Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

pH-Independent two-photon fluorescent lysotrackers for real-time monitoring autophagy

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List of Contents

Experimental Section

Materials and Equipments

All reagents and solvents were commercially purchased. LysoTracker Red DND-99 and MitoTracker Red FM were purchased from Invitrogen (USA). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker Avance II spectrometer at 400 and 100 MHz respectively. HRMS spectra were recorded by a LTQ Orbitrap XL of mass spectrometer. UV-vis absorption spectra and fluorescence spectra were recorded on a UV-1800 spectrophotometer (Shimadzu) and a F-7000 Fluorescence spectrometer (Hitachi) respectively. The fluorescence quantum yields were detected by HORIBA FluoroMax-4P (HORIBA Jobin Yvon). All pH measurements were performed using a Model PHS-3C meter calibrated at room temperature (23 ± 2 ^{o}C) with standard buffers of pH 9.21 and 4.01.

For confocal microscopy, cells were imaged on a Zeiss LSM 710 META upright confocal laser-scanning microscope using magnification 40x, 20x oil immersion lenses for monolayer cultures and tissue sections. Image data acquisition and processing was performed using Zeiss LSM Image Browser, Zeiss LSM Image Expert and Image J.

Preparation of Test Solution

Solutions of **Lyso-MCO** and **Lyso-NCO** (1 mM) were prepared in DMSO. Phosphate-buffered saline (PBS) buffer solution: 20 mM NaCl, Na_2HPO_4, $NaH_2PO_4{\cdot}2H_2O$, pH = 7.4. The test solution of **Lyso-MCO and Lyso-NCO** (10 μM) in different solvents were prepared. Analyte solutions of NaCl, KI, GSH, Cys, NaHS, $NaHSO_3$, Na_2SO_3, NaClO, H_2O_2, $NaNO_2$ and $NaIO_3$ were prepared by dissolving them in distilled water to final concentrations of 100 mM for NaCl and KCl, 0.2 mM for GSH and Cys, and 25 mM for the other analytes.

Measurement of Fluorescence Quantum yield (Φ)

The fluorescence quantum yields of probe **Lyso-MCO** (λ_{ex} = 318 nm, λ_{em} = 409 nm), **Lyso-NCO** (λ_{ex} = 328 nm, λ_{em} = 444 nm) (10 μM) were detected in the DMSO solution, respectively. And that of **Lyso-MCO** (λ_{ex} = 318 nm, λ_{em} = 427 nm), **Lyso-NCO** (λ_{ex} = 328 nm, λ_{em} = 450 nm) (10 μM) were recorded in the PBS solution (containing 20% DMSO), respectively. All calculations are automatically performed by the Quantum Yield and Color Calculator software of the device.

Measurement of Two-Photon Absorption Cross Sections (δ)

Two-photon excited fluorescence (TPEF) spectra were measured using femtosecond laser pulse and Ti: sapphire system (680~1080 nm, 80 MHz, 140 fs, Chameleon II) as the light source. All

measurements were carried out in air at 20 °C. Two-photon absorption cross sections were measured using two-photon-induced fluorescence measurement technique and thus the cross sections can be calculated by means of the following equation:[1]

$$\delta = \delta_{ref} \frac{\Phi_{ref}}{\Phi} \frac{c_{ref}}{c} \frac{n_{ref}}{n} \frac{F}{F_{ref}}$$

Here, the subscripts *ref* stands for the reference molecule. δ is the two-photon absorption cross sections value, c is the concentration of solution, n is the refractive index of the solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The Φ_{ref} value of reference (fluorescein 0.97) is taken from the literature.[2] The two-photon absorption cross sections were tested with 0.1 mM **Lyso-MCO and Lyso-NCO** by using optically matching solutions of fluorescein as a standard. The δ_{ref} value of reference is taken from the literature,[3] and the relative error of δ value in the experiment is about ±15%.

Cell Cytotoxicity Experiment

To appraise the cytotoxicity of **Lyso-MCO** and **Lyso-NCO**, MTT (5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assays were performed as reported in the past.[4] MCF-7 cells and HeLa cells were passed and plated to ca. 70% confluence in 96-well plates 24 h before treatment. Ahead of **Lyso-MCO/Lyso-NCO** treatment, DMEM (Dulbecco's Modified Eagle Medium) with 10% FCS (Fetal Calf Serum) was removed and replaced with fresh DMEM, and the final concentrations of **Lyso-MCO/Lyso-NCO** range from 0, 2, 10, 15 to 20 μM were added respectively. The treated cells were incubated for 24 h at 37 °C under 5% CO_2. Afterwards, cells were treated with 5 mg/mL MTT (40 μL /well) and incubated for another 4 h (37 °C, 5% CO_2). Then use of DMSO (150 μL/well) for dissolving the cells, and the absorbance was recorded to be 570 nm. The cell viability (%) was analyzed based on the following equation:

$$\text{Cell viability\%} = OD_{570}\,(\text{sample})/OD_{570}\,(\text{control}) \times 100$$

where OD_{570} (sample) stands for the optical density of the wells loaded with various concentration of **Lyso-MCO/Lyso-NCO** and OD_{570} (control) stands for that of the wells treated with DMEM plus 10% FCS. The percent of cell survival values is relative to untreated control cells.

Cell Culture and Two-Photon Fluorescence Imaging

For two-photon bio-imaging, MCF-7 cells and HeLa cells were cultured in DMEM supplemented with 10% FCS at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Cytotoxicity assays

showed that **Lyso-MCO** and **Lyso-NCO** were safe enough for two-photon bio-imaging at low concentrations, so that the cells were pretreated with 2 μM **Lyso-MCO** and **Lyso-NCO** respectively, then washed 3-time with PBS buffer. At last, the LysoTracker Red DND-99 (LTR, final concentration: 0.5 μM) was added for another 15 min at 37 ºC, washed 3-time with PBS buffer. Another group of the cells were pretreated with 2 μM **Lyso-MCO** and **Lyso-NCO** respectively, washed 3-time with PBS buffer. Next MCF-7 cells were co-stained with MitoTracker Red FM (MTR, final concentration: 0.5 μM) for another 30 min at 37 ºC, washed 3-time with PBS buffer. Then, cells imaging were recorded on a confocal microscope (Zeiss LSM 710 Meta NLO with a 40/63 oil lens) with a mode-locked titanium-sapphire laser source set at wavelength 700 nm.

