Conformational Restraint as a Strategy for Navigating towards Lysosomes

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SUPPORTING INFORMATION

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General Experimental for chemistry

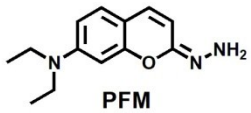
Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Anhydrous toluene was distilled from Na prior to use. Dry CH_2Cl_2 were distilled from CaH_2. Reactions were monitored by thin layer chromatography using TLC Silica gel 60 F254 supplied by Qingdao Puke Seperation Meterial Corporation, Qingdao, P. R. China. Silica gel for column chromatography was 200-300 mesh and was supplied by Qingdao Marine Chemical Factory, Qingdao, P. R. China. Characterization of intermediates and final compounds was done using NMR spectroscopy and mass spectrometry. Proton NMR spectra was recorded on a Brucker 500 (400 or 500 MHz) spectrometer. ^{13}C NMR was recorded on a Brucker 500 (100 or 125 MHz) spectrometer. The spectra were calibrated using residual undeuterated solvent as the internal reference ($CDCl_3$: ^1H NMR=7.26, ^{13}C NMR=77.16). The following abbreviations were used to designate multiplicities: s=singlet, d=doublet, t=triplet, m=multiplet. All spectra were recorded at 25°C and chemical shifts were given in ppm and coupling constants (*J*) in Hz. High-resolution mass data were obtained on an Agilent 6224 TOF LC/MS spectrometer using ESI-TOF (electrospray ionization-time of flight). UV-Vis spectra were taken on a HITACHI U-3010 Spectrophotometer. Fluorescence measurements were performed on an Agilent Cary Eclipse Fluorescence Spectrophotometer with slit widths to be 5 and 5 nm for excitement and emission respectively except otherwise indicated, and the photomultiplier (PMT) detector voltage was set at medium.

General procedures for probe synthesis

The coumarin with appropriate 7-amino substitution (1.0 eq, 0.12 M) was reacted with Lawesson's reagent (2.0 eq) in dry toluene under reflux with an inert atmosphere of nitrogen for 6 h. After being cooled to ambient temperature, the volatile parts was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel eluted with CH_2Cl_2 to give the intermediate with the lactone group thionated, which may be used directly for the next step.

The thionated intermediate (1.0 eq, 0.08 M) was dissolved in ethanol, to which under an inert atmosphere of nitrogen was added hydrazine hydrate (99%, 4.0 eq). The mixture was heated to reflux and kept under reflux for 2 h, then was cooled to ambient temperature. After removal of ethanol by rotary evaporation, the residue was purified by column chromatography on silica gel eluted with CH_2Cl_2 to give the desired probe as a reddish brown solid.

Probe structure characterization



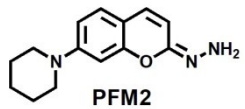
PFM

Yield: 66% in two steps

^1H NMR (500 MHz, $CDCl_3$, 25°C): δ 6.98 (d, *J* = 8.8 Hz, 1H), 6.65 (d, *J* = 9.6 Hz, 1H), 6.37 (m, 2H), 5.98 (d, *J* = 9.7 Hz, 1H), 3.37 (q, *J* = 7.1 Hz, 4H), 1.19 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (125 MHz, $CDCl_3$, 25°C): δ 154.60, 148.99, 146.10, 128.51, 127.84, 113.43, 108.88, 106.82, 97.90, 44.64, 12.66.

ESI-HRMS (*m/z*): $[M+H]^+$ calc'd. for $C_{13}H_{18}N_3O$: 232.1450, found 232.1458.



PFM2

Yield: 83% in two steps

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 (d, *J* = 9.1 Hz, 1H), 6.60 (m, 3H), 6.01 (d, *J* = 9.7 Hz, 1H), 5.08 (brs, 2H), 3.40 – 3.08 (m, 4H), 1.81 – 1.46 (m, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 154.31, 152.88, 145.83, 128.18, 127.53, 115.18, 111.47, 110.72, 101.79, 49.59, 25.50, 24.39.

ESI-HRMS (*m/z*): $[M+H]^+$ calc'd. for $C_{13}H_{18}N_3O$: 244.1450, found 244.1448.

Yield: 70% in two steps

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.71 (s, 1H), 6.62 (d, *J* = 8.4 Hz, 1H), 6.31 (s, 1H), 5.97 (d, *J* = 9.3 Hz, 1H), 3.35 (m, 4H), 2.68 (m, 2H), 1.93 (m, 2H), 1.6 (m, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 153.30, 147.01, 146.34, 129.05, 127.06, 117.93, 113.13, 108.76, 96.83, 48.46, 45.75, 27.52, 22.18, 10.94..

ESI-HRMS (*m/z*): $[M+H]^+$ calc'd. for $C_{13}H_{18}N_3O$: 244.1450, found 244.1452.

Yield: 86% in two steps

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.59-6.57 (m, 2H), 5.93 (d, *J* = 9.7 Hz, 1H), 3.18-3.15 (m, 4H), 2.82 (t, *J* = 6.6 Hz, 2H), 2.69 (t, *J* = 6.4 Hz, 2H), 2.02 – 1.90 (m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 149.37, 146.65, 144.17, 129.22, 124.60, 116.35, 113.00, 108.98, 107.65, 50.07, 49.54, 27.28, 21.96, 21.13, 20.49.

ESI-HRMS (*m/z*): $[M+H]^+$ calc'd. for $C_{15}H_{18}N_3O$: 256.1450, found 256.1498.

General experimental for photophysical property characterization

All the photophysical characterization experiments were carried out at ambient temperature. Deionized water was used to prepare all aqueous solutions. Phosphate buffer saline (PBS, 10 mM) was purged with nitrogen for 5 min before use. **PFM4** was dissolved in DMSO to make a 5 mM stock solution. Stock solutions of FA and other bio-relevant species were prepared by dissolving commercial chemicals in deionized water or DMSO.

To test the fluorescent response of **PFM4** towards FA or other reactive species, aliquots of probe stock solutions were diluted with PBS and treated with analytes to make sure both probes and analytes were kept at desired final concentrations. After quick and vigorous shaking, the mixture was allowed standing in the dark for desired time and then the fluorescence spectra were taken under excitation at 451 nm. All fluorometric experiments were performed in triplicate.

Cell culture

The human umbilical vein cell line, EA.hy926, was purchased from ATCC (CRL-2922). The EA cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fatal bovine serun (Invitrogen), penicillin (100 U/mL, Invitrogen), and streptomycin (100 U/mL, Invitrogen). The cultures were maintained at 37 ^oC in a 95% humidified atmosphere with 5% CO_2.

Cytotoxicity assay

EA cells were seeded in 96-well plates at a density of 5×10^3 cells/well at 37 °C in a 95% humidified atmosphere with 5% CO_2 for 24 h. After washing with PBS twice, **PFM4** with concentration of 1 μM, 5 μM, 10 μM, 20 μM, and 40 μM were added to the cells, which were allowed an incubation period of 24 h. After introducing 10 μL of CCK8 solution for 1 h, the absorption at 450 nm was measured by Microplate Spectrophotometer (MD I3X). Each experiment was repeated three times, and the average values were taken in analyses.

