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A biocompatible two-photon absorbing fluorescent mitochondrial probe for deep *in vivo* bioimaging

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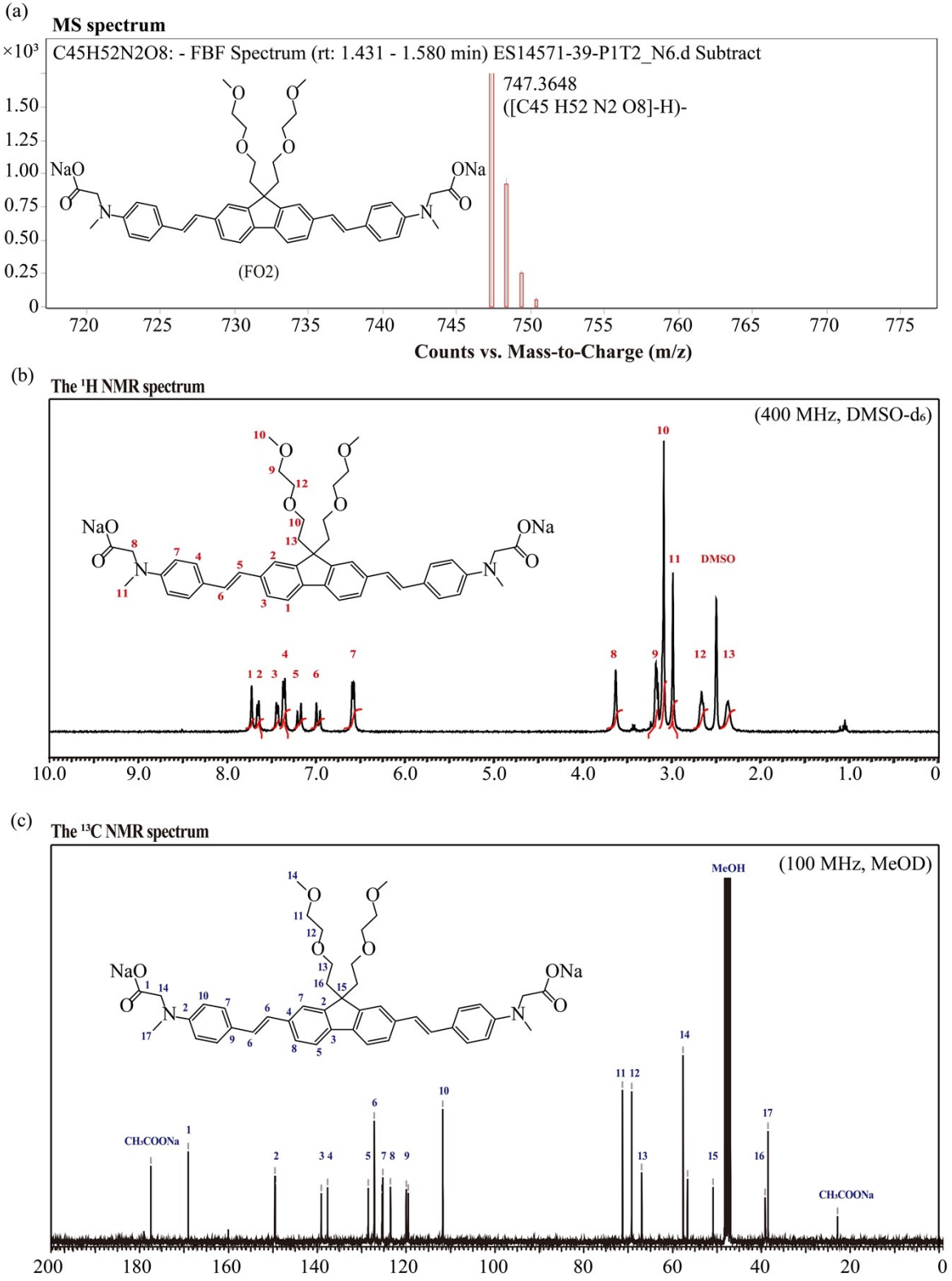

Figure S1. Structure representations of FO2. (a) The HRMS spectrum, (b) the ^{1}H NMR spectroscopy, and (c) the ^{13}C NMR spectrum of FO2. Relative peaks of hydrogen or carbon and the other peaks were labeled by numerals.