Cell Uptake

MCF-7 cells were incubated with 2 μM **Lyso-MCO/Lyso-NCO** at 37 ºC under 5% CO_2 for 12 min, at 4 ºC under 5% CO_2 for 12 min respectively, then washed 3-time with PBS buffer. Another two groups, MCF-7 cells were pretreated with endocytic inhibitors NH_4Cl (final concentration: 50 mM), and chloroquine (final concentration: 50 μM) respectively, and then treated with 2 μM **Lyso-MCO/Lyso-NCO** at 37 °C under 5% CO_2 for 12 min, then washed 3-time with PBS buffer. Finally, cells imaging were recorded on a confocal microscope (Zeiss LSM 710 Meta NLO with a 40/63 oil lens) with a mode-locked titanium-sapphire laser source set at wavelength 700 nm.

Photostability Experiments

MCF-7 cells were incubated with 2 μM **Lyso-MCO/Lyso-NCO** at 37 ºC under 5% CO_2 for 12 min, then washed 3-time with PBS buffer. Afterwards, the LysoTracker Red DND-99 (LTR, final concentration: 0.5 μM) was added for another 15 min at 37 ºC, washed 3-time with PBS buffer. Finally, cells imaging were recorded at different time points (0, 2, 5, 8, 10, 15, 20 min) on a confocal microscope (Zeiss LSM 710 Meta NLO with a 40/63 oil lens) with a mode-locked titanium-sapphire laser source set at wavelength 700 nm.

Preparation of Fresh Mouse Liver Slices and Two-Photon Fluorescence Imaging

All steps involving animals were approved by and followed the guidelines of the Anhui University Animal Care Committee, Faculty of life science. We have tried our best to reduce the number of animal used in these studies and also taken effort to reduce animal suffering from pain and discomfort.

For TP bio-imaging, slices were prepared from the liver of 7-day-old mouse. Slices were cut to 180 μm thickness by using a vibrating-blade microtome in 10 mM PBS buffer (pH = 7.4). Slices were

respectively stained with 10 μM **Lyso-MCO** and **Lyso-NCO** (final concentration: 0.5 μM) in PBS buffer bubbled with 95% air and 5% CO_2 for 30 min at 37 °C. Then slices were washed 3 times with PBS buffer, transferred to the glass-bottomed dishes. Mouse liver slices imaging was recorded on a confocal microscope (Zeiss LSM 710 Meta NLO with a 40/63 oil lens) with a mode-locked titanium–sapphire laser source set at wavelength 700 nm.

Western Blotting

MCF-7 cells were seeded at a density of 1.0×10^5 per well in cell culture plate. After 24 h, some cells were incubated for different times (0, 0.3, 0.6, 1, 2, 4 h) with 6-OHDA and other cells were incubuted 4 h with normal condition, with 6-OHDA in the absence and presence of 100 μM 3-Methyladenine (3-MA). Both adherent and floating cells were collected and lysed with protein lysis buffer containing 0.5% Triton X 100, 100 mM Tris–HCl, 150 mM NaCl, 0.1 U/mL aprotinin for 10 s on ice and centrifuged at 3000 g for 5 min. Protein concentration was determined by Bradford assay and equal protein resolved on 15% SDS–PAGE. Protein was electroblotted on a polyvinylidene-difluoride (PVDF) membrane at 200 mA for 1.5 h at 4 °C. The membrane was blocked using 5% skimmed milk for 1.5 h at room temperature, incubated overnight at 4 °C with primary antibody against the LC3 proteins in TBS + Tween 20 (TBST) at a dilution of 1:1000, using GAPDH (primary antibody dilution, 1:1000) as loading control. After washing 3 times, 12 min each time, with TBST, the membrane was incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:3000 in skimmed milk) at room temperature for 1.0 h. The membrane was then incubated with enhanced chemiluminescence reagent (ECL) solution for certain time. The membrane was washed three times with TBST and signal visualized by using EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel) by exposing to X-ray film.

Real-Time Dual-Color Monitoring Intercellular Autophagy by Lyso-NCO in 6-hydroxydopamine (6-OHDA) Treated Cells

MCF-7 cells were incubated with 2 μM **Lyso-MCO/Lyso-NCO** at 37 °C under 5% CO_2 for 12 min, then washed 3-time with PBS buffer. Afterwards, the MitoTracker Red FM (MTR, final concentration: 0.5 μM) was added for another 30 min at 37 °C, washed 3-time with PBS buffer. Another two groups, MCF-7 cells pre-treatment with 6-hydroxydopamine (6-OHDA, autophagy inducer, final concentration: 40 μM) in the absence and presence of 3-Methyladenine (3-MA, autophagy inhibitor, final concentration: 100 μM) respectively, and then treated with 2 μM

Lyso-MCO/Lyso-NCO at 37 °C under 5% CO_2 for 12 min, then washed 3-time with PBS buffer. Subsequently, the MitoTracker Red FM (MTR, final concentration: 0.5 μM) was added for another 30 min at 37 °C, washed 3-time with PBS buffer. Finally, cells imaging were recorded at different time points (0, 30, 60, 90, 120, 150, 180, 210 and 240 min) on a confocal microscope (Zeiss LSM 710 Meta NLO with a 40/63 oil lens) with a mode-locked titanium-sapphire laser source set at wavelength 700 nm.

Synthesis of Compound 1[5]

The mixture of 3,6-diiodocarbazole (5 mmoL, 2.095 g) and KOH (10 mmol, 0.560 g), 2-(2-(2-methoxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate ($Me(CH_2CH_2O)_3OTs$, 2.40 g, 7.55 mmol) and DMF (30 mL) were stirred for 24 h at room temperature. Then 100 mL of H_2O was added. The aqueous solution was extracted with CH_2Cl_2 (DCM, 100 mL) and the DCM solution was washed with H_2O (3×100 mL), dried over anhydrous Na_2SO_4. The mixture was rotary evaporated, and the solvent was removed. The residue was purified by column chromatography (silica gel) using petroleum ether/ethyl acetate (v/v, PE:EA) = 6:1 as eluent to afford compound **1**, as a light yellow solid in 60.0% yield, m.p. 72.4-74.6 °C. FT-IR (KBr, cm^{-1}) : 2884, 1474,1422, 1292, 1123, 798.