Flow cytometric analysis

Samples of EA cells for flow cytometry were prepared by passaging and seeding in 6-well plates before experiments. Cells of 70% confluency were trypsinized, pelleted *via* centrifugation, resuspended in medium. For analysis of exogenous FA in live system, cells were first incubated with 40 μM, 200 μM, 400 μM, 1 mM, 2 mM FA at 37 °C for 30 min, and then stained with 5 μM **PFM4** for 15 min at 37 °C. For analysis of endogenous FA in living EA cells, cells were incubated with 5 μM **PFM4** with or without 200 μM $NaHSO_3$ pre-treatment. Excitation was provided by the 488 nm HeNe laser. For analysis of endogenous FA in living EA cells upon Amyloid-β (Aβ) (1-42) treatment, cells were pre-treated with 20 μM Aβ(1-42), 10 mM N-acetyl-L-cysteine (NAC), or co-incubation of Aβ(1-42) and NAC for 24h. After that, cells were washed with PBS (pH 7.4) and then treated with **PFM4** (5 μM) for 15 min at 37℃. Each plot represented 10,000 viable cells, non-viable cells were excluded from flow cytometry analysis by appropriate gating. All data analyses were carried out using FCS Express V3 (De Novo Software).

Confocal fluorescence imaging

For confocal fluorescent imaging experiments to detect exogenous FA in living system, cells were incubated with 200 μM FA for 30 min, and then washed with PBS (pH 7.4), following treated with 5 μM **PFM4** for 15 min. For the fluorescence imaging experiments of endogenous FA in living cells, EA cells were incubated without or with 200 μM $NaHSO_3$ for 30 min, and then washed with PBS (pH 7.4), followed by 5 μM **PFM4** incubation for 15 min. The residual probe was washed three times by PBS (pH 7.4) before imaging. Fluorescence was obtained with a confocal laser scanning microscope (Olympus, FV1000). For confocal microscopy imaging of subcellular distribution of **PFM4** in living cells, EA Cells were incubated with 5 μM **PFM4** for 15 min at 37 °C and followed by staining with 1 μM of Lyso-trackers/Mito-tracker/ER-tracker for 15min, then washed with PBS (pH 7.4). The **PFM4** fluorescence was monitored at λ_{em}475-560nm (λ_{ex}=458 nm). Fluorescent signal from Lyso-tracker Red/Mito-tracker/ER-tracker was obtained at λ_{em} 587-680nm (λ_{ex}=543 nm). For visualization of endogenous FA in EA cells native or upon ER stress, cells were incubated with 5 μM **PFM4** after being treated with thapsigargin (TG, 5 μM), and fluorescence were obtained with a confocal laser scanning microscope. For confocal fluorescent imaging experiments to detect endogenous FA in living EA cells upon Aβ(1-42) treatment, cells were pre-treated with 20 μM Aβ(1-42), 10 mM NAC, or co-incubation of Aβ(1-42) and NAC for 24h. After that, cells were washed with PBS (pH 7.4) and then treated with **PFM4** (5 μM, green) for 15 min at 37 °C. Fluorescence was obtained after **PFM4** incubation for 15 min. Digital images were captured using the FV10-ASW 3.0 viewer software (Olympus). Cell counts were performed using a 40× or 60× objective in at least five fields of view randomly selected from each coverslip. At least 3 independent experiments were counted. The fluorescence density was analyzed using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was carried out by using GraphPad InStat 6.0 software. Unpaired two-tailed Student's *t*-test was used for comparing data from two populations, and one-way analysis of variance (ANOVA) was used for multiple group comparisons. Data were expressed as mean ± (SD or SEM, where appropriate). The value $P<0.05$ was considered statistically significant.

Supplementary figures

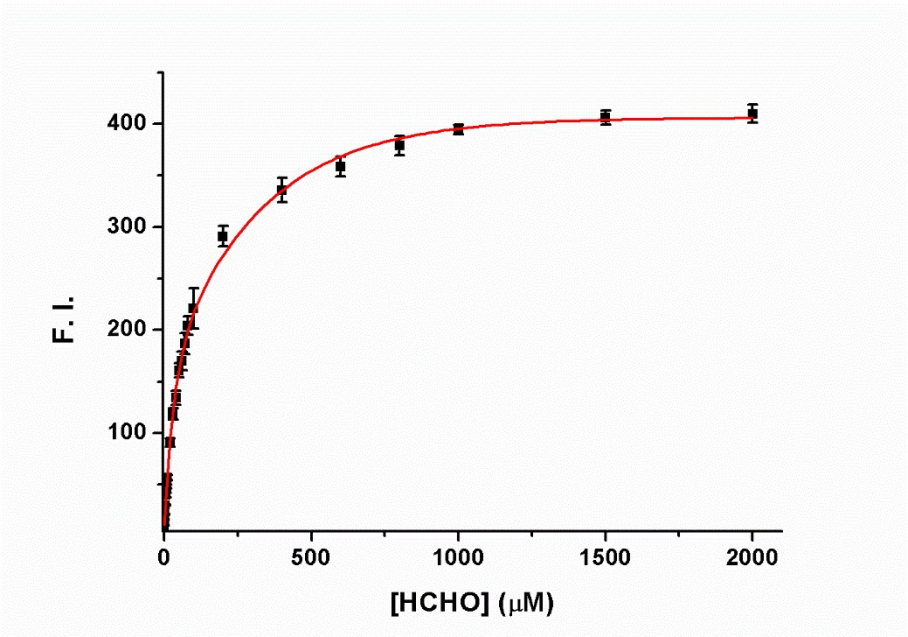


Fig. S1 FA-dose dependent increase of **PFM4** fluorescence. Data shown were the emission (520 nm) of **PFM4** (5 μM) in PBS (pH 7.4, 10 mM) after being incubated with FA of indicated concentration for 30 min.

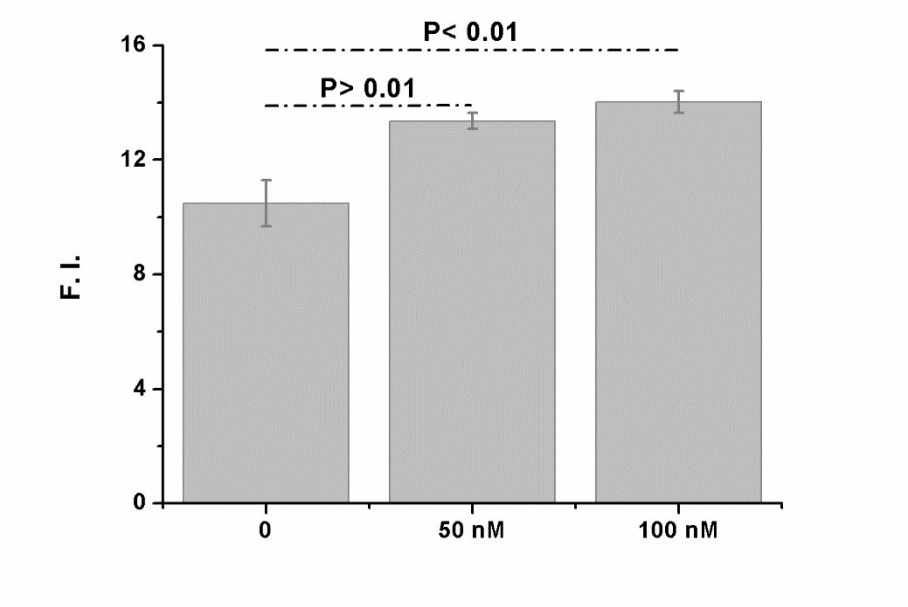


Fig. S2 Limit of detection of **PFM4**. Results were obtained as the concentration of FA that induced a statistically significant increase in fluorescence intensity at 520 nm compared with a blank control after 30 min with a *p*-value < 0.01. Experiments were carried out by incubating **PFM4** (10 μM) with FA (0, 50, 100 nM) in PBS (10 mM, pH 7.4) at ambient temperature for 30 min and then collecting the emission at 520 nm by excitation at 451 nm. Statistical analyses were performed with a two-tailed Student's *t*-test (n = 3). Error bars are standard deviation.