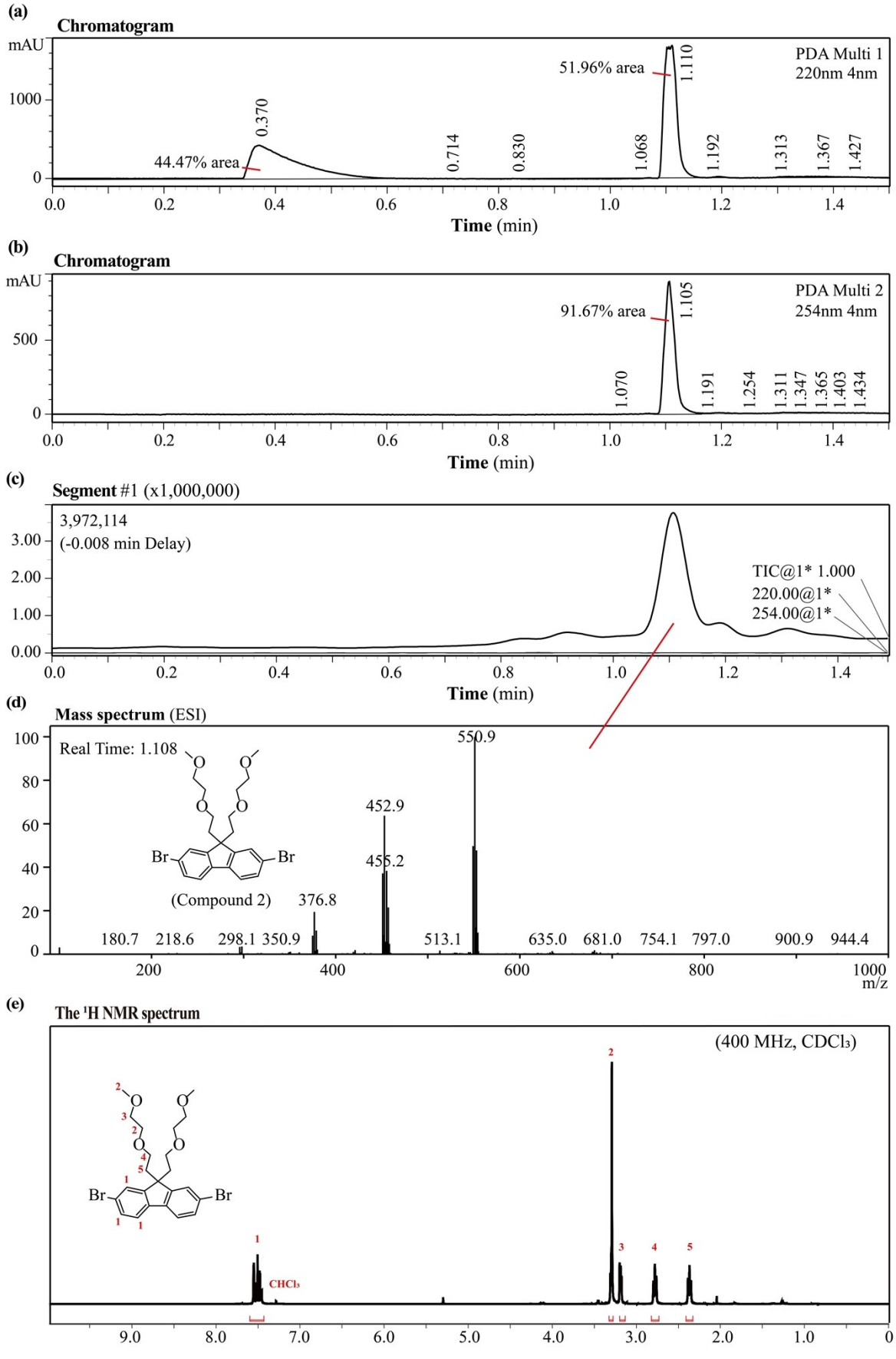
(a)
Chromatogram
mAU
PDA Multi 1
220nm 4nm
51.96% area
44.47% area
0.370
0.714
0.830
1.068
1.110
1.192
1.313
1.367
1.427
Time (min)
(b)
Chromatogram
mAU
PDA Multi 2
254nm 4nm
91.67% area
1.070
1.105
1.191
1.254
1.311
1.347
1.365
1.403
1.434
Time (min)
(c)
Segment #1 (x1,000,000)
3,972,114
(-0.008 min Delay)
TIC@1* 1.000
220.00@1*
254.00@1*
Time (min)
(d)
Mass spectrum (ESI)
Real Time: 1.108
(Compound 2)
550.9
452.9
455.2
376.8
180.7
218.6
298.1
350.9
513.1
635.0
681.0
754.1
797.0
900.9
944.4
m/z
(e)
The ¹H NMR spectrum
(400 MHz, CDCl₃)
CHCl₃

