Supporting Information

Photoactivatable fluorophores for durable labelling of individual cells

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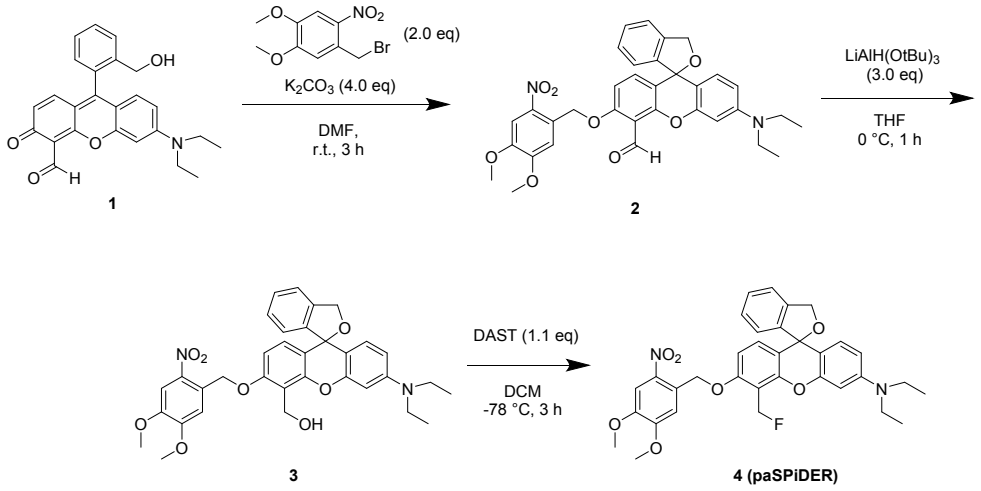
Synthesis and characterization of compounds

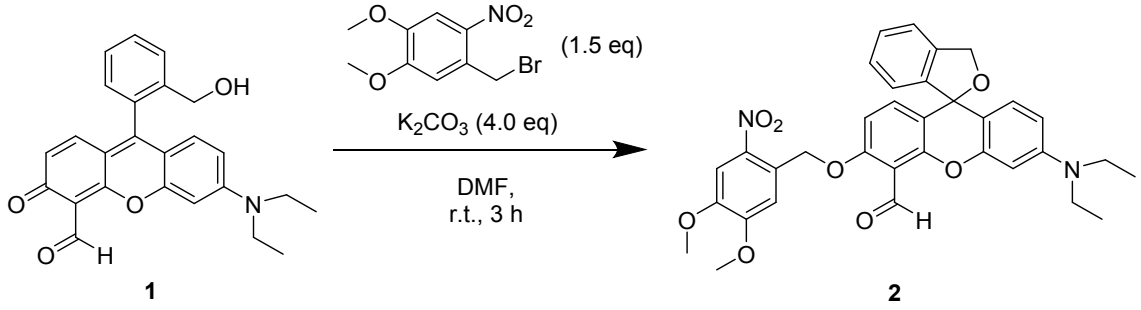
Materials and general information

All chemical reagents and dry solvents for synthesis were purchased from commercial suppliers (Wako Pure Chemical, Tokyo Chemical Industries, Sigma-Aldrich Japan) and were used without further purification. The composition of mixed solvents is given as volume ratio (v/v). Dimethyl sulfoxide (DMSO, fluorometric grade) used for the spectrometric measurements was purchased from Dojindo. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on an AVANCE III 400 Nanobay (Bruker, 400 MHz for ^{1}H NMR, 101 MHz for ^{13}C NMR). Mass spectra (MS, ESI-TOF) were measured with a MicroTOF (Bruker). High-resolution MS (HRMS) was measured using sodium formate as an external standard. Flash chromatographic purifications were performed on an automated silica gel flash column chromatography system YFLC AI-580 (Yamazen). Preparative reversed-phase HPLC using an Inertssustain C18 4.6 mm x 150 mm column (GL Science) or an Inertsil ODS-3 20 mm x 250 mm column (GL Science) was performed on a PU-2080 system equipped with a MD-2010 detector (JASCO) or a PU-2087 system equipped with a MD-2010 detector (JASCO). Solvent A: 99% H_2O, 1% CH_3CN and 0.1% TFA. B: 99% CH_3CN and 1% H_2O.



Scheme S1. Synthetic scheme of **paSPiDER-1.**

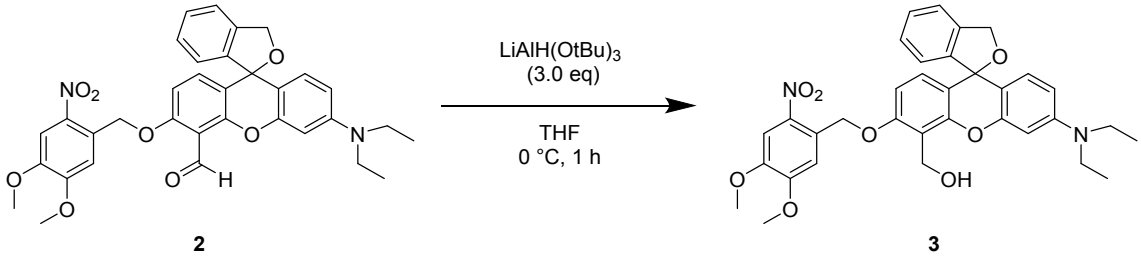
4-CHO-caged HMDER, 2



Scheme S1-2. Synthesis of 4-CHO-caged HMDER 2.

Compound **1** was synthesized as previously described[1]. To a solution of compound **1** (104 mg, 0.25 mmol) in dry DMF (3 mL), potassium carbonate (173 mg, 1.25 mmol), 4,5-dimethoxy-2-nitrobenzyl bromide (143 mg, 0.50 mmol), and a small amount of sodium sulfate were added, and the solution was stirred at r.t. for 3 h. Then, the reaction mixture was evaporated and the residue was dissolved in water. The resulting solution was extracted with CH_2Cl_2 (3 x), and the combined organic layer was evaporated. The residue was purified by silica gel flash column chromatography (hexane/EtOAc = 67/33) to obtain the desired product **2** as a red solid (99.8 mg, y. 67%). A sample was identified by ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17 (6H, t, *J* = 7.0 Hz), 3.36 (4H, q, *J* = 7.0 Hz,), 3.96 (3H, s), 4.25 (3H, s), 5.25 (1H, d, *J* = 12.4 Hz), 5.29 (1H, d, *J* = 12.4 Hz), 5.50 (2H, s), 6.42 (1H, dd, *J* = 8.8, 2.6 Hz), 6.47 (1H, d, *J* = 2.6 Hz), 6.77 (1H, d, *J* = 8.8 Hz), 6.83 (1H, d, *J* = 8.8 Hz), 6.94 (1H, d, *J* = 7.6 Hz), 7.16 (1H, d, *J* = 8.8 Hz), 7.28 (1H, m), 7.38 (2H, m), 7.76 (1H, s), 8.27 (1H, s), 10.93 (1H, s); ^{13}C NMR (101 MHz, $CDCl_3$): δ 12.6, 44.5, 56.4, 57.3, 67.9, 71.9, 83.3, 97.8, 107.5, 107.7, 109.0, 110.7, 110.9, 112.6, 119.0, 120.8, 123.9, 128.3, 128.5, 129.2, 129.5, 136.6, 138.2, 139.6, 144.4, 147.8, 149.0, 151.2, 154.5, 154.8, 158.3, 188.5; HRMS (ESI+): calcd for $[M+Na]^+$, 619.20509; found, 619.20507 (0.02 mmu).

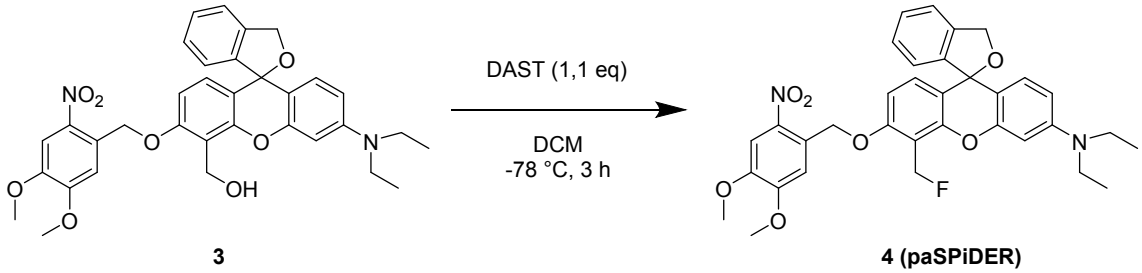
4-CH_2OH-caged HMDER, 3



Scheme S1-3. Synthesis of 4-CH_2OH-caged HMDER 3.