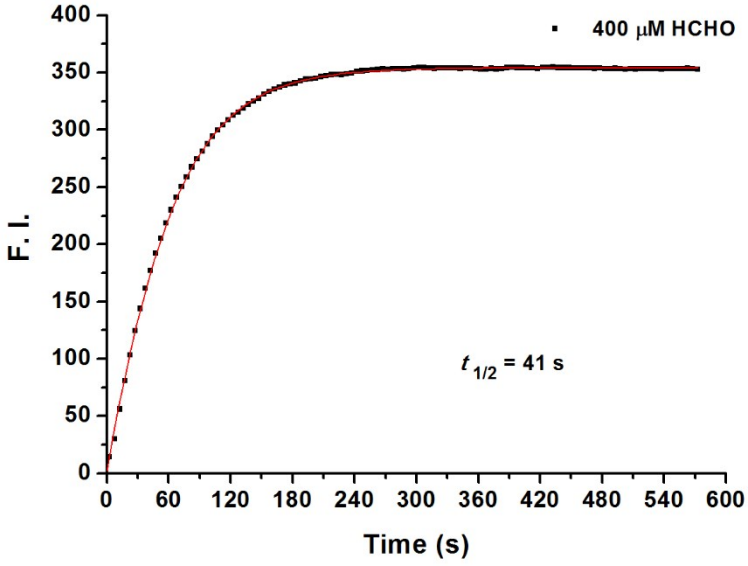


Fig. S3 Detection kinetic of **PFM4** (5 μM) towards FA at 400 μM. Data were the time-lapsed emission (520 nm) of **PFM4** (5 μM) in PBS (pH 7.4, 10 mM) after being treated with FA of indicated concentration.

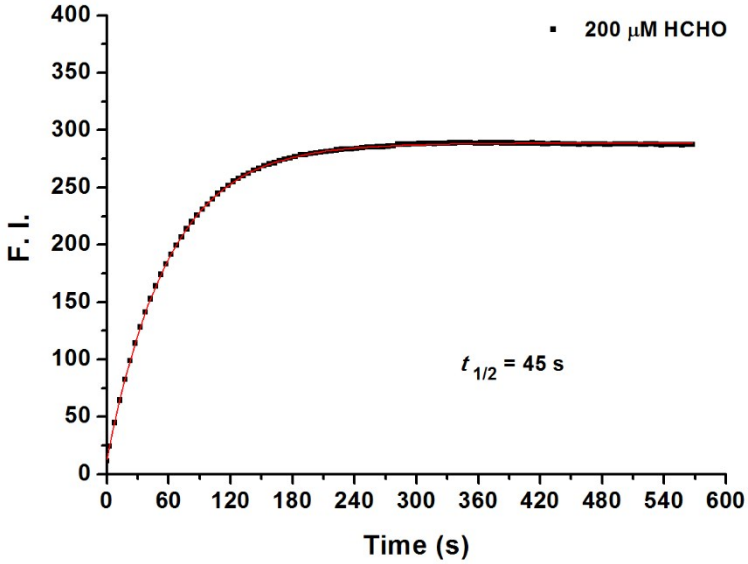


Fig. S4 Detection kinetic of **PFM4** (5 μM) towards FA at 200 μM. Data were the time-lapsed emission (520 nm) of **PFM4** (5 μM) in PBS (pH 7.4, 10 mM) after being treated with FA of indicated concentration

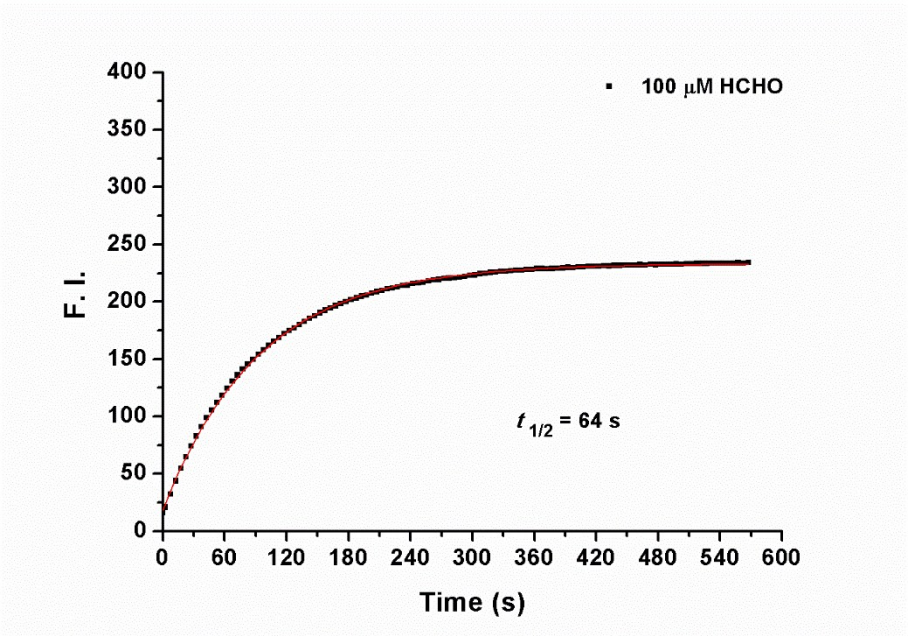


Fig. S5 Detection kinetic of **PFM4** (5 μM) towards FA at 100 μM. Data were the time-lapsed emission (520 nm) of **PFM4** (5 μM) in PBS (pH 7.4, 10 mM) after being treated with FA of indicated concentration