Figure S2. Structure representations of Compound 2. (a-d) The LC-MS spectra of Compound 2 (2,7-dibromo-9H-fluorene and 1-(2-bromoethoxy)-2-methoxy-ethane). (b-c) One main peak of the liquid chromatogram at 1.11 min (d) with its desired mass, showing that the core precursor, 2,7-dibromo-9H-fluorene, was completely consumed. (e) The ^{1}H NMR spectrum of Compound 2, relative peaks and solvent peak were labeled by numerals.

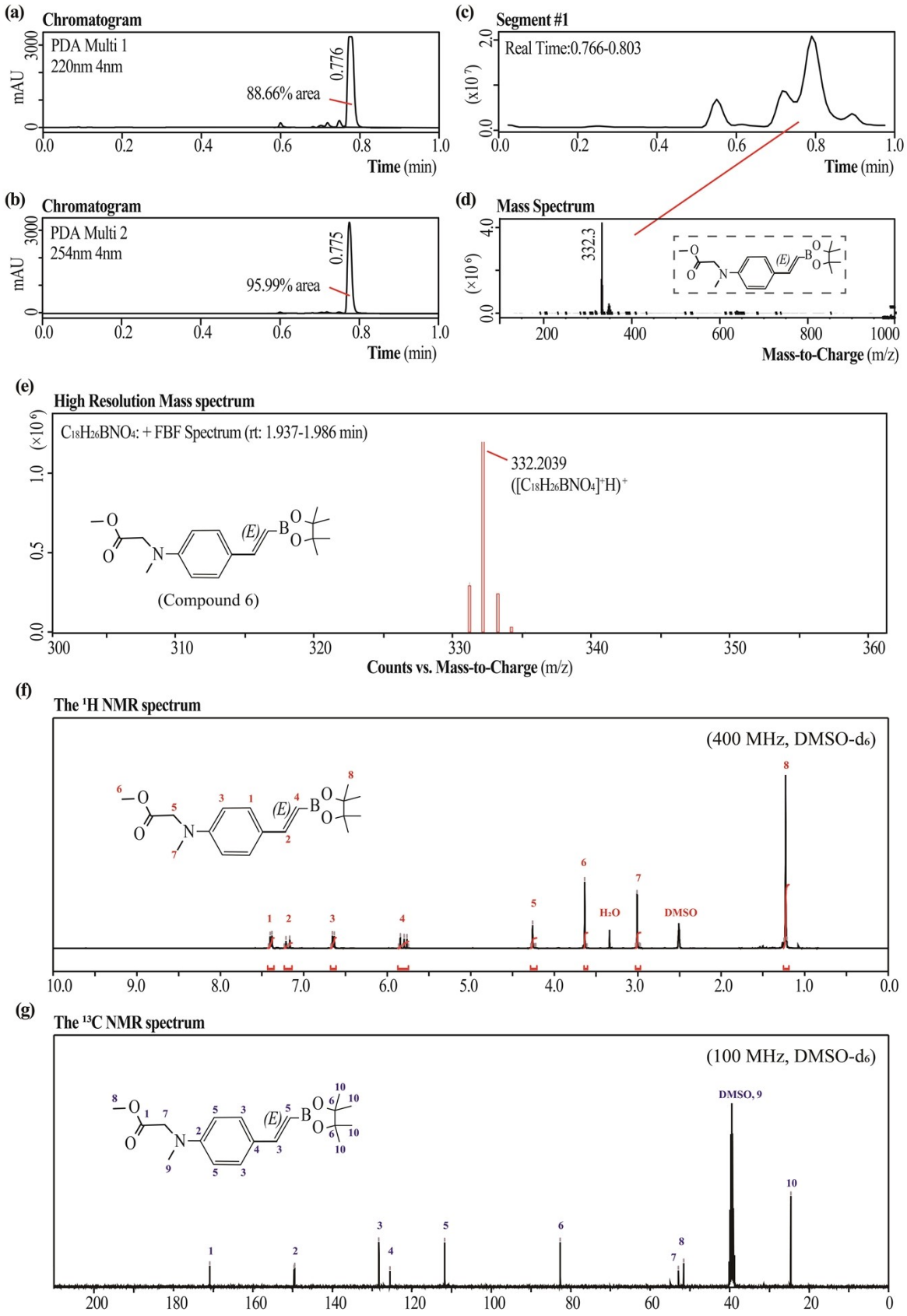

Figure S3. Structure representations of Compound 6. (a-d) The LC-MS spectra of Compound 6 (methyl N-(4-bromophenyl)-N-methylglycinate). One main peak of the liquid chromatogram from 0.766 min to 0.803 min (d) with its desired mass, showing that the core

precursor was completely consumed. (e) The high-resolution mass spectrum of Compound 6, calcd. $C_{18}H_{26}BNO_4$, 331.1955, and found 330.1997. (f) The ^{1}H NMR spectrum and (g) the ^{13}C NMR spectrum of Compound 6, with relative peaks and other peaks were labeled.