^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.29 (d, J = 1.6 Hz, 2H), 7.69 (dd, J = 8.6, 1.6 Hz, 2H), 7.22 (d, J = 8.6 Hz, 2H), 4.40 (t, J = 5.6 Hz, 2H, CH_2), 3.81 (t, J = 5.6 Hz, 2H, CH_2), 3.52 – 3.37 (m, 8H), 3.33 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 139.76, 134.50, 129.21, 124.03, 111.32, 81.94, 77.37, 77.25, 77.05, 76.73, 71.84, 70.99, 70.63, 70.56, 69.33, 59.05, 43.48; HRMS (ESI, *m/z*)*:* Calcd for $C_{19}H_{21}I_2NO_3$ ($[M + H]^+$) 565.9709, found 565.9689; Anal. Calcd for $C_{19}H_{21}I_2NO_3$: C 40.37, H 3.74, I 44.90, N 2.47, O 8.49; found C 40.58, H 3.64, N 2.26.

Synthesis of Compound Lyso-MCO

Compound **1** (1.412 g, 2.5 mmol) and 1-ethynyl-4-methoxybenzene (0.990 g, 7.5 mmol) were added into a flask containing mixture of $Pd(PPh_3)_2Cl_2$ (0.070 g, 0.1 mmol), CuI (0.048 g, 0.25 mmol). Then Tetrahydrofuran (THF, 12 mL) and triethylamine (Et_3N, 6 mL) was added into above solution. Under the protection of argon, the system was heated at 50 °C for 12 h and cooled. Then, the mixture was filtered to remove salts. The solvent was distilled off under vacuum and the residue was purified by column chromatography (silica gel, PE:EA:DCM =5:1:1) to afford 1.08 g **Lyso-MCO**, as a light yellow solid in 75.20% yield, m.p. 123.5-125.4 °C. FT-IR (KBr, cm^{-1}): 2870, 2520, 1604, 1360, 1246, 1021.

^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.23 (d, J = 0.8 Hz, 2H), 7.62 (dd, J = 8.5, 1.4 Hz, 2H), 7.51 (d, J = 8.7 Hz, 4H), 7.41 (d, J = 8.5 Hz, 2H), 6.90 (d, J = 8.8 Hz, 4H), 4.47 (t, J = 5.6 Hz, 2H, CH_2), 3.87 (t, J = 5.8 Hz, 2H), 3.84 (s, 6H, CH_3), 3.46 (m, J = 8.7Hz, 8H, CH_2), 3.33 (s, 3H, CH_3); ^{13}C NMR (100MHz, $CDCl_3$, ppm): δ 159.36, 140.50, 132.92, 129.58, 123.81, 122.58, 115.93, 114.41, 114.01, 109.30, 89.11, 87.70, 77.35, 77.24, 77.04, 76.72, 71.84, 71.02, 70.66, 70.58, 69.37, 59.02, 55.32, 43.53. HRMS (ESI, *m/z*): Calcd for $C_{37}H_{35}NO_5$ $[M + H]^+$ 574.2611, found 574.2593; Anal. Calcd for $C_{37}H_{35}NO_5$: C 77.47, H 6.15, N 2.44, O 13.94; found C 77.25, H 6.11, N 2.26.

Synthesis of Compound Lyso-NCO

Compound **1** (1.412 g, 2.5 mmol) and 4-ethynyl-*N*,*N*-dimethylaniline (1.088 g, 7.5 mmol) were added into a flask containing mixture of $Pd(PPh_3)_2Cl_2$ (0.070 g, 0.1 mmol), CuI (0.048 g, 0.25 mmol). Then 12 mL THF and 6 mL Et_3N was added the above solution. Under the protection of argon, the system was heated at 50 ^{o}C for 12 h and cooled. Then, the mixture was filtered to remove salts. The solvent was distilled off and the residue was purified by column chromatography (silica gel, PE:EA:DCM =4:1:1) to afford 1.14 g **Lyso-NCO**, as a light yellow solid in 76.2% yield, m.p. 145.2-147.6 ^{o}C. FT-IR (KBr, cm^{-1}): 2884, 2807, 2207, 1610, 1512, 1360, 1130.

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.22 (s, 2H), 7.60 (d, J = 8.5 Hz, 2H), 7.46 (d, J = 8.7 Hz, 4H), 7.39 (d, J = 8.5 Hz, 2H), 6.69 (d, J = 8.4 Hz, 4H), 4.47 (t, J = 5.6 Hz, 2H, CH_2), 3.86 (t, J = 5.7 Hz, 2H, CH_2), 3.55 (m, 6H, CH_2), 3.41 (dd, J = 3.1 Hz, 2H, CH_2), 3.34 (s, 3H, CH_3), 2.99 (s, 12H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 149.86, 140.23, 132.56, 129.43, 123.56, 122.59, 114.88, 111.97, 110.74, 109.16, 88.71, 88.32, 71.81, 70.99, 70.61, 70.53, 69.34, 58.98, 43.45, 40.28. HRMS (ESI, *m/z*): Calcd for $C_{39}H_{41}N_3O_3$ $[M + H]^+$ 600.3250, found 600.3226; Anal. Calcd for $C_{39}H_{41}N_3O_3$: C 78.10, H 6.89, N 7.00, O 7.99; found C 78.08, H 6.89, N 6.95.

References

(1) Xu, C.; Webb, W. W. *J. Opt. Soc. Am. B* **1996**, 13, 481-491.

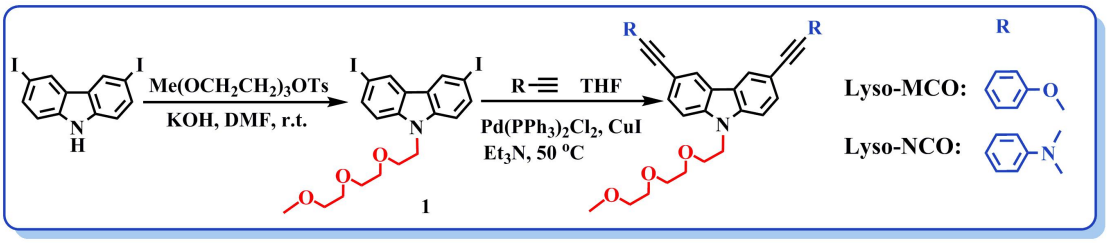
(2) Crosby, G. A.; Demas, J. N. *J. Phys. Chem.* **1971**, 75, 991-1024.

(3) Makarov, N. S.; Drobizhev, M.; Rebane, A. *Opt. Express* **2008**, 16, 4029-4047.

(4) Yao, D. F.; Lin, Z.; Wu, J. C. *ACS Appl. Mater. Inter.* **2016**, 8, 5847-5856.

(5) Gross, D. E.; Moore, J. S. *Macromolecules* **2011**, 44, 3685-3687.

Supporting Figures



Scheme S1 Synthetic routes of lysotrackers **Lyso-MCO** and **Lyso-NCO**.

