Supporting Information for

Development of a mitochondrial-targeted two-photon fluorescence turn-on probe for formaldehyde and its bio-imaging applications in living cells and tissue

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Materials and instruments

All of the reagents used are commercially available and purchased from commercial suppliers and used directly without further purification. All chemical reagents mentioned have potential danger and harm to health, they should be manipulated carefully and recovered, and should not be discard for environmental protection. The solvents were dried with sodium or calcium hydride and purified by distillation before use. Double distilled water was used throughout all experiments and test procedure. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals. The ^{1}H NMR and ^{13}C NMR data was collected on AVANCE III 400 MHz Digital NMR Spectrometer and tetramethylsilane (TMS) was used as internal standard. Proton chemical shifts are reported in parts per million downfield form TMS. High resolution electrospray mass (HRMS) were recorded on Apex-Ultra. Electronic absorption spectra were recorded on Shimadzu UV-2700 UV–vis spectrophotometer, fluorescent spectra were measured by Hitachi F4600 Fluorescent Spectrophotometer. Cell imaging was performed on Nikon A1MP Ti-e Fluorescence Microscopy. The pH value was determined on Mettler-Toledo pH Meter.

General procedure for the spectrum measurement

Unless otherwise noted, all the measurements were made according to the following procedure. The concentration of the probe stock solution was 1.0 mM in DMSO, and the analytes stock solutions were prepared in the ultrapure water at the appropriate concentration. In 5 mL volumetric flask, the test solution was prepared by placing 25 μL probe stock solution and 225 μL DMSO, requisite amount of analyte stock solution, then adjusted the final volume to 2 mL with PBS buffer. The spectrum tests were recorded with a 1 cm standard quartz cell at room temperature. The absorption spectra were obtained on a Shimadzu UV-2700 Power spectrometer. The

photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer. The excitation wavelength was 440 nm, the excitation slit widths were 5 nm, and the emission slit widths were 5 nm.

Cells culture

The HeLa cells were cultured in 35 mm glass-bottom culture dishes with DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. Before the imaging experiments, the cells were washed with PBS for 2-3 times.

Fluorescence imaging of FA in living cells

We were divided into three groups for the exogenous FA imaging experiments: the first group of cells were cultured with 5 μM **MT-FA** for 20 min; the second group of cells were pre-cultured with 150 μM FA for 20 min, and then cultured with 5 μM **MT-FA** for another 40 min; the third group of cells were pre-cultured with 150 μM FA and 300 μM $NaHSO_3$ for 20 min, then the cells cultured with 5 μM **MT-FA** for another 40 min.

The endogenous FA imaging experiments were also divided into three groups: the first group of cells were cultured with 15 μM Thapsigargin (TG) for 30 min; the second group of cells were pre-cultured with 15 μM Thapsigargin (TG) for 30 min, and then cultured with 5 μM **MT-FA** for further 40 min; the third group of cells were pre-cultured with 15 μM Thapsigargin (TG) and 300 μM $NaHSO_3$ for further 30 min, then the cells were cultured with 5 μM **MT-FA** for another 40 min.

For the colocalization fluorescence imaging experiment, after the HeLa cells were incubated with 150 μM FA incubated for 20 min, the culture medium of the cells was changed to a fresh media containing 5 μM **MT-FA** and further incubated for 40 min. Then changed to a fresh media containing 500 nM Mito-tracker Deep Red for more 5 min. The residual probe was removed by washing three times using PBS

before the imaging experiment.

Cytotoxicity assays

HeLa cell line were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with10% FBS and 100 U/mL of penicillin and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were then seeded into 96-well plates, and then 0-50.0 μM of **MT-FA** (99.9% DMEM and 0.1% DMSO) was added respectively. Subsequently, the cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air for 24 h. Finally, the absorbance of the solution was acquired by using the microplate reader at 570 nm. The toxicity of **MT-FA** was calculated by the following formula.

$$\text{The cell viability (\%)} = (OD_s\text{-}OD_b)/(OD_c\text{-}OD_b)\times 100\ \%.$$

OD_s denotes the cells incubated with various concentrations of the probe, OD_c denotes the cells without the probe, OD_b denotes the wells containing only the culture medium.

