## Electronic Supplementary Information (ESI) for

# Modular Development of Organelle-targeting Fluorescent Probes for Imaging Formaldehyde in Live Cells

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## Supplementary figures

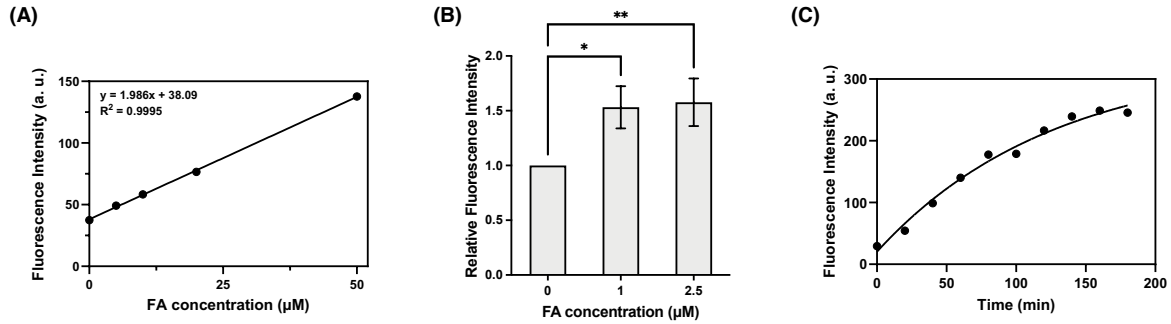



**Figure S1.** Detection of FA with FFP551-Nuc in aqueous solutions. (A) Fluorescence of FFP551-Nuc in the presence of FA at low concentrations (0–50 μM). (B) The detection limit of FFP551-Nuc for FA. (C) Time-dependent fluorescence responses of FFP551-Nuc to FA (0.5 mM). FFP551-Nuc (10 μM) was treated with FA of indicated concentrations in PBS (20 mM, pH 7.4) at 37 °C for 2 h. The fluorescence intensity at 551 nm was measured with excitation at 441 nm. Data in (B) are shown as mean ± standard deviation (*n* = 3). Statistical analysis for multiple comparisons was performed with one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

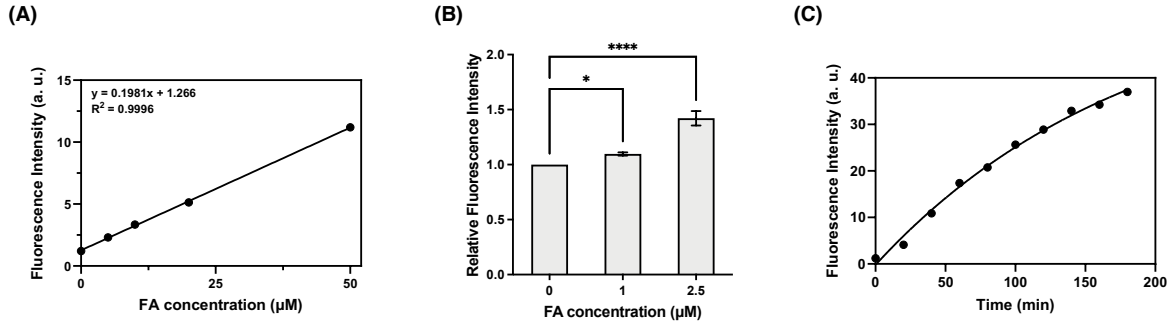



**Figure S2.** Detection of FA with FFP551-ER in aqueous solutions. (A) Fluorescence of FFP551-ER in the presence of FA at low concentrations (0–50 μM). (B) The detection limit of FFP551-ER for FA. (C) Time-dependent fluorescence responses of FFP551-ER to FA (0.5 mM). FFP551-ER (10 μM) was treated with FA of indicated concentrations in PBS (20 mM, pH 7.4) at 37 °C for 2 h. The fluorescence intensity at 551 nm was measured with excitation at 441 nm. Data in (B) are shown as mean ± standard deviation (*n* = 3). Statistical analysis for multiple comparisons was performed with one-way ANOVA. * $p < 0.05$, **** $p < 0.0001$.

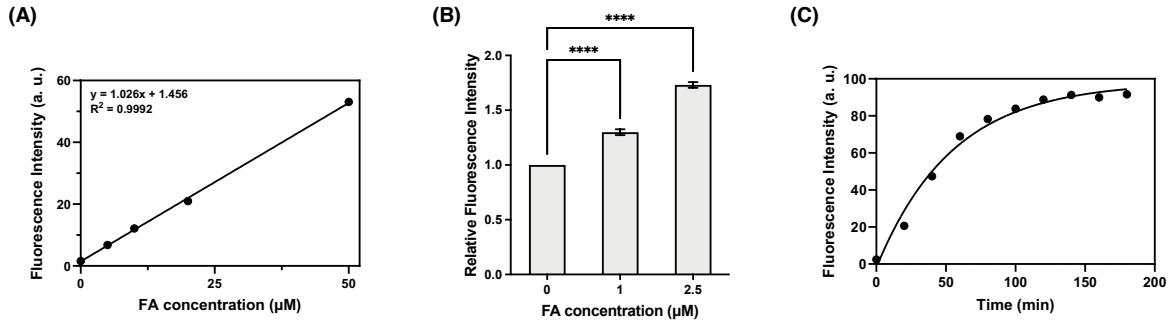



**Figure S3.** Detection of FA with FFP551-Mito in aqueous solutions. (A) Fluorescence of FFP551-Mito in the presence of FA at low concentrations (0–50 μM). (B) The detection limit of FFP551-Mito for FA. (C) Time-dependent fluorescence responses of FFP551-Mito to FA (0.5 mM). FFP551-Mito (10 μM) was treated with FA of indicated concentrations in PBS (20 mM, pH 7.4) at 37 °C for 2 h. The fluorescence intensity at 551 nm was measured with excitation at 441 nm. Data in (B) are shown as mean ± standard deviation (*n* = 3). Statistical analysis for multiple comparisons was performed with one-way ANOVA. **** $p < 0.0001$.

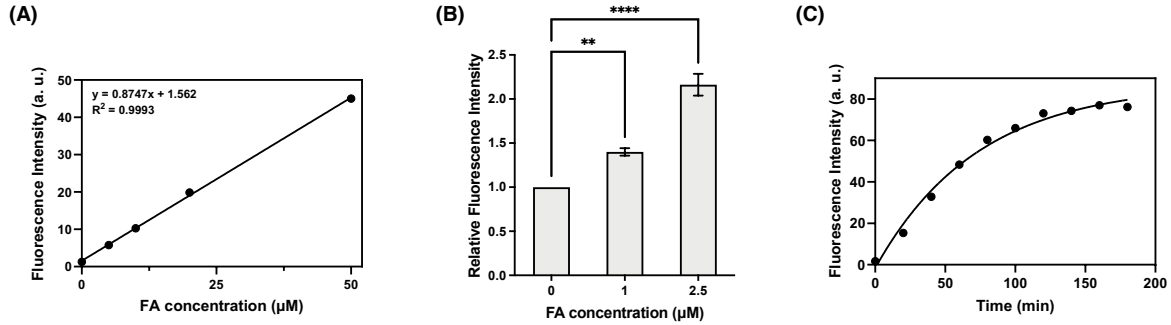



**Figure S4.** Detection of FA with FFP551-Lyso in aqueous solutions. (A) Fluorescence of FFP551-Lyso in the presence of FA at low concentrations (0–50 μM). (B) The detection limit of FFP551-Lyso for FA. (C) Time-dependent fluorescence responses of FFP551-Lyso to FA (0.5 mM). FFP551-Lyso (10 μM) was treated with FA of indicated concentrations in PBS (20 mM, pH 7.4) at 37 °C for 2 h. The fluorescence intensity at 551 nm was measured with excitation at 441 nm. Data in (B) are shown as mean ± standard deviation (*n* = 3). Statistical analysis for multiple comparisons was performed with one-way ANOVA. ** $p < 0.01$, **** $p < 0.0001$.

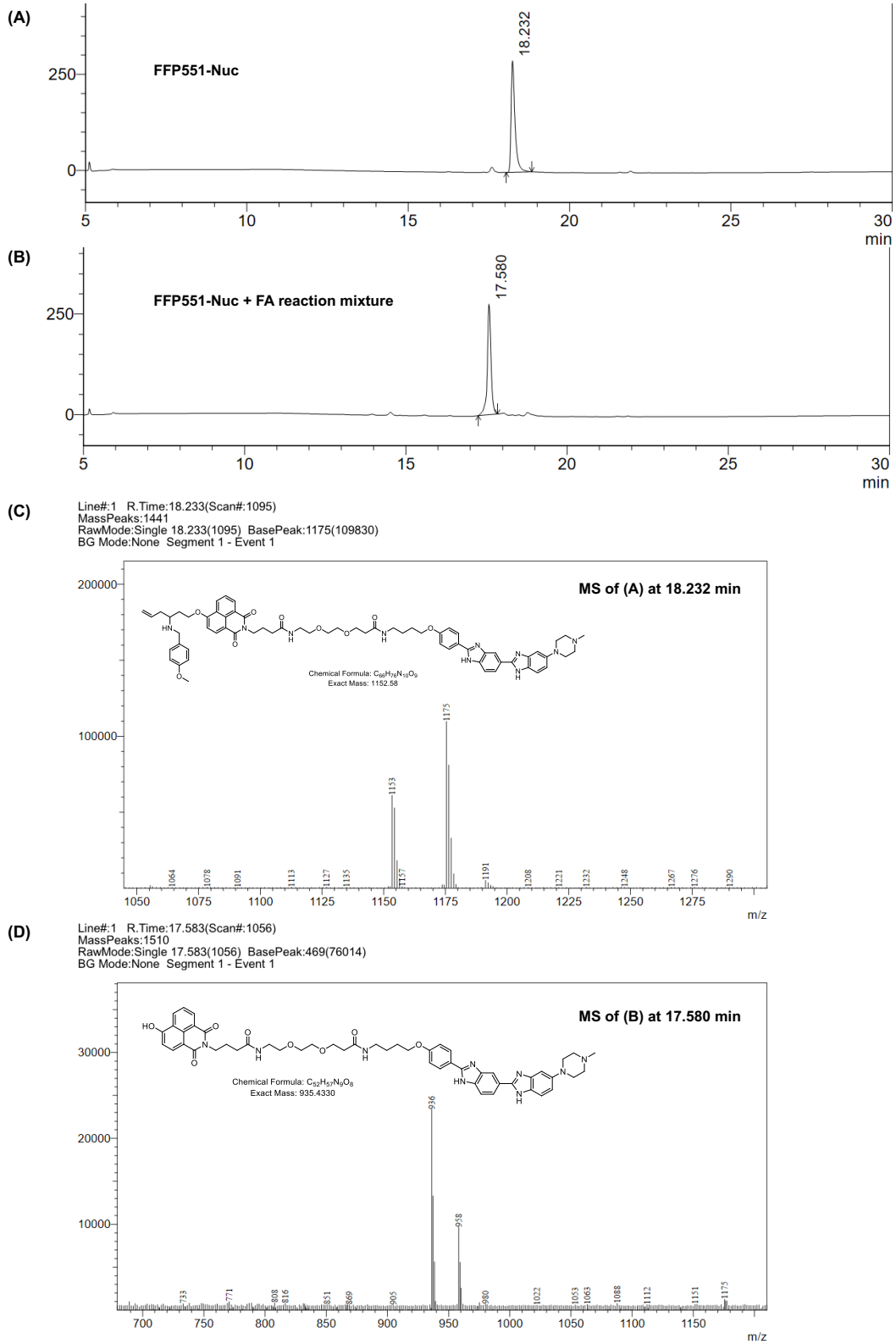



**Figure S5.** LC-MS analysis of the reaction mixture between FFP551-Nuc and FA. FFP551-Nuc (250 μM) was reacted with FA (5 mM) in PBS/$CH_3CN$ (v/v = 1:1) at 37 °C for 2 h and the mixture was analyzed by LC-MS. (A) LC analysis of FFP551-Nuc. (B) LC analysis of the reaction mixture of FFP551-Nuc with FA. (C) Mass spectrum at 18.232 min in (A). (D) Mass spectrum at 17.580 min in (B).

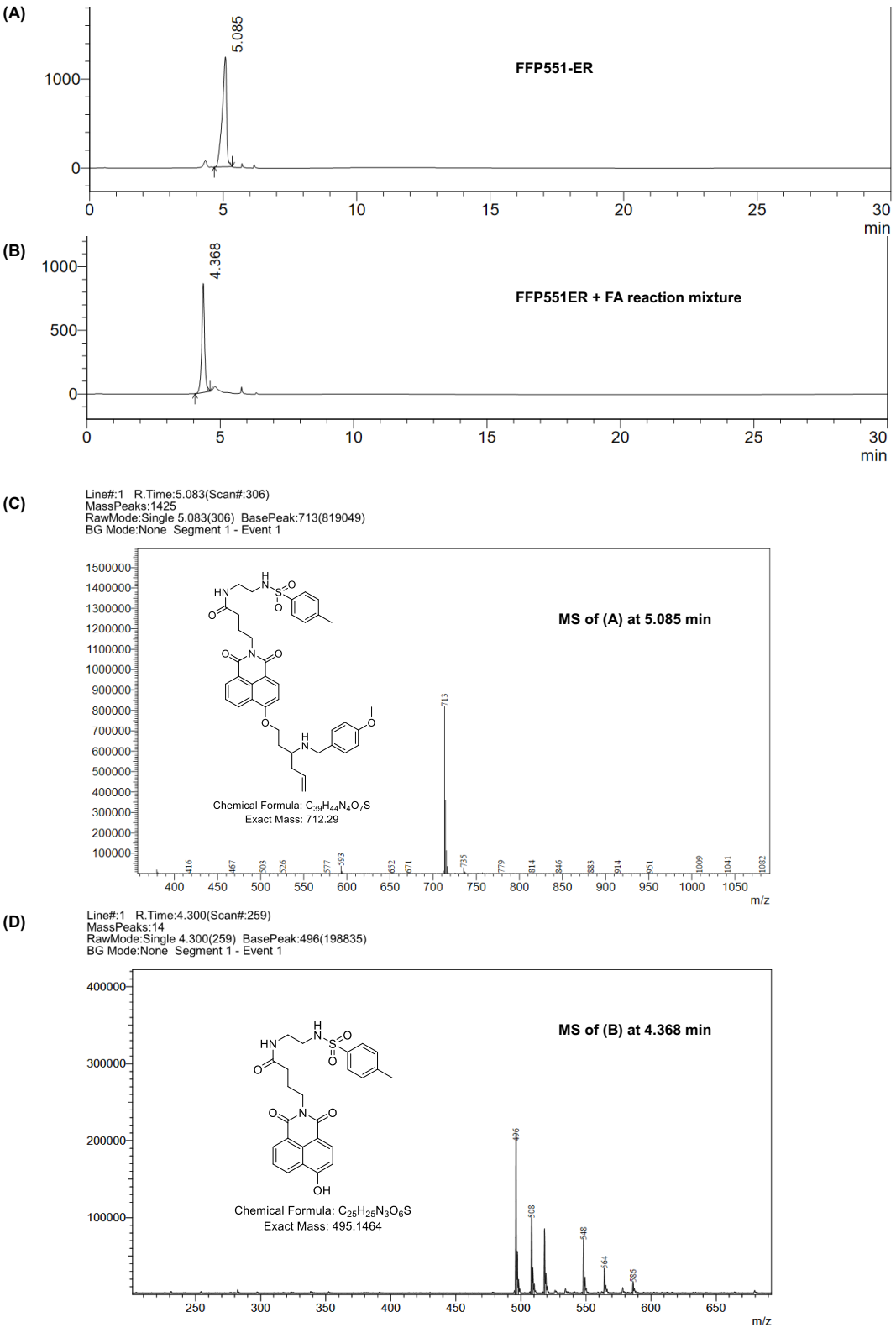



**Figure S6.** LC-MS analysis of the reaction mixture between FFP551-ER and FA. FFP551-ER (250 μM) was reacted with FA (5 mM) in PBS/$CH_3CN$ (v/v = 1:1) at 37 °C for 2 h and the mixture was analyzed by LC-MS. (A) LC analysis of FFP551-ER. (B) LC analysis of the reaction mixture of FFP551-ER with FA. (C) Mass spectrum at 5.085 min in (A). (D) Mass spectrum at 4.368 min in (B).