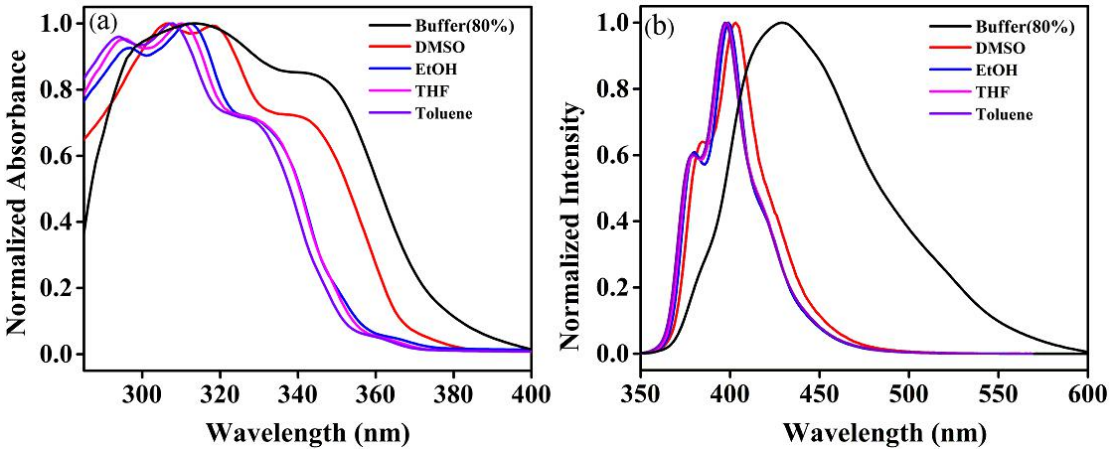


Fig. S1 Normalized absorption (a) and emission (b) spectra of **Lyso-MCO** in PBS Buffer (containing 20% DMSO), DMSO, EtOH, THF and toluene, respectively.

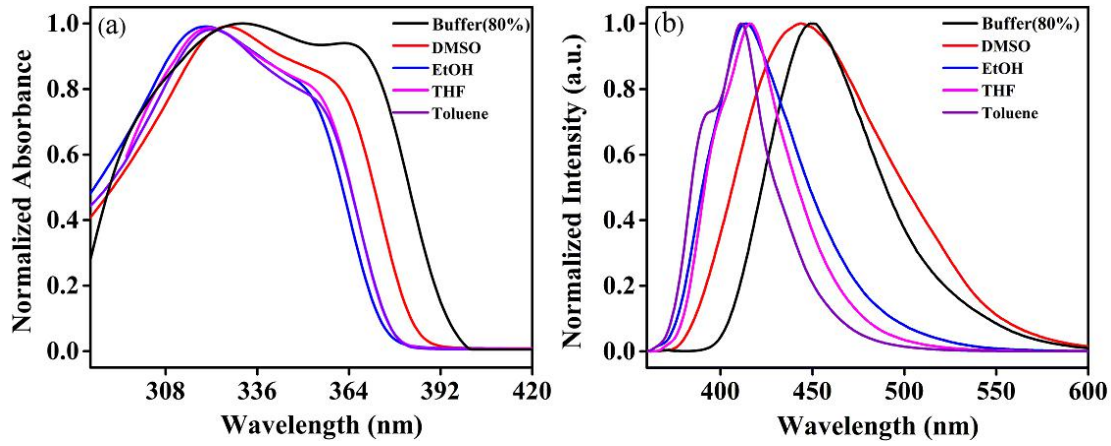


Fig. S2 Normalized absorption (a) and emission (b) spectra of **Lyso-NCO** in PBS Buffer (containing 20% DMSO), DMSO, EtOH, THF and toluene, respectively.

Table S1 Photophysical properties of **Lyso-MCO/Lyso-NCO** in different solvents.

Compound	Solvents	$\lambda_{ab}^{a}(\varepsilon^{b})$	λ_{em}^{c}	$\Delta\lambda_{Stokes}^{d}$	Φ^{e}	$\delta\Phi^{f}$
Lyso-MCO	Buffer (containing 20% DMSO)	318 (4.75)	429	108	10	/
	DMSO	315 (12.98)	403	85	32	76
	Ethyl alcohol	312 (14.82)	399	87	/	/
	THF	310 (15.17)	398	80	/	/
	Toluene	308 (13.51)	397	89	/	/
Lyso-NCO	Buffer (containing 20% DMSO)	329 (5.58)	450	121	19	/
	DMSO	328 (18.43)	444	116	43	215
	Ethyl alcohol	317 (19.43)	414	95	/	/
	THF	321 (18.73)	416	92	/	/
	Toluene	321 (18.40)	411	90	/	/

[a] Peak position of the maximum absorption band (10 μM). [b] Maximum molar absorbance in 10^4 mol^{-1} L cm^{-1}. [c] Peak position of the maximum emission band. [d] Stokes shift in nm. [e] Fluorescent quantum yield (10 μM in DMSO and PBS buffer containing 20% DMSO). [f] Maximum two-photon action cross-sections (0.1 mM in DMSO).

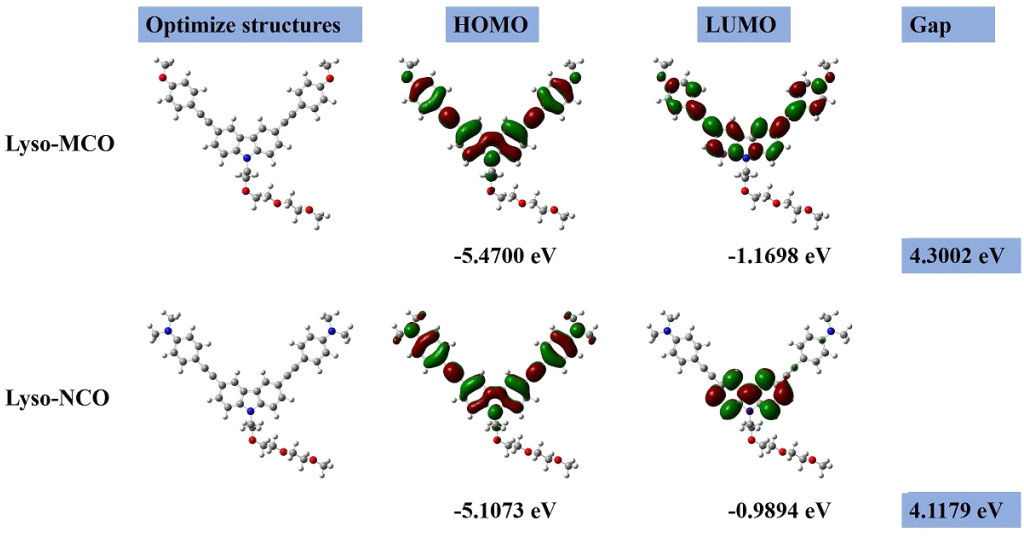


Fig. S3 Spatial distributions of calculated HOMOs and LUMOs and HOMO/LUMO energy gaps (units, eV) of **Lyso-MCO** and **Lyso-NCO**.