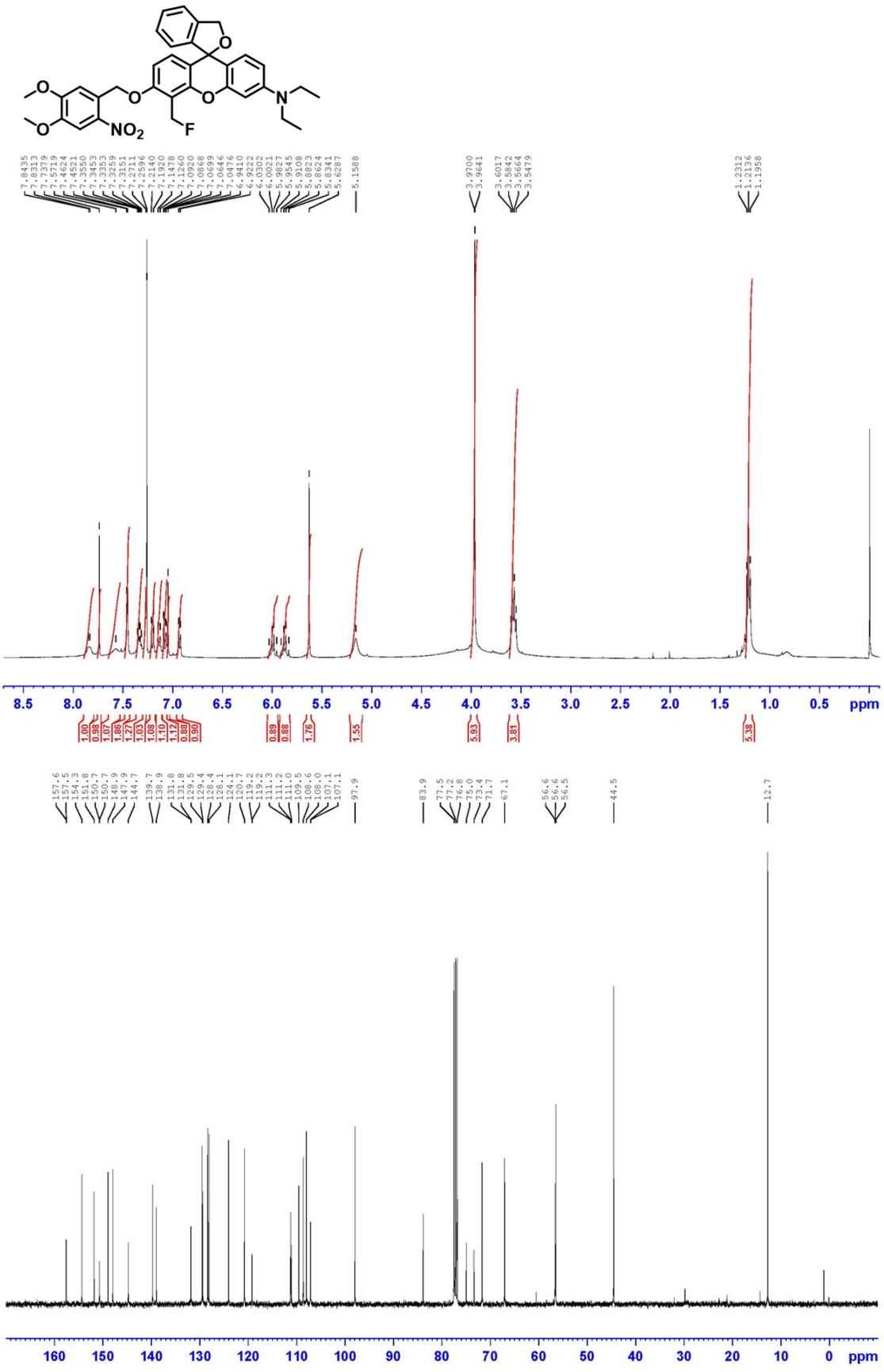
Compound **2** (20 mg, 0.0 mmol) was dissolved in dry THF (4 mL) and the solution was stirred at 0 ˚C. 1.0 M LiAlH(O*t*Bu)3 in THF (0.5 mL) was added dropwise and stirring was continued at 0 ˚C for 1 h. Saturated NH_4Cl aqueous solution (3 mL) was added to the solution on ice to quench the reaction. EtOAc (7 mL) was added and the solution was stirred at room temperature for 1 h, then the organic phase was separated. Saturated potassium sodium tartrate aqueous solution and CH_2Cl_2 were added to the aqueous phase. The reaction mixture was stirred, and the organic phase was separated (2 x). The combined organic phase was evaporated and the residue was purified by silica gel flash column chromatography (CH_2Cl_2/EtOAc = 98/2) to obtain the desired product **3** as an orange solid. A sample was identified by ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17 (6H, t, *J* = 7.0 Hz), 3.36 (4H, q, *J* = 7.0 Hz), 3.96 (3H, s), 3.98 (3H, s), 5.14 (2H, s), 5.20 (1H, d, *J* = 12.4 Hz), 5.24 (1H, d, *J* = 12.4 Hz), 5.53 (2H, s), 6.40 (1H, dd, *J* = 8.8, 2.6 Hz), 6.46 (1H, d, *J* = 2.6 Hz), 6.64 (1H, d, *J* = 8.8 Hz), 6.75 (1H, d, *J* = 8.8 Hz), 6.85 (1H, d, *J* = 8.8 Hz), 6.93 (d, 1H, *J* = 7.6 Hz), 7.27 (1H, m), 7.35 (2H, m), 7.42 (1H, s), 7.75 (1H, s); ^{13}C NMR (101 MHz, $CDCl_3$): δ 12.7, 44.5, 54.9, 56.6, 67.5, 71.6, 77.3, 83.9, 97.8, 107.6, 108.1, 108.6, 109.6, 111.3, 116.0, 119.3, 120.7, 124.1, 128.1, 128.3, 129.2, 129.6, 129.8, 139.1, 139.8, 144.6, 148.0, 148.9, 150.0, 151.9, 154.2, 156.9; HRMS (ESI+): calcd for [M+H]+, 599.23879; found, 599.23958 (0.8 mmu).

paSPiDER-1, 4.



Scheme S1-4. Synthesis of paSPiDER-1 4.

Compound **3** (10 mg, 0.017 mmol) was dissolved in dry CH_2Cl_2 (1 mL), and a solution of DAST (3.3 μL, 0.018 mmol) in CH_2Cl_2 was added dropwise at -78 ˚C. The mixture was stirred at -78 ˚C for 3 h. MeOH and H_2O were added at r.t., and the resulting mixture was extracted with CH_2Cl_2 (3 x). The combined organic phase was evaporated, and the residue was purified by silica gel flash column chromatography (Hexane/EtOAc = 67/33) and HPLC (A/B = 10/90 to 90/10) to obtain paSPiDER (**4**) as a red solid (2.5 mg, y. 25%). A sample was identified by ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17 (6H, t, *J* = 7.0 Hz), 3.36 (4H, q, *J* = 7.0 Hz), 3.96 (3H, s), 3.97 (3H, s), 5.22 (1H, d, *J* = 12.4 Hz), 5.25 (1H, d, *J* = 12.4 Hz), 5.56 (2H, s), 5.90 (1H, dd, *J* = 48.4, 16.4 Hz), 5.92 (1H, dd, *J* = 48.4, 16.4 Hz), 6.40 (1H, dd, *J* = 8.8, 2.6 Hz), 6.48 (1H, d, *J* = 2.6 Hz), 6.65 (1H, d, *J* = 8.8 Hz), 6.75 (1H, d, *J* = 8.8 Hz), 6.92 (1H, d, *J* = 7.8 Hz), 6.94 (1H, dd, *J* = 8.9, 2.6 Hz), 7.27 (1H, m), 7.36 (2H, m), 7.49 (1H, s), 7.75 (1H, s); ^{13}C NMR (101 MHz, $CDCl_3$): δ 12.7, 44.5, 56.4, 56.6(J_{CF} = 1.01 Hz), 67.0, 71.7, 74.1 (J_{CF} = 161.6 Hz), 83.8, 97.9, 107.1 (J_{CF} = 2.939 Hz), 108.0, 108.6, 109.5, 111.1 (**J_{CF}** = 10.1 Hz), 111.2, 119.2 (J_{CF} = 2.868 Hz), 120.7, 124.1, 128.1, 128.4, 129.4, 129.5, 131.8 (J_{CF} = 4.272 Hz), 138.9, 139.7, 144.7, 147.9, 148.9, 150.7 (J_{CF} = 2.989 Hz), 151.8, 154.4, 157.5 (J_{CF} = 2.80 Hz); HRMS (ESI+): calcd for [M+H]+, 601.23446; found, 601.23339 (1.1 mmu).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O
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NO2
F
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1.2136
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0.98
1.07
1.85
1.71
1.03
1.08
1.10
1.14
0.89
0.80
0.89
0.88
1.76
1.55
5.93
3.81
5.38
157.6
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154.3
151.8
150.7
150.2
148.9
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138.9
131.8
131.8
129.5
129.4
128.4
128.1
124.1
120.7
119.2
111.3
111.2
111.0
109.5
108.6
108.0
107.1
107.1
97.9
83.9
77.5
77.2
76.8
75.0
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56.6
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56.5
44.5
12.7
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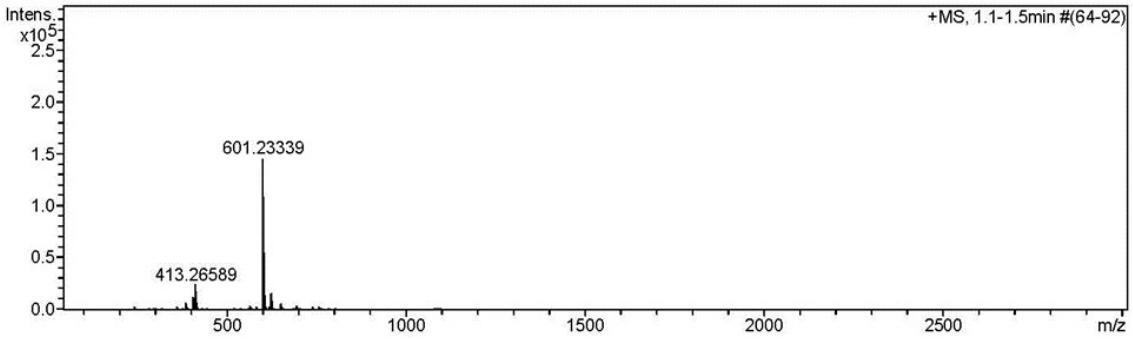
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2.0
1.5
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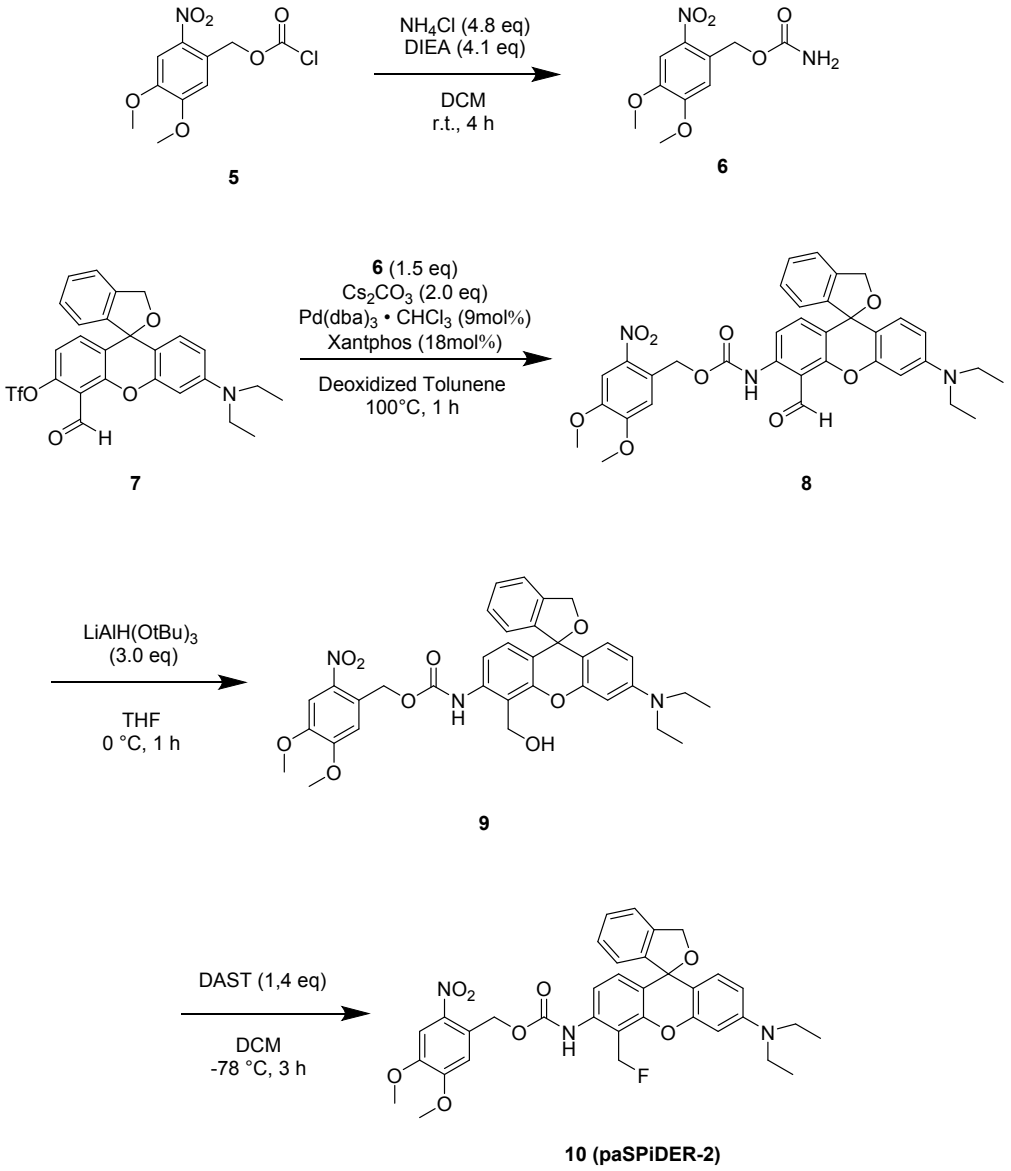
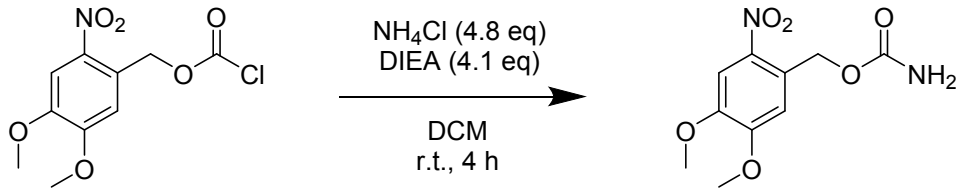
m/z



