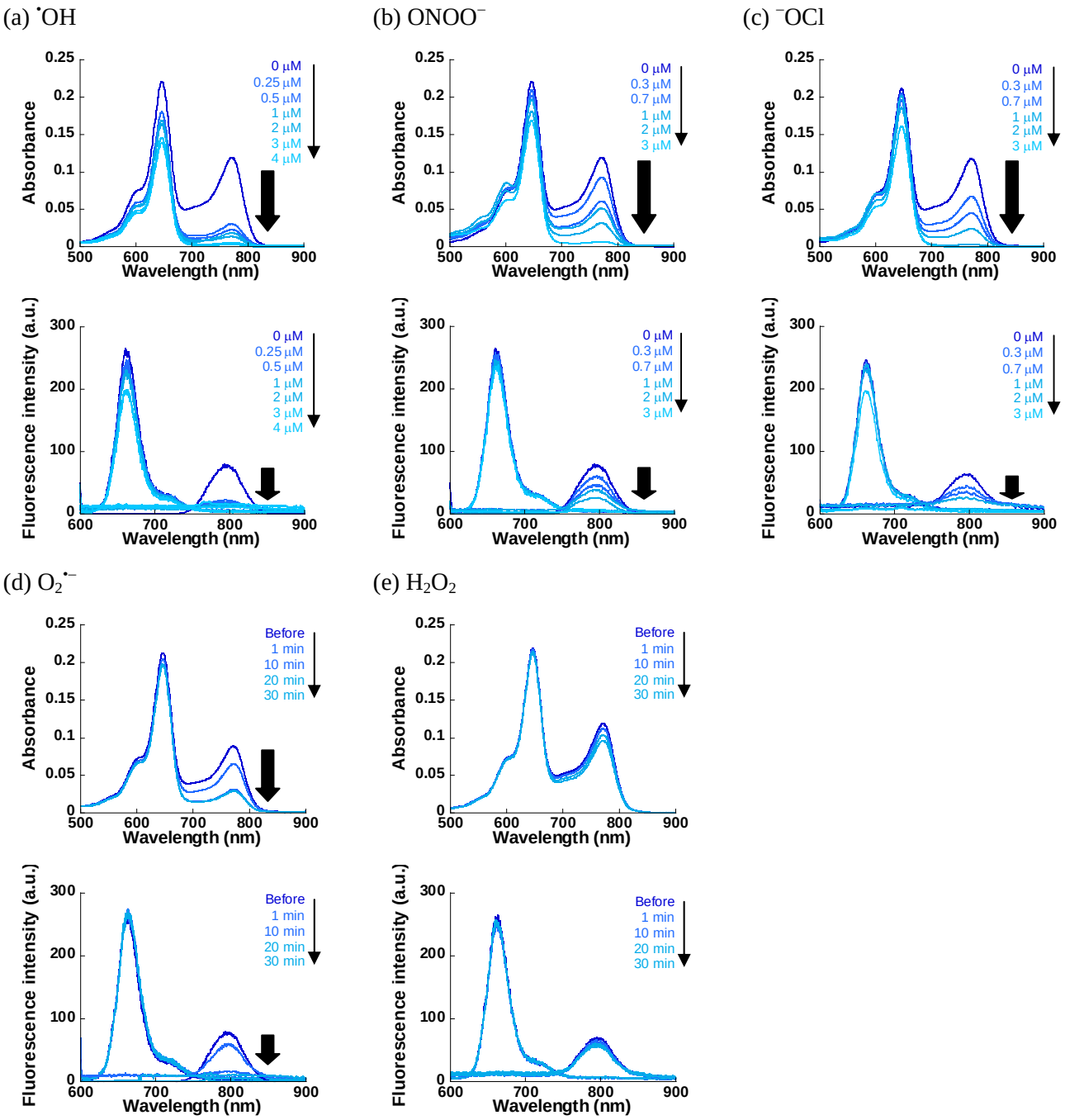

Figure S2. The absorption (upper) and fluorescence (lower) spectra of a 1 μM solution of $Cy5SO_3H$ (Dye **9**) and IR786S (Dye **1**) after reaction with various ROS. For details, see the experimental procedure.