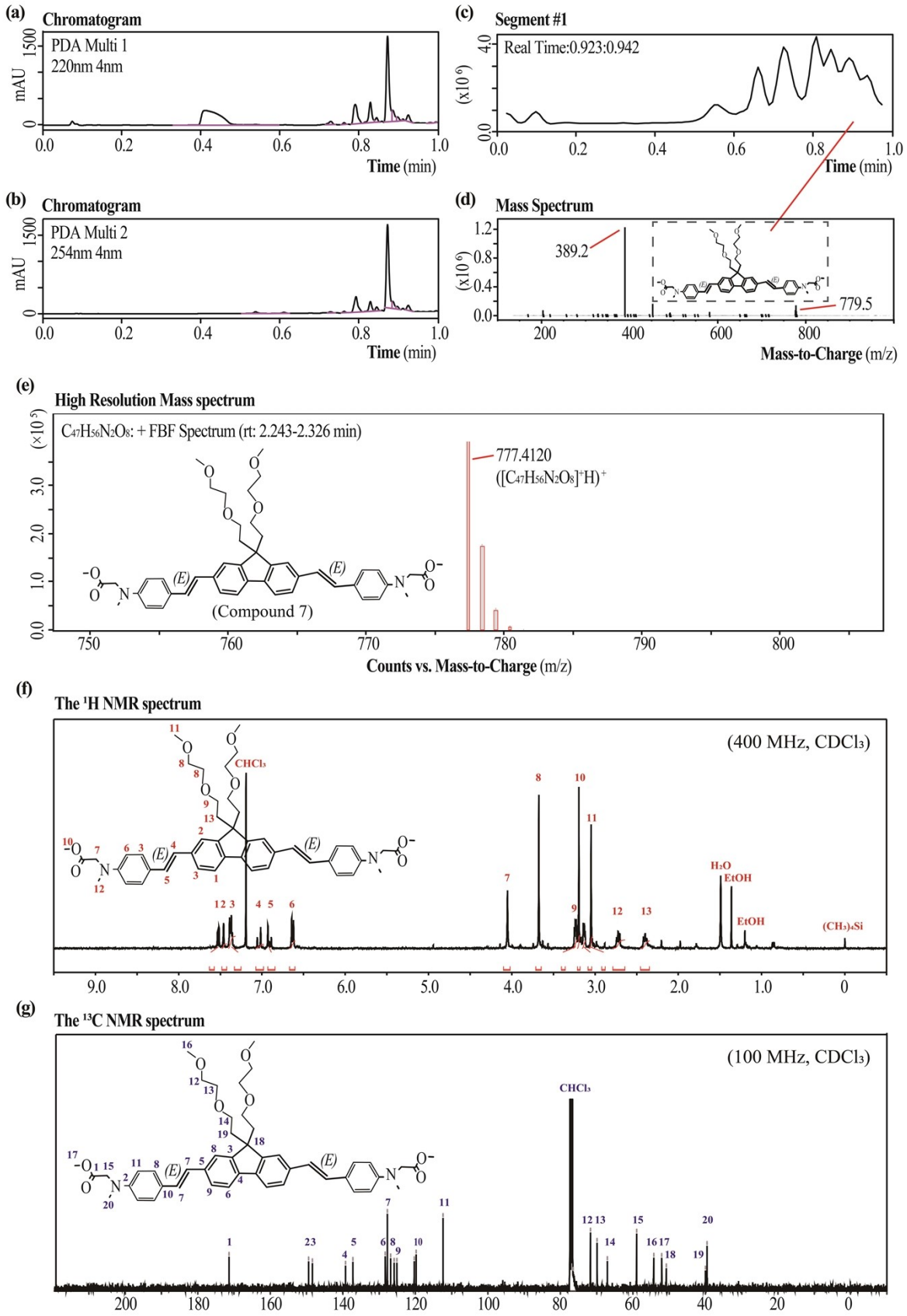

Figure S4. Structure representations of Compound 7. (a-d) The LC-MS spectra of Compound 7 (dimethyl 2,2'-((((1E,1'E)-(9,9-bis(2-(2-methoxyethoxy)ethyl)-9H-fluorene-2,7-diyl)bis(ethene-2,1-diyl))bis(4,1-phenylene))bis(methylazanediyl))diacetate). One main peak

of the liquid chromatogram with its desired mass, showing that the core precursor was completely consumed. (e) The high-resolution mass spectrum of Compound 7, calcd. $C_{47}H_{56}N_2O_8$, 776.4037, and found 776.4046. (f) The ^{1}H NMR spectrum and (g) the ^{13}C NMR spectrum of Compound 7, with relative peaks and other peaks, were labeled.