Scheme S2. Synthetic scheme of **paSPiDER-2.**

4,5-Dimethoxy-2-nitrobenzyl amideformate, 6.

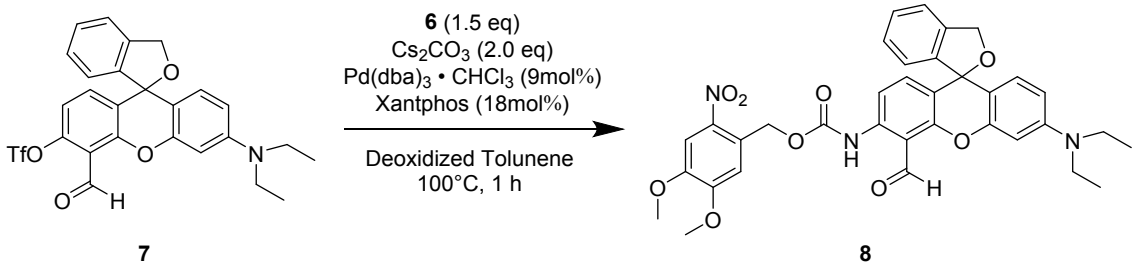


5 **6**

Scheme S2-1. Synthesis of 4,5-dimethoxy-2-nitrobenzyl amideformate, 6.

To a solution of 4,5-dimethoxy-2-nitrobenzyl chloroformate **5** (434 mg, 1.7 mmol) in dichloromethane (10 mL), ammonium chloride (439 mg, 8.2 mmol), and N,N-diisopropylethylamine (1.2 mL, 6.9 mmol) were added, and the mixture was stirred vigorously at r.t. under an Ar atmosphere for 4 h. A mixed solvent (H_2O 5 mL, iPrOH 1.5 mL, and dichloromethane 3.5 mL) was added at r.t., and the solution was stirred for 10 min, then filtered and evaporated. The residue was purified by silica gel flash column chromatography (hexane/EtOAc = 67/33) to obtain the desired product 6 as a yellow solid (308 mg, y. 71%). A sample was identified by ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.87 (3H, s), 3.90 (3H, s), 5.29 (2H, s), 6.62 (1H, br), 6.91 (1H, br), 7.19 (1H, s), 7.69 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6): δ 56.0, 56.2, 61.9, 108.0, 110.5, 127.9, 139.3, 147.6, 153.2, 156.1; HRMS (ESI+): calcd for [M+Na]+, 279.05876; found, 279..05745 (1.3 mmu).

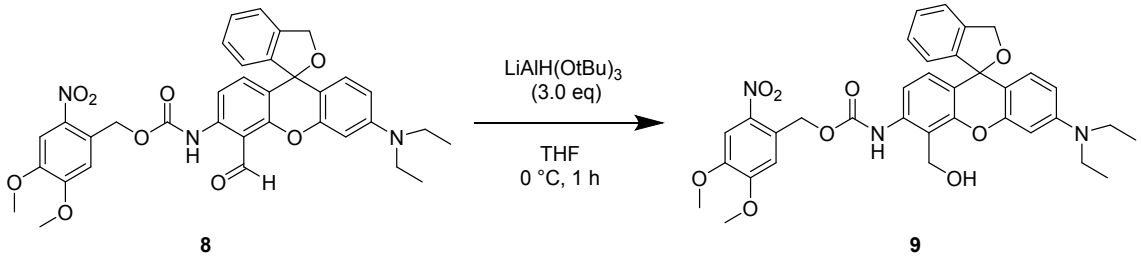
4-CHO-caged HMDiEtR, 8.



Scheme S2-2. Synthesis of 4-CHO-caged HMDiEtR 8.

Compound **7** was synthesized as previously described[2]. To a solution of compound **7** (100 mg, 0.19 mmol) in deoxygenated toluene (5 mL), cesium carbonate (122 mg, 0.38 mmol), 4,5-dimethoxy-2-nitrobenzyl amideformate 6 (72 mg, 0.28 mmol), xantphos (20 mg, 0.033 mmol), and tris(dibenzylideneacetone)dipalladium(0) chloroform adduct (18 mg, 0.017 mol) were added. The solution was stirred vigorously at 100°C under an Ar atmosphere for 1.5 h, then filtered and evaporated. The residue was purified by silica gel flash column chromatography (Hexane/EtOAc = 67/33) to obtain the desired product **8** as a yellow solid (85 mg, y. 71%). A sample was identified by ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17 (6H, t, *J* = 7.0 Hz), 3.36 (4H, q, *J* = 7.0 Hz), 3.96 (3H, s), 4.25 (3H, s), 5.25 (1H, d, *J* = 12.4 Hz), 5.29 (1H, d, *J* = 12.4 Hz), 5.50 (2H, s), 6.42 (1H, dd, *J* = 8.8, 2.6 Hz), 6.47 (1H, d, *J* = 2.6 Hz), 6.77 (1H, d, *J* = 8.8 Hz), 6.83 (1H, d, *J* = 8.8 Hz), 6.94 (1H, d, *J* = 7.6 Hz), 7.16 (1H, d, *J* = 8.8 Hz), 7.28 (1H, m), 7.38 (2H, m), 7.76 (1H, s), 8.27 (1H, s), 10.93 (1H, s); HRMS (ESI+): calcd for $[M+Na]^+$, 662.21090; found, 662.21090 (0.0 mDa).

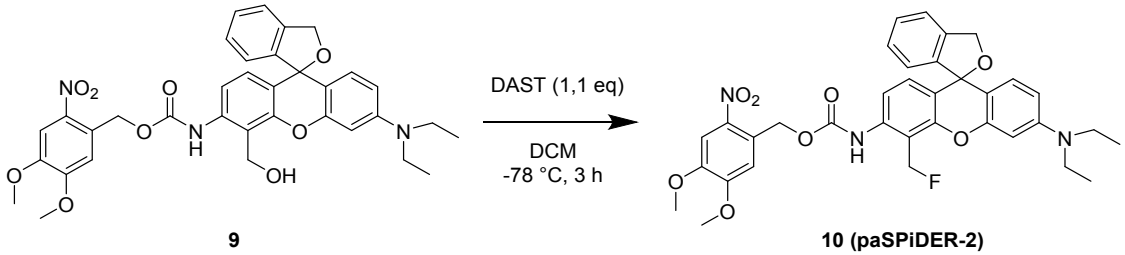
4-CH_2OH-caged HMDiEtR, 9.



Scheme S2-3. Synthesis of 4-CH_2OH-caged HMDiEtR 9.