Mice liver tissue slices preparation and fluorescent imaging

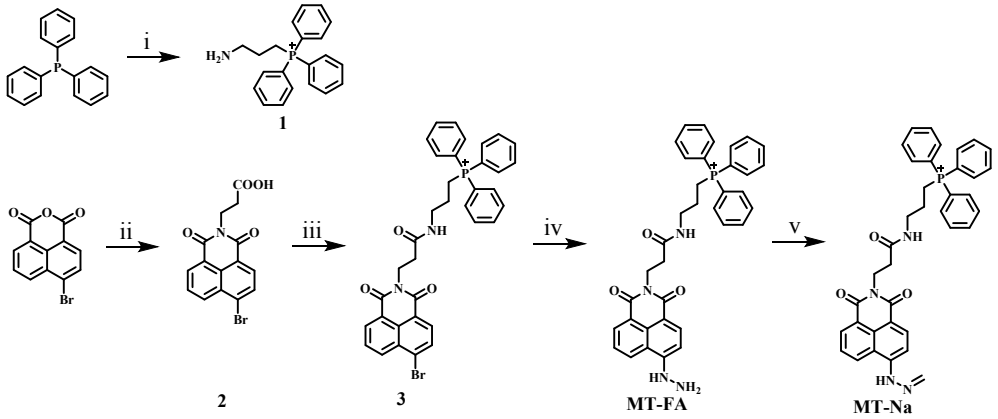
4 weeks old Kunming mice were purchased from Shandong University Laboratory Animal Centre (Shandong, China). The mice were killed by cervical vertebra dislocation, the liver tissues were cut into about 500 μm in size. For the control group, the liver tissue slices treated with **MT-FA** (10 μM) for 60 min. For the experimental group, the liver tissue slices pretreated with FA (300 μM) for 40 min, and then treated with **MT-FA** (10 μM) for another 60 min. Then the imaging experiments were carried out.

The fluorescence images were acquired by a Nikon A1MP Ti-e confocal microscopy. The OP and TP fluorescence emission were obtained at 500-550 nm under excitation of 488 nm and 800 nm, respectively.

Synthesis

The synthetic route of the probe **MT-FA** was shown in Scheme S1. The compound **1**, compound **2**, compound **3** were synthesized according to the previous literature[1].

Scheme S1. Synthesis of the probe **MT-FA.**



Reagents and conditions: (i) 3-Bromopropylamine Hydrobromide, acetonitrile, reflux, 24h; (ii) *β*-alanine, ethanol, 80 °C, 8h; (iii) Compound **1**, DCC and DMAP, dichloromethane, ambient temperature, 12 hours; (iv) 80% Hydrazine Hydrate, ethanol, 80 °C, 8h; (v) Formaldehyde, ethanol, ambient temperature, 60 min.

[1] B. Deng, M. Ren, J.-Y. Wang, K. Zhou and W. Lin, *Sens. Actuators, B.* 2017, **248**, 50-56.

Synthesis of compound MT-FA

Compound **3** (0.74 g, 1 mmol) was added into ethanol. Then 80 % hydrazine hydrate (0.16 g, 5 mmol) was added dropwise to the mixture with vigorous stirring. Then the reaction system stirred at 80 °C for 8 hours. After cooling to ambient temperature, the mixture was filtered and the crude product was washed three times. The crude product was purified by flash chromatography on silica gel (DCM/MeOH = 30:1) and re-crystallized from ethanol to afford an orange solid with ethanol to get a yellow powder as compound **MT-FA** with a yield of 79 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.62 (d, J = 8.4 Hz, 1H), 8.26 (d, J = 6.8 Hz, 1H), 8.12 (m, 2H), 7.92 (m, 3H), 7.80 (m, 12H), 7.59 (t, J = 7.9 Hz, 1H), 7.19 (d, J = 8.8 Hz, 1H), 4.70 (s, 2H),

4.21 (t, J = 7.3 Hz, 2H), 3.59 (m, 2H), 3.20 (m, 2H), 2.42 (t, J = 7.3 Hz, 2H), 1.67 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.83, 164.19, 163.27, 153.65, 135.45, 135.42, 134.61, 134.12, 134.02, 130.92, 130.81, 130.69, 129.80, 128.95, 124.46, 122.09, 119.28, 118.88, 118.43, 107.56, 104.43, 55.41, 36.64, 34.47, 22.69. HRMS m/z calcd for $C_{36}H_{34}N_4O_3P^+$ [M^+]: 601.2323; found 601.2362.

Synthesis of the compound MT-Na

The compound **MT-FA** (136 mg, 0.2 mmol) was dissolved in 95 % ethanol (1 mL), and then FA (75 mg, 1 mmol, 40 % solution in H_2O) was added. The suspension was stirred at room temperature for 60 min. Subsequently, the mixture was concentrated under vacuum, and the resulting residue was purified by silica gel column chromatography (DCM/MeOH = 20:1 to 10:1) to afford orange solid of the compound **MT-Na** with a yield of 57 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (d, J = 8.4 Hz, 1H), 8.65 (t, J = 5.4 Hz, 1H), 7.80 (m, 2H), 7.71 (m, 8H), 7.62 (m, 8H), 7.05 (m, 2H), 6.41 (d, J = 11.6 Hz, 1H), 4.31 (t, J = 7.0 Hz, 2H), 3.65 (m, 2H), 3.40 (s, 3H), 2.73 (t, J = 7.0 Hz, 2H), 1.81 (m, 3H). HRMS m/z calcd for $C_{37}H_{34}N_4O_3P^+$ [M^+]: 613.2363; found 613.2365.