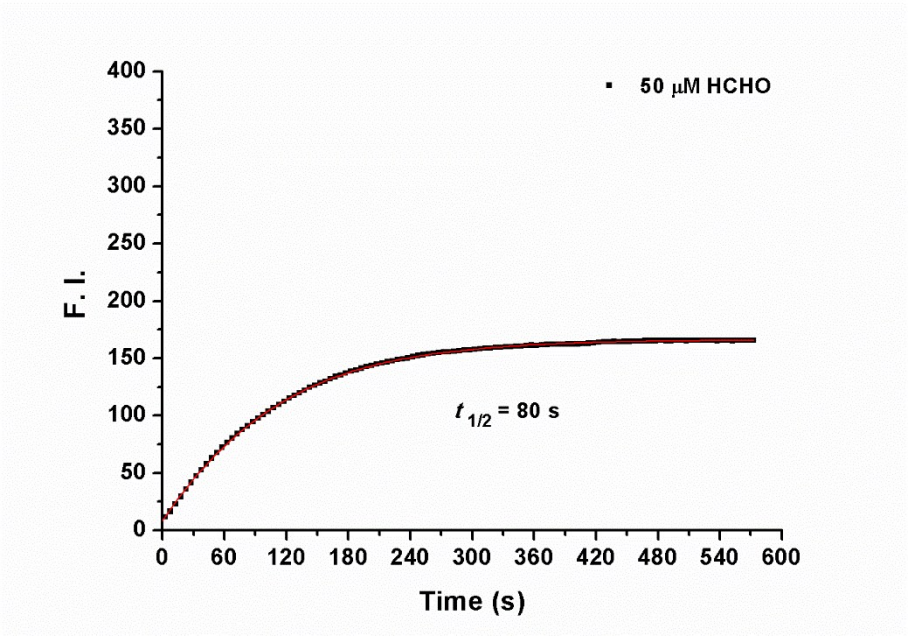


Fig. S6 Detection kinetic of **PFM4** (5 μM) towards FA at 50 μM. Data were the time-lapsed emission (520 nm) of **PFM4** (5 μM) in PBS (pH 7.4, 10 mM) after being treated with FA of indicated concentration

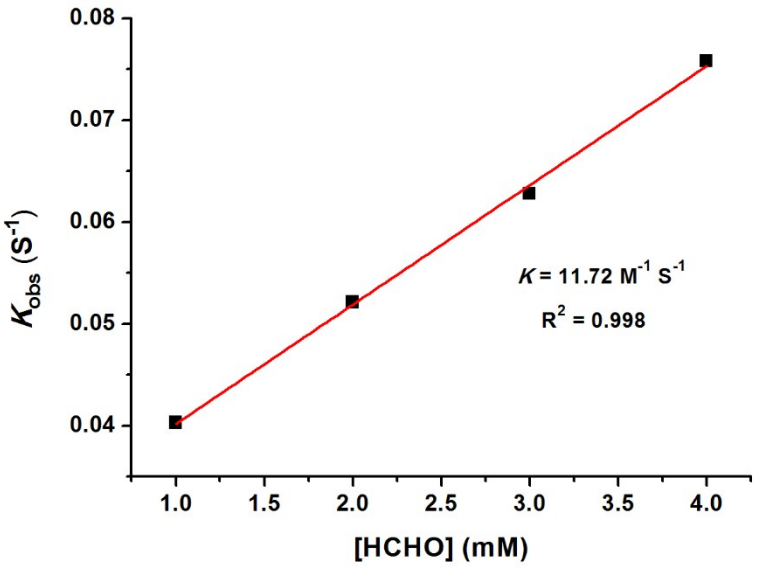


Fig. S7 Response kinetics of **PFM4** towards FA. Reactivity of **PFM4** with FA in PBS at ambient temperature displaying the linear dependence of the observed rates on FA concentration.

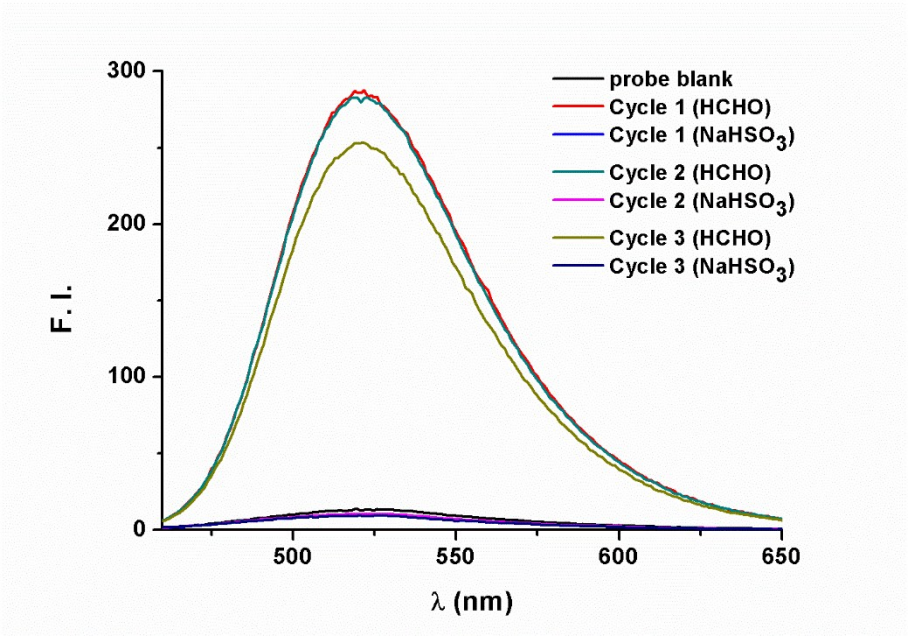


Fig. S8 Fluorescent spectra of **PFM4** (5 μM) in PBS (10 mM, pH 7.4) after repetitively subsequent treatment of FA (200 μM) and $NaHSO_3$ (200 μM).

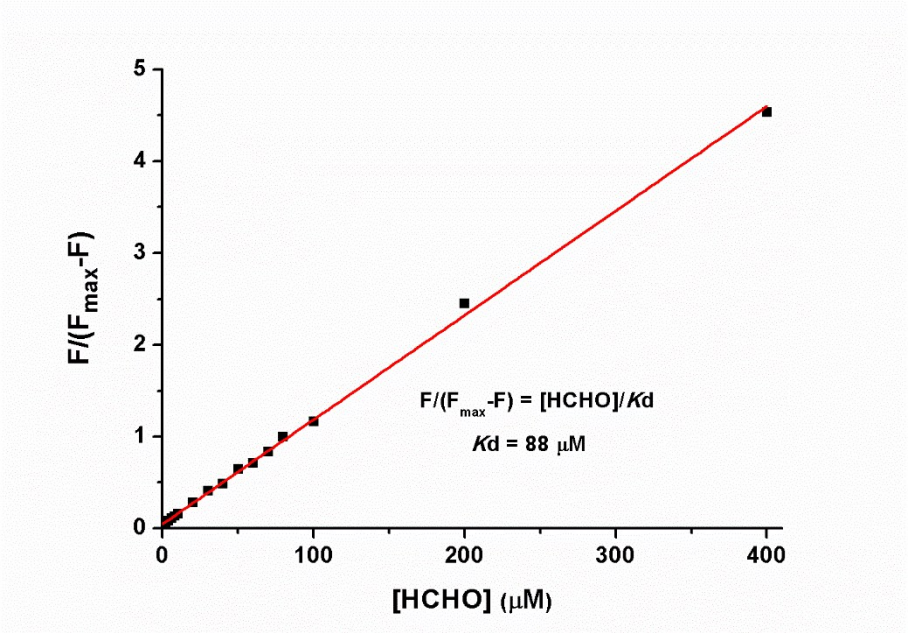


Fig. S9 Plot of Kd. F_{max} was the maxium fluorescent intensity (520 nm) of **PFM4** (5 μM) after the treatment of a large enough amount of FA, and F is the fluorescence (520 nm) after the treatment of indicated amount of FA.