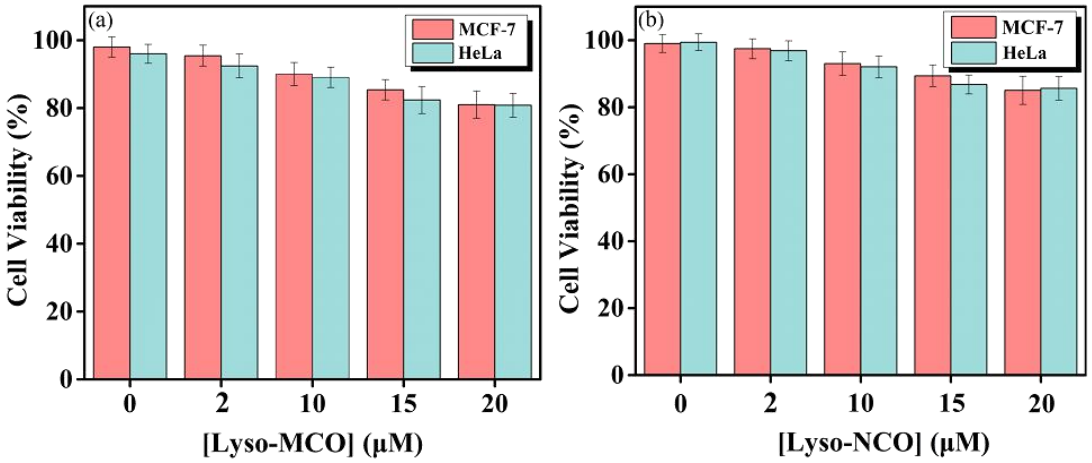


Fig. S4 Cytotoxicity data of **Lyso-MCO** (a) and **Lyso-NCO** (b) (MCF-7 cells and HeLa cells incubated for 24 h).

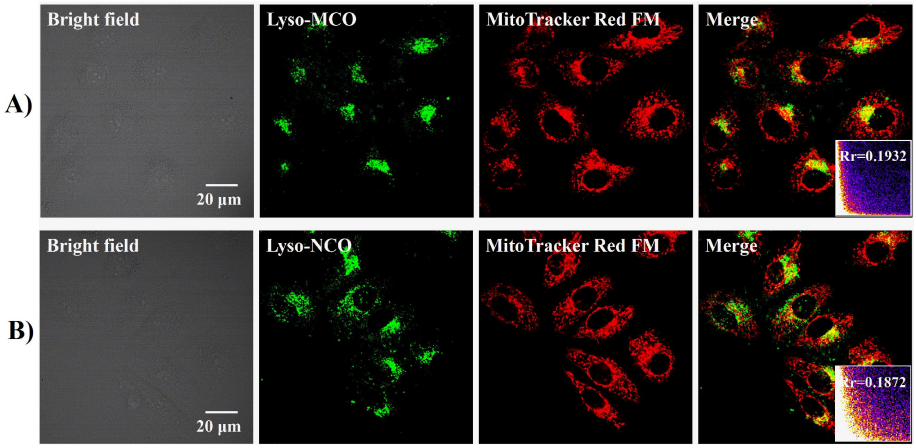


Fig. S5 Confocal fluorescence images of MCF-7 cells. Cells were treated with **Lyso-MCO**/**Lyso-NCO** (2 μM) for 12 min and subsequently with MitoTracker Red FM (MTR, 0.5 μM) for 30 min. Green channel (**Lyso-MCO**, 426 ± 20 nm; **Lyso-NCO**, 450 ± 20 nm), λ_{ex} = 700 nm; red channel (599 ± 20 nm), λ_{ex} = 579 nm; (Merge) overlap image of green channel and red channel. Inset: colocalization profile between probes and MTR; Scale bars: 20 μm.

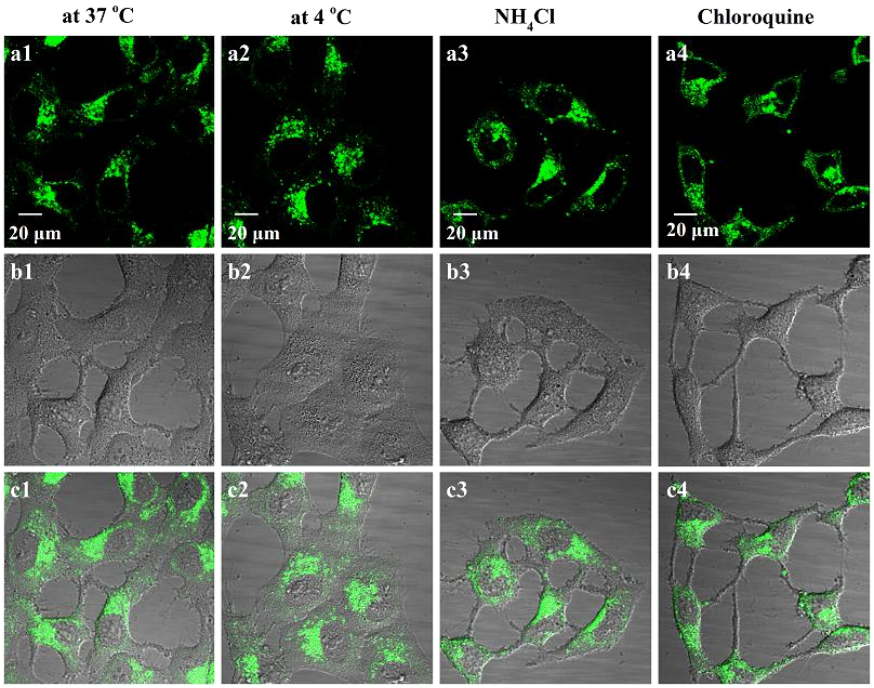


Fig. S6 TPM images of MCF-7 cells treated with **Lyso-MCO** under different conditions. (a) The cells were treated with **Lyso-MCO** at 37 °C for 12 min (a1~c1), at 4 °C for 12 min. (a2~c2). The cells were pretreated with endocytic inhibitors NH_4Cl (a3~c3), and chloroquine (a4~c4) respectively, and then treated with **Lyso-MCO** at 37 °C for 12 min. Green channel (**Lyso-MCO**, 426 ± 20 nm), λ_{ex} = 700 nm. Scale bar: 20 μm.