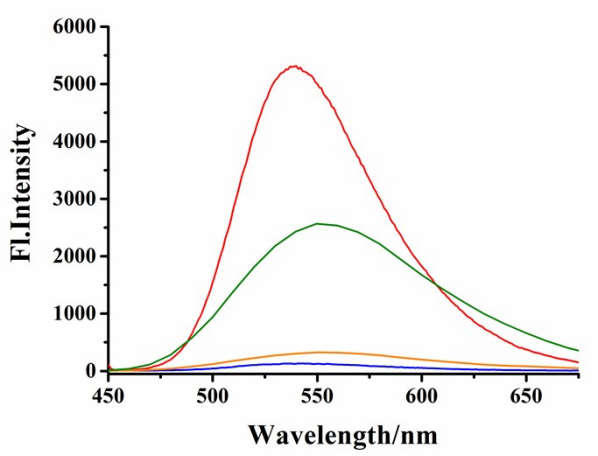


Fig. S1. The one-photon fluorescence of **MT-FA** (5 μM) in absence (the blue curve) and presence (the red curve) of FA (150 μM) and the two-photon fluorescence of MT-FA (5 μM) in absence (the orange curve) and presence (the green curve) of FA (150 μM) in PBS buffer (pH 7.4, 5 % DMSO). OP excitation at 539 nm; TP excitation at 800 nm

Detection limit: The detection limit was determined from the fluorescence titration data based on a reported method.[2] The probe **MT-FA** (5 μM) was titrated with FA (0-150 μM) for 40 min. The fluorescent intensity data at 539 nm were normalized between the minimum intensity and the maximum intensity. A linear regression curve was then fitted to the normalized fluorescent intensity data and the point at which this line crossed the axis (Fig. S2) was considered as the detection limit (4.9×10^{-6} M).

[2] M.Shortreed, R.Kopelman, M. Kuhn, B. Hoyland. *Anal. Chem.*, 1996, **68**, 1414-1418.

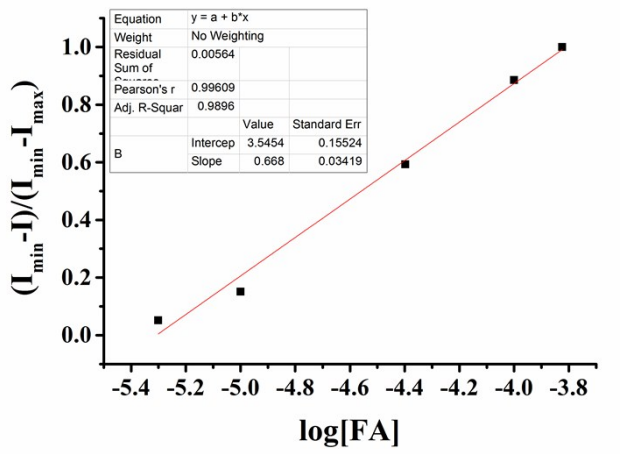


Fig. S2. Normalized response of the fluorescence signal by changing the concentration of FA.

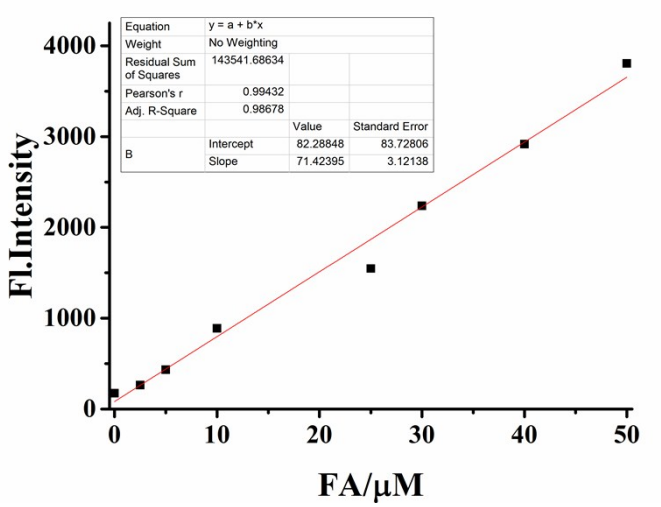


Fig. S3. Relationship between fluorescence intensity at 539 nm of **MT-FA** (5 μM) and the amount of FA (0-50 μM).