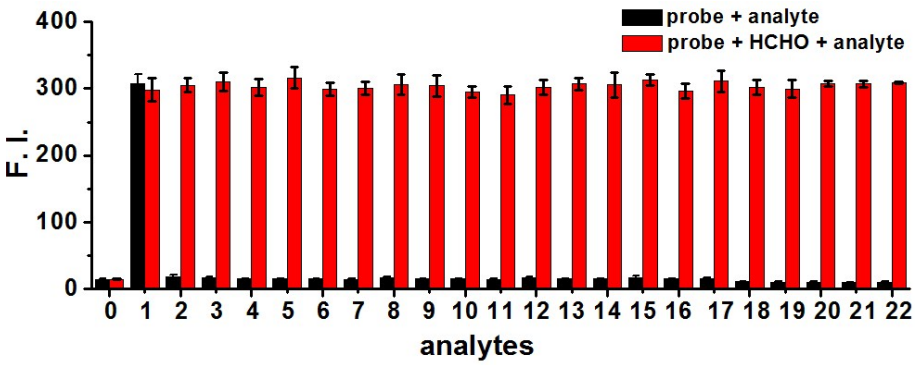


Fig. S10 Emission (520 nm) of **PFM4** (5 μM) upon treatment with various analytes (200 μM): (0) probe blank (1) FA (2) acetaldehyde (3), malonaldehyde (4) ascorbic acid (5) glucose (6) glucosone (7) oxalic acid (8) pyruvate (9) methylglyoxal (10) glyoxal (11), *p*-methoxybenzaldehyde (12) trichloroacetaldehyde (13) *p*-nitrobenzaldehyde (14) acetone (15) HClO (16) H_2O_2 (17) GSH (18) α-Ketoglutaric acid (19) malic acid (20) Citric Acid (21) Succinic Acid (22) lactic acid.

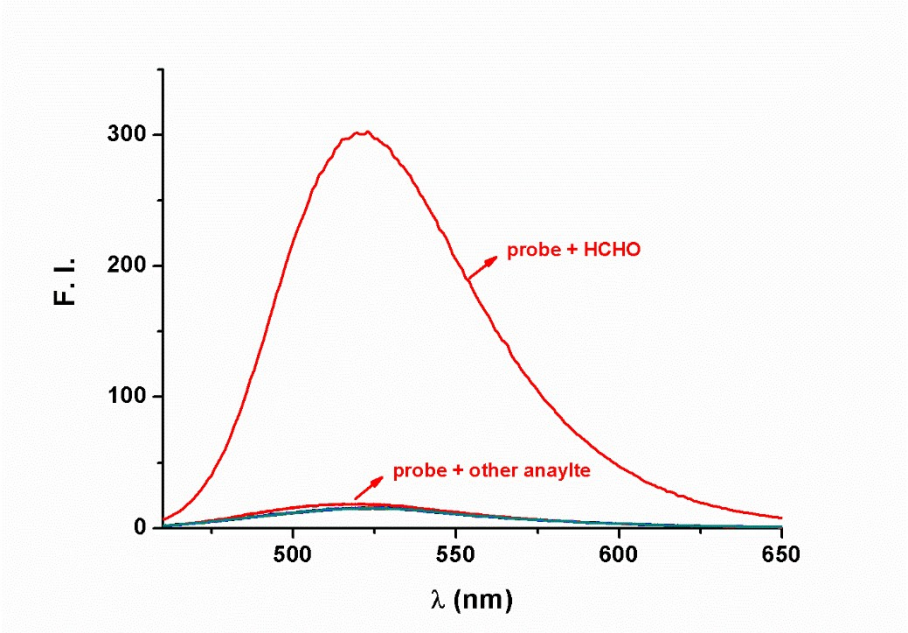


Fig. S11 Fluorescence spectra of **PFM4** (5 μM) in PBS (10 mM, pH 7.4) after the treatment of various analytes.

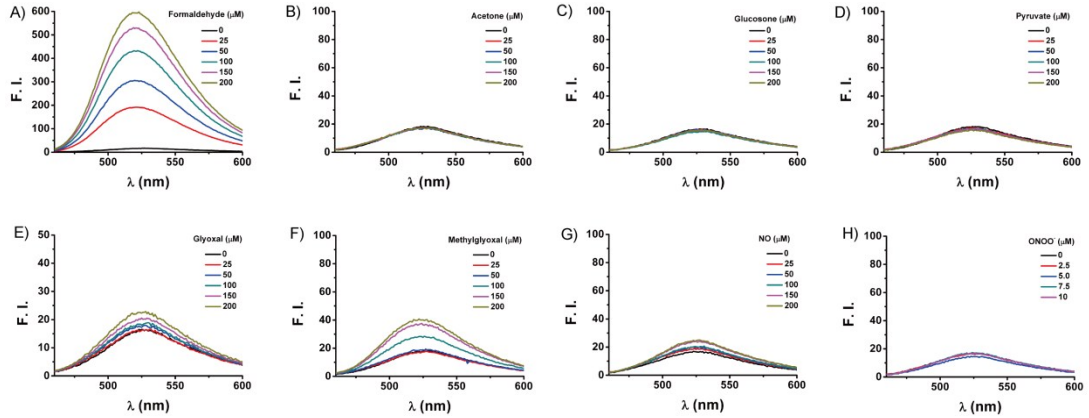


Fig. S12 Fluorescence spectra of **PFM4** (10 μM) after the treatment of various analytes at indicated concentrations. Spectra were taken after 30 min of incubation in PBS (10 mM, pH 7.4) at ambient temperature.

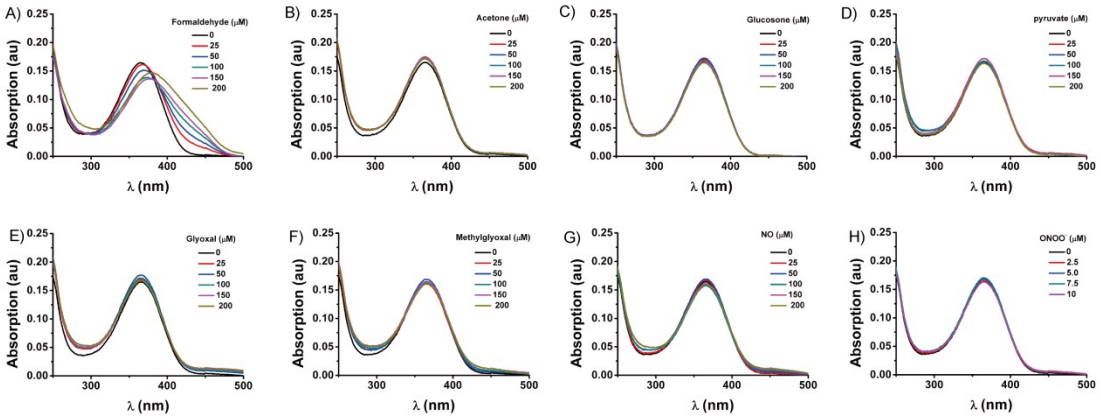


Fig. S13 UV-Vis spectra of **PFM4** (10 μM) after the treatment of various analytes at indicated concentrations. Spectra were taken after 30 min of incubation in PBS (10 mM, pH 7.4) at ambient temperature.