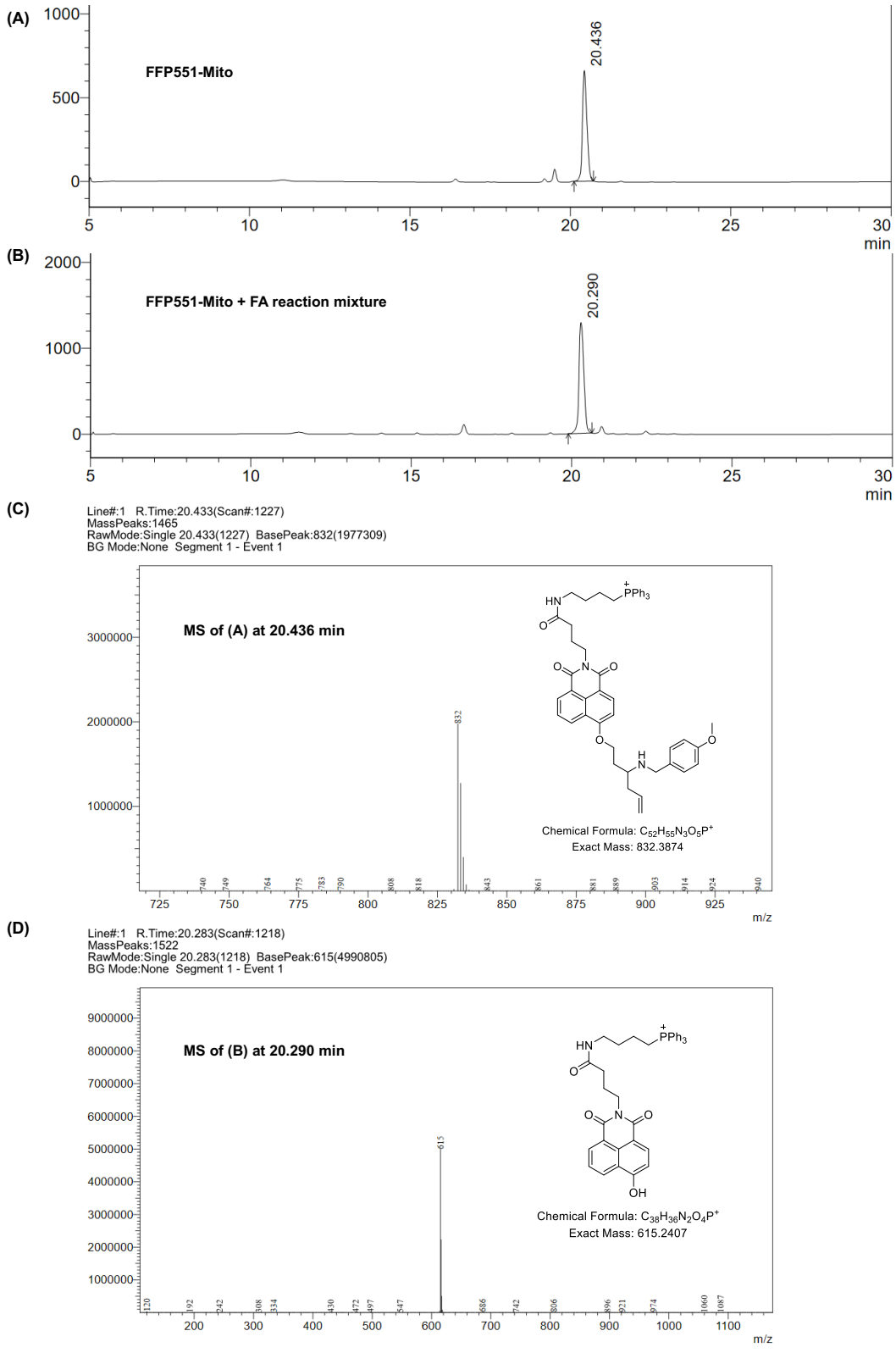



**Figure S7.** LC-MS analysis of the reaction mixture between FFP551-Mito and FA. FFP551-Mito (250 μM) was reacted with FA (5 mM) in PBS/$CH_3CN$ (v/v = 1:1) at 37 °C for 2 h and the mixture was analyzed by LC-MS. (A) LC analysis of FFP551-Mito. (B) LC analysis of the reaction mixture of FFP551-Mito with FA. (C) Mass spectrum at 20.436 min in (A). (D) Mass spectrum at 20.290 min in (B).

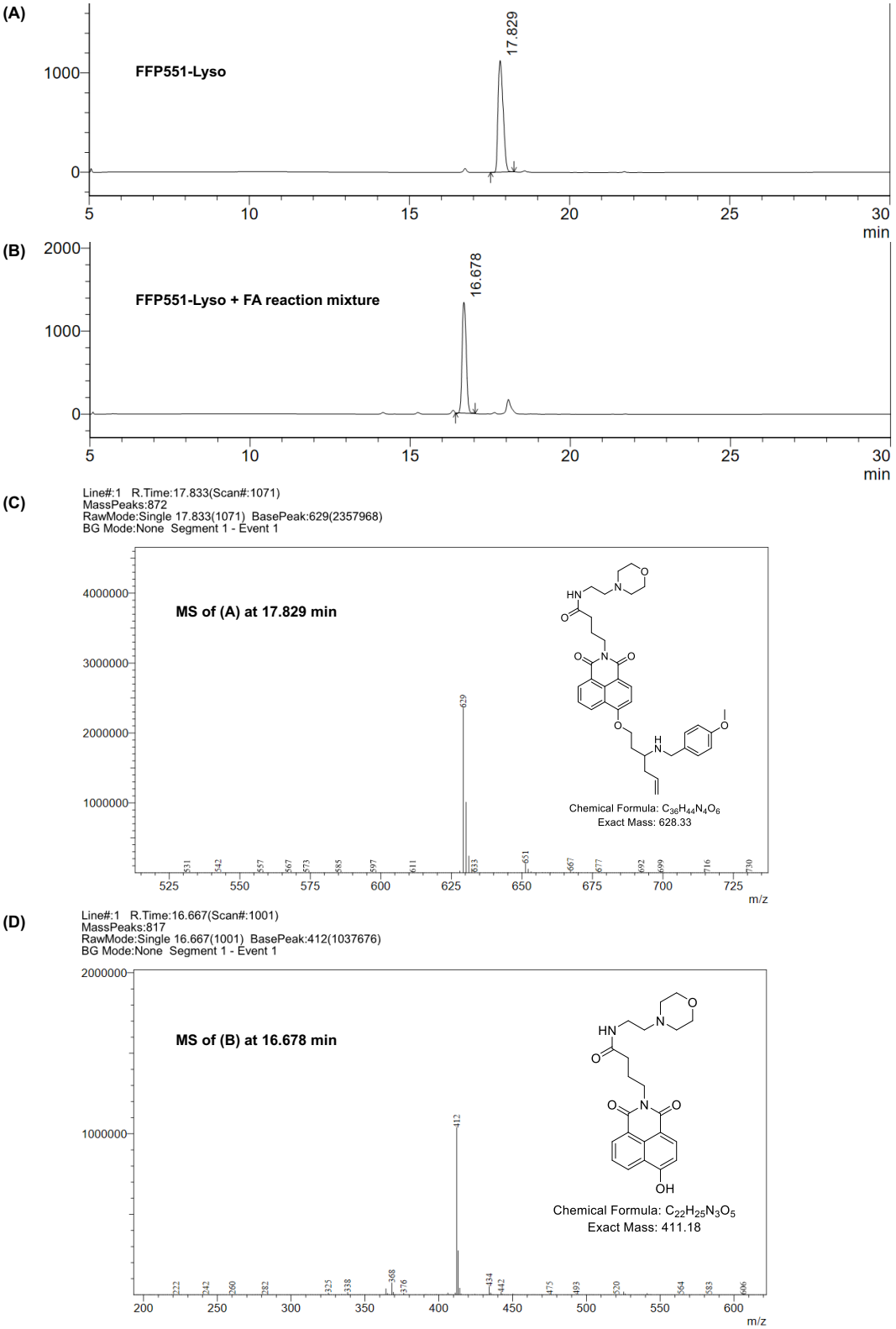



**Figure S8.** LC-MS analysis of the reaction mixture between FFP551-Lyso and FA. FFP551-Lyso (250 μM) was reacted with FA (5 mM) in PBS/$CH_3CN$ (v/v = 1:1) at 37 °C for 2 h and the mixture was analyzed by LC-MS. (A) LC analysis of FFP551-Lyso. (B) LC analysis of the reaction mixture of FFP551-Lyso with FA. (C) Mass spectrum at 17.829 min in (A). (D) Mass spectrum at 16.678 min in (B).

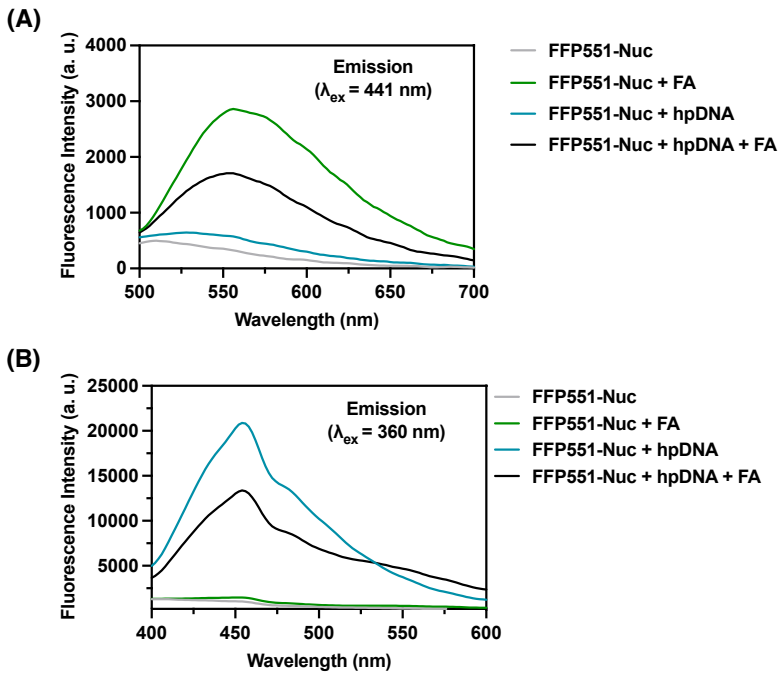



**Figure S9.** Fluorescence emission spectra of FFP551-Nuc in the presence of hpDNA and FA with excitation at (A) 441 nm and (B) 360 nm. FFP551-Nuc (10 μM) was treated with hpDNA (10 μM) and FA (1 mM) in PBS (20 mM, pH 7.4) at 37 °C for 1 h. Fluorescence spectra in (A) show the emission of FFP551-Nuc, while fluorescence spectra in (B) show the emission of Hoechst 33258.