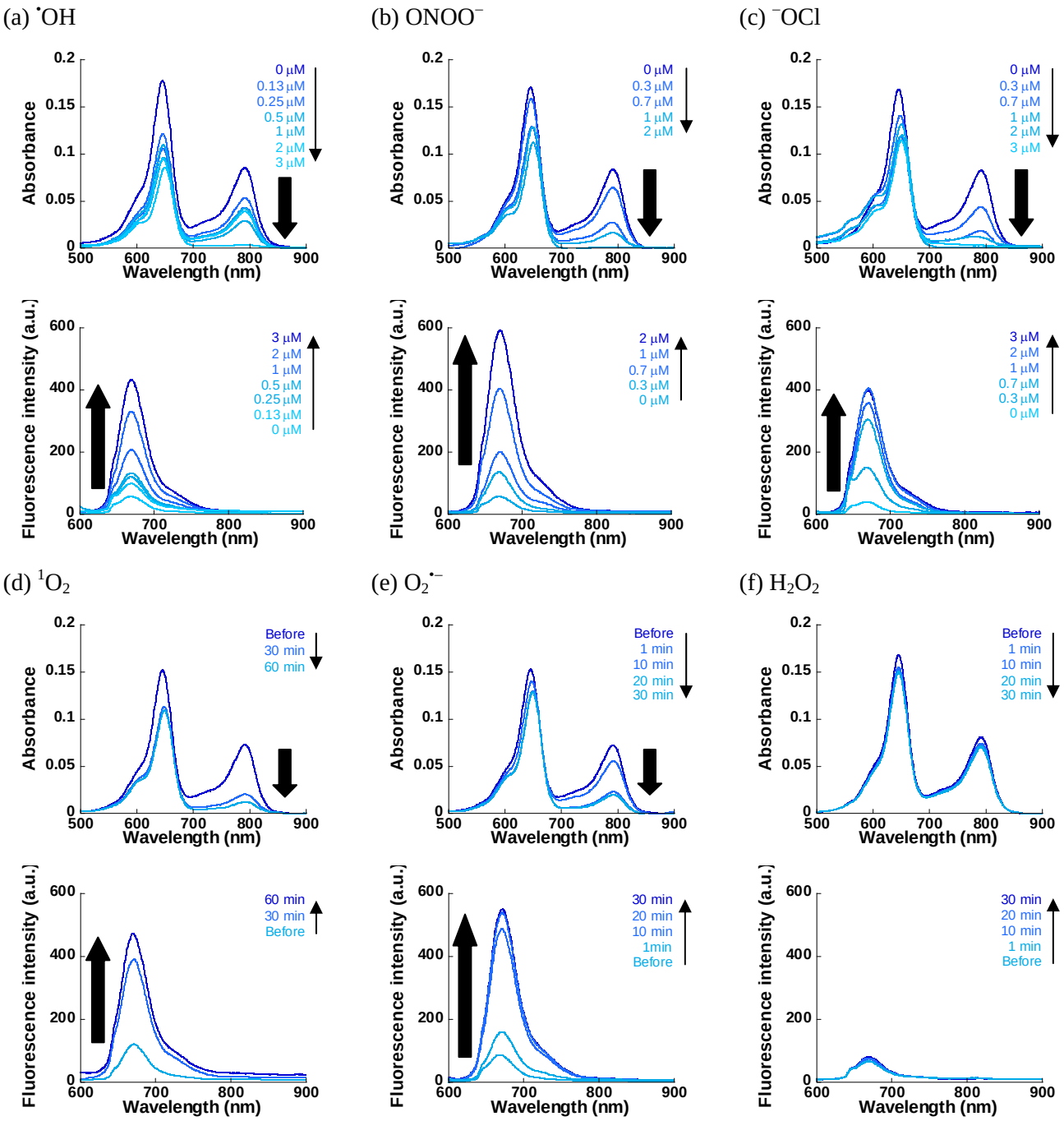

Figure S3. The absorption (upper) and fluorescence (lower) spectra of a 1 μM solution of FOSCY-1 after reaction with various ROS. For details, see experimental procedure.

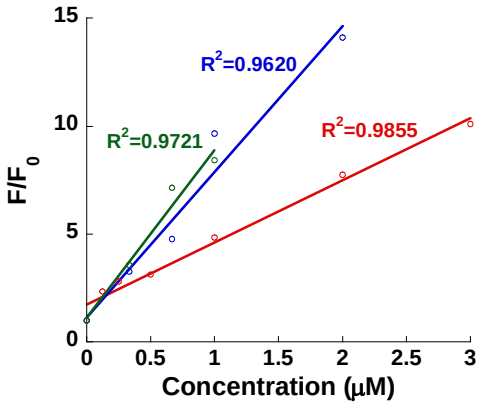

Figure S4. Relationship between the concentrations of added $^{\bullet}$OH (red), $ONOO^{-}$ (blue), or ^{-}OCl (green) to a 1 μM solution of FOSCY-1 and the augmentation of the fluorescence intensity. Data represent the ratio of the fluorescence intensity after the addition (F) to the initial fluorescence intensity (F_0).