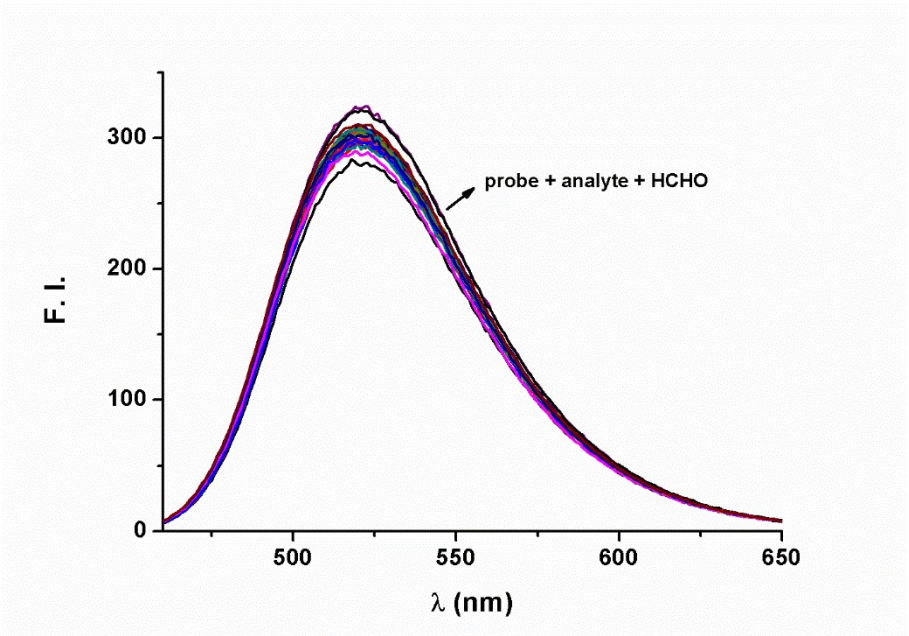


Fig. S14 Fluorescence spectra of **PFM4** (5 μM) in PBS (10 mM, pH 7.4) in the co-presence of FA and other analytes.

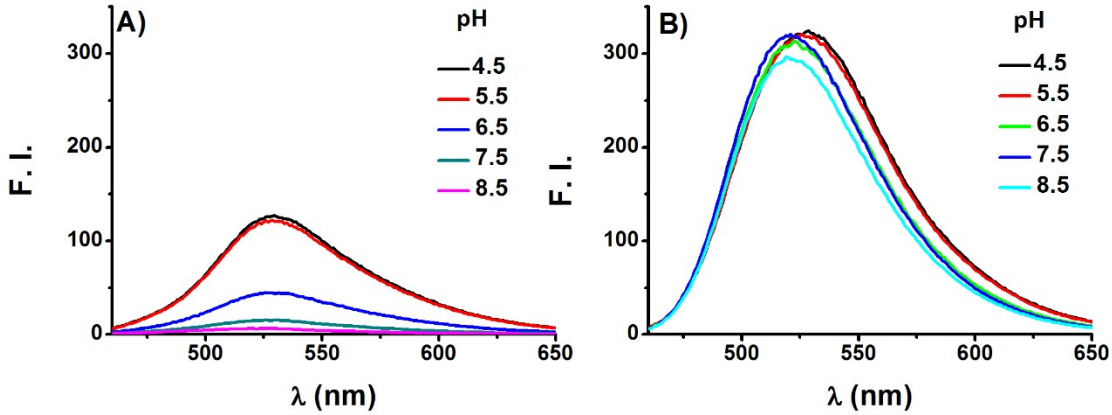


Fig. S15 (A) Fluorescence spectra of **PFM4** (5 μM) in PBS of various pH. (B) Fluorescence spectra of **PFM4** (5 μM) after the treatment of FA (200 μM) in PBS of various pH.

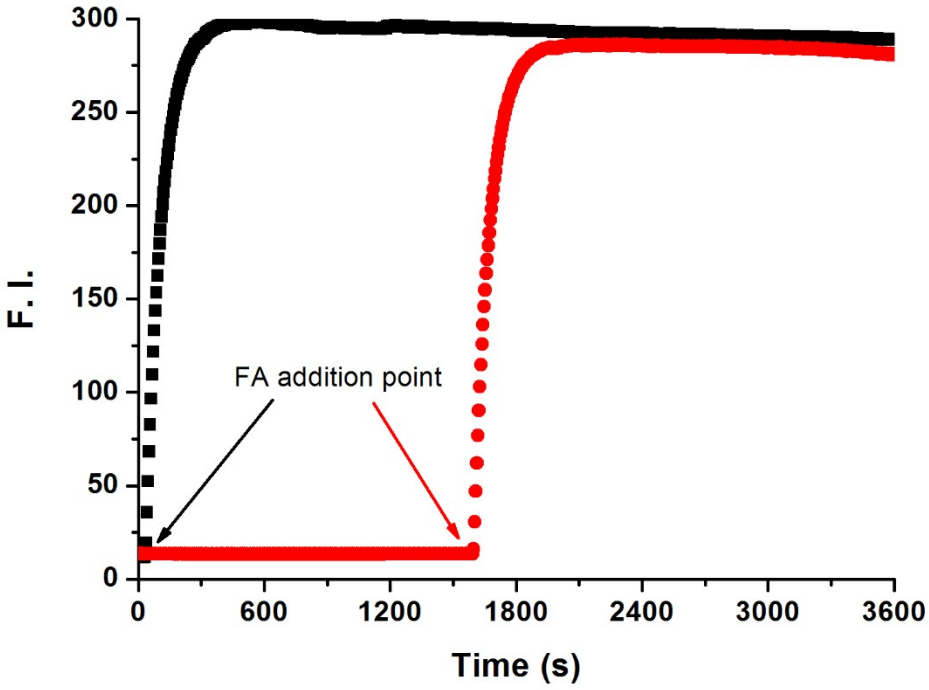


Fig. S16 Emission (520 nm) of **PFM4** (5 μM) before and after the treatment of FA (200 μM) under continuous irradiation.

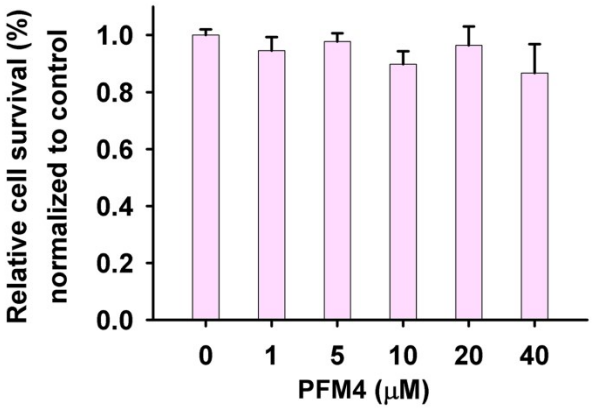


Fig. S17 Cytotoxicity of **PFM4** was assessed in EA cells by CCK8 assays. **PFM4** (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM) were added and incubated for 24 h in EA cells. After introducing CCK8 (10 μL) solution in a 95% humidified atmosphere with 5% CO_2 (37 ^oC) for 1 h, the absorption at 450 nm was measured by SpectraMax i3x.