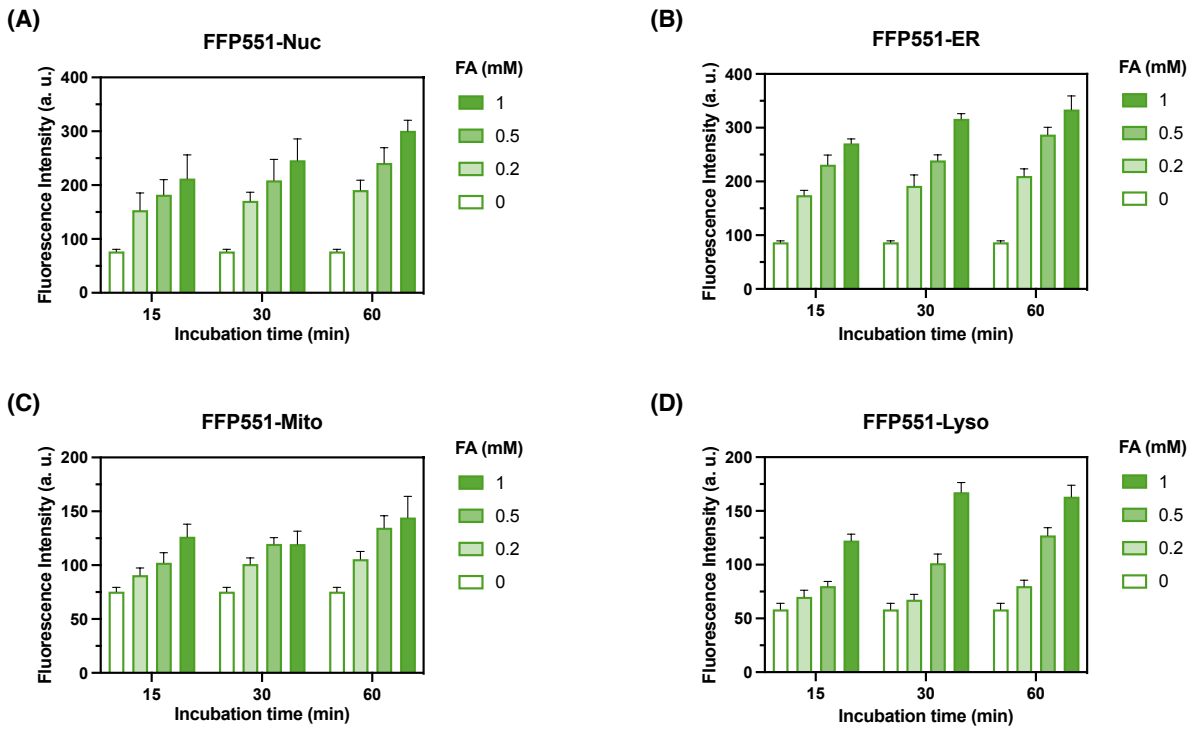



**Figure S10.** Dose- and time-dependent detection of FA with (A) FFP551-Nuc, (B) FFP551-ER, (C) FFP551-Mito, and (D) FFP551-Lyso in HeLa cells. HeLa cells were loaded with FFP551-Nuc, FFP551-ER, FFP551-Mito, or FFP551-Lyso for 30 min and treated with FA at different concentrations for indicated periods. Cells were washed and intracellular fluorescence was measured with a plate reader. Probes were excited at 441 nm and fluorescence at 551 nm was measured. Fluorescence intensities are shown as mean ± standard deviation ($n$ = 5).

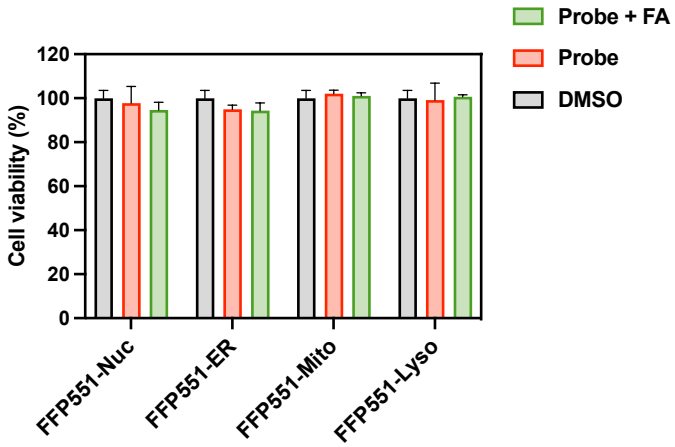



**Figure S11.** MTT analysis of the cytotoxicity of probes. HeLa cells were incubated with FFP551-Nuc (40 μM), FFP551-ER (40 μM), FFP551-Mito (80 μM), or FFP551-Lyso (40 μM) for 24 h, treated with or without FA (1 mM) at 37 °C for 1 h, and subjected to the MTT assay. Cell viabilities are shown as mean ± standard deviation ($n$ = 5).

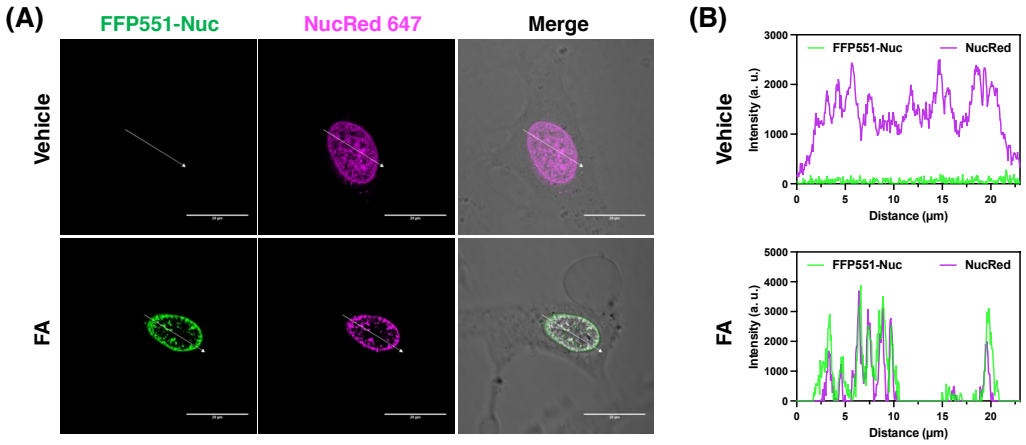



**Figure S12.** Imaging of FFP551-Nuc colocalization with NucRed Live 647 in live HeLa cells. (A) HeLa cells were loaded with FFP551-Nuc for 30 min, treated with vehicle (buffer only) or FA (1 mM) for 1 h, and stained with NucRed Live 647. Scale bars are 20 μm. (B) Intensity profiles of FFP551-Nuc and NucRed Live 647 within the regions of interest shown in (A).

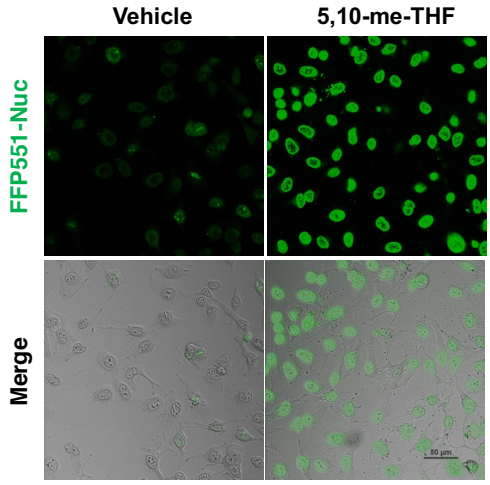
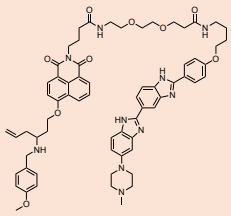
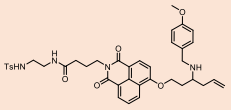
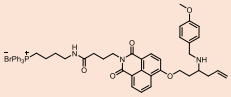
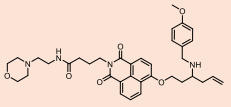
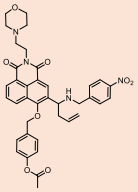
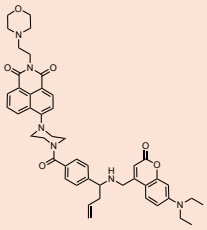



**Figure S13.** Fluorescence imaging of endogenous FA generated from 5,10-me-THF metabolism using FFP551-Nuc in live HeLa cells. Cells were loaded with FFP551-Nuc for 30 min, treated with vehicle (buffer only) or 5,10-me-THF (1 mM) for 1 h, and imaged with confocal fluorescence microscopy. The scale bar represents 50 μm.

## Supplementary tables

**Table S1.** Summary of reported organelle-targeting FA fluorescent probes, including two nucleus-targeting, one ER-targeting, two mitochondria-targeting, and eleven lysosome-targeting probes.[a, b]

| Probe | Buffer[c] | $\lambda_{ex}/\lambda_{em}$ (nm) | LOD (μM)[d] | Enhancement[e] | Targeted organelle | Biological application | Ref |
|---|---|---|---|---|---|---|---|
|  FFP551-Nuc | 10 μM in PBS/0.1% DMSO | 441/551 | 1 | 5.6-fold (0.1 mM FA, 2 hr) | Nucleus | Imaging endogenous FA in live cells (HeLa) | This work |
|  FFP551-ER | 10 μM in PBS/0.1% DMSO | 441/551 | 1 | 16.8-fold (0.1 mM FA, 2 hr) | ER | Imaging exogenous FA in live cells (HeLa) | This work |
|  FFP551-Mito | 10 μM in PBS/0.1% DMSO | 441/551 | 1 | 44.3-fold (0.1 mM FA, 2 hr) | Mitochondria | Imaging exogenous FA in live cells (HeLa) | This work |
|  FFP551-Lyso | 10 μM in PBS/0.1% DMSO | 441/551 | 1 | 48.6-fold (0.1 mM FA, 2 hr) | Lysosome | Imaging exogenous FA in live cells (HeLa) | This work |
|  NBC | 1 μM in PBS | 485/525 | 0.27 | 9.5-fold (0.08 mM FA, 35 min) | Lysosome | Imaging exogenous FA in live cells (HeLa) and mouse | 1 |
|  Lyso-TPFP | 10 μM in PBS/1% DMSO | 390/566, 506 | 3 | 3-fold (0.25 mM FA, 3 hr) | Lysosome | Imaging endogenous FA in live cells (HepG2) and abdomen tissues | 2 |

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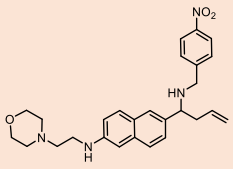
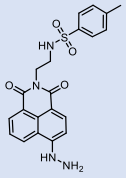
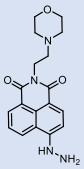
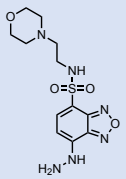
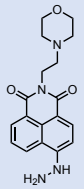
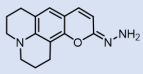
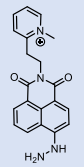
|  FA-Lyso | 5 μM in PBS/0.1% DMSO | 380/530 | 0.65 | 280-fold (2 mM FA, 60 min, pH = 5) | Lysosome | Imaging endogenous FA in live cells (HeLa, WI38) | 3 |
|  Na-FA-ER | 5 μM in PBS/1% DMSO | 440/543 | 0.71 | 140-fold (0.25 mM FA, 50 min) | ER | Imaging endogenous FA in live cells (HeLa) | 4 |
|  Na-FA-Lyso | 5 μM in PBS/1% DMSO | 440/543 | 5.02 | 350-fold (0.2 mM FA, 30 min) | Lysosome | Imaging endogenous FA in live cells (HeLa) | 5 |
|  FAP1 | 10 μM in Tris-HCl/60% $CH_3CN$ | 430/560 | 0.016 | 3.1-fold (0.001 mM FA, 2 hr) | Lysosome | Imaging exogenous FA in live cells (EC1) | 6 |
|  NP-Hy | 10 μM in PBS/1% DMSO | 440/540 | 0.35 | >600-fold (0.05 mM FA,30 min) | Lysosome | Imaging endogenous FA in live cells (HeLa) | 7 |
|  PFM4 | 5 μM in PBS/0.1% DMSO | 451/520 | 0.1 | >200-fold (0.25 mM, 30 min) | Lysosome | Imaging endogenous FA in live cells (EA) | 8 |
|  Mito-FA-FP | 5 μM in PBS/0.5% DMSO | 440/550 | 12.4 | 12-fold (0.2 mM FA, 30 min) | Mitochondria | Imaging endogenous FA in live cells (HeLa) and zebrafishes | 9 |

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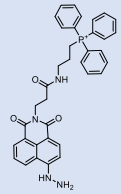
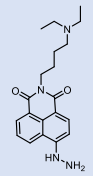
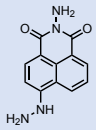
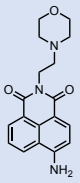
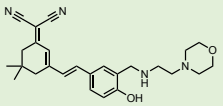
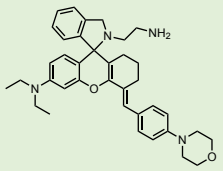
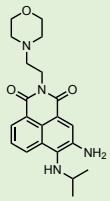
|  MT-FA | 5 μM in PBS/5% DMSO | 440/539 | 4.9 | 43.5-fold (0.15 mM, 40 min) | Mitochondria | Imaging endogenous FA in live cells (HeLa) and liver tissues | 10 |
|  Nu-FA | 5 μM in PBS/1% DMSO | 440/558 | 2.17 | 246-fold (0.35 mM FA, 25 min) | Nucleus | Imaging endogenous FA in live cells (HeLa) | 11 |
|  Nu-DH | 10 μM in PBS/1% DMSO | 440/538 | 0.7 | 120-fold (0.3 mM FA, 12 min) | Nucleus | Imaging endogenous FA in live cells (HeLa) and zebrafishes | 12 |
|  AMNT | 10 μM in PBS | 435/540, 528 | 1.77 | ~5-fold (1 mM FA, 30 min) | Lysosome | Imaging endogenous FA in live cells (HeLa) | 13 |
|  SWJT-6 | 10 μM in PBS/50% DMSO | 435/571, 661 | 5.65 | 3.7-fold (1 mM FA, 1 hr) | Lysosome | Imaging endogenous FA in live cells (HeLa) and zebrafishes | 14 |
|  NIR-Lyso-FA | 10 μM in PBS/20% ethanol | 630/770 | 0.48 | 19-fold (0.25 mM FA,1 hr) | Lysosome | Imaging endogenous FA in live cells (PD), mice and zebrafishes | 15 |
|  NP-Lyso | 10 μM in PBS | 380/444 | 0.27 | 60-fold (2 mM FA, 1 hr) | Lysosome | Imaging endogenous FA in live cells (L929 and HeLa) | 16 |

[a] This table only summarizes organelle-targeting FA fluorescent probes.

[b] Probes in the orange background are based on the 2-aza-Cope mechanism. Probes in the blue background are based on the formimine-formation mechanism. Probes in the green background are based on the aminal-formation mechanism.

[c] PBS: phosphate-buffered saline, pH = 7.4.

[d] LOD: limit of detection.

[e] Experimental conditions are shown in parentheses.