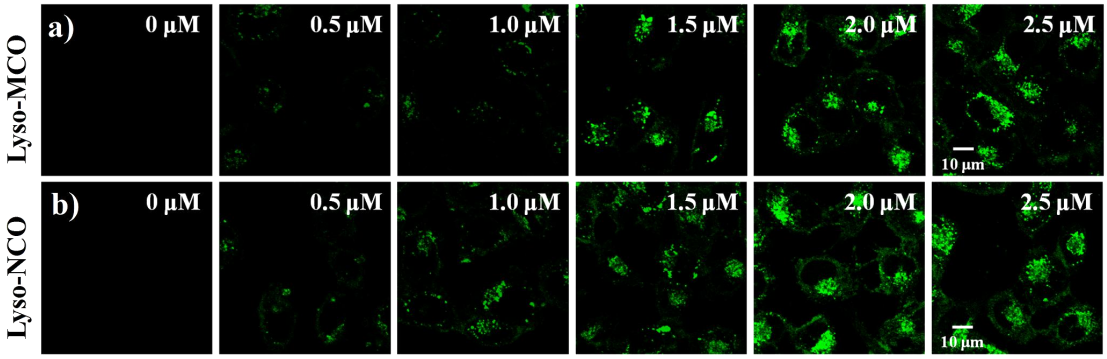


Fig. S7 Two-photon confocal fluorescence microscopy images of MCF-7 cells stained with different concentrations (0~2.5 μM) of **Lyso-MCO** (a) and **Lyso-NCO** (b). Scale bar: 10 μm.

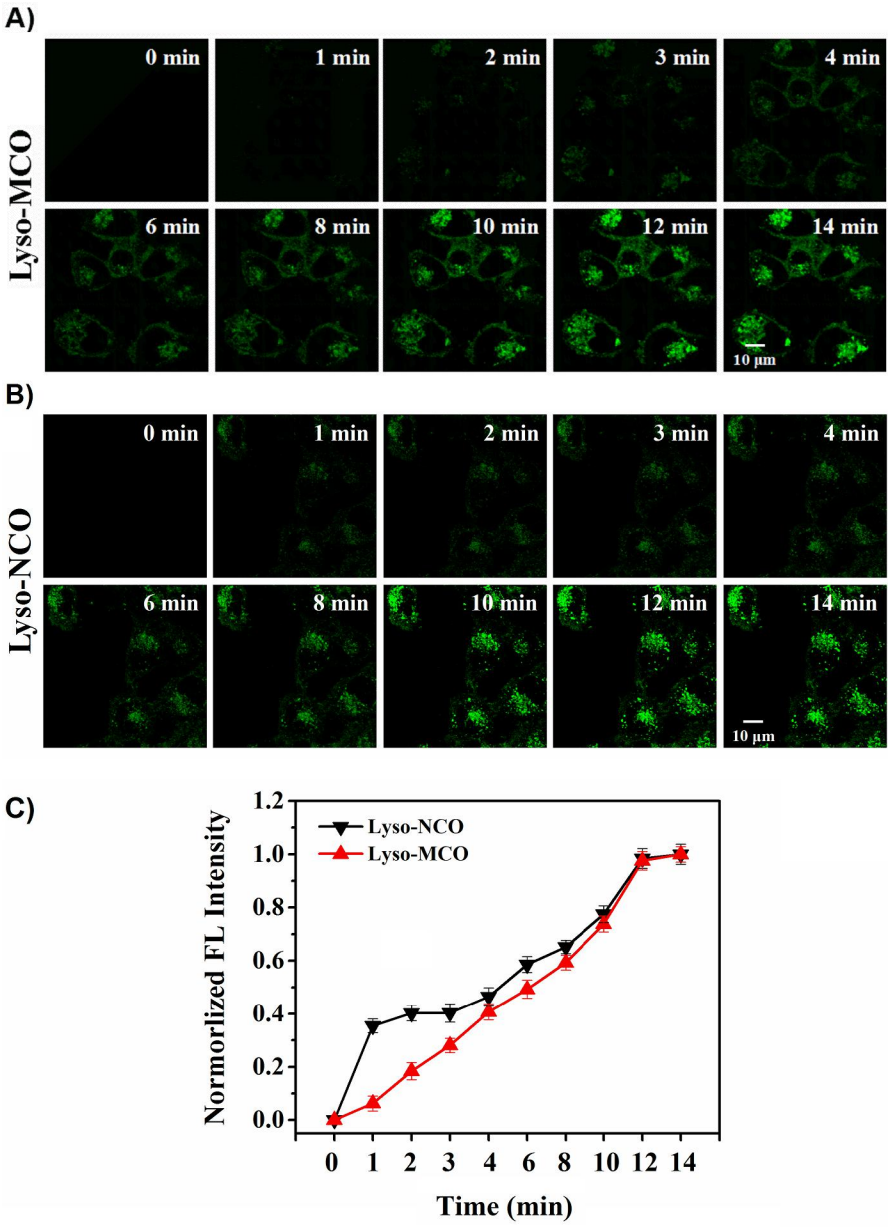


Fig. S8 Two-photon confocal fluorescence microscopy images of MCF-7 cells stained with **Lyso-MCO** (2 μM, A) and **Lyso-NCO** (2 μM, B) at different time points (0-14 min). Green channel (**Lyso-MCO**, 426 ± 20 nm; **Lyso-NCO**, 450 ± 20 nm), λ_{ex} = 700 nm. (C) Fluorescence enhancement curves of **Lyso-MCO** and **Lyso-NCO** after different time scans. (**Lyso-MCO**, 426 ± 20 nm; **Lyso-NCO**, 450 ± 20 nm), λ_{ex} = 700 nm. Scale bar: 10 μm.