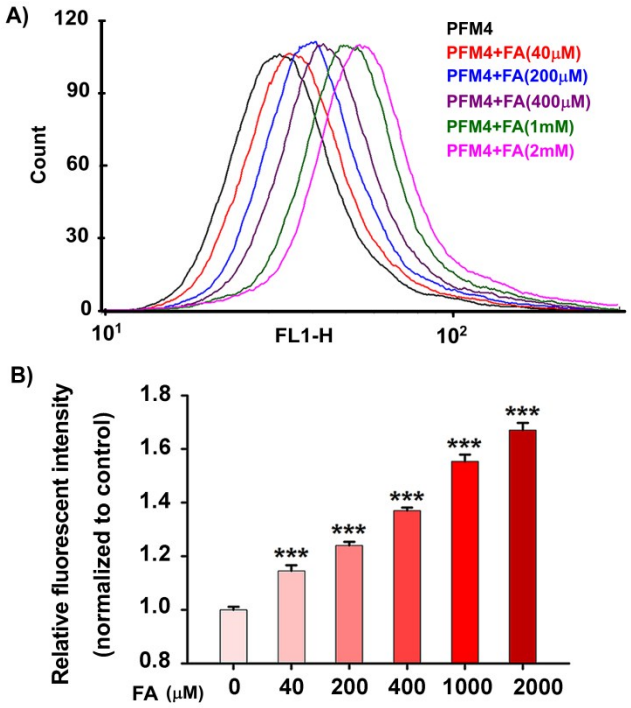


Fig. S18 Flow cytometry analysis of exogenous FA in living EA cells. Cells were stained with FA (40 μM, 200 μM, 400 μM, 1 mM and 2 mM) at 37 °C for 30 min, and then incubated with **PFM4** (5 μM) for 15 min. A) The **PFM4** fluorescence was monitored at 515-545 nm (λ_{ex}=488 nm). B) The endogenous FA in live cells quantified as the percentage of relative fluorescence intensity by flow cytometry analysis. Each plot represented 10,000 viable cells (non-viable cells were excluded from flow cytometry analysis by appropriate gating). Data were expressed as mean ± S.E.M., *n*=5, ***P < 0.001 *versus* **PFM4**.

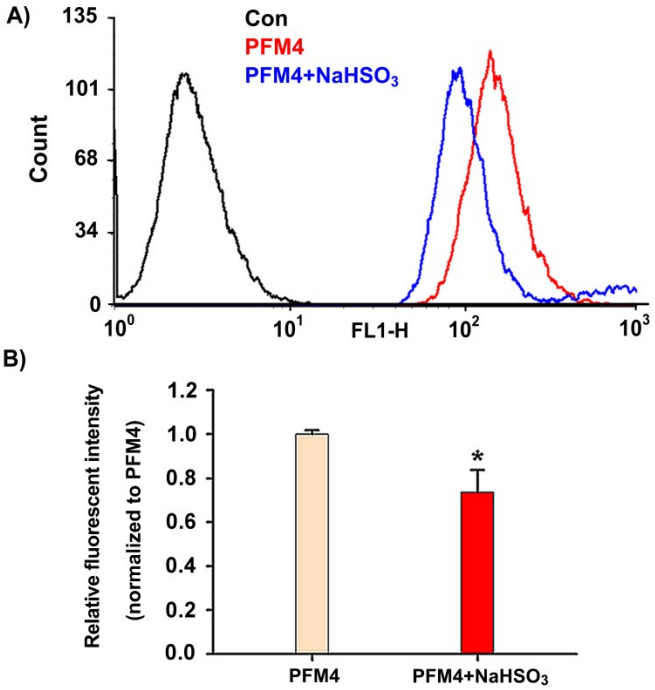


Fig. S19 Flow cytometry analysis of endogenous FA in living EA cells. A) Cells were stained with **PFM4** (5 μM) for 15 min at 37 °C, or pretreatment with $NaHSO_3$ (200 μM) for 1 h at 37 °C, and then stained with **PFM4** (5 μM) for 15 min. The **PFM4** fluorescence was monitored at 515-545 nm (λ_{ex}=488 nm). B) Quantification of fluorescence

intensity by flow cytometry analysis. Each plot represented 10,000 viable cells (non-viable cells were excluded from flow cytometry analysis by appropriate gating). Data were expressed as mean ± S.E.M., n=5, *P < 0.05 *versus* **PFM4**.

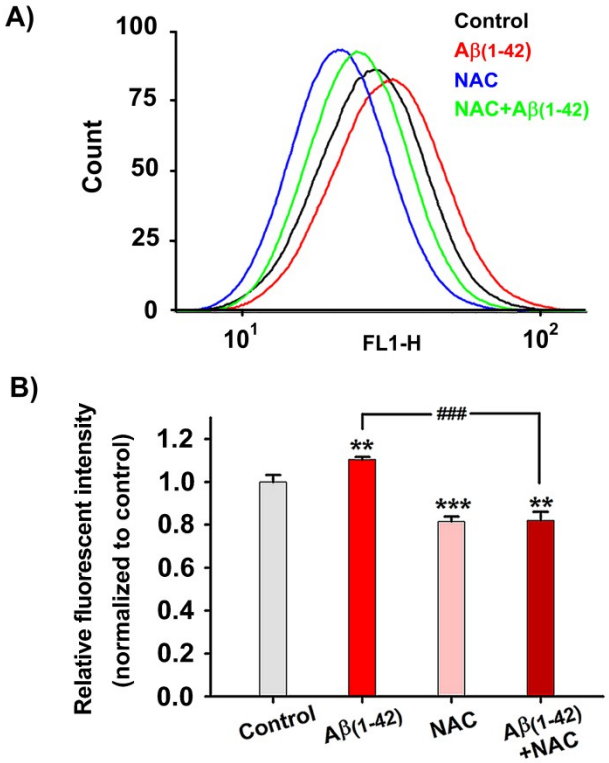


Fig. S20 Flow cytometry analysis of endogenous FA in living EA cells upon Aβ(1-42) treatment. EA cells were pre-treated with 20 μM Aβ(1-42), NAC, or co-incubation of Aβ(1-42) and 10 mM NAC for 24 h. After that, cells were washed with PBS (pH 7.4) and then treated with **PFM4** (5 μM, green) for 15 min at 37 °C. A) The **PFM4** fluorescence was monitored at 515-545 nm (λ_{ex}=488 nm). B) Changes of endogenous FA in live cells quantified as the percentage of relative fluorescence intensity by flow cytometry analysis. Each plot represented 10,000 viable cells (non-viable cells were excluded from FACS analysis by appropriate gating). Data were expressed as mean ± S.E.M., n=5, **P<0.01, ***P<0.001 *versus* **PFM4** alone, ###P<0.001 *versus* Aβ(1-42).