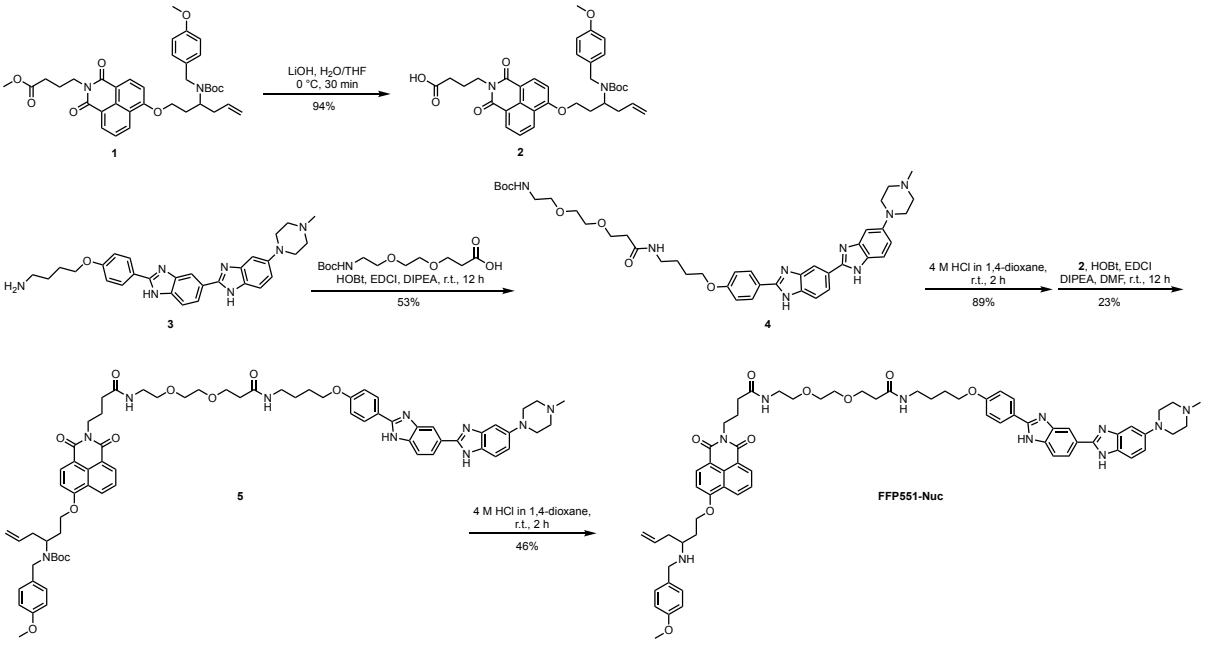
**General synthetic materials**

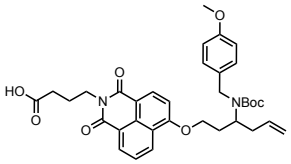
Unless otherwise specified, chemicals were obtained from commercial sources and used as received without further purification. When air- or moisture-sensitive reagents were used, chemical reactions were set up in oven-dried flasks under the protection of a $N_2$ atmosphere. Anhydrous tetrahydrofuran (THF) was distilled from sodium/benzophenone. Anhydrous dichloromethane ($CH_2Cl_2$) was distilled from calcium hydride. $GF_{254}$ silica gel plates for analytical TLC analyses were purchased from Qingdao Haiyang Chemical. TLC spots were monitored by UV illumination at 254 nm and stained with iodine or phosphomolybdic acid (PMA). Silica gel (230-400 mesh, reagent grade) for flash column chromatography was purchased from Qingdao Haiyang Chemical. $^1H$ and $^{13}C$ NMR spectra were recorded in deuterated solvents ($CDCl_3$ or $CD_3OD$) at room temperature with Bruker Avance NMR spectrometers. Chemical shifts are reported in $\delta$ ppm, and $J$ values are reported in Hz. $^1H$ NMR chemical shifts are calibrated with tetramethylsilane (TMS, $\delta$ = 0.00 ppm) in $CDCl_3$ or $CD_3OD$ ($CD_3$, $\delta$ = 3.31 ppm). $^{13}C$ NMR chemical shifts are calibrated with $CDCl_3$ ($\delta$ = 77.16 ppm) or $CD_3OD$ ($\delta$ = 49.00 ppm). Splitting patterns are shown as follows: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet. High-resolution mass spectra were recorded with a Q-Extractive Plus mass spectrometer. LC-MS analyses were performed with a Shimadzu LCMS-2020 system.

**Synthesis of FFP551-Nuc**

Probe FFP551-Nuc was synthesized according to Scheme S1.

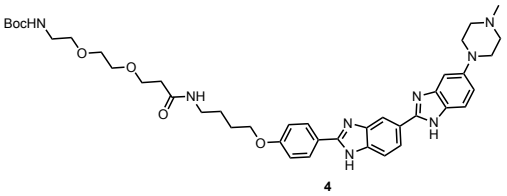
Scheme S1. Synthesis of probe FFP551-Nuc.





2

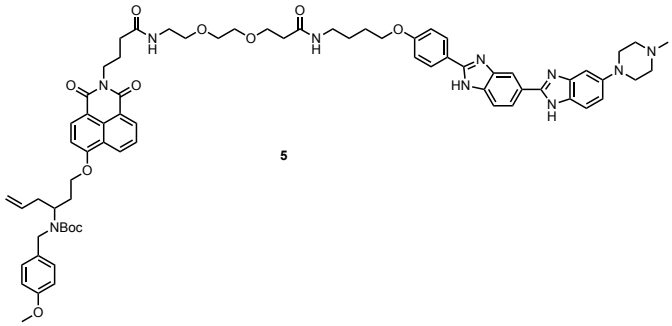
**4-(6-((3-((tert-butoxycarbonyl)(4-methoxybenzyl)amino)hex-5-en-1-yl)oxy)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)butanoic acid (2).** Compound **1** was synthesized as previously reported.[17] To a stirred solution of compound **1** (312 mg, 0.5 mmol) in THF (5 mL) was added LiOH (50 mg, 2.1 mmol) in water (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then acidified with 1 N HCl to pH = 1. The mixture was diluted with isopropanol (20 mL) and extracted with DCM (30 mL). The organic layer was washed with brine (30 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc = 4:1) to afford compound **2** (287 mg, 94% yield) as a white solid. $^1$H NMR (500 MHz, $CD_3OD$) δ 8.26 (t, *J* = 8.3 Hz, 1H), 8.14 (d, *J* = 8.4 Hz, 1H), 8.10 (s, 1H), 7.50 (t, *J* = 7.8 Hz, 1H), 7.10 (d, *J* = 8.1 Hz, 2H), 6.60 (d, *J* = 8.3 Hz, 1H), 6.53 (s, 2H), 5.89 – 5.73 (m, 1H), 5.16 – 5.02 (m, 2H), 4.57 – 4.41 (m, 1H), 4.07 (t, *J* = 7.1 Hz, 2H), 4.02 – 3.76 (m, 4H), 3.53 (s, 3H), 2.67 – 2.48 (m, 1H), 2.48 – 2.23 (m, 4H), 2.06 (ddt, *J* = 13.7, 9.4, 5.1 Hz, 1H), 1.97 (p, *J* = 7.4 Hz, 2H), 1.54 – 1.34 (m, 9H). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 175.28, 164.38, 163.79, 159.92, 158.75, 135.34, 133.07, 130.95, 130.87, 129.08, 128.78, 128.48, 125.38, 123.03, 121.65, 116.47, 113.96, 113.34, 108.50, 105.85, 55.47, 54.19, 39.13, 37.38, 31.25, 29.26, 27.51, 26.69, 23.36. HRMS calcd for $C_{35}H_{40}N_2NaO_8$ $[M+Na]^+$ 639.2682, found 639.2678.



4

***tert*-butyl (2-(2-(3-((4-(4-(5-(4-methylpiperazin-1-yl)-1*H*,1'*H*-[2,5'-bibenzo[*d*]imidazol]-2'-yl)phenoxy)butyl)amino)-3-oxopropoxy)ethoxy)ethyl)carbamate (4).** Compound **3** was synthesized according to the literature.[18] To a stirred solution of compound **3** (79 mg, 0.16 mmol), HOBt (33 mg, 0.24 mmol), EDCI (47 mg, 0.24 mmol), and 2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatetradecan-14-oic acid (Sigma-Aldrich; 67 mg, 0.24 mmol) in DMF (10 mL) was added DIPEA (130 µL, 0.8 mmol) at 0 °C. The reaction mixture was stirred at 25 °C for 12 h, quenched with water, and then extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with 0.1 N HCl (3 × 15 mL) and brine (20 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH/DCM = 1:20) to afford compound **4** (64 mg, 53% yield) as a yellow

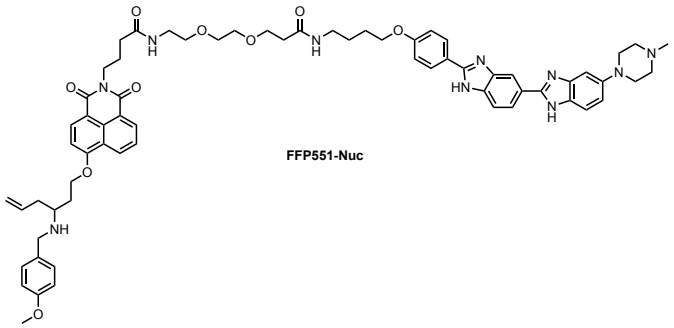
solid. $^1$H NMR (500 MHz, $CD_3OD$) δ 8.12 (s, 1H), 8.01 (t, *J* = 5.0 Hz, 1H), 7.91 (d, *J* = 8.2 Hz, 2H), 7.84 (d, *J* = 7.5 Hz, 1H), 7.58 (d, *J* = 8.1 Hz, 1H), 7.46 (d, *J* = 8.2 Hz, 1H), 7.07 (s, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 6.91 (d, *J* = 8.4 Hz, 2H), 3.90 (t, *J* = 6.1 Hz, 2H), 3.72 (t, *J* = 6.1 Hz, 2H), 3.56 (t, *J* = 3.0 Hz, 4H), 3.47 (t, *J* = 5.7 Hz, 2H), 3.35 (s, 3H), 3.30 – 3.26 (m, 4H), 3.26 – 3.20 (m, 2H), 3.19 (t, *J* = 5.5 Hz, 2H), 2.92 – 2.77 (m, 4H), 2.45 (t, *J* = 6.1 Hz, 2H), 1.81 – 1.69 (m, 2H), 1.69 – 1.57 (m, 2H), 1.40 (s, 9H). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 172.49, 161.09, 157.04, 153.91, 152.35, 147.04, 138.46, 134.08, 129.46, 128.22, 124.37, 123.26, 121.16, 117.36, 115.21, 114.91, 114.59, 112.58, 111.07, 101.49, 78.70, 69.94, 69.83, 69.71, 67.41, 66.98, 53.77, 48.50, 42.71, 42.00, 39.91, 38.70, 36.35, 29.36, 27.43, 26.26, 25.68. HRMS calcd for $C_{41}H_{55}N_8O_6$ $[M+H]^+$ 755.4245, found 755.4241.




5

***tert*-butyl (4-methoxybenzyl)(1-((2-(19-(4-(5-(4-methylpiperazin-1-yl)-1*H*,1'*H*-[2,5'-bibenzo[*d*]imidazol]-2'-yl)phenoxy)-4,14-dioxo-8,11-dioxa-5,15-diazanonadecyl)-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl)oxy)hex-5-en-3-yl)carbamate (5).** To a stirred solution of compound **4** (64 mg, 0.09 mmol) was added 3 mL of HCl solution (4 N in 1,4-dioxane). The reaction mixture was stirred at 25 °C for 2 h. The solvent was removed under reduced pressure to afford the deprotected compound as a yellow solid (56 mg, 89% yield) which was used directly without further purification. To a stirred solution of this crude product (56 mg, 0.08 mmol) in DMF (5 mL) was added HOBt (18 mg, 0.13 mmol), EDCI (25 mg, 0.13 mmol), compound **2** (52 mg, 0.1 mmol), and DIPEA (57 μL, 0.34 mmol) successively at 0 °C. The reaction mixture was stirred at 25 °C for 12 h, quenched with water, and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with 0.1 N HCl (3 × 15 mL) and brine (20 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH/DCM = 1:20) to afford compound **5** (24 mg, 23% yield) as a yellow solid. $^1$H NMR (400 MHz, $CD_3OD$) δ 8.35 (s, 1H), 8.18 (s, 2H), 8.08 (s, 1H), 7.88 – 7.76 (m, 3H), 7.66 – 7.48 (m, 3H), 7.14 (s, 1H), 7.07 – 6.96 (m, 3H), 6.90 (d, *J* = 8.7 Hz, 2H), 6.57 (d, *J* = 8.2 Hz, 1H), 6.51 – 6.30 (m, 2H), 5.78 – 5.61 (m, 1H), 5.03 – 4.96 (m, 2H), 4.56 – 4.27 (m, 1H), 4.05 – 3.84 (m, 6H), 3.77 – 3.64 (m, 5H), 3.58 (s, 5H), 3.54 – 3.46 (m, 5H), 3.40 – 3.32 (m, 5H), 3.24 (t, *J* = 6.6 Hz, 2H), 3.15 (s, 3H), 2.73 (s, 3H), 2.51 – 2.37 (m, 4H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.26 – 2.13 (m, 2H), 1.98 – 1.88 (m, 2H),

1.81 – 1.73 (m, 2H), 1.72 – 1.60 (m, 2H), 1.33 (s, 9H). $^{13}$C NMR (101 MHz, $CD_3OD$) δ 191.04, 174.10, 173.27, 172.74, 172.66, 172.61, 164.49, 163.91, 161.08, 160.03, 158.70, 156.81, 155.81, 153.69, 152.47, 147.44, 133.29, 131.02, 129.56, 129.13, 128.93, 128.71, 128.18, 125.67, 125.49, 123.15, 121.74, 121.29, 121.07, 116.50, 115.47, 115.20, 114.64, 113.98, 113.27, 112.34, 105.83, 101.55, 70.00, 69.95, 69.44, 67.53, 67.03, 54.18, 49.21, 43.37, 39.29, 39.07, 38.79, 36.36, 33.28, 31.77, 29.53, 29.45, 29.36, 29.02, 27.50, 26.81, 26.37, 25.80, 24.18, 22.45, 18.04, 13.19. HRMS calcd for $C_{71}H_{85}N_{10}O_{11}$ $[M+H]^+$ 1253.6399, found 1253.9393.