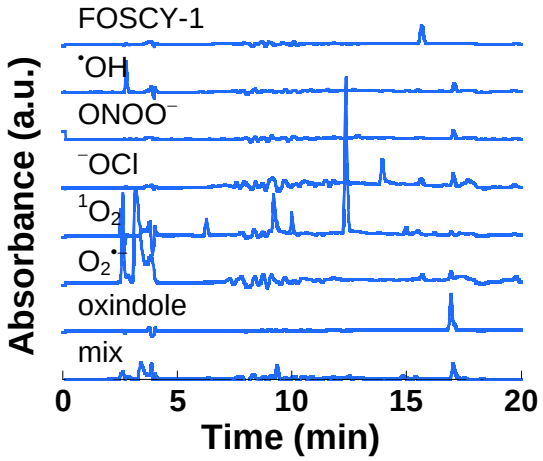

Figure S5. HPLC chromatograms of FOSCY-1, the reaction mixture of FOSCY-1 with $^{\bullet}$OH, $ONOO^{-}$, ^{-}OCl, $^{1}O_2$, or $O_2^{\bullet-}$, 1,3,3-trimethyloxindole, and a mixture of them. Samples were analyzed by HPLC with linear gradient elution (eluent A/B = 80/20 - 20 min - 0/100; flow rate = 1.0 mL/min). The monitored wavelength was 250 nm.

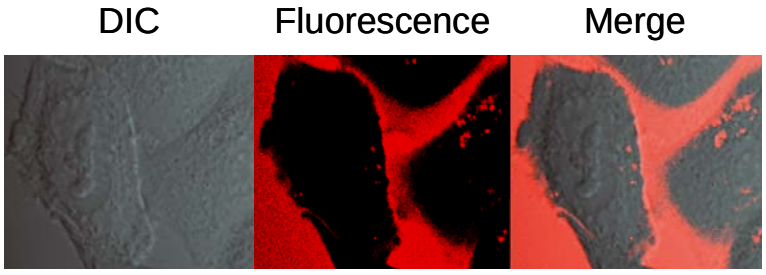

Figure S6. Confocal fluorescence microscopy images of HeLa cells loaded with FOSCY-1.

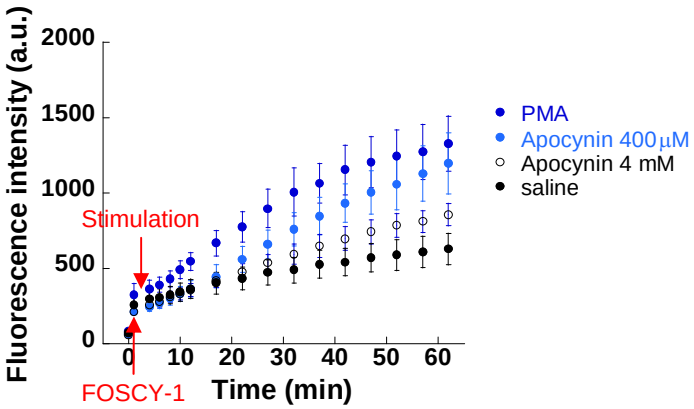

Figure S7. Time courses of average fluorescence intensity observed in the abdominal region of a mouse model of peritonitis. Data are shown as mean ± standard deviation (n = 6 for "PMA" and "saline" samples or n = 3 for "Apocynin 400 μM" and "Apocynin 4 mM" samples). The monitored wavelength was 680 nm.

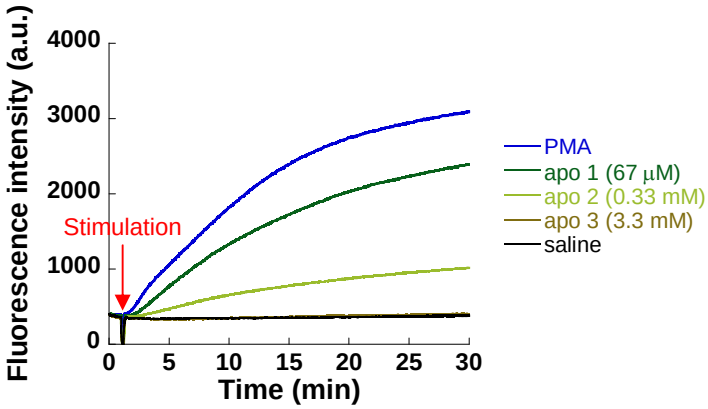

Figure S8. Time courses of fluorescence intensity observed with FOSCY-1 in the presence of PMA-stimulated peritoneal exudate cells obtained from a mouse model of peritonitis. Representative data are shown (n = 2). For details, see the experimental procedure. The monitored wavelength was 668 nm.