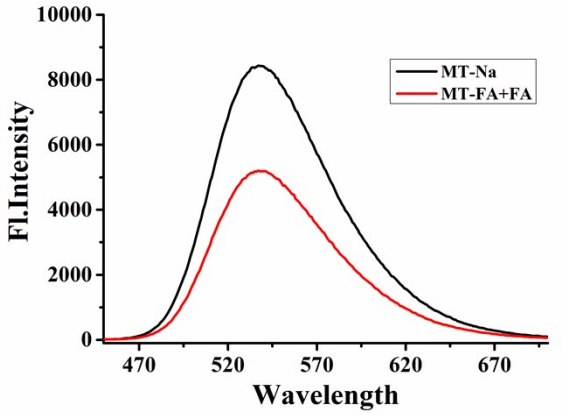


Fig. S4. The fluorescence response of the probe **MT-FA** (5 μM) to FA (150 μM) (The red curve) and compound **MT-Na** (5 μM) (The black curve) in PBS buffer (pH 7.4, 5 % DMSO).

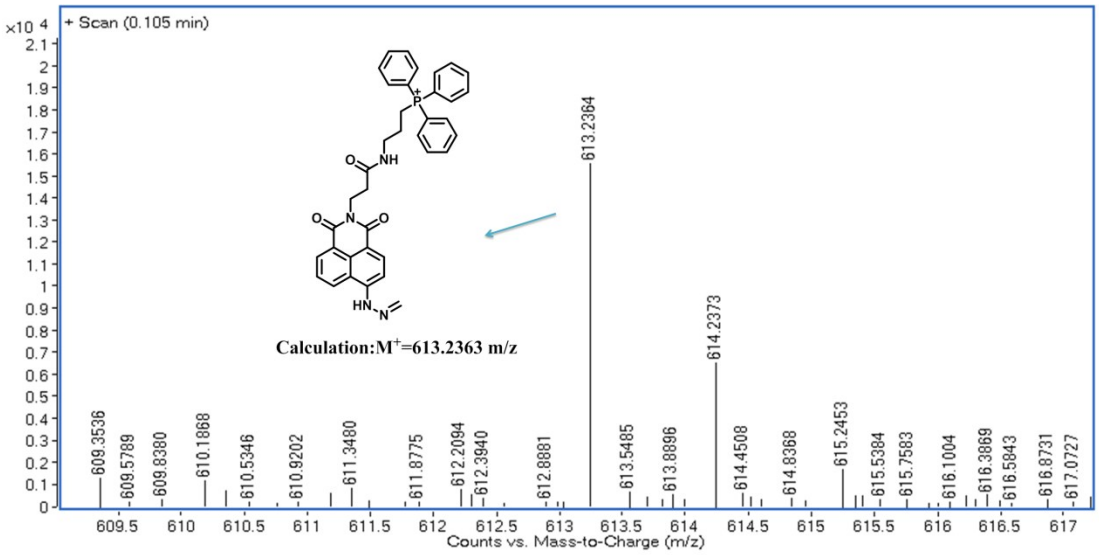


Fig. S5. HR-MS spectrum of 5 μM **MT-FA** treated with 150 μM FA in 10 mM PBS buffer (pH 7.4, 5 % DMSO).

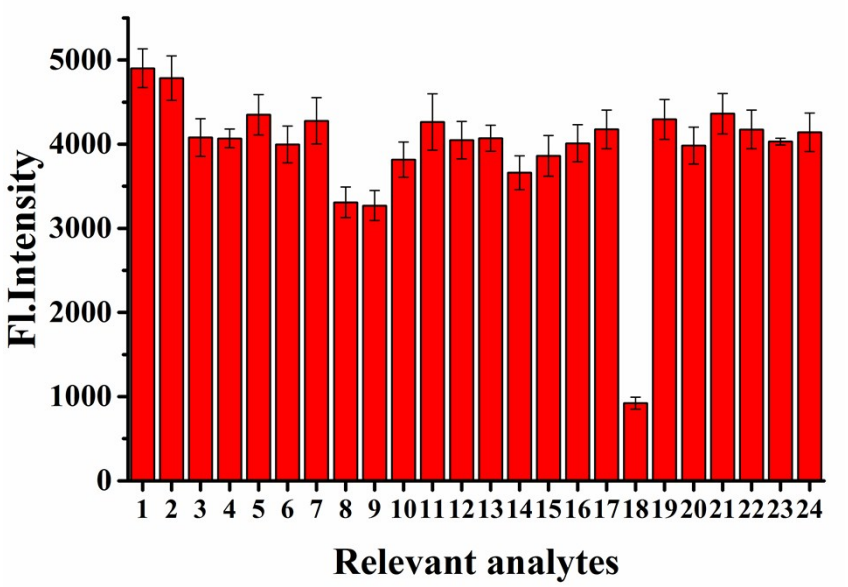


Fig. S6. the fluorescence intensity of **MT-FA** (5μM, at 539 nm) responding to FA (150 μM) in presence (red) of various interfering species (150 μM) in PBS buffer (pH 7.4, 5 % DMSO).: (1) PBS; (2) glyoxal; (3) methylglyoxal; (4) sodium pyruvate; (5) 4-dimethylaminobezaldehyde; (6) trichloroacetaldehyde; (7) acetaldehyde; (8) 4-nitro-benzaldehyde; (9) acetone; (10) NaClO; (11) H_2O_2; (12) DTBP; (13) TBHP; (14) NO; (15) $CaCl_2$; (16) $MgCl_2$; (17) KNO_3; (18) $NaHSO_3$; (19) Na_2SO_4; (20) $NaNO_2$ (21) Na_2S; (22) GSH; (23) Hcy; (24) Cys;. λ_{ex} = 440 nm.