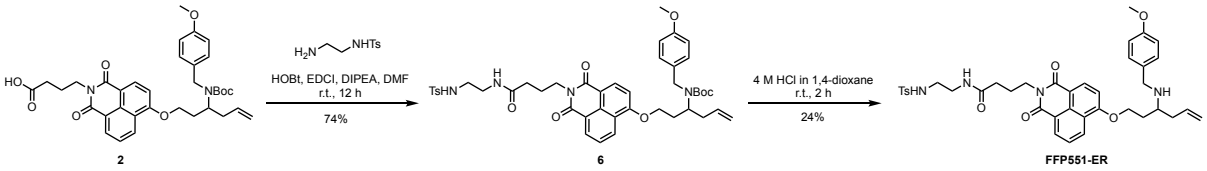


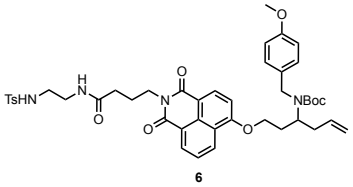

**4-(6-((3-((4-methoxybenzyl)amino)hex-5-en-1-yl)oxy)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-*N*-(2-(2-(3-((4-(4-(5-(4-methylpiperazin-1-yl)-1*H*,1'*H*-[2,5'-bibenzo[*d*]imidazol]-2'-yl)phenoxy)butyl)amino)-3-oxopropoxy)ethoxy)ethyl)butanamide (FFP551-Nuc).** To a stirred solution of compound **5** (24 mg, 0.019 mmol) in 1,4-dioxane was added 3 mL of HCl solution (4 N in 1,4-dioxane). The reaction mixture was stirred at 25 °C for 2 h. The solvent was removed under reduced pressure and the residue was purified with preparative TLC (MeOH/DCM = 1:20) to afford probe FFP551-Nuc as a yellow solid (10 mg, 46% yield). $^1$H NMR (400 MHz, $CD_3OD$) δ 8.36 (dd, *J* = 7.3, 1.2 Hz, 1H), 8.26 (d, *J* = 8.3 Hz, 1H), 8.18 – 8.02 (m, 2H), 7.95 – 7.79 (m, 3H), 7.60 – 7.46 (m, 3H), 7.22 – 7.15 (m, 2H), 7.14 (d, *J* = 2.3 Hz, 1H), 7.03 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.98 – 6.91 (m, 2H), 6.88 (d, *J* = 8.3 Hz, 1H), 6.69 – 6.59 (m, 2H), 5.79 (ddt, *J* = 17.2, 10.1, 7.1 Hz, 1H), 5.21 – 5.09 (m, 2H), 4.21 – 4.07 (m, 2H), 4.06 – 3.94 (m, 4H), 3.83 (q, *J* = 13.1 Hz, 2H), 3.73 (t, *J* = 5.9 Hz, 2H), 3.59 (d, *J* = 4.1 Hz, 6H), 3.51 (t, *J* = 5.5 Hz, 2H), 3.36 – 3.32 (m, 2H), 3.28 – 3.22 (m, 5H), 3.14 – 3.03 (m, 1H), 2.77 (t, *J* = 5.0 Hz, 4H), 2.50 – 2.42 (m, 5H), 2.41 – 2.34 (m, 2H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.23 – 2.16 (m, 1H), 2.07 – 1.97 (m, 3H), 1.97 – 1.89 (m, 2H), 1.85 – 1.77 (m, 2H), 1.74 – 1.64 (m, 2H). $^{13}$C NMR (101 MHz, $CD_3OD$) δ 179.30, 175.36, 173.94, 165.72, 165.13, 162.36, 161.17, 160.75, 154.94, 153.62, 149.39, 134.97, 134.62, 132.30, 131.40, 130.82, 130.22, 129.71, 129.44, 129.21, 126.89, 125.41, 124.37, 123.09, 122.61, 122.33, 119.38, 116.31, 115.94, 115.53, 114.94, 107.35, 71.26, 71.21, 70.70, 68.81, 68.29, 66.95, 56.00, 55.61, 54.32, 51.44, 50.18, 45.77, 40.57, 40.32, 40.04, 37.74, 37.62, 36.53, 34.53, 33.03, 32.58, 30.80, 28.07, 27.62, 27.07, 26.90, 25.41. HRMS calcd for $C_{66}H_{77}N_{10}O_9$ $[M+H]^+$ 1153.5875, found 1153.5873.

**Synthesis of FFP551-ER**

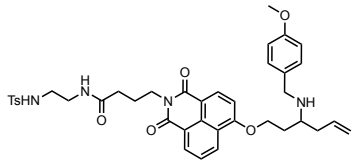
Probe FFP551-ER was synthesized according to Scheme S2.

Scheme S2. Synthesis of probe FFP551-ER.




***tert*-butyl (4-methoxybenzyl)(1-((2-(4-((2-((4-methylphenyl)sulfonamido)ethyl)amino)-4-oxobutyl)-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl)oxy)hex-5-en-3-yl)carbamate (6).** To a stirred solution of compound **2** (154 mg, 0.25 mmol), HOBt (51 mg, 0.38 mmol), EDCI (72 mg, 0.38 mmol), and *N*-(2-aminoethyl)-4-methylbenzenesulfonamide (65 mg, 0.3 mmol) in DMF (10 mL) was added DIPEA (0.16 mL, 1 mmol) at 0 °C. The reaction mixture was stirred at 25 °C for 12 h, quenched with water, and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with 0.1 N HCl (3 × 15 mL) and brine (20 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH/DCM = 1:20) to afford compound **6** (150 mg, 74% yield) as a yellow solid. $^1$H NMR (500 MHz, $CD_3OD$) δ 8.26 (s, 1H), 8.11 (d, *J* = 8.2 Hz, 2H), 7.70 (d, *J* = 8.2 Hz, 2H), 7.47 (s, 1H), 7.29 (d, *J* = 8.1 Hz, 2H), 7.11 (d, *J* = 7.6 Hz, 2H), 6.71 – 6.43 (m, 3H), 5.78 (s, 1H), 5.15 – 5.00 (m, 2H), 4.59 – 4.32 (m, 1H), 4.08 (dd, *J* = 11.3, 4.0 Hz, 1H), 4.00 (t, *J* = 6.6 Hz, 2H), 3.97 – 3.89 (m, 2H), 3.89 – 3.78 (m, 1H), 3.53 (s, 3H), 3.21 (t, *J* = 5.9 Hz, 2H), 2.97 (t, *J* = 6.1 Hz, 2H), 2.67 – 2.46 (m, 1H), 2.45 – 2.36 (m, 2H), 2.34 (s, 3H), 2.22 (t, *J* = 7.3 Hz, 2H), 2.12 – 2.00 (m, 1H), 2.00 – 1.88 (m, 2H), 1.51 – 1.34 (m, 9H). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 175.46, 165.70, 165.11, 161.26, 160.07, 144.61, 138.80, 136.67, 134.51, 132.27, 130.73, 130.06, 129.86, 128.01, 126.73, 124.30, 122.90, 117.80, 115.20, 114.69, 107.22, 67.52, 56.73, 55.58, 49.85, 43.35, 40.52, 40.40, 34.34, 28.85, 25.19, 21.47. HRMS calcd for $C_{44}H_{52}N_4NaO_9S$ $[M+Na]^+$ 835.3353, found 835.3377.



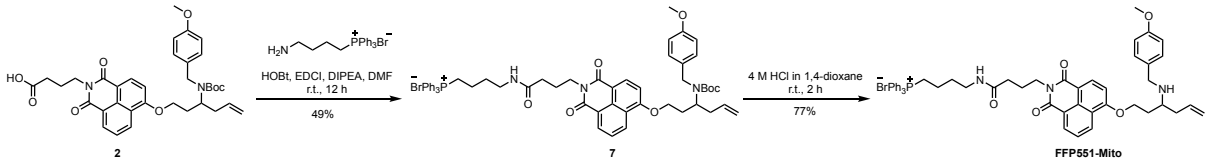
FFP551-ER

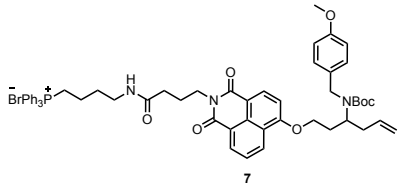
**4-(6-((3-((4-methoxybenzyl)amino)hex-5-en-1-yl)oxy)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-*N*-(2-((4-methylphenyl)sulfonamido)ethyl)butanamide (FFP551-ER).** To a stirred solution of compound **6** (150 mg, 0.184 mmol) in 1,4-dioxane was added 5 mL of HCl solution (4 N in 1,4-dioxane). The reaction mixture was stirred at 25 °C for 2 h. The solvent was removed under reduced pressure and the residue was purified with preparative TLC (MeOH/DCM = 1:20) to afford probe FFP551-ER as a yellow solid (32 mg, 24% yield). $^{1}$H NMR (500 MHz, $CD_3OD$) δ 8.21 (d, *J* = 7.2 Hz, 1H), 8.16 (d, *J* = 8.2 Hz, 1H), 8.04 (d, *J* = 8.3 Hz, 1H), 7.69 (d, *J* = 8.2 Hz, 2H), 7.43 (t, *J* = 7.8 Hz, 1H), 7.30 (d, *J* = 8.2 Hz, 2H), 7.27 (d, *J* = 8.5 Hz, 2H), 6.90 (d, *J* = 8.4 Hz, 1H), 6.73 (d, *J* = 8.5 Hz, 2H), 5.91 (ddt, *J* = 17.2, 10.1, 7.1 Hz, 1H), 5.24 (dd, *J* = 25.2, 13.7 Hz, 2H), 4.35 – 4.22 (m, 2H), 4.03 – 3.88 (m, 4H), 3.64 (s, 3H), 3.27 – 3.22 (m, 1H), 3.19 (t, *J* = 6.1 Hz, 2H), 2.94 (t, *J* = 6.1 Hz, 2H), 2.53 (q, *J* = 6.5, 6.0 Hz, 2H), 2.35 (s, 3H), 2.28 – 2.13 (m, 4H), 1.97 – 1.84 (m, 2H). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 175.50, 165.67, 165.10, 161.18, 160.85, 144.70, 138.78, 135.06, 134.59, 132.30, 131.38, 130.75, 130.08, 129.63, 129.44, 128.00, 126.91, 124.29, 122.97, 119.44, 115.47, 115.11, 107.52, 67.18, 55.73, 54.59, 50.40, 43.36, 40.51, 40.45, 37.88, 34.35, 32.81, 25.17, 21.44. HRMS calcd for $C_{39}H_{45}N_4O_7S$ $[M+H]^+$ 713.3009, found 713.2997.

**Synthesis of FFP551-Mito**

Probe FFP551-Mito was synthesized according to Scheme S3.

Scheme S3. Synthesis of probe FFP551-Mito.

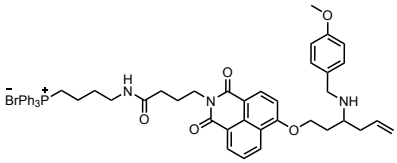


7

**(4-(4-(6-((3-((*tert*-butoxycarbonyl)(4-methoxybenzyl)amino)hex-5-en-1-yl)oxy)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)butanamido)butyl)triphenylphosphonium**

**bromide (7).** To a stirred solution of compound **2** (150 mg, 0.29 mmol), HOBt (71 mg, 0.52 mmol), EDCI (100 mg, 0.52 mmol), and (4-aminobutyl)triphenylphosphonium bromide (116 mg, 0.35 mmol) in DMF (10 mL) was added DIPEA (0.23 mL, 1.4 mmol) at 0 °C. The reaction mixture was stirred at 25 °C for 12 h, quenched with water, and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with 0.1 N HCl (3 × 15 mL) and brine (20 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH/DCM = 1:20) to afford compound **7** (144 mg, 49% yield) as a yellow solid. $^1$H NMR (400 MHz, $CD_3OD$) δ 8.33 (s, 1H), 8.28 – 8.14 (m, 2H), 7.81 – 7.73 (m, 9H), 7.71 – 7.67 (m, 5H), 7.64 – 7.54 (m, 1H), 7.42 – 7.26 (m, 1H), 7.14 (d, *J* = 7.1 Hz, 2H), 6.74 (d, *J* = 7.1 Hz, 1H), 6.62 – 6.43 (m, 2H), 5.98 – 5.68 (m, 1H), 5.19 – 4.97 (m, 2H), 4.48 (d, *J* = 14.9 Hz, 1H), 4.12 (d, *J* = 15.0 Hz, 1H), 4.03 – 3.91 (m, 5H), 3.59 – 3.39 (m, 5H), 3.24 (t, *J* = 5.4 Hz, 2H), 2.66 – 2.49 (m, 1H), 2.49 – 2.27 (m, 2H), 2.21 (t, *J* = 7.4 Hz, 2H), 2.10 (s, 1H), 1.88 (p, *J* = 7.3 Hz, 2H), 1.83 – 1.63 (m, 4H), 1.50 – 1.34 (m, 9H). $^{13}$C NMR (101 MHz, $CD_3OD$) δ 175.20, 165.47, 164.87, 161.29, 159.95, 136.66, 136.16, 136.13, 134.80, 134.70, 134.44, 132.22, 131.49, 131.36, 130.04, 126.91, 126.82, 126.32, 124.33, 122.92, 120.14, 119.28, 118.60, 117.85, 114.58, 111.81, 107.24, 55.50, 49.85, 40.42, 38.75, 34.68, 31.31, 31.14, 28.81, 25.61, 22.59, 22.07, 20.68, 20.64. HRMS calcd for $C_{57}H_{63}N_3O_7P^+$ $[M]^+$ 932.4398, found 932.4175.