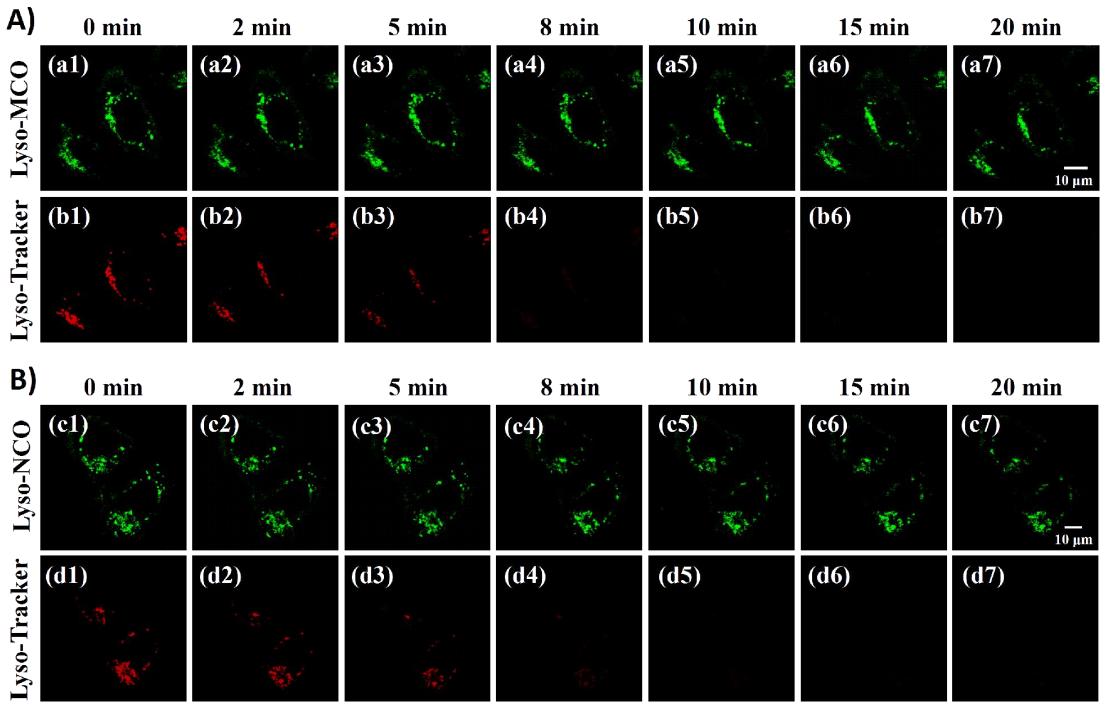


Fig. S9 (A) Two-photon confocal fluorescence microscopy images of MCF-7 cells co-stained with **Lyso-MCO** (2 μM) and LTR (0.5 μM) at different time points (0-20 min), (a1~a7) green channel (426 ± 20 nm), λ_{ex} = 700 nm; (b1~b7) red channel (590 ± 20 nm), λ_{ex} = 559 nm; (b) Two-photon confocal fluorescence microscopy images of MCF-7 cells stained with **Lyso-NCO** (2 μM) and LTR (0.5 μM) at different time points (0-20 min). (c1~c7) green channel (450 ± 20 nm), λ_{ex} = 700 nm; (d1~d7) red channel (590 ± 20 nm), λ_{ex} = 559 nm. Scale bar: 10 μm.

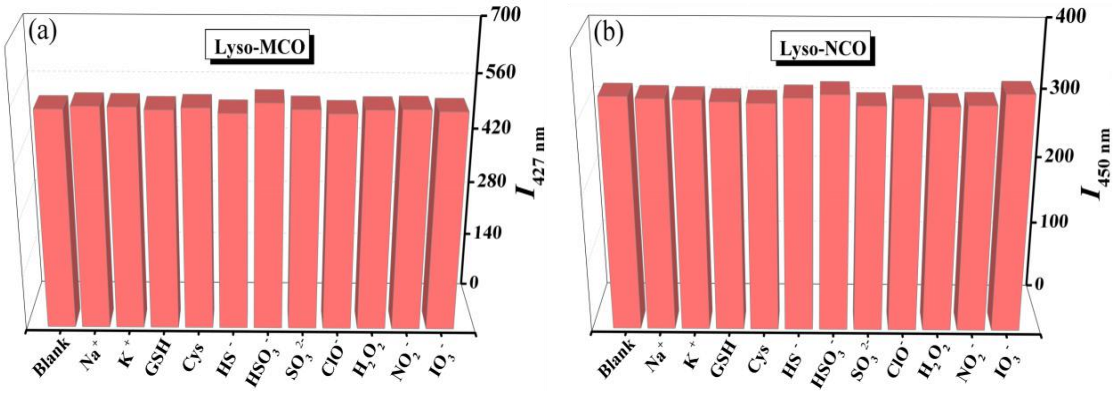


Fig. S10 Fluorescence intensities of **Lyso-MCO** and **Lyso-NCO** (10 μM) to various analytes (left-right): Blank, Na^+, K^+, GSH, Cys, HS^-, HSO_3^-, SO_3^{2-}, ClO^-, H_2O_2, NO_2^- and IO_3^- in DMSO/PBS buffer (2/8, v/v, 20 mM, pH 7.4). (a, λ_{ex} = 318 nm; b, λ_{ex} = 328 nm).

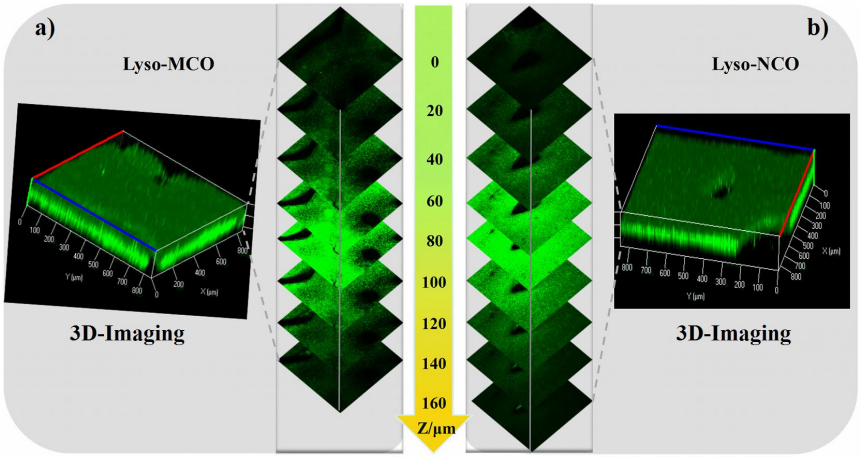


Fig. S11 Two-photon fluorescence images of a fresh rat liver slice incubated with **Lyso-MCO** (a, 10 μM) at depths of approximately 0 to 140 μm and **Lyso-NCO** (b, 10 μM) at depths of approximately 0 to 160 μm with a magnification of 20 X. Green channel (**Lyso-MCO**, 426 ± 20 nm; **Lyso-NCO**, 450 ± 20 nm), λ_{ex} = 700 nm.