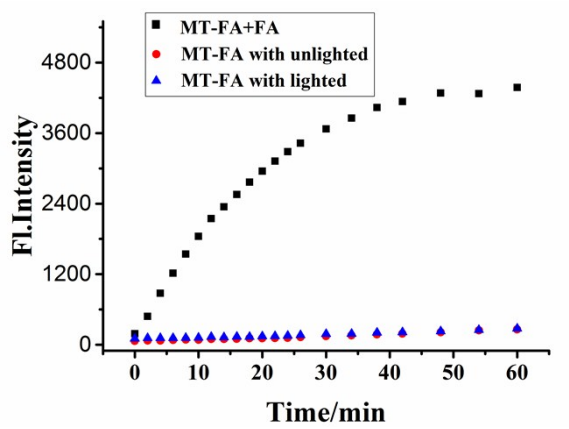


Fig. S7. Photostability profiles of **MT-FA** (5 μM) in the absence or presence of UV-irradiated (365 nm). The fluorescence intensities at 539 nm were continuously monitored at time intervals in PBS (10 mM, pH 7.4, 5 % DMSO).

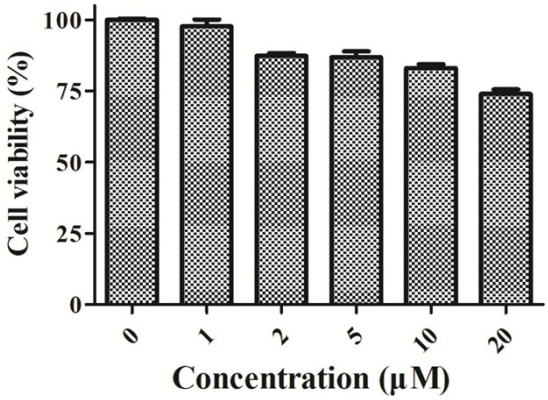


Fig. S8. Effects of the probe **MT-FA** with varied concentrations (0-20 μM) on the viability of the Hela cells. The probe with varied concentrations was incubated with the cells for 24 h. The viability of the cells in the absence of the probe is defined as 100 %, and the data are the mean standard deviation of five separate measurements.

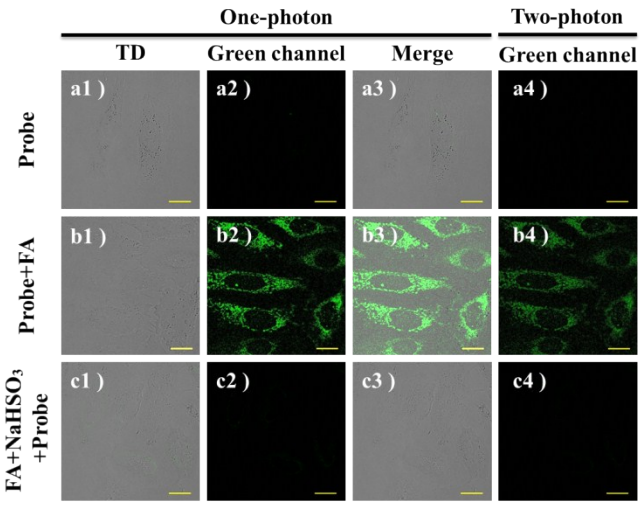


Fig. S9. Fluorescence imaging of the exogenous FA in HeLa cells. a1)-a4) The image of the HeLa cells treated with **MT-FA** (5 μM); b1)-b4) The image of the HeLa cells treated with FA (150 μM) and **MT-FA** (5 μM); c1)-c4) The image of the HeLa cells treated with FA (150 μM), $NaHSO_3$ (300 μM) and **MT-FA** (5 μM). OP-excitation was at 488 nm, TP-excitation was at 800 nm and emission collection was from 500 - 550 nm. Scale bar: 20 μm.