FFP551-Mito

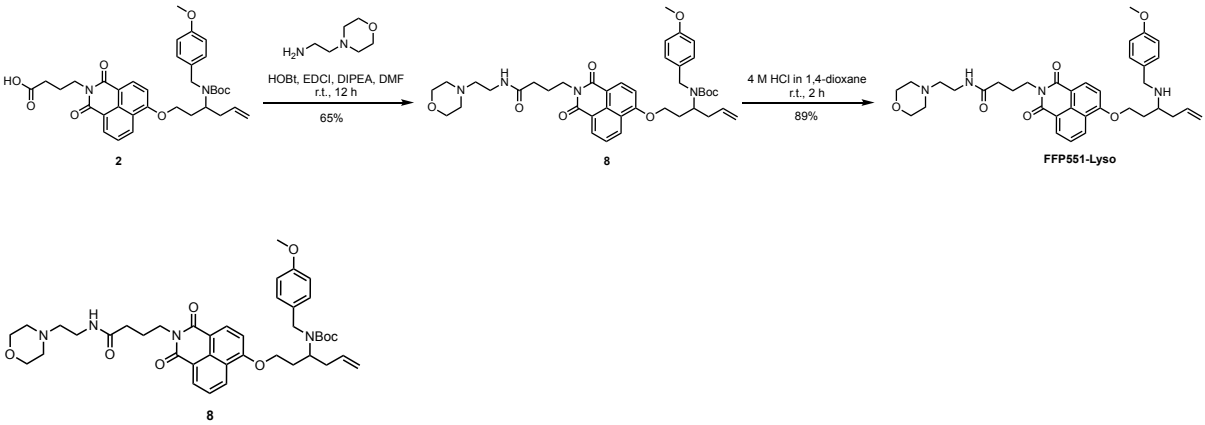
**(4-(4-(6-((3-((4-methoxybenzyl)amino)hex-5-en-1-yl)oxy)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)butanamido)butyl)triphenylphosphonium bromide (FFP551-Mito).** To a stirred solution of compound **7** (144 mg, 0.142 mmol) in 1,4-dioxane was added 4 mL of HCl solution (4 N in 1,4-dioxane). The reaction mixture was stirred at 25 °C for 2 h. The solvent was removed under reduced pressure and the residue was purified with preparative TLC (MeOH/DCM = 1:20) to afford probe FFP551-Mito as a yellow solid (100 mg, 77% yield). $^1$H NMR (400 MHz, $CD_3OD$) δ 8.34 (dd, *J* = 7.3, 1.0 Hz, 1H), 8.31 – 8.21 (m, 2H), 7.81 – 7.72 (m, 9H), 7.72 – 7.65 (m, 6H), 7.63 – 7.54 (m, 1H), 7.41 (d, *J* = 8.7 Hz, 2H), 7.09 (d, *J* = 8.4 Hz, 1H), 6.79 (d, *J* = 8.7 Hz, 2H), 5.95 (ddt, *J* = 17.2, 10.1, 7.1 Hz, 1H), 5.42 – 5.14 (m, 2H), 4.49 – 4.35 (m, 2H), 4.22 (q, *J* = 13.2 Hz, 2H), 3.97 (t, *J* = 7.2 Hz, 2H), 3.67 (s, 3H), 3.60 – 3.51 (m, 1H), 3.51 – 3.40 (m, 2H), 3.23 (t, *J* = 6.0 Hz, 2H), 2.77 – 2.61 (m, 2H), 2.50 – 2.30 (m, 2H), 2.21 (t, *J* = 7.4 Hz, 2H), 1.88 (q, *J* = 7.3 Hz, 2H), 1.82 – 1.65 (m, 4H). $^{13}$C NMR (101 MHz, $CD_3OD$) δ 175.30, 165.49, 164.89, 161.47, 161.02, 136.21, 136.18, 134.85, 134.75, 134.57, 133.83, 132.32, 132.24, 131.53, 131.40, 130.11, 129.77, 127.11, 126.05, 124.33,

123.09, 120.48, 120.21, 119.35, 115.69, 115.30, 107.73, 66.67, 55.74, 54.97, 49.85, 40.47, 38.82, 36.37, 34.70, 25.62, 22.65, 22.14, 20.75. HRMS calcd for $C_{52}H_{55}N_3O_5P^+$ $[M]^+$ 832.3874, found 832.3887.

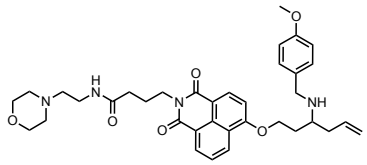
**Synthesis of FFP551-Lyso**

Probe FFP551-Lyso was synthesized according to Scheme S4.

Scheme S4. Synthesis of probe FFP551-Lyso.




***tert*-butyl (4-methoxybenzyl)(1-((2-(4-((2-morpholinoethyl)amino)-4-oxobutyl)-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl)oxy)hex-5-en-3-yl)carbamate (8).** To a stirred solution of compound **2** (133 mg, 0.26 mmol), HOBt (53 mg, 0.39 mmol), and EDCI (75 mg, 0.39 mmol) in DMF (10 mL) was added 2-morpholinoethan-1-amine (0.04 mL, 0.3 mmol) and DIPEA (60 µL, 0.35 mmol) at 0 °C. The reaction mixture was stirred at 25 °C for 12 h, quenched with water, and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with 0.1 N HCl (3 × 15 mL) and brine (20 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH/DCM = 1:20) to afford compound **8** (104 mg, 65% yield) as a yellow solid. $^1$H NMR (500 MHz, $CD_3OD$) δ 8.38 (s, 1H), 8.31 – 8.15 (m, 2H), 7.61 (d, *J* = 6.7 Hz, 1H), 7.14 (d, *J* = 8.2 Hz, 2H), 6.75 (d, *J* = 8.1 Hz, 1H), 6.55 (s, 2H), 5.95 – 5.71 (m, 1H), 5.22 – 5.00 (m, 2H), 4.63 – 4.45 (m, 1H), 4.20 – 4.10 (m, 3H), 4.09 – 3.99 (m, 2H), 3.99 – 3.91 (m, 1H), 3.70 – 3.65 (m, 4H), 3.56 (s, 3H), 3.35 – 3.31 (m, 2H), 2.67 – 2.54 (m, 1H), 2.54 – 2.49 (m, 4H), 2.49 – 2.43 (m, 3H), 2.44 – 2.38 (m, 1H), 2.32 (t, *J* = 7.5 Hz, 2H), 2.17 – 2.07 (m, 1H), 2.03 (p, *J* = 7.3 Hz, 2H), 1.55 – 1.36 (m, 9H). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 175.29, 165.77, 165.18, 161.35, 160.06, 157.07, 136.69, 134.50, 132.28, 130.45, 130.22, 130.00, 126.82, 124.44, 123.11, 117.83, 115.34, 114.63, 109.87, 107.25, 67.58, 58.52, 55.49, 54.56, 40.53, 37.08, 34.59, 28.81, 25.41. HRMS calcd for $C_{41}H_{53}N_4O_8$ $[M+H]^+$ 729.3863, found 729.3860.



FFP551-Lyso

**4-(6-((3-((4-methoxybenzyl)amino)hex-5-en-1-yl)oxy)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-*N*-(2-morpholinoethyl)butanamide (FFP551-Lyso).** To a stirred solution of compound **8** (65 mg, 0.09 mmol) in 1,4-dioxane was added 3 mL of HCl solution (4 N in 1,4-dioxane). The reaction mixture was stirred at 25 °C for 2 h. The solvent was removed under reduced pressure and the residue was purified with preparative TLC (MeOH/DCM = 1:20) to afford probe FFP551-Lyso as a yellow solid (50 mg, 89% yield). $^{1}$H NMR (500 MHz, $CD_3OD$) δ 8.36 (d, *J* = 7.2 Hz, 1H), 8.28 (dd, *J* = 16.7, 8.3 Hz, 2H), 7.61 (t, *J* = 7.8 Hz, 1H), 7.50 (d, *J* = 8.5 Hz, 2H), 7.12 (d, *J* = 8.3 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 2H), 5.98 (ddt, *J* = 17.3, 10.2, 7.1 Hz, 1H), 5.48 – 5.27 (m, 2H), 4.45 (s, 2H), 4.35 (q, *J* = 13.2 Hz, 2H), 4.09 (t, *J* = 6.9 Hz, 4H), 3.86 (s, 2H), 3.81 – 3.64 (m, 6H), 3.60 (t, *J* = 5.6 Hz, 2H), 3.39 – 3.33 (m, 2H), 3.22 (s, 2H), 2.77 (dq, *J* = 22.0, 7.4, 6.6 Hz, 2H), 2.64 – 2.48 (m, 1H), 2.48 – 2.38 (m, 1H), 2.35 (t, *J* = 7.1 Hz, 2H), 2.01 (p, *J* = 6.8 Hz, 2H). $^{13}$C NMR (126 MHz, $CD_3OD$) δ 176.76, 165.71, 165.13, 161.98, 160.89, 134.63, 132.97, 132.69, 132.43, 130.15, 129.74, 127.18, 124.30, 123.98, 123.06, 121.16, 115.78, 115.55, 107.81, 66.34, 65.11, 58.65, 55.83, 55.35, 53.58, 40.32, 35.52, 35.05, 33.85, 30.88, 24.76. HRMS calcd for $C_{36}H_{45}N_4O_6$ $[M+H]^+$ 629.3339, found 629.3326.

**Photophysical characterization**

Fluorescent probes were dissolved in DMSO (10 mM) and diluted with phosphate-buffered saline (PBS; 20 mM, pH 7.4) to 10 μM concentrations (containing 0.1% DMSO) for photophysical characterization.

Ultraviolet-visible spectroscopy was recorded with a UV-visible spectrophotometer (Techcomp UV-1100). Fluorescence spectroscopy was recorded with a fluorescence spectrophotometer (Hitachi F-4600), with slit widths for both excitation and emission at 2.5 nm and the photomultiplier voltage at 700 V. Samples in multiwell plates were measured on a microplate reader (BioTek Cytation 5).

For FA response assays, FA solutions of different concentrations were slowly added to the probe solutions. The mixtures were incubated at 37°C for indicated periods with vigorous stirring before the fluorescence was measured. For selectivity assays, the probe solutions were incubated with the analytes of interest (500 μM unless specified) at 37 °C for indicated periods with vigorous stirring. Sources of the analytes of interest were reported previously.[17, 19]

**LC-MS analysis**

The LC-MS analysis procedures on the reactions of fluorescent probes with FA were described previously.[17] Briefly, probes were dissolved in a mixed solvent of PBS and $CH_3CN$ (v/v = 1:1) at 250 μM concentrations and incubated with FA (5 mM) at 37 °C for 2 h with vigorous stirring. The mixture was filtered and analyzed by LC-MS. Samples were loaded onto a reverse-phase C18 column (4.6 x 250 mm, 5 μm) and eluted with a linear gradient of water (containing 0.1 % TFA) and $CH_3CN$ (from 0 to 70% $CH_3CN$ in 15 min) at 0.8 mL/min. Compounds were detected by UV absorption at 254 nm with 360 nm as the reference. Electrospray ionization (ESI) was used for mass analysis.

**Preparation of hairpin DNA**

Hairpin-forming oligonucleotide[18, 20] 5'-CGCGAATTCGCGTTTTCGCGAATTCGCG-3' (28 bp) was used for binding with FFP551-Nuc. This synthetic DNA was dissolved in ultrapure water at 1 mM concentration. To form hairpin structures, the DNA solution was heated at 98 °C for 5 min and then slowly cooled down to room temperature.

**Cell culture**

HeLa cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C with 5% $CO_2$.

**Confocal fluorescence imaging**

For exogenous FA imaging, HeLa cells were plated in 35-mm cover-slip dishes. After overnight culture, cells were washed twice with the BSS buffer (136.9 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 0.44 mM $KH_2PO_4$, 0.335 mM $Na_2HPO_4$, 10 mM PIPES, pH 7.2) and loaded with FFP551-Nuc (40 μM), FFP551-ER (40 μM), FFP551-Mito (80 μM), or FFP551-Lyso (40 μM) in the BSS buffer for 30 min at 37 °C/5% $CO_2$. Cells were washed with the BSS buffer, treated with FA (0 mM or 1 mM) in the BSS buffer for 1 h, and again washed with the BSS buffer three times. For co-localization experiments, cells were stained with Hoechst 33342, ER-tracker Red, MitoTracker Red, or LysoTracker Red. Cells were imaged in FluoroBrite DMEM (ThermoFisher) on a confocal fluorescence microscope (Nikon A1R) equipped with a 100x oil immersion objective.

For endogenous FA imaging, HeLa cells were plated in 35-mm cover-slip dishes. After overnight culture, cells were pretreated with GSK-LSD1 (1 μM) or TCP (20 μM) for 24 h, incubated with FFP551-Nuc (40 μM) in the BSS buffer for 30 min at 37 °C/5% $CO_2$, washed with the BSS buffer three times, stained with Hoechst 33342, and finally imaged in FluoroBrite DMEM (Thermo Fisher Scientific) on a confocal fluorescence microscope (Nikon A1R). For

5,10-methylene-tetrahydrofolate (5,10-me-THF) treatment, cells were pretreated with 1 mM 5,10-me-THF in the BSS buffer and incubated with FFP551-Nuc (40 μM) in the BSS buffer for 30 min.

FFP551-Nuc, FFP551-ER, FFP551-Mito, and FFP551-Lyso were excited with the 488 nm laser, and fluorescence was collected at 510–590 nm. Hoechst 33342 was excited with the 405 nm laser, and fluorescence was collected at 425–475 nm. MitoTracker Red, ER-Tracker Red, and LysoTracker Red were excited with the 561 nm laser, and fluorescence was collected at 590–640 nm. NucRed Live 647 was excited with the 640 nm laser, and fluorescence was collected at 650–690 nm.

Imaging analyses were performed with the NIS-Elements (Nikon) and ImageJ (NIH) software. Fluorescence intensities of the images were quantified in ImageJ. For every condition, multiple wells (reported as *n*) were processed and analyzed in parallel. The mean fluorescence intensities of these wells were calculated and subjected to statistical analysis. Statistical analyses for paired and multiple comparisons were performed with Student's t-test and one-way ANOVA, respectively, in Prism (GraphPad Software).