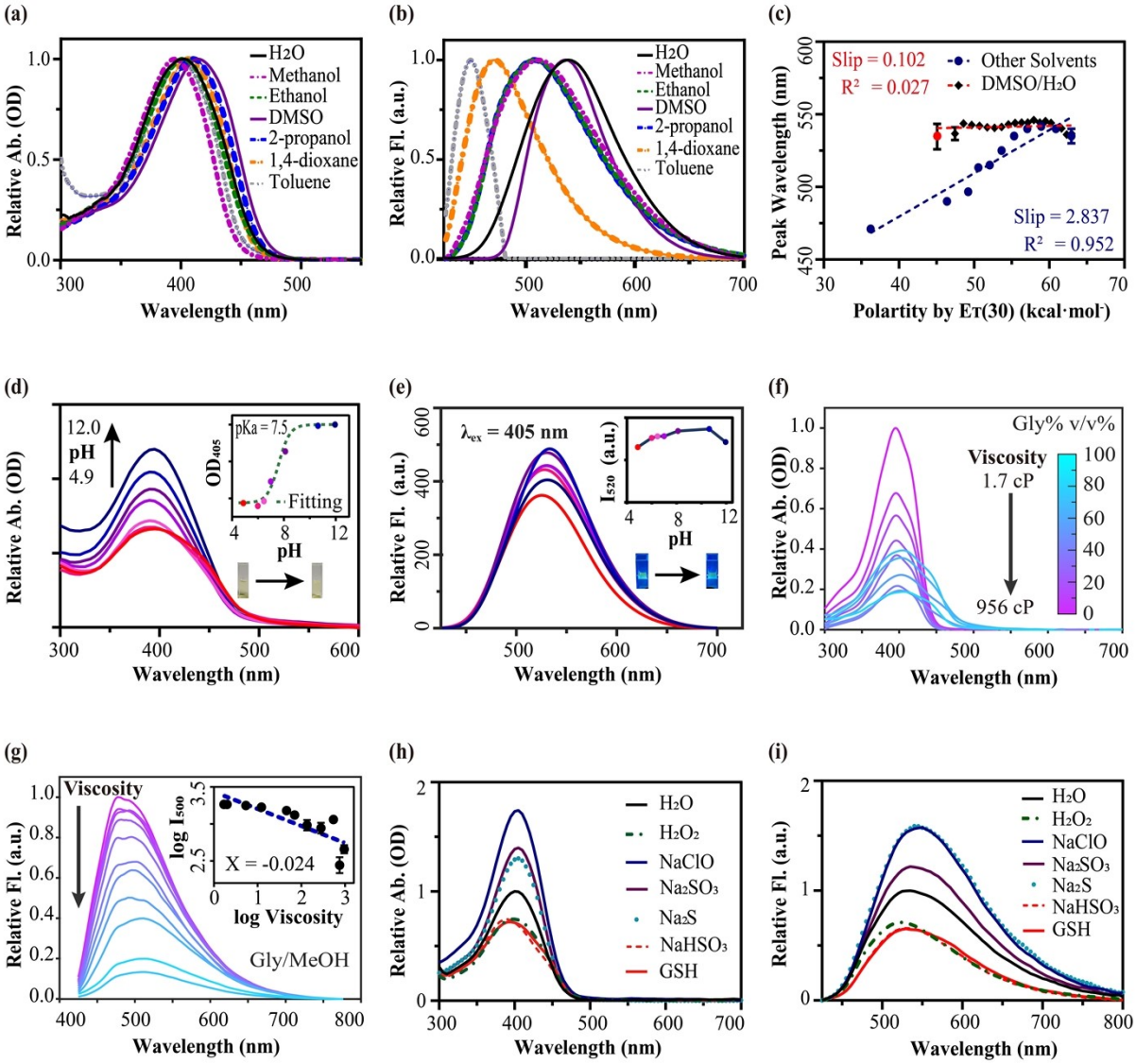

Figure S5. The solvent environment response of FO2.

(a-c) Solvents of various polarities.[1] A stored FO2 (in deionized water, 4 mM) was added to prepare working solutions (20 μM). (a) The absorption and (b) the emission spectra (λ_{Ex} = 405 nm) were recorded (T = 37 °C). (c) The $E_T(30)$ was employed to indicate solvent polarity[2], and the linearity of polarity and emission bands (blue dot) was shown (Slip = 2.837, R^2 = 0.952) excluding an outlier (DMSO, red dot). Then the polarity response of FO2 in the DMSO/H_2O system (black diamond) with different volume ratios was tested (Slip = 0.102, R^2 = 0.027), where the polarity in the binary solvent mixtures were calculated according to a reported formulation. Each test was replicated for 3 times, represented as mean ± SD.[3]

(d-e) Solvents of different pH environment in phosphate buffers. A stored FO2 (in deionized water, 1 mM) was added to the phosphate buffer saline with different pH values to prepare working solutions of various pH (10 μM). Then absorption and emission curves in solutions of various pH values were recorded in a cuvette filled with FO2 working solution. (d) The ultraviolet absorption spectra and a sigmoid curve on the right panel fitting scatters of optical density at 405 nm, with an inset depicting the color of detected solutions (pH 4.9, 11.3). (g) The emission spectra under irradiation light of 405 nm and the fluorescent signals at 520 nm

of these emission spectra were recorded on the up-right panel, with an inset depicting the color (excited by a straight light laser at 405 nm) of detected solutions (pH 4.9, 11.3). (f-g) Solvents of different viscosity tested by the glycerol/methanol system, which has a set of well-distributed viscosity.[4] A stored solution of FO2 (deionized water, 10 mM) was added to prepare working solutions (20 μM). (f) The absorption spectra (25 ℃) shift from the viscosity of pure methanol to pure glycerol (viscosity data obtained from literature).[5] And (g) relevant emission spectra with a right-up panel of the viscosity sensitive analysis (the linear regression coefficient of log Viscosity and log fluorescence at 500 nm), where the Forster-Hoffmann value ($X = -0.024$, $R^2 = 0.706$) illustrated a negative effect of fluorescence intensity with viscosity (each test replicated for 3 times, represented as mean ± SD).[6] (h-i) The selectivity of various bio-analytes by (h) absorption and (i) emission spectra. A stored solution of FO2 (deionized water, 10 mM) was added to prepare working solutions (20 μM, deionized water). Solvents of different bio-analytes, including nucleophiles potentially respondent towards FO2,[7] were selected and measured the absorption and emission spectra (200 μM) after mixing for 10 minutes. The GSH represents reduced glutathione.