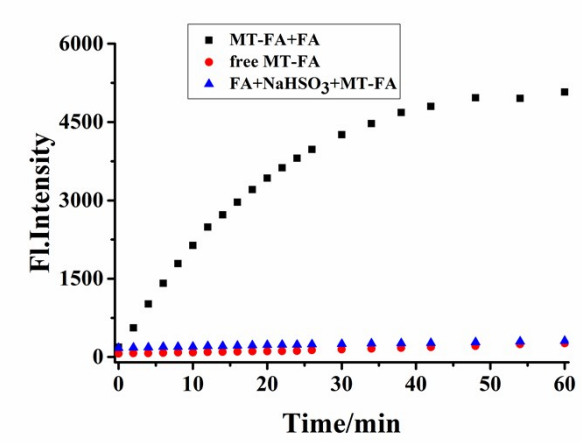


Fig. S10. Reaction-time profiles of **MT-FA** treated with FA [■], free **MT-FA** [●], or FA pre-incubated with $NaHSO_3$ then treated with **MT-FA** [▲]. The fluorescence intensities at 539 nm were continuously monitored for 60 min in PBS (10 mM, pH 7.4, 5 % DMSO). The concentrations for **MT-FA**, FA, and $NaHSO_3$ were 5 μM, 150 μM, 300 μM, respectively. λ_{ex} = 440 nm.

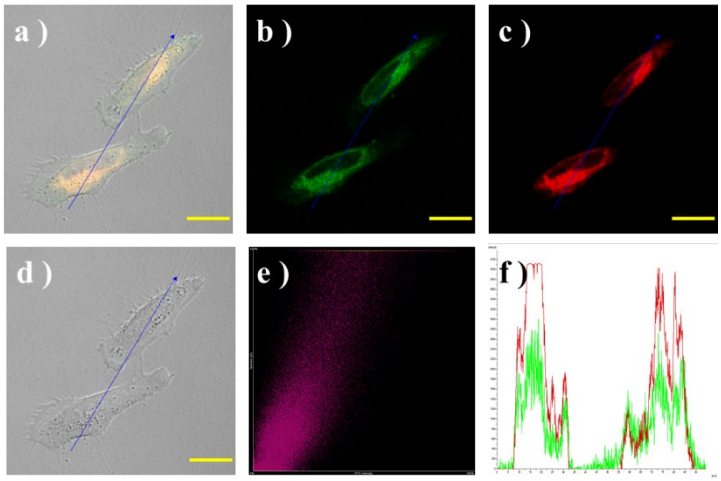


Fig. S11. The images of the living HeLa cells co-incubated with **MT-FA** (5 μM) and FA (150μM) and Mito-Tracker Deep Red (500 nM). a) Bright-field image of the HeLa cells treated with **MT-FA** (5 μM) and FA (150μM) and Mito-Tracker Deep Red (500 nM); b) The fluorescence image of the green channel; c) The fluorescence image of the red channel; d) The merged image of a, b, and c; e) Intensity scatter plot of the green and red channels. f) Intensity scatter plot of the green and red channels in the ROI of d). Scale bar: 20 μm.

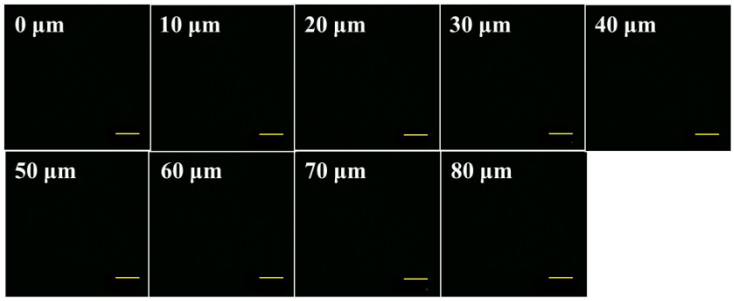


Fig. S12. Two-photon fluorescence imaging of the liver slides treated with **MT-FA**. Fluorescence images of the liver slides incubated with MT-FA (10 μM). Excitation was at 800 nm by the femtosecond laser and the emission collection was from 500-550 nm. Scale bar: 20 μm.