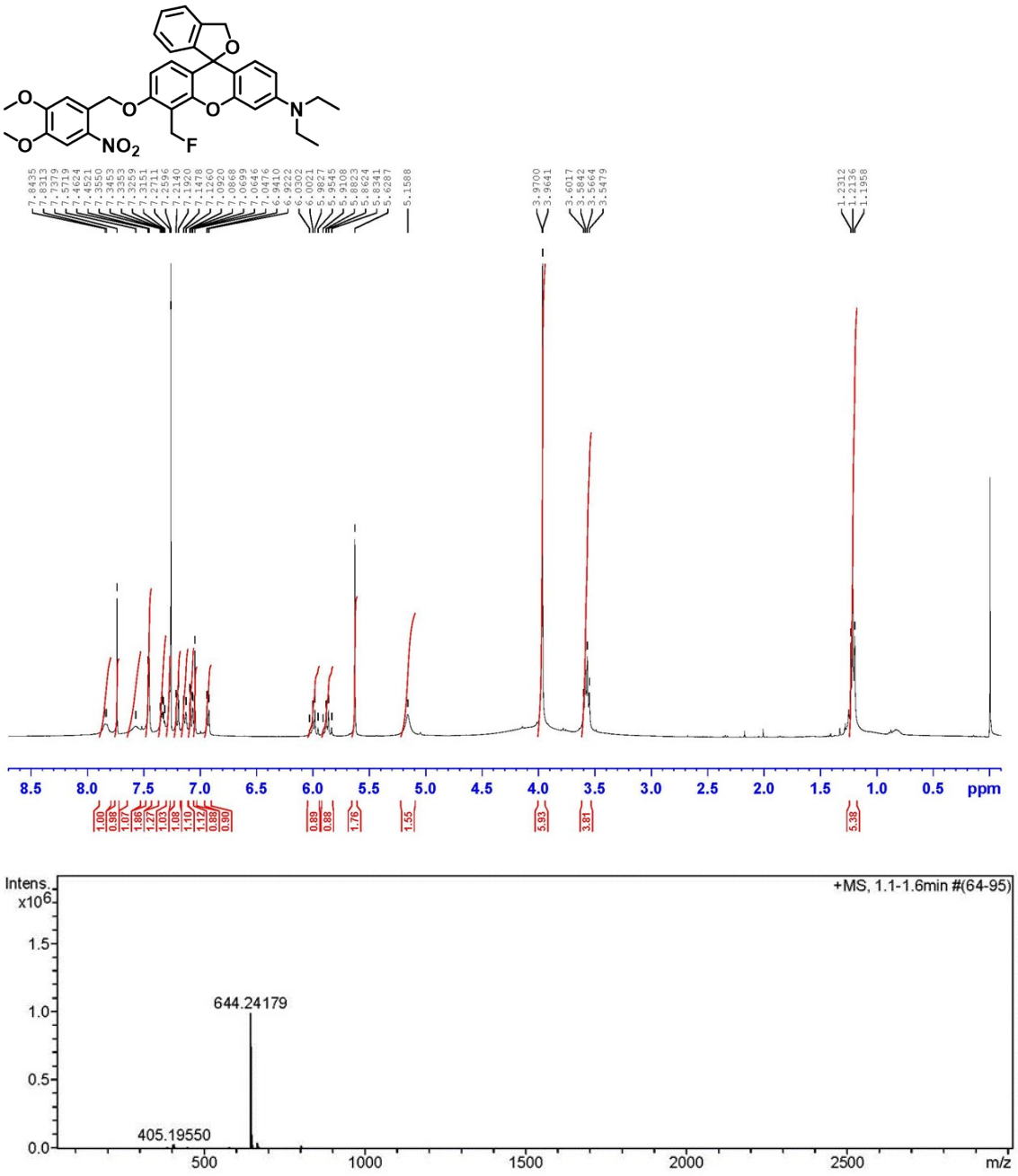
Compound **8** (85 mg, 0.13 mmol) was dissolved in dry THF (5 mL) and the solution was stirred at 0 ˚C. 1.0 M LiAlH(O*t*Bu)$_3$ in THF (0.5 mL) was added dropwise and the resulting solution was stirred at 0 ˚C for 1 h. Saturated $NaHCO_3$ aqueous solution (3 mL) was added on ice to quench the reaction. EtOAc (7 mL) was added and the mixture was stirred at room temperature for 1 h, then the organic phase was separated. Saturated potassium sodium tartrate aqueous solution and CH_2Cl_2 were added to the aqueous phase. The mixture was stirred, and the organic phase was separated (2 x). The combined organic phase was evaporated and the residue was purified by silica gel flash column chromatography (hexane/EtOAc = 70/30) to obtain the desired product **9** as an orange solid (85 mg, y. 99%). A sample was identified by ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.16 (6H, t, *J* = 7.0 Hz), 3.34 (4H, q, *J* = 7.0 Hz), 3.94 (3H, s), 3.96 (3H, s), 4.91 (2H, s), 5.22 (1H, d, *J* = 12.5 Hz), 5.26 (1H, d, *J* = 12.5 Hz), 5.58 (1H, d, *J* = 14.1 Hz), 5.64 (1H, d, *J* = 14.1 Hz), 6.38 (1H, s), 6.42 (1H, d, *J* = 8.4 Hz), 6.72 (d, 1H, *J* = 8.8 Hz), 6.79 (1H, d, *J* = 8.4 Hz), 6.85 (1H, d, *J* = 8.8 Hz), 6.90 (1H, d, *J* = 7.6 Hz), 7.25 (1H, m), 7.35 (2H, m), 7.52 (1H, br), 7.70 (1H, s), 8.30 (1H, s); HRMS (ESI+): calcd for [M+H]+, 642.24461 found, 662.24551 (0.9 mDa).

paSPiDER-2, 10.



Scheme S2-4. Synthesis of paSPiDER-2 10.

Compound **9** (85 mg, 0.13 mmol) was dissolved in dry CH_2Cl_2 (1 mL), and a solution of DAST (8.8 μL, 0.066 mmol) in CH_2Cl_2 was added dropwise at -78 °C. The mixture was stirred at -78 °C for 3 h. MeOH and H_2O were added at r.t., and the resulting mixture was extracted with CH_2Cl_2 (3 x). The combined organic phase was evaporated, and the residue was purified by silica gel flash column chromatography (hexane/EtOAc = 67/33) and HPLC (A/B = 10/50 to 50/10) to obtain paSPiDER-2 **10** as a red solid (12 mg, y. 14%). A sample was identified by ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.21 (6H, t, *J* = 7.1 Hz), 3.58 (4H, q, *J* = 7.0 Hz), 3.96 (3H, s), 3.97 (3H, s), 5.16 (2H, br), 5.63 (2H, s), 5.91 (1H, dd, *J* = 48.1, 19.4 Hz), 5.95 (1H, dd, *J* = 48.1, 19.4 Hz), 6.93 (1H, d, *J* = 7.5 Hz), 7.04 (1H, s), 7.10 (1H, dd, *J* = 8.8, 2.1 Hz), 7.13 (1H, d, *J* = 6.3 Hz), 7.20 (1H, d, *J* = 8.8 Hz), 7.27 (1H, m), 7.34 (1H, m), 7.45 (2H, m), 7.57 (1H, br), 7.74 (1H, s), 7.84 (1H, m); HRMS (ESI+): calcd for [M+H]+, 644.24027; found, 644.24179 (1.5 mDa).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405.19550
m/z
ppm

Methods

Spectral measurements (Fig. 1b, Table 1)

UV-visible absorption spectra were measured using a UV-2450 spectrophotometer (Shimadzu), and fluorescence spectra were measured using a F-7000 fluorescence spectrophotometer (Hitachi). The probes were first dissolved in DMSO (fluorometric grade, Dojindo) to obtain stock solutions. Absolute fluorescence quantum efficiency was determined with an absolute PL quantum yield spectrometer, Quantaurus-QY (Hamamatsu Photonics). For photoirradiation experiments in vitro, probe solutions (1 μM) in 200 mM sodium phosphate buffer, pH 7.4, in a quartz cuvette were illuminated at 405 nm (18.3 or 19.3 mW/cm^2) using a Xe lamp (Asahi Spectra Inc., MAX-303) equipped with a 405 nm bandpass filter (FWHM 10 nm) with stirring at room temperature. The absorption and fluorescence spectra were recorded every 15 or 30 minutes.

Determination of pK_{cycl} or pK_a values of compounds (Fig. S2, S3, Table 1)

Absorption and fluorescence emission spectra of compounds were measured in 200 mM sodium phosphate buffer at different pH values, containing <1% (v/v) DMSO as a cosolvent. For compounds with n acid-base equilibria (n = 1 or 2), pH profiles of absorbance (Abs) or fluorescence intensity (FI) were fitted to the following formula to determine pK_a values.

$$Abs\ or\ FI = \frac{c_0 + \sum_{k=1}^{n} c_k \cdot 10^{k \cdot pH - \sum_{l=1}^{k} pK_{al}}}{1 + \sum_{k=1}^{n} 10^{k \cdot pH - \sum_{l=1}^{k} pK_{al}}}$$

(pK_{a1} < pK_{a2} < ••• < pK_{an}; c_n = constant).

LC-MS analysis of photoreaction products (Fig. S4)

A 100 μM solution of paSPiDERs in 200 mM sodium phosphate buffer, pH 7.4, containing 0.1% DMSO as a co-solvent was irradiated at room temperature for 3 h. LC-MS analyses of the photoreaction solution were performed on a Waters Acquity UPLC (H class)/QDa quadrupole MS analyzer equipped with an Acquity UPLC BEH C18 column (Waters). Eluent A (H_2O containing 0.1 % formic acid) and eluent B (acetonitrile) were used for UPLC analyses.

SDS-PAGE of photoreaction products in the presence of BSA (Fig. S5)

Solutions of BSA (1 mg/ml) and probes in 200 mM sodium phosphate buffer, pH 7.4 were incubated and irradiated for 5 h. SDS-PAGE analyses were performed using Mini-PROTEAN TGX Gels (Bio-Rad). Fluorescence images of the gels were obtained on an Image Quant LAS

4000 (GE Healthcare) with a 497 nm LED for excitation and a 520 nm long pass filter for emission. The gels were stained with Coomassie brilliant blue (CBB) solution.

Cell line and culture

A549 cells were cultured in DMEM (Wako) containing 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2.