**MTT assay**

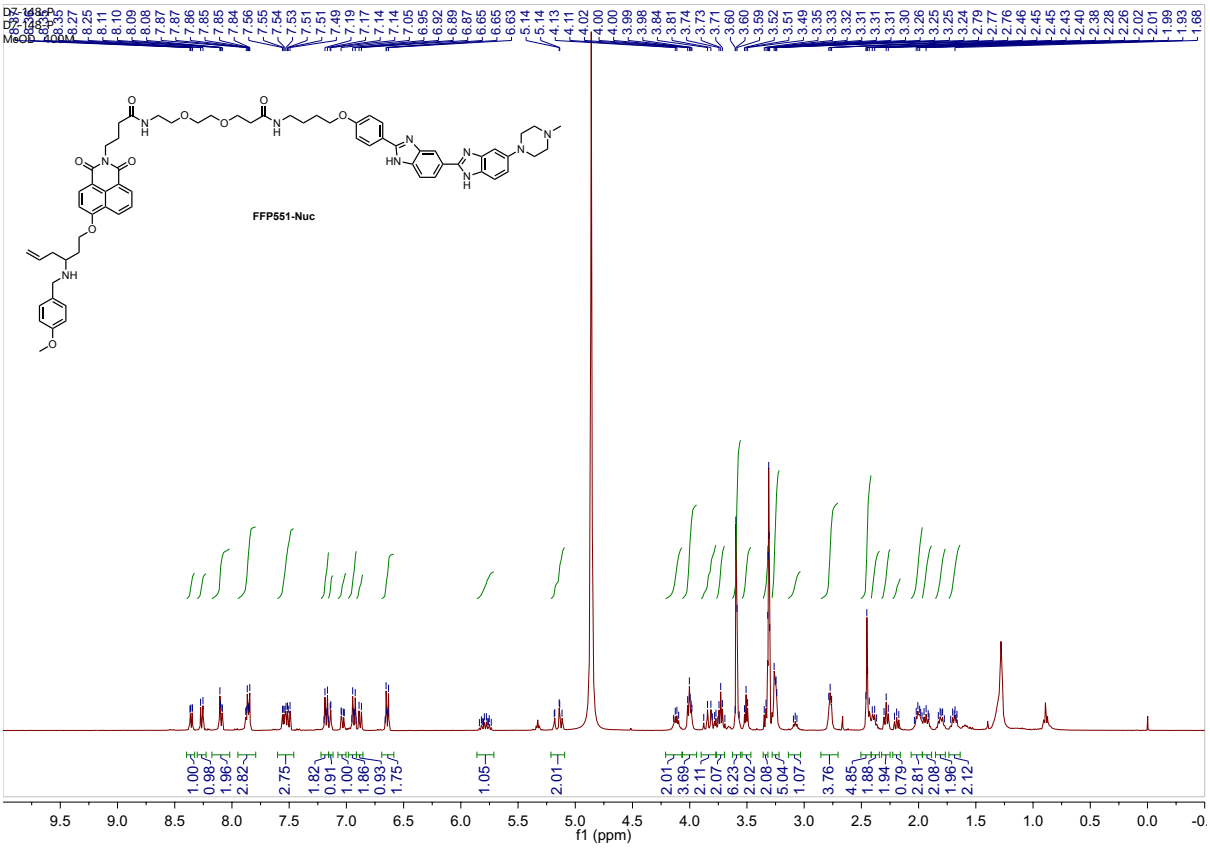
MTT assay was performed to examine the cytotoxicity of probes and FA treatment. Briefly, HeLa cells were plated in 96-well plates and cultured overnight. Cells were incubated with individual probes for 24 h, washed with the BSS buffer, and treated with FA for 1 h. After that, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solutions (Beyotime, C0009) were added into the wells and incubated with the cells for 4 h at 37 °C. Formazan solubilization solutions were then added into the wells and incubated for another 4 h at 37 °C. The absorbance of each well at 570 nm was measured with a Cytation 5 plate reader.

**Reference**


1. J. Liu, K. Li, P. Xue and J. Xu, *Anal. Biochem.*, 2022, **652**, 114749.
2. X. Xie, F. Tang, X. Shangguan, S. Che, J. Niu, Y. Xiao, X. Wang and B. Tang, *Chem. Commun.*, 2017, **53**, 6520-6523.
3. Y. Jiang, S. Huang, M. Liu, Z. Li, W. Xiao, H. Zhang, L. Yang and H. Sun, *Biosensors*, 2022, **12**, 855.
4. Y. Tang, Y. Ma, A. Xu, G. Xu and W. Lin, *Methods Appl. Fluoresc.*, 2017, **5**, 024005.
5. Y. Tang, X. Kong, Z. R. Liu, A. Xu and W. Lin, *Anal. Chem.*, 2016, **88**, 9359-9363.
6. M. Wang, X. Zhang, L. Wang, J. Zhang, N. Liu and D. Zhang, *Microchem. J.*, 2023, **191**, 108761.
7. Z. Xu, X. Yang, Z. Liu and M.-X. Zhang, *J. Photochem. Photobiol. A: Chem.*, 2022, **426**, 113731.

8. X.-G. Liang, J. Cheng, S. Qin, L.-X. Shao, M.-Z. Huang, G. Wang, Y. Han, F. Han and X. Li, *Chem. Commun.*, 2018, **54**, 12010-12013.
9. F. Xin, Y. Tian, C. Gao, B. Guo, Y. Wu, J. Zhao, J. Jing and X. Zhang, *Analyst*, 2019, **144**, 2297-2303.
10. A. Xu, Y. Tang and W. Lin, *New J. Chem.*, 2018, **42**, 8325-8329.
11. J. F. Li, D. H. Ding, W. H. Song, J. Y. Wang, W. Quan, L. Huang and W. Y. Lin, *Sensors Actuators B: Chem.*, 2022, **368**, 132136.
12. J. Li, D. Ding, J. Wang, L. Xu, D. Tan and W. Lin, *Chem. Eng. J.*, 2022, **448**, 137634.
13. T. Cao and H. Ma, *RSC Adv.*, 2022, **12**, 18093-18101.
14. H. Xu, L.-Z. Xu, R.-P. Huai, C. Zhang, S.-N. Suo, Y.-Q. Li, Y. Peng and Y.-W. Wang, *Org. Biomol. Chem.*, 2023, **21**, 6405-6409.
15. W. Quan, G. Zhang, Y. Li, W. Song, J. Zhan and W. Lin, *Anal. Chem.*, 2023, **95**, 2925–2931.
16. S. Cai, C. Liu, J. Gong, S. He, L. Zhao and X. Zeng, *Spectrochim. Acta, Pt. A: Mol. Biomol. Spectrosc.*, 2021, **245**, 118949.
17. Y. Du, Y. Zhang, M. Huang, S. Wang, J. Wang, K. Liao, X. Wu, Q. Zhou, X. Zhang, Y. D. Wu and T. Peng, *Chem. Sci.*, 2021, **12**, 13857-13869.
18. G. Lukinavičius, C. Blaukopf, E. Pershagen, A. Schena, L. Reymond, E. Derivery, M. Gonzalez-Gaitan, E. D'Este, S. W. Hell, D. Wolfram Gerlich and K. Johnsson, *Nat. Commun.*, 2015, **6**, 8497.
19. Y. Zhang, Y. Du, M. Li, D. Zhang, Z. Xiang and T. Peng, *Angew. Chem. Int. Ed.*, 2020, **59**, 16352-16356.
20. S. Y. Breusegem, R. M. Clegg and F. G. Loontiens, *J. Mol. Biol.*, 2002, **315**, 1049-1061.