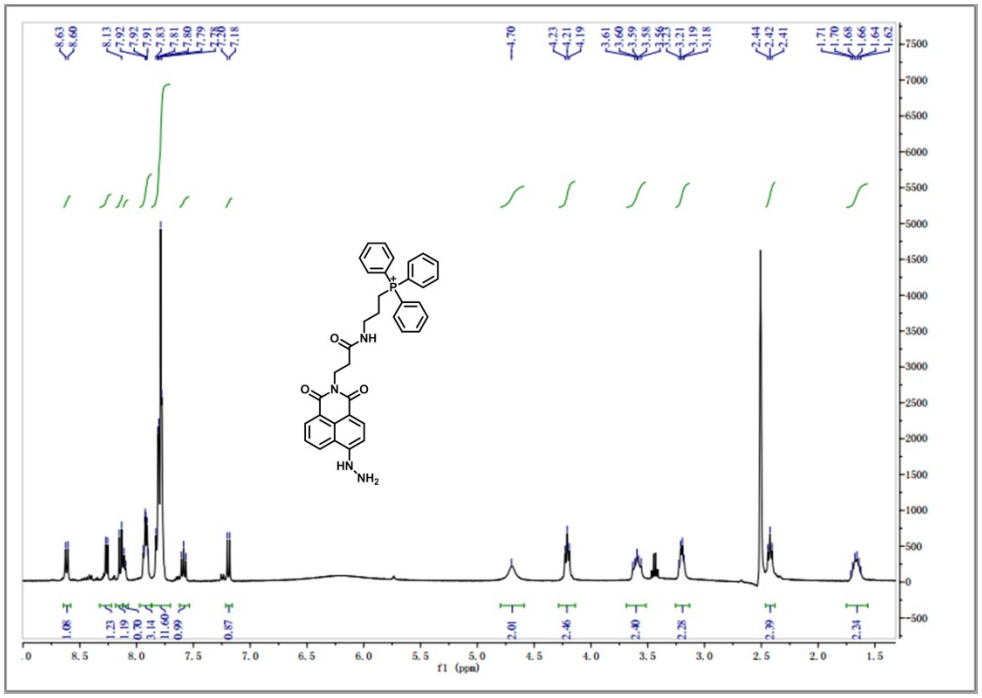


Fig. S13. ^{1}H NMR spectrum of the probe **MT-FA**.

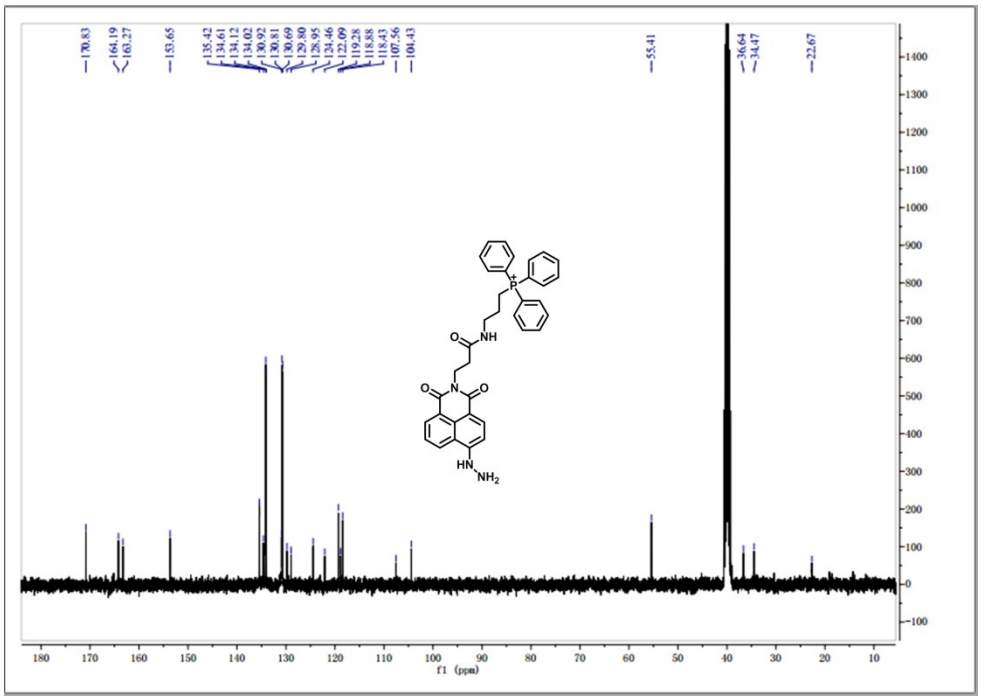


Fig. S14. ^{13}C NMR spectrum of the probe **MT-FA**.

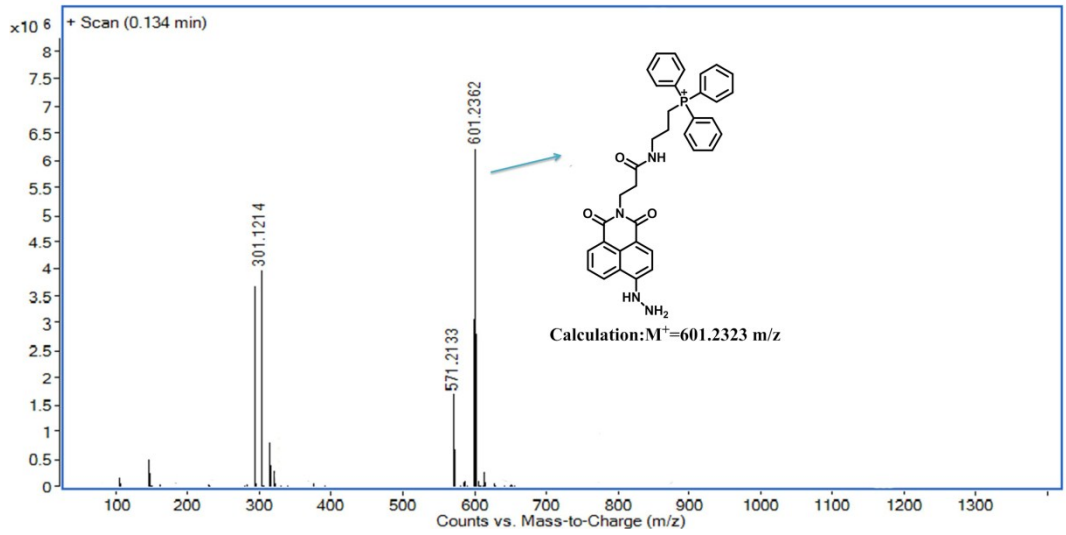


Fig. S15. HR-MS spectrum of the probe **MT-FA**.