Confocal imaging of cells (Fig. 2-5, Fig. S6-S10, S12-S13)

A549 cells were seeded on 8-chamber plates (Ibidi, μ-slide) and cultured overnight. The medium was replaced with paSPiDERs (10 μM) in phenol red-free DMEM, and the cells were further incubated for 1 hour at 37 °C under 5% CO_2. The cells were photo-irradiated with a 405 nm laser under a confocal microscope (for single-cell labeling : 5%, 25 sec for paSPiDER-1, 50%, 20 sec for paSPiDER-2, others : 100%, 30 sec). For fixation, the light-irradiated cells were incubated with 4% paraformaldehyde (PFA) or MeOH for 15 minutes at room temperature and washed with PBS. For evaluation of phototoxicity, the medium was replaced with DMEM containing 2 μM ethidium homodimer-1 (EthD-1). DIC and fluorescence images were acquired with a confocal fluorescence microscope (for single-cell labeling : TCS SP5 X, others : TCS SP8 STED, Leica) equipped with a white-light laser, a 405 nm laser and an objective lens (for single-cell labeling : HCX PL APO CS 63x/1.40 Oil, others : HCX PL APO CS 40x/1.25 Oil, Leica). For time-lapse imaging, fluorescence images were captured every 1 h for 24 h. For evaluation of phototoxicity, excitation and emission wavelengths were 520 nm/535–575 nm for paSPiDER-1, 561 nm/600–650 nm for EthD-1. Others, ex/em = 525 nm/535-595 nm for paSPiDERs.

Cell viability assay (Fig. S11)

A549 cells were seeded in a plastic-bottomed 96-well plate and cultured overnight. The medium was exchanged to fresh medium containing paSPiDER-1 or CellTracker™ Red CMTPX dye (product number C34552, ThermoFisher) at various concentrations, containing 1% DMSO as a cosolvent, followed by incubation for 12 h at 37°C. The medium in each well was replaced with 100 μL of fresh medium. The cells were further incubated in medium containing 10% Cell Counting Kit-8 (Dojindo), and the absorbance at 450 nm was measured using an Envision plate reader (Perkin Elmer) to determine the cell viability. Values from wells containing cells without dyes were taken as representing 100% cell viability.

Imaging of *Drosophila* wing disc (Fig. 5)

Wing imaginal discs of third-instar larvae from *Drosophila melanogaster* Canton S were dissected in PBS. Dissected tissues were incubated with 50 μM paSPiDER-1 in Schneider's *Drosophila* Medium (Gibco, 21720001) supplemented with 10% FBS for 30 min at room temperature, then flush-washed with PBS, mounted on a glass-bottomed dish (Matsunami, D111300), and incubated with the medium. UV irradiation was performed using an Leica SP8

microscope with a 405 nm laser at 20% intensity for 15 sec. The irradiated tissue was fixed in 4 % PFA for 30 min. After washing with PBST (0.1 % Triton-X100), the tissue was incubated with blocking buffer (PBST (0.1% Triton-X100) with 5 % normal donkey serum) for 30 min, followed by incubation with anti-phosphohistone (S28) H3 rat monoclonal antibody (Abcam, ab10543) diluted 1:200 in the blocking buffer for 60 minutes. After washing with PBST, second antibody anti-rat IgG 633 (Thermo Fisher Scientific, A-21094) was used to visualise the pH3 signal under a Leica SP8 microscope. Excitation/emission wavelengths were 488 nm/500-550 nm for paSPiDER-1 and 633 nm/645-695 nm for second antibody anti-rat IgG 633, respectively.

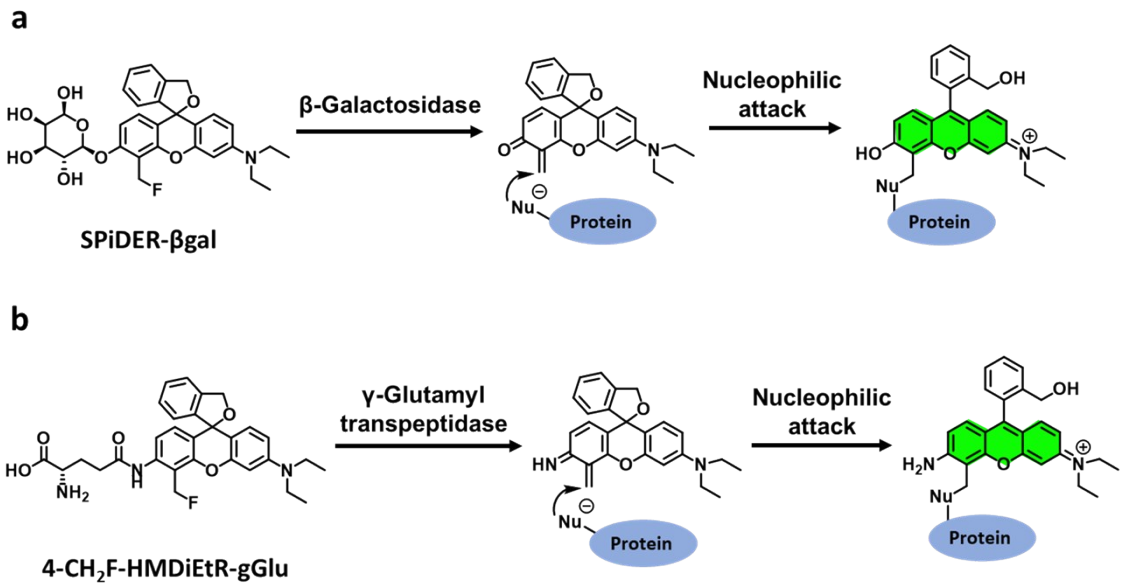
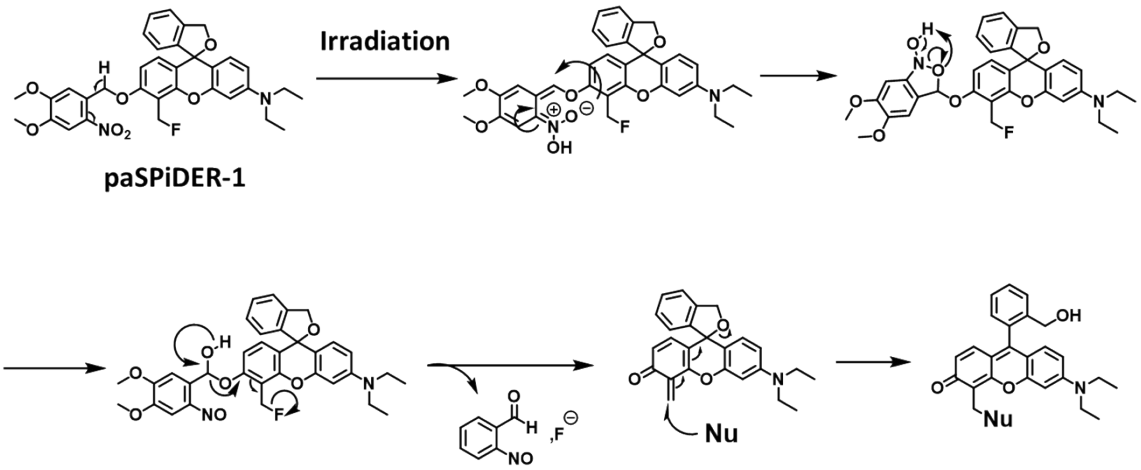
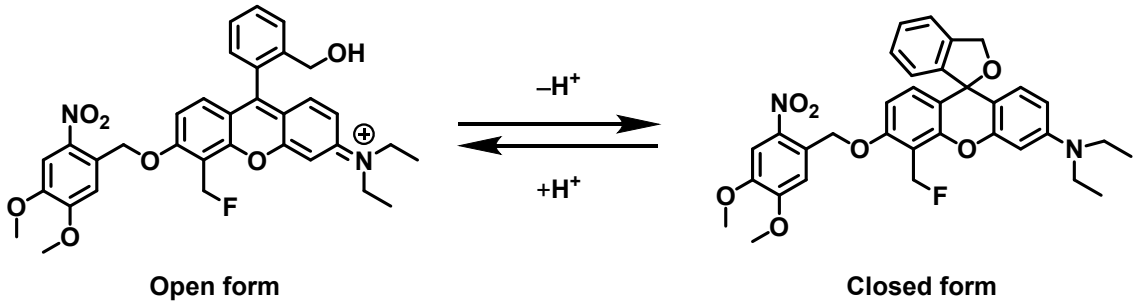


Figure S1. The reaction scheme of SPiDER-βGal (a) and 4-CH_2F-HMDiEtR-gGlu (b)



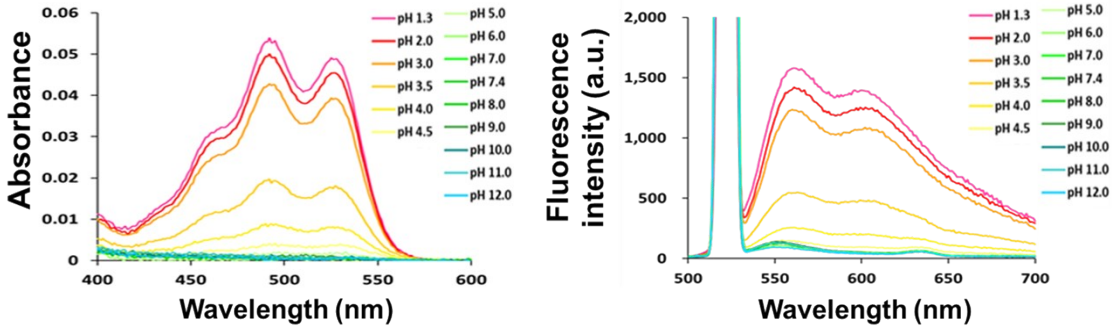
Scheme S3. Reaction scheme of paSPiDER-1 in response to light irradiation, showing the activation mechanism of fluorescence and covalent binding to intracellular nucleophiles.