## NMR spectra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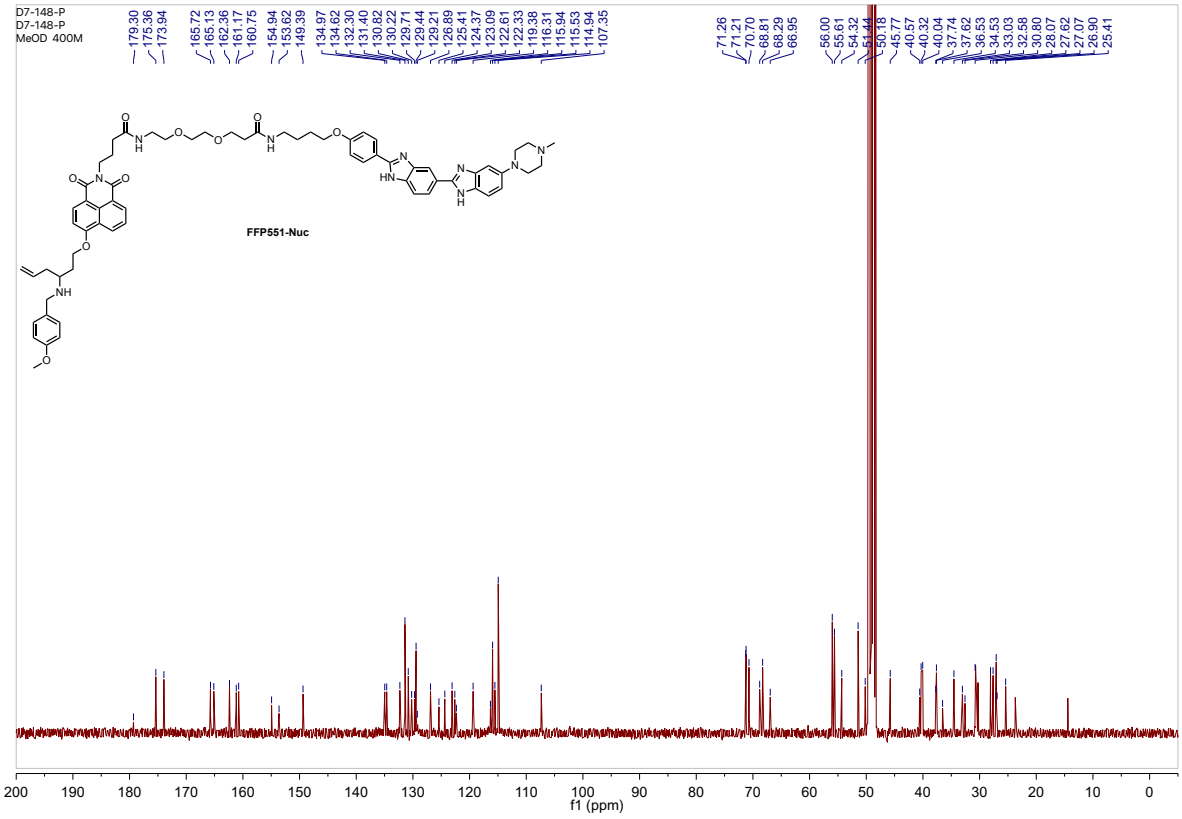
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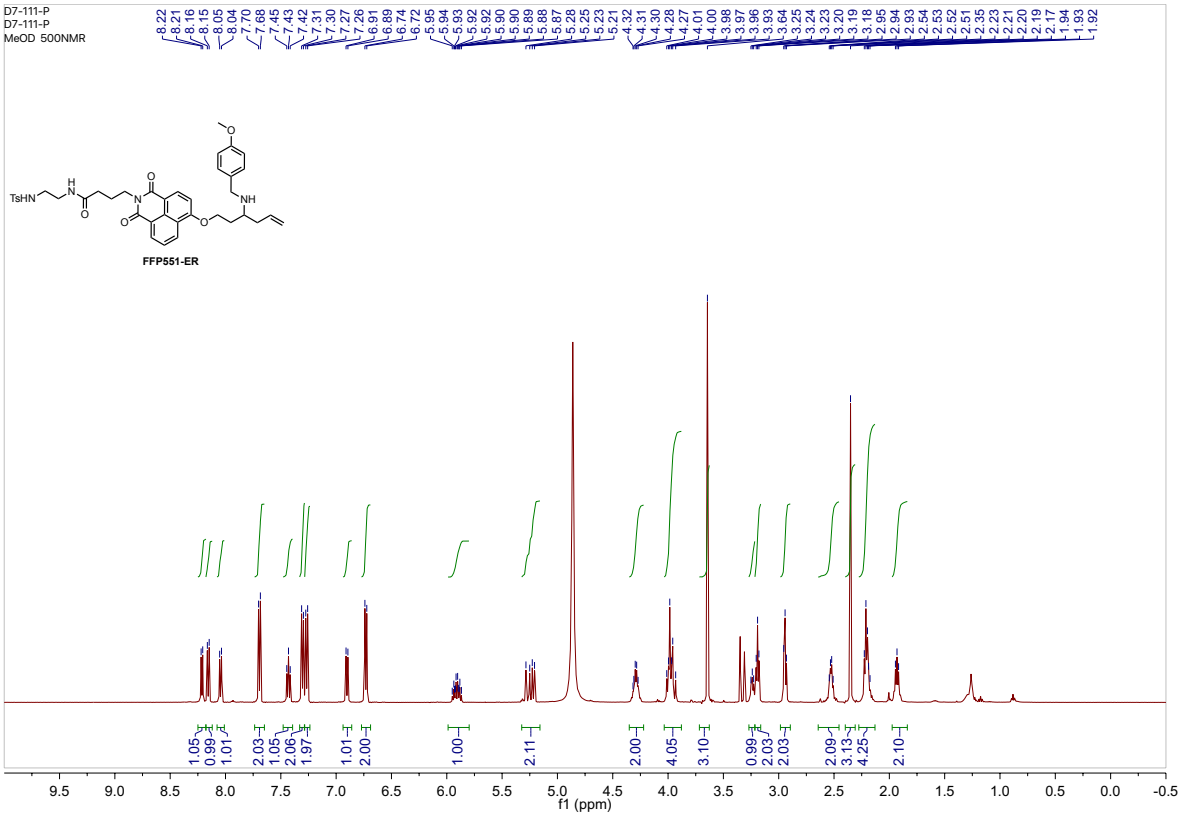
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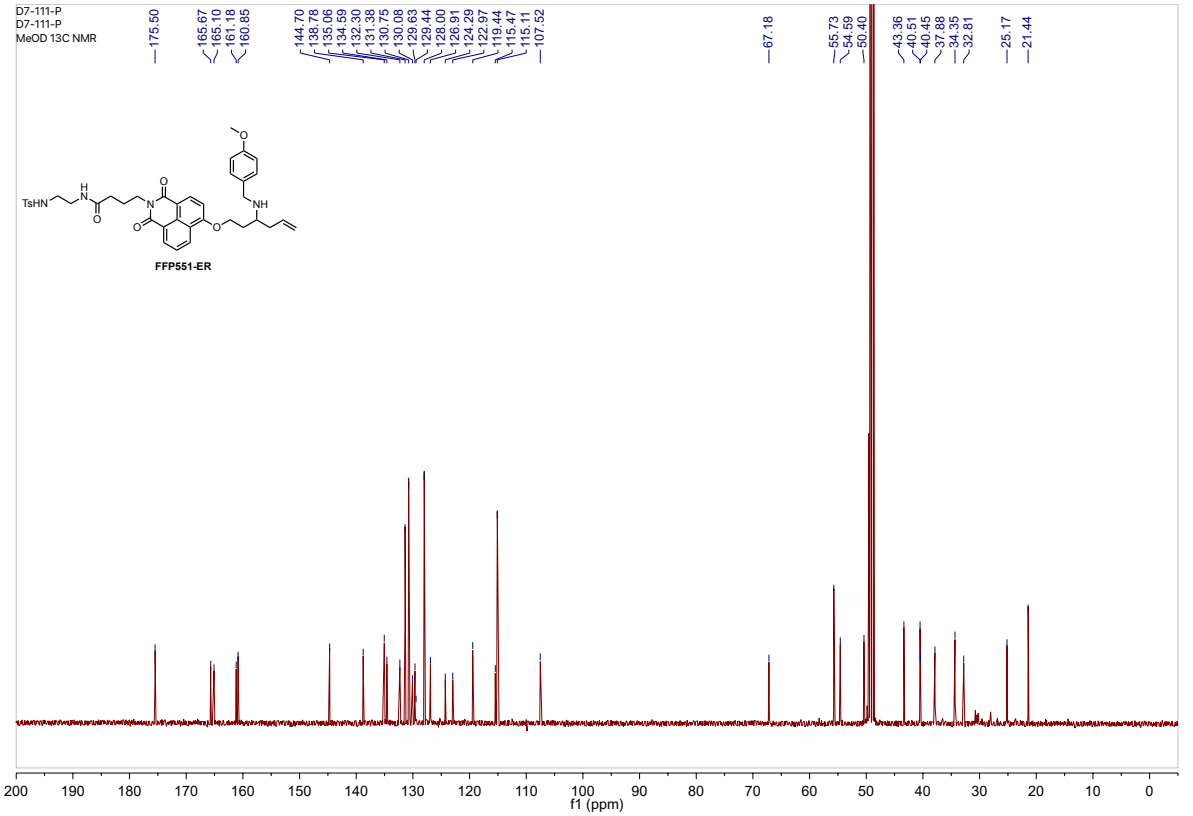
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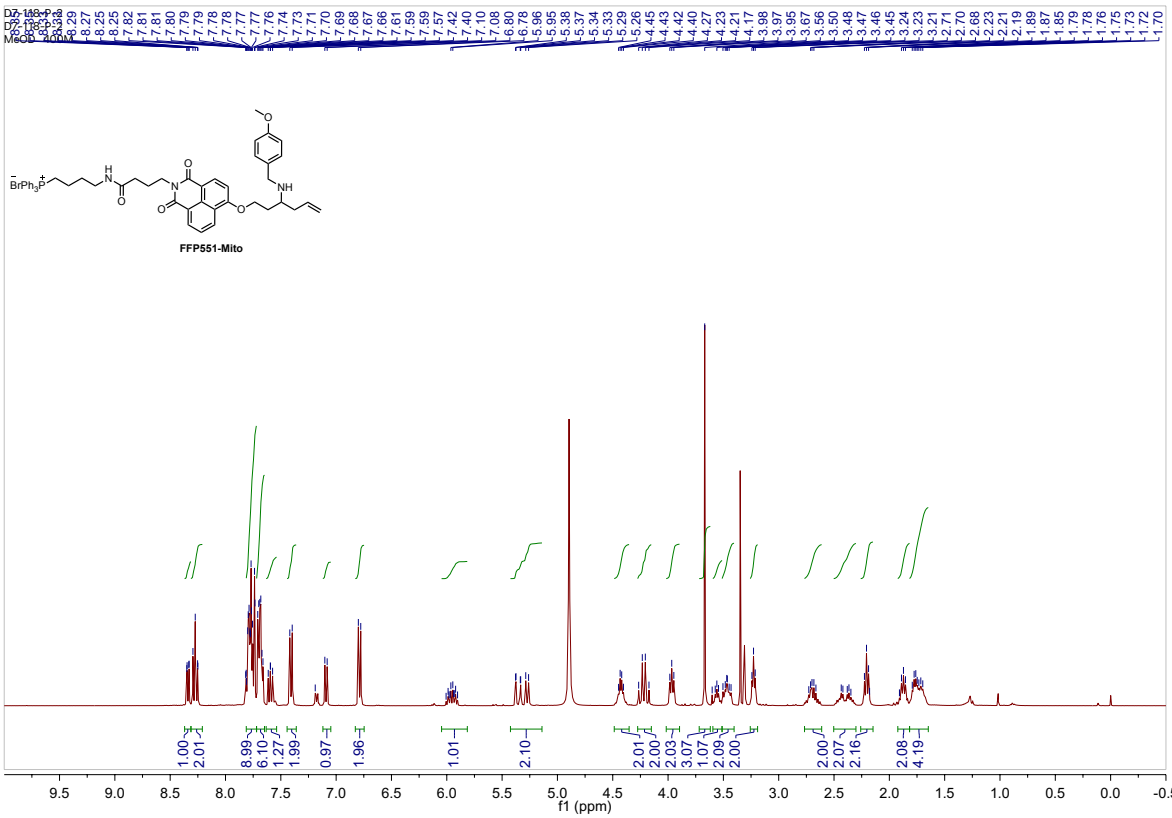
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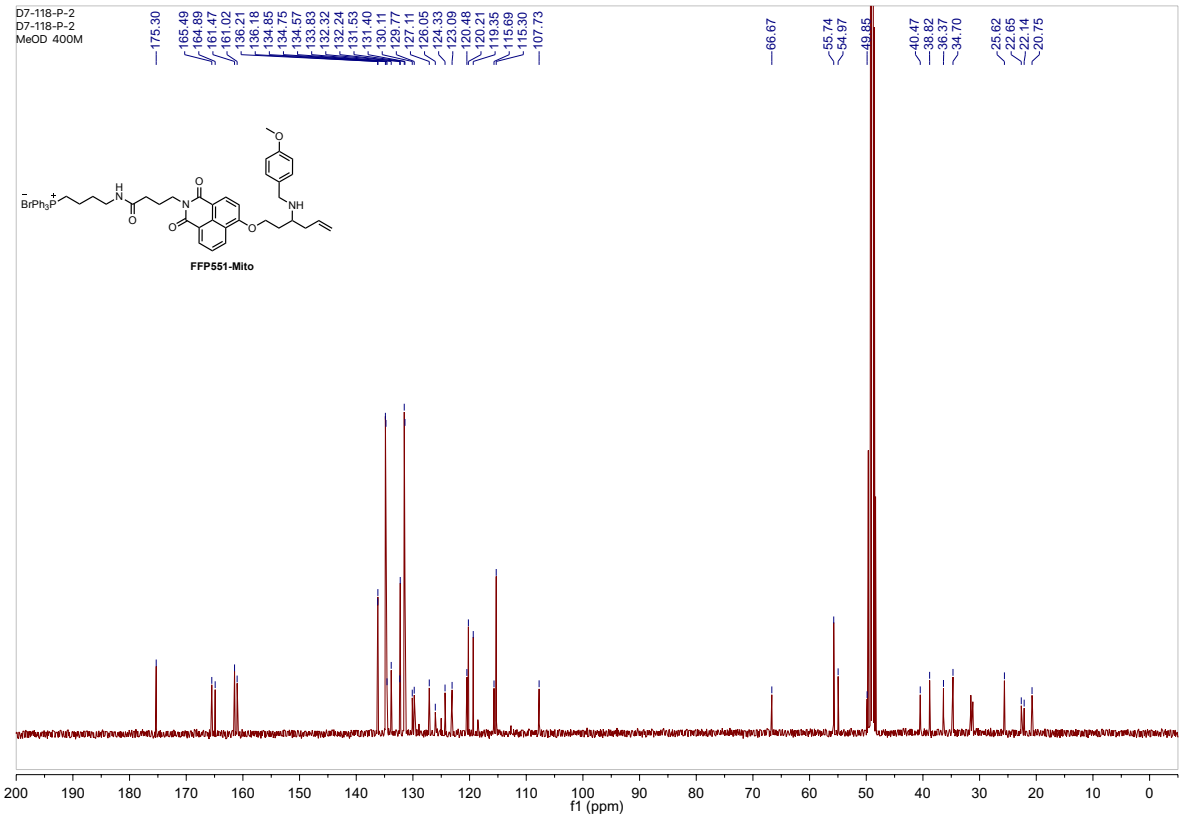
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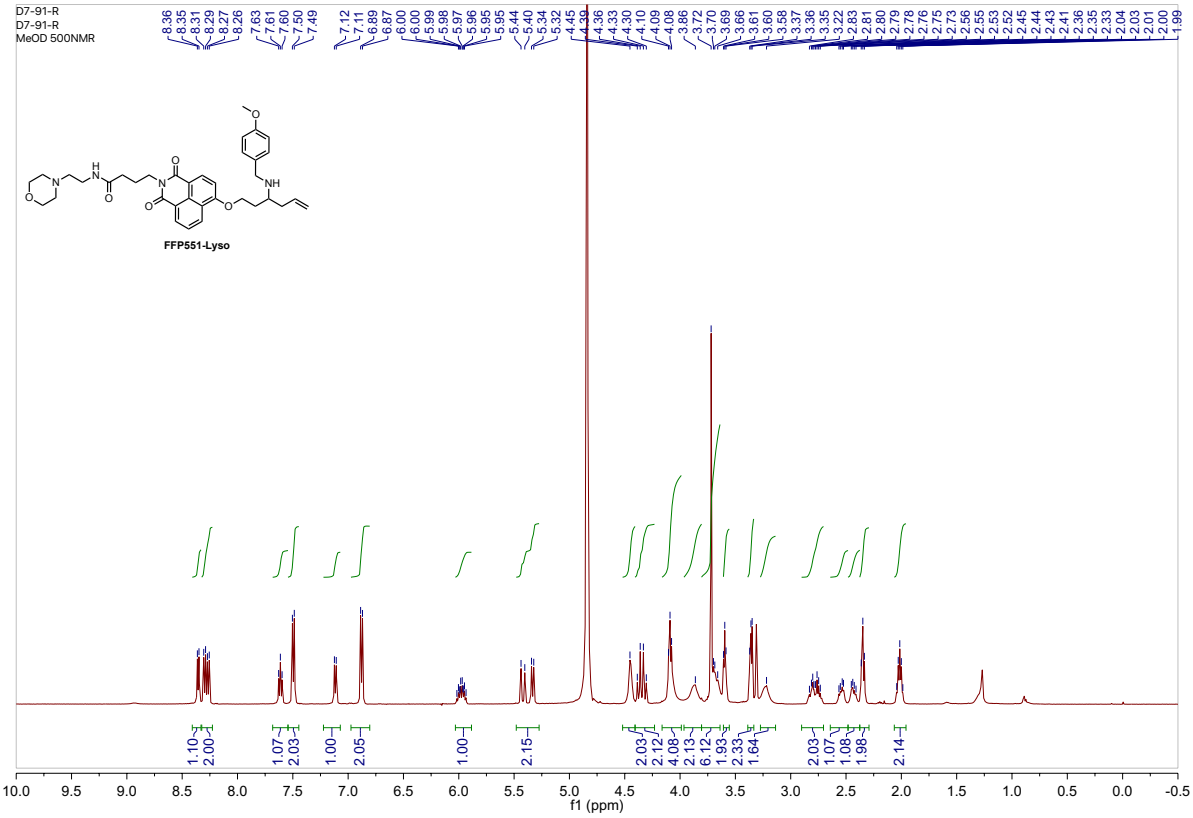
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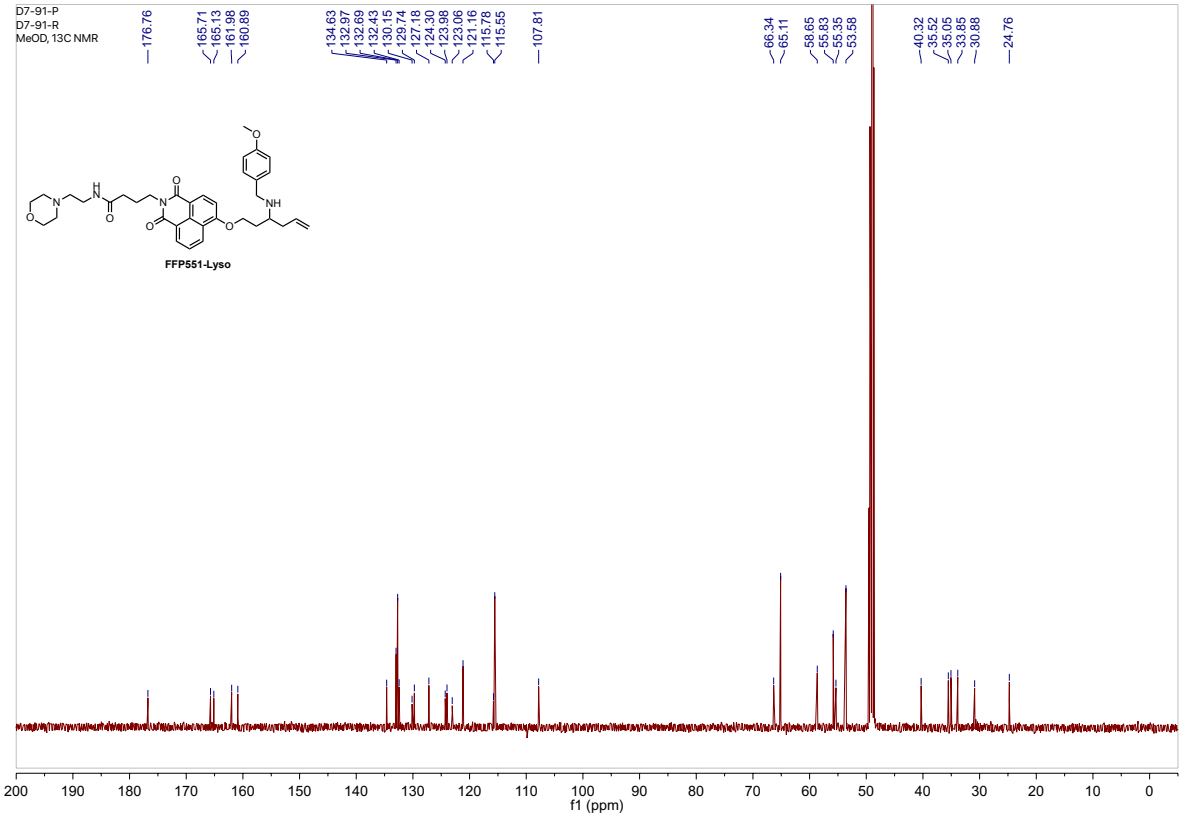
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f1 (ppm)