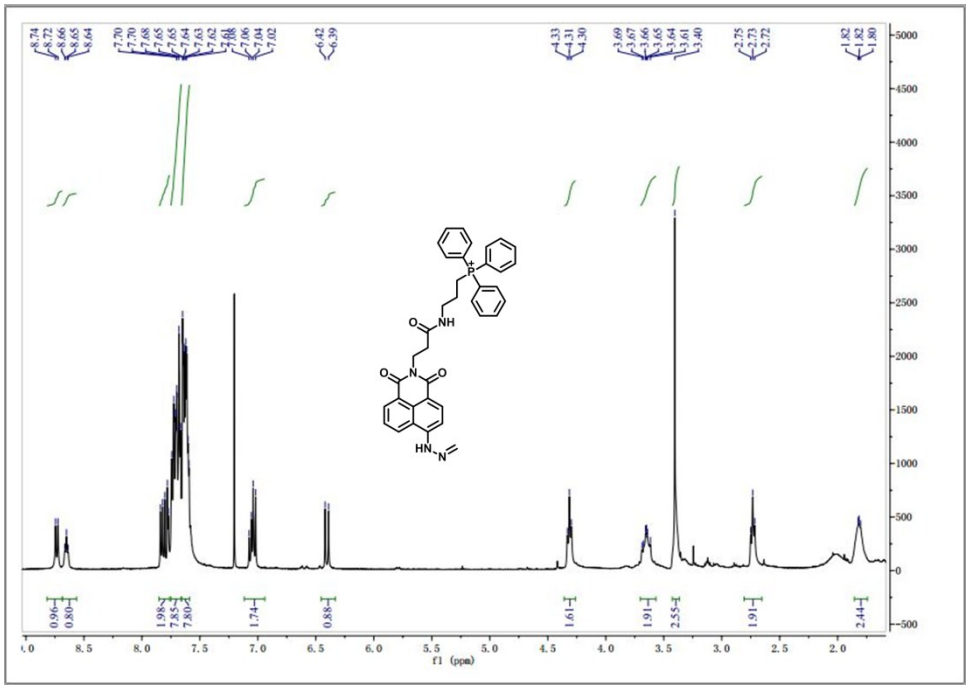


Fig. S16. ^{1}H NMR spectrum of the compound **MT-Na**.

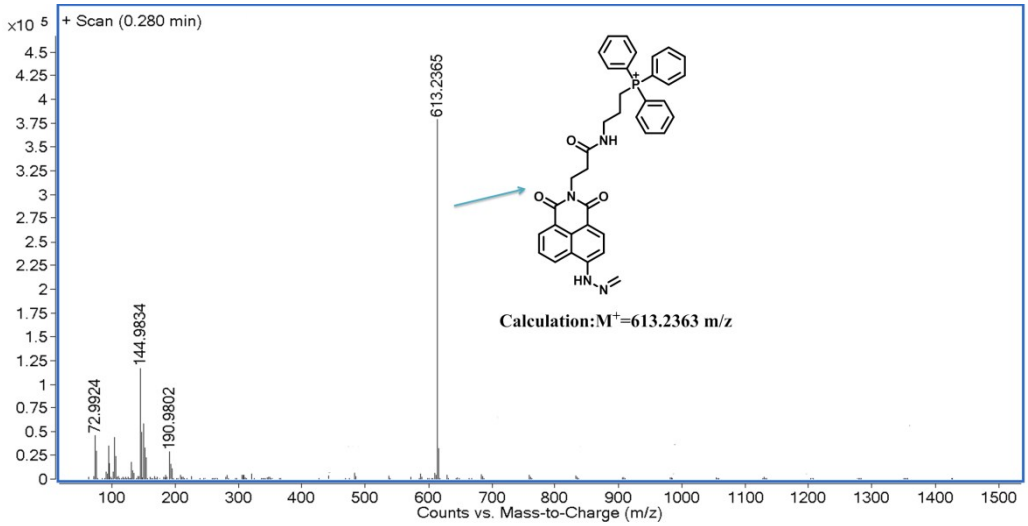


Fig. S17. HR-MS spectrum of the compound **MT-Na**.