(a)



Open form Closed form

(b)



(c)

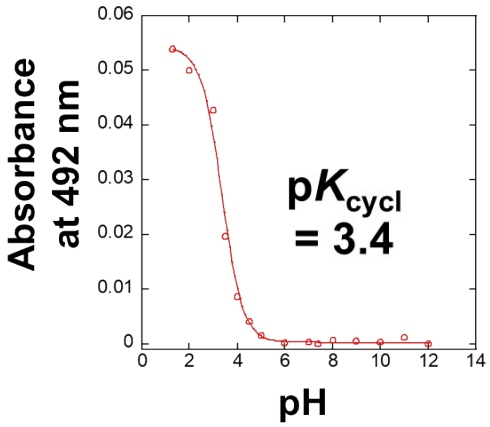
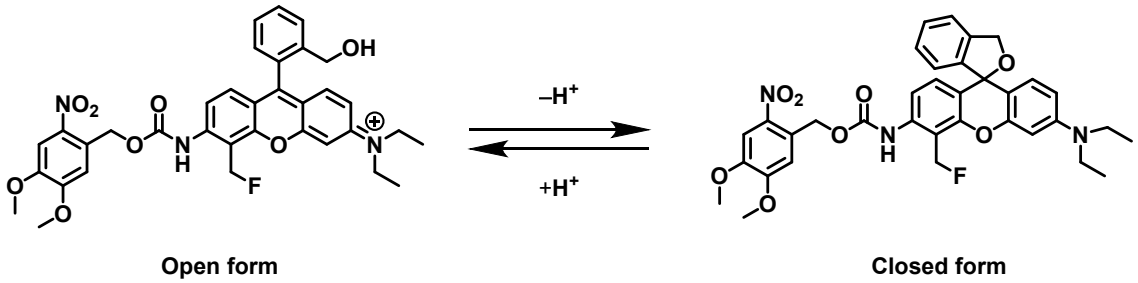
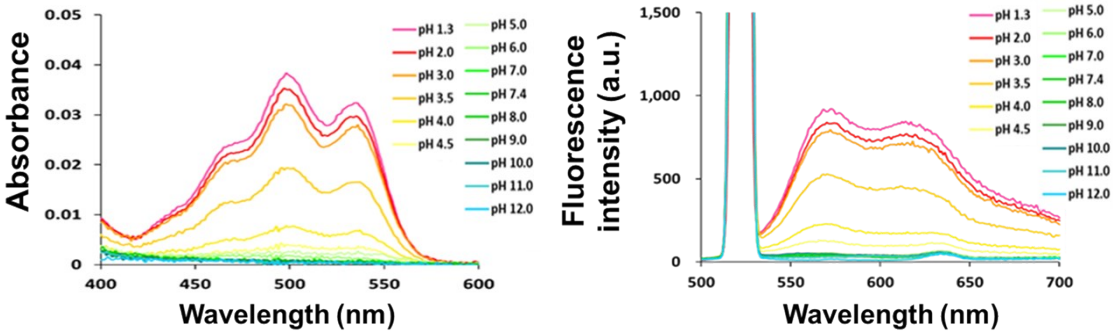


Figure S2. pH dependency of absorption and fluorescence spectra. (a) Acid-base equilibrium and pH-dependent spirocyclization of **paSPiDER-1**. (b) pH dependency of absorption (left) and fluorescence (right) spectra. Absorption and fluorescence spectra of 1 μM paSPiDER in 200 mM sodium phosphate buffer of various pH values, containing 1% DMSO as a cosolvent. (c) Maximum absorbance at 492 nm was plotted against pH to determine the pK_{cycl} value.

(a)



(b)



(c)

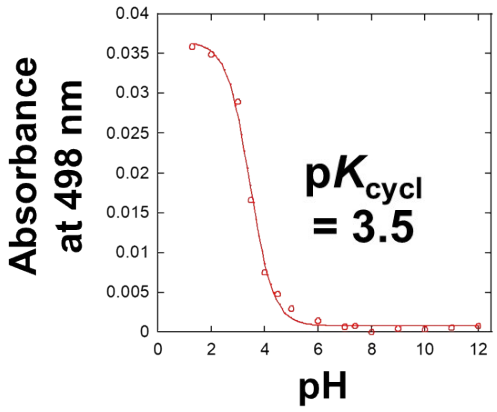


Figure S3. pH dependency of absorption and fluorescence spectra. (a) Acid-base equilibrium and pH-dependent spirocyclization of **paSPiDER-2**. (b) pH dependency of absorption (left) and fluorescence (right) spectra. Absorption and fluorescence spectra of 1 µM paSPiDER-2 in 200 mM sodium phosphate buffer of various pH values, containing 0.1% DMSO as a cosolvent. (c) Maximum absorbance at 498 nm was plotted against pH to determine the pK_{cycl} value.

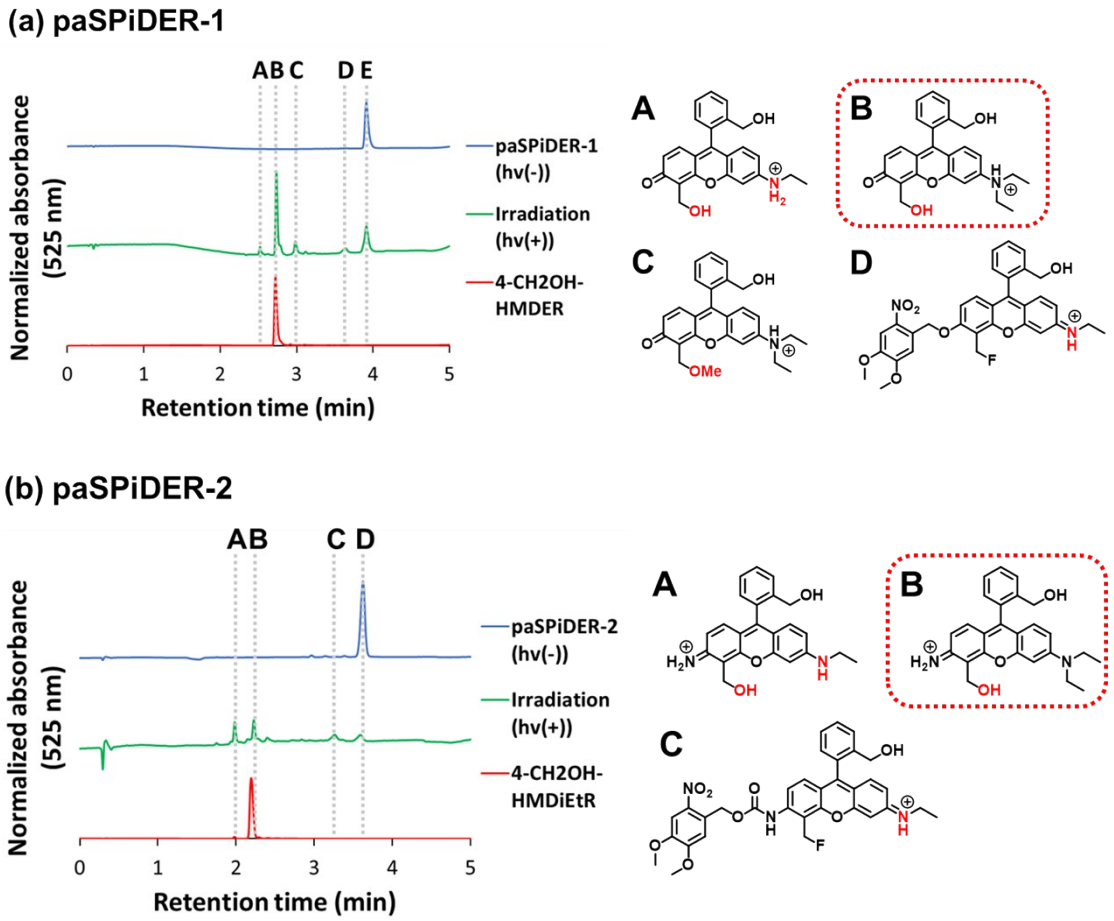


Figure S4. LC-MS analysis of photoreaction products of **paSPiDER-1** (a) and **paSPiDER-2** (b). 100 μM solutions of paSPiDERs in 200 mM sodium phosphate buffer, pH 7.4, containing 1% DMSO (v/v), were irradiated at 405 nm (18.7 mW / cm^2 for 3 h for paSPiDER-1, and 18.3 mW / cm^2 for 1 h for paSPiDER-2). Absorbance at 525 nm was monitored. (top) Non-irradiated paSPiDER-1 or paSPiDER-2, (middle) probe solutions after 405 nm light irradiation, (bottom) major hydrolysis products (4-CH_2OH-HMDER or 4-CH_2OH-HMDiEtR).

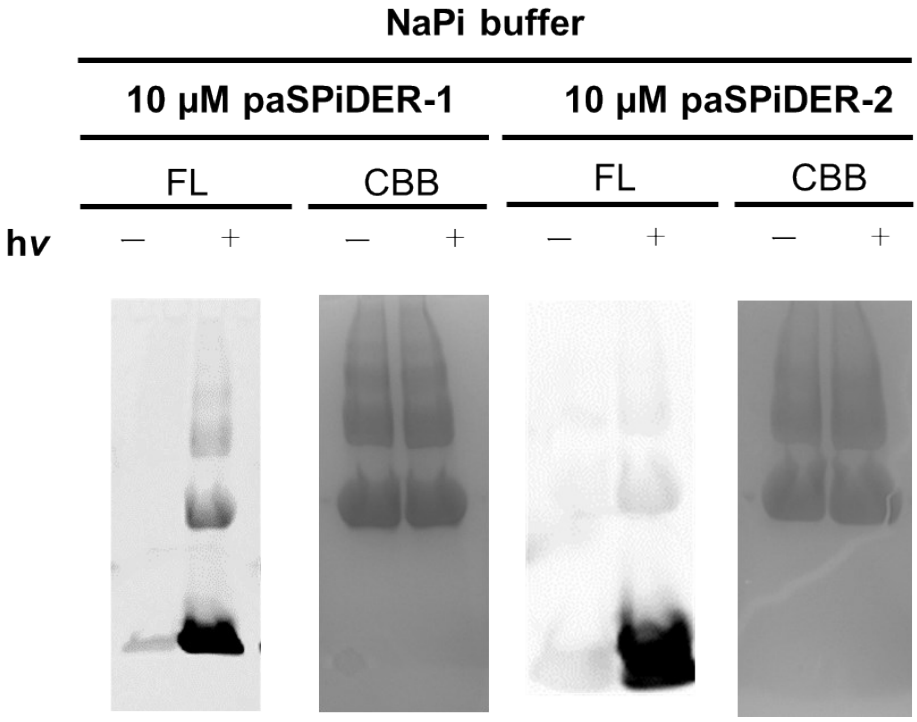


Figure S5. SDS-PAGE analysis of photo-irradiation-induced labeling of BSA with paSPiDERs. A solution of 1 mg/mL BSA and 100 μM paSPiDER-1 or paSPiDER-2 in 200 mM sodium phosphate buffer, pH 7.4 containing 1% DMSO (*v*/*v*) was irradiated at 405 nm (18.7 mW / cm^2) for 4 h, then subjected to SDS-PAGE analysis. Ex/Em = 497/520 nm.