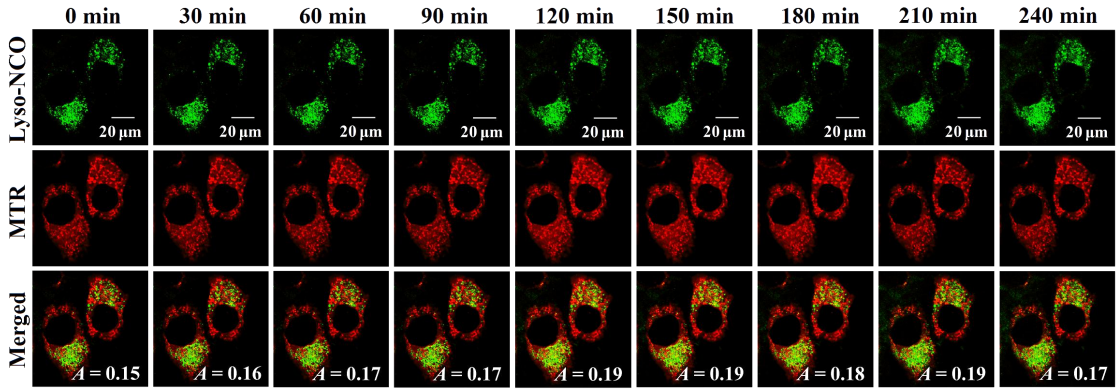


Fig. S12 Dual-color TPM images of MCF-7 cells in rich-nutrient condition (control) at different time nodes. MCF-7 cells were pretreated with **Lyso-NCO** (2 μM), then co-labeled with 0.5 μM MTR for 30 min before imaging. **Lyso-NCO**, 450 ± 20 nm; MTR, 599 ± 20 nm; Merged images of green channel and red channel. The numbers in the bottom column stand for the co-localization coefficients

Lyso-NCO and MTR (A value), λ_{ex} = 700 nm. Scale bar: 20 μm. Cells shown are representative images from replicate experiments (n = 5).

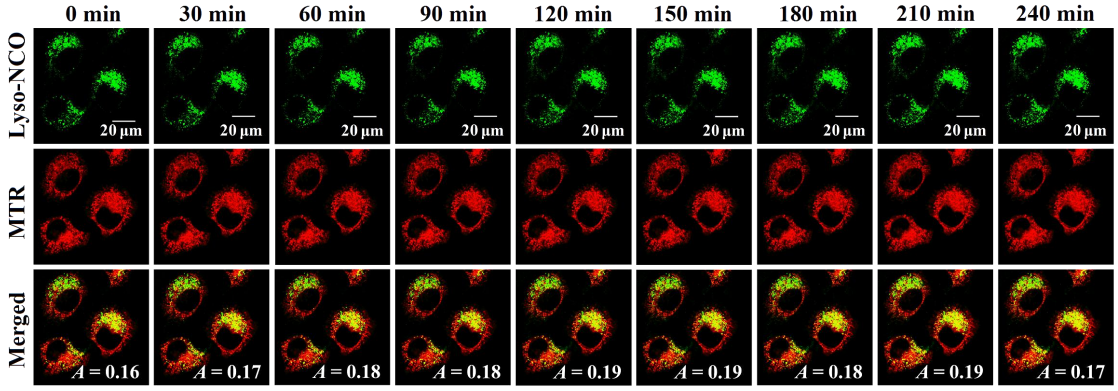


Fig. S13 Dual-color TPM images of MCF-7 cells in 6-OHDA + 3-MA (inhibition of autophagy) at different time nodes. MCF-7 cells were pretreated with **Lyso-NCO** (2 μM), then co-labeled with 0.5 μM MTR for 30 min before imaging. **Lyso-NCO**, 450 ± 20 nm; MTR, 599 ± 20 nm; Merged images of green channel and red channel. The numbers in the bottom column stand for the co-localization coefficients between **Lyso-NCO** and MTR (A value), λ_{ex} = 700 nm. Scale bar: 20 μm. Cells shown are representative images from replicate experiments (n = 5).

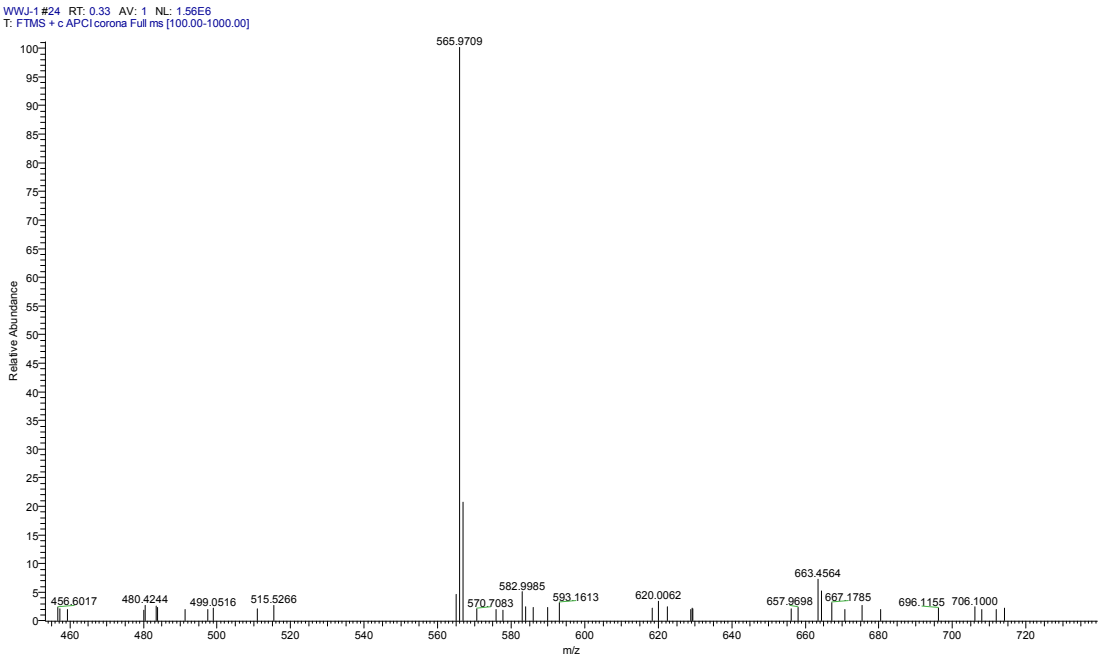


Fig. S14 ESI-TOF mass spectra of compound **1**.