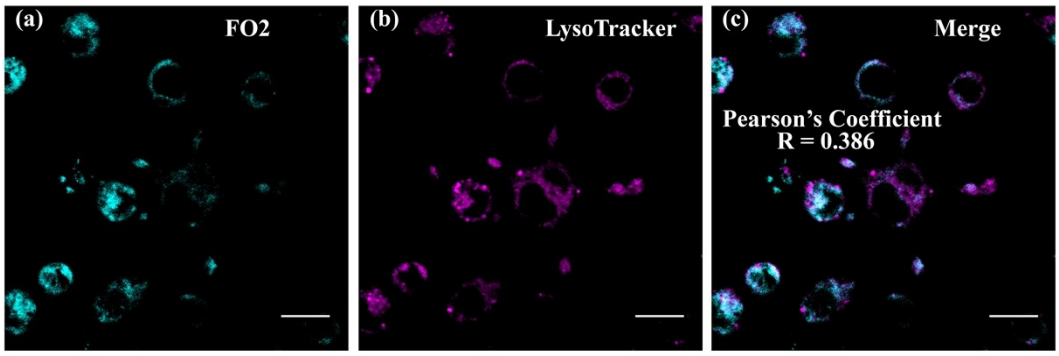

Figure S6. Colocalization analysis of FO2 and lysosome. (a) The FO2 (800 nm, λ_{ex} = 405 nm; λ_{em} = 430/530 nm) and (b) LysoTracker™ Deep Red FO2 (100 nm, λ_{ex} = 635 nm; λ_{em} 655/755 nm) were mixed and added to incubate C6 cells for 30 min before one-photon laser excitation. Confocal microscopy under an oil lens was recorded (X60, Oil Zoom ×2). Scale bar = 10 μm.