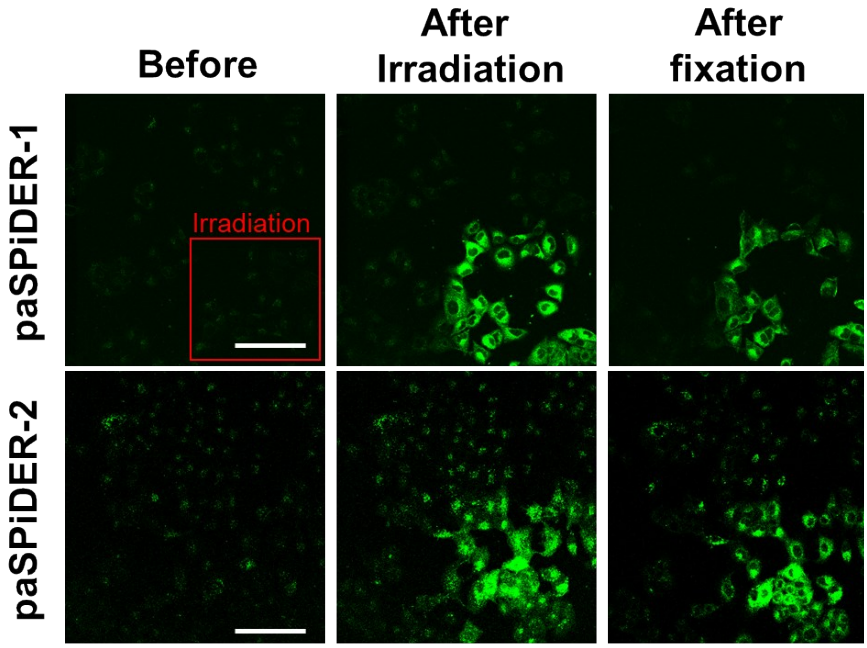


Figure S6. Fluorescence images of A549 cells treated with 10 μM paSPiDER-1 and paSPiDER-2 for 1 h. Cells in the region indicated by the red box were irradiated with a 405 nm laser, then fixed using 4% PFA solution for 10 min. The cell passage number is more than ten. Ex/Em = 525 nm/535–595 nm. Scale bars, 100 μm.

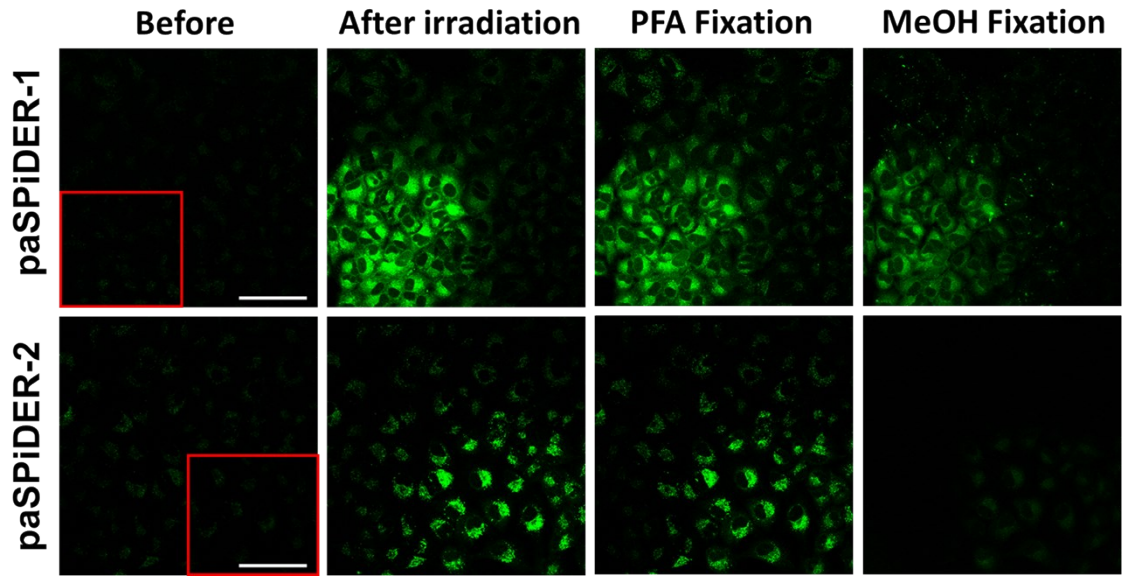


Figure S7. Fluorescence images of A549 cells with low cell passage number. The cells were treated with 10 μM paSPiDER-1 and paSPiDER-2 for 1 h. Then, cells in the region indicated by the red box were irradiated with a 405 nm laser, followed by fixation using 4% PFA solution and MeOH for 15 min each. The cell passage number is three. Ex/Em = 520 nm/535–595 nm. Scale bars, 75 μm.

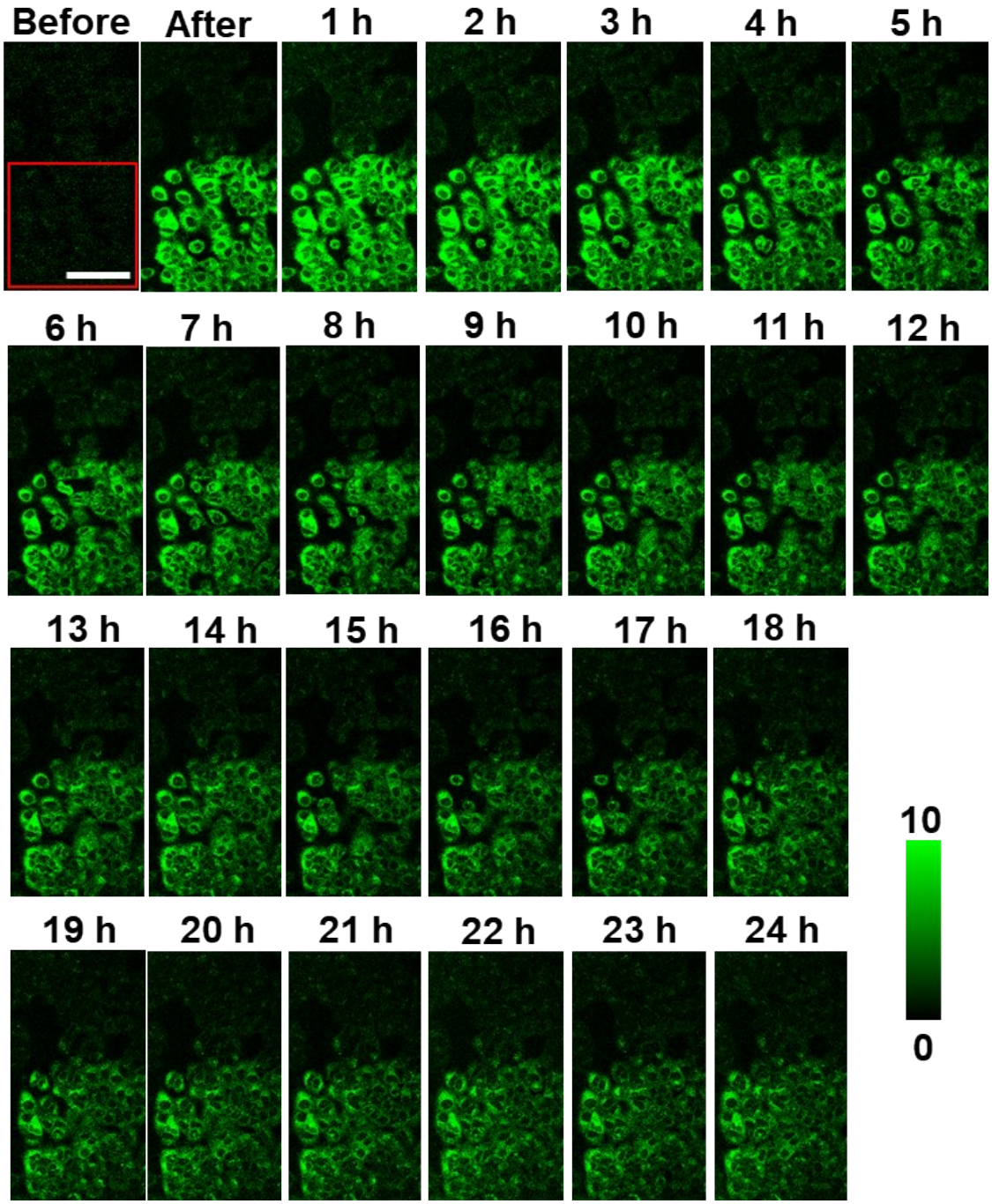


Figure S8. Fluorescence images of A549 cells treated with paSPiDER 10 μM **paSPiDER-1** for 1 h. Cells were observed for 24 h after irradiation with a 405 nm laser in the region indicated by the red box (Before panel). Images were acquired every 1 h. Ex/Em = 525 nm/535–595 nm. Scale bars, 100 μm.