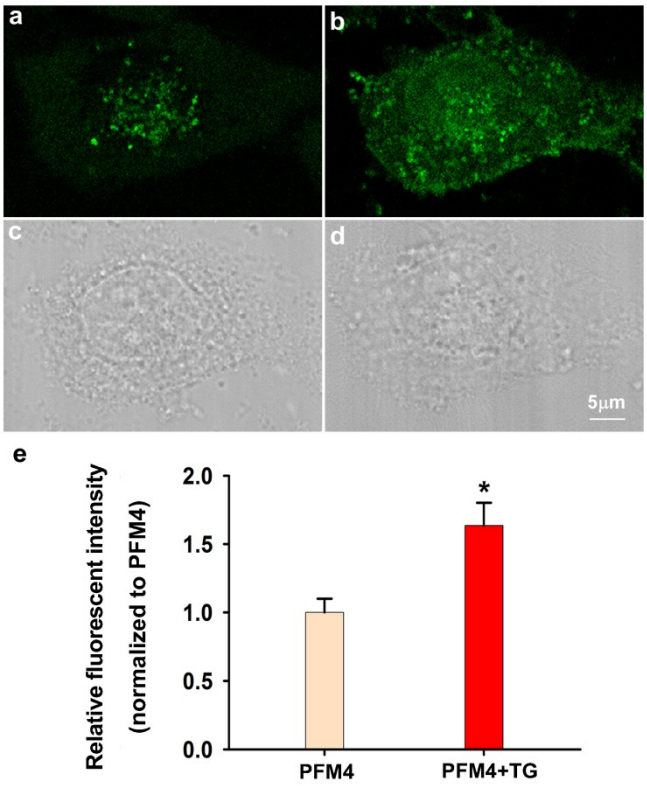
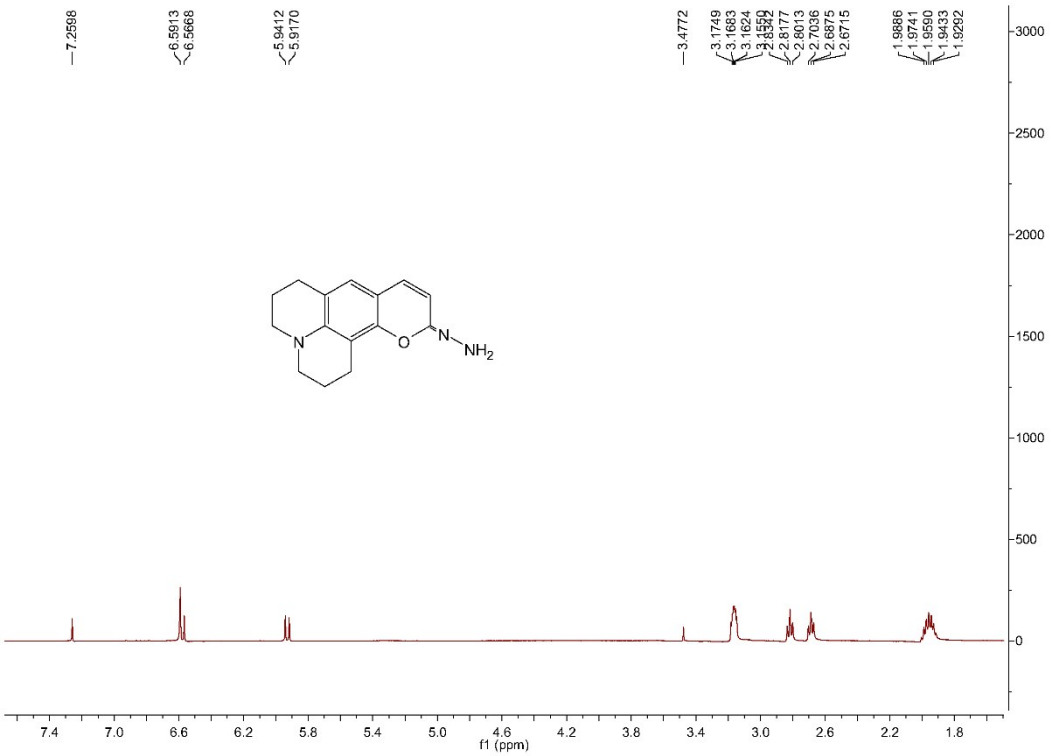
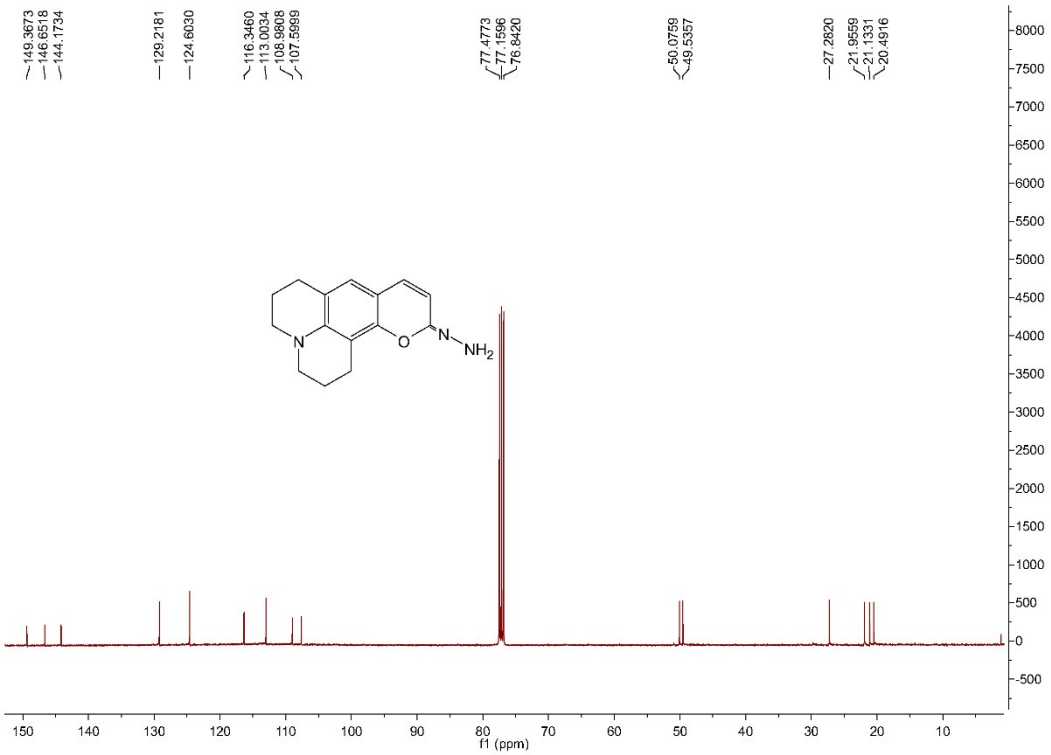


Fig. S21 Imaging endogenous FA in living EA cells upon ER stress. EA cells were incubated with **PFM4** (5 μM, green) for 15 min at 37 °C, and then washed with PBS (pH 7.4) followed by thapsigargin (TG, 5 μM) incubation for 60 min. Fluorescence was obtained after TG incubation. a, c) EA cells treated with **PFM4** only. b, d) EA cells treated with **PFM4,** and following incubation with TG. Scale bar=5 μm. e) Quantification of image data. Data were expressed as mean ± S.E.M., a minimum of 3 images for each condition were quantified and averaged, **P*<0.05 *versus* **PFM4** alone. The **PFM4** fluorescence was monitored at 515-545 nm (λ_{ex}=458 nm).





NMR traces of PFM4