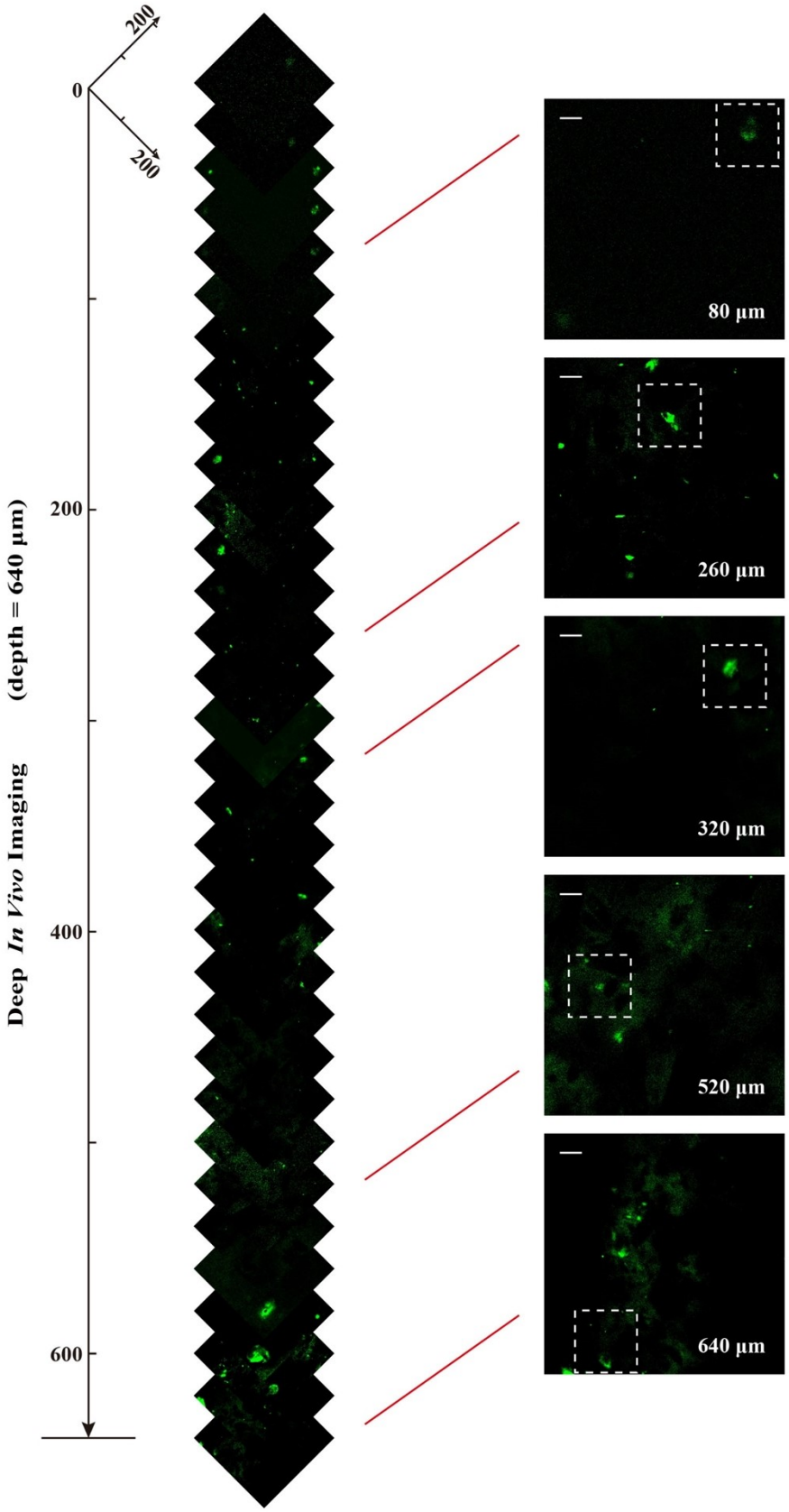

Figure S7. The stack of FO2 *in vivo* imaging in mice cortex. A circular craniotomy opening (1.5~2.0 mm diameter) was performed, and FO2 was stereotactically injected (80 nL per site, 0.2 mM in disinfected normal saline) in various depths (200 μm, 400 μm, 600 μm).

Two-photon images were recorded with excitation lasers at 810 nm, and each picture in the stack differed by 20 μm. The white rectangular dashed box showed typical mitochondria figures in cortex cells. Scale bar = 20 μm.

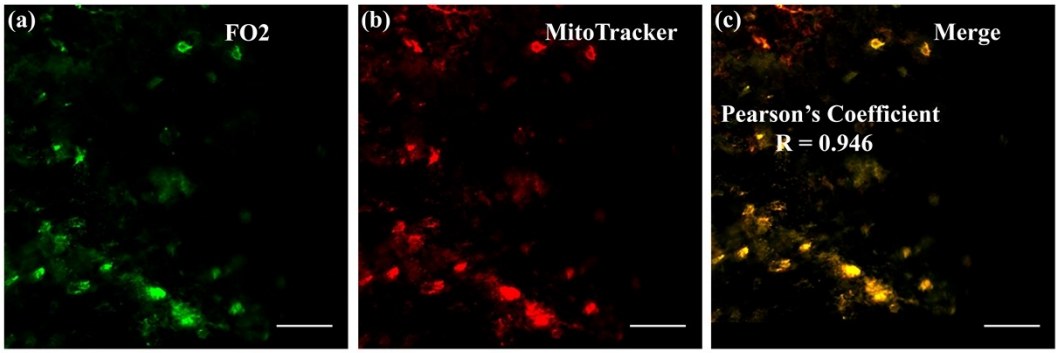

Figure S8. Two-photon Colocalization in vivo. Two-photon fluorescent signals from mice cortex and excited by a femtosecond laser at 810 nm were collected, including (a) FO2 signals (200 μM, 30 min; 495nm-540nm), (b) mitochondria by commercial dye MitoTracker Red (20 μM, 30 min; 575nm-645nm), and (c) the merged image of (a) and (b). Colocalization results suggested a high affinity of FO2 to mitochondria. Bar scale = 50 μm.

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