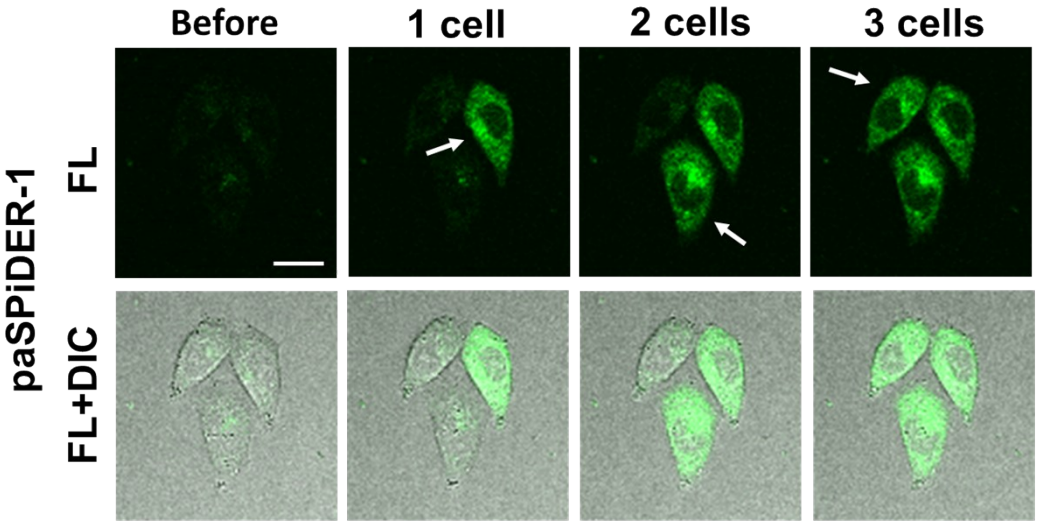


Figure S9. Fluorescence and DIC images of A549 cells treated with 10 μM paSPiDER-1 for 1 h, and then successively irradiated with a 405 nm laser at the whole individual cell indicated by the white arrow in each panel. Ex/Em = 525 nm/535–595 nm. Scale bars, 20 μm.

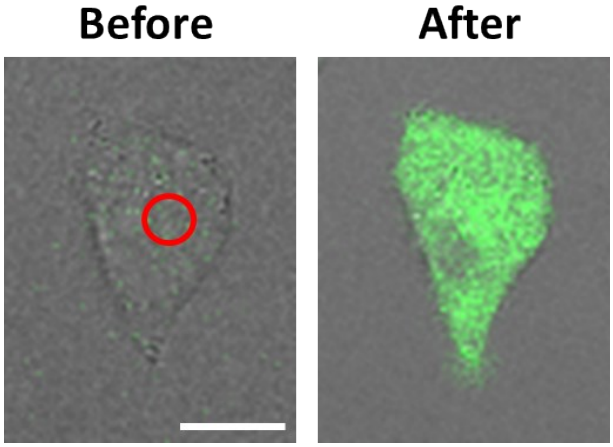


Figure S10. Fluorescence and DIC images of A549 cells treated with 10 μM paSPiDER-1 for 1 h, and then successively irradiated with a 405 nm laser at the part of single-cell indicated by the red circle (n = 10). The fluorescence image was captured 15 seconds after light irradiation. Ex/Em = 525 nm/535–595 nm. Scale bars, 20 μm.

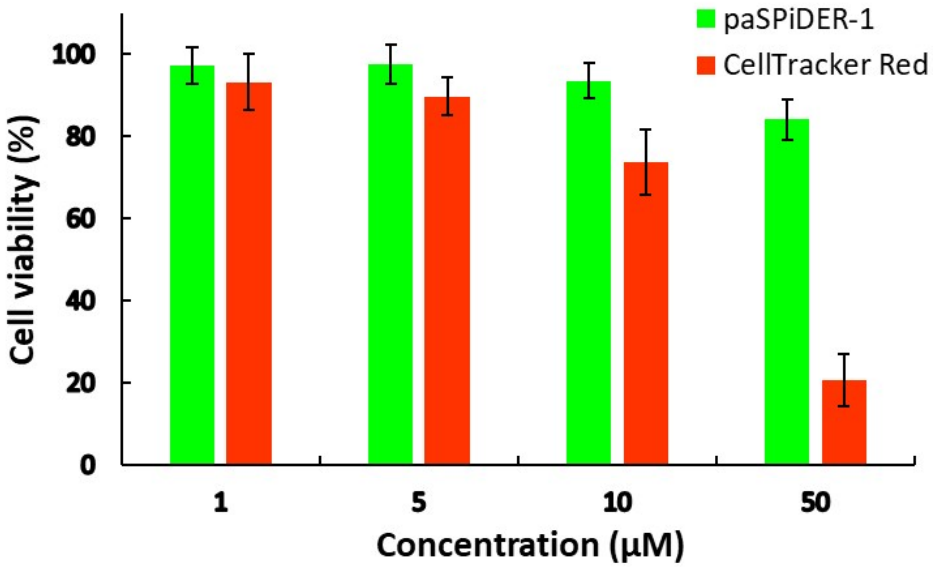


Figure S11. Comparison of the dark toxicity of paSPiDER-1 and CellTracker™ Red CMTPX dye to A549 cells. A549 cells were incubated with the indicated concentrations of paSPiDER-1 and CellTracker Red for 12 h at 37 °C under 5% CO_2, and the cell viability was determined by CCK-8 assay. Data represent mean ± s.d. from two independent experiments in quadruplicate.

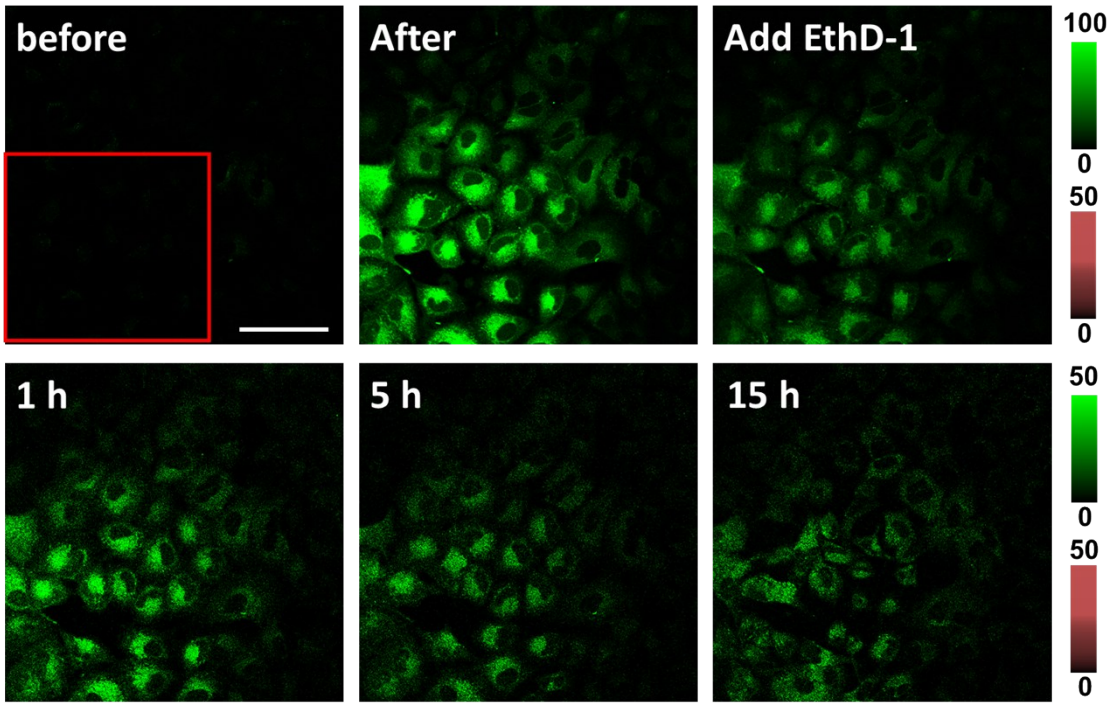


Figure S12. Evaluation of phototoxicity of paSPiDER-1. A549 cells were treated with 10 μM paSPiDER-1 for 1 h, and cells in the region indicated by the red box were irradiated with a 405 nm laser. Then, fluorescence images were captured for 15 h after replacement of the medium with DMEM containing 2 μM EthD-1. paSPiDER-1: ex/em = 520 nm/535–575 nm, EthD-1: ex/em = 561 nm/600–650 nm. Scale bars, 75 μm.

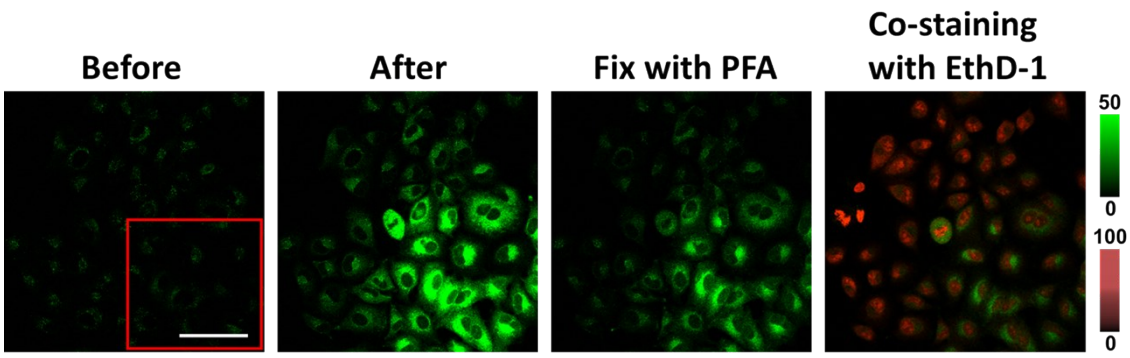


Figure S13. EthD-1 staining of fixed cells. A549 cells were treated with 10 μM paSPiDER-1 for 1 h, and cells in the region indicated by the red box were irradiated with a 405 nm laser. Then, the cells were fixed using 4% PFA solution for 15 min, and the solution was replaced with DMEM containing 2 μM EthD-1. paSPiDER-1: ex/em = 520 nm/535–575 nm, EthD-1: ex/em = 561 nm/600–650 nm. Scale bars, 75 μm.

1. T. Doura, M. Kamiya, F. Obata, Y. Yamaguchi, T. Y. Hiyama, T. Matsuda, A. Fukamizu, M. Noda, M. Miura and Y. Urano, *Angew Chem Int Ed Engl*, 2016, **55**, 9620-9624.
2. R. Obara, M. Kamiya, Y. Tanaka, A. Abe, R. Kojima, T. Kawaguchi, M. Sugawara, A. Takahashi, T. Noda and Y. Urano, *Angew Chem Int Ed Engl*, 2021, **60**, 2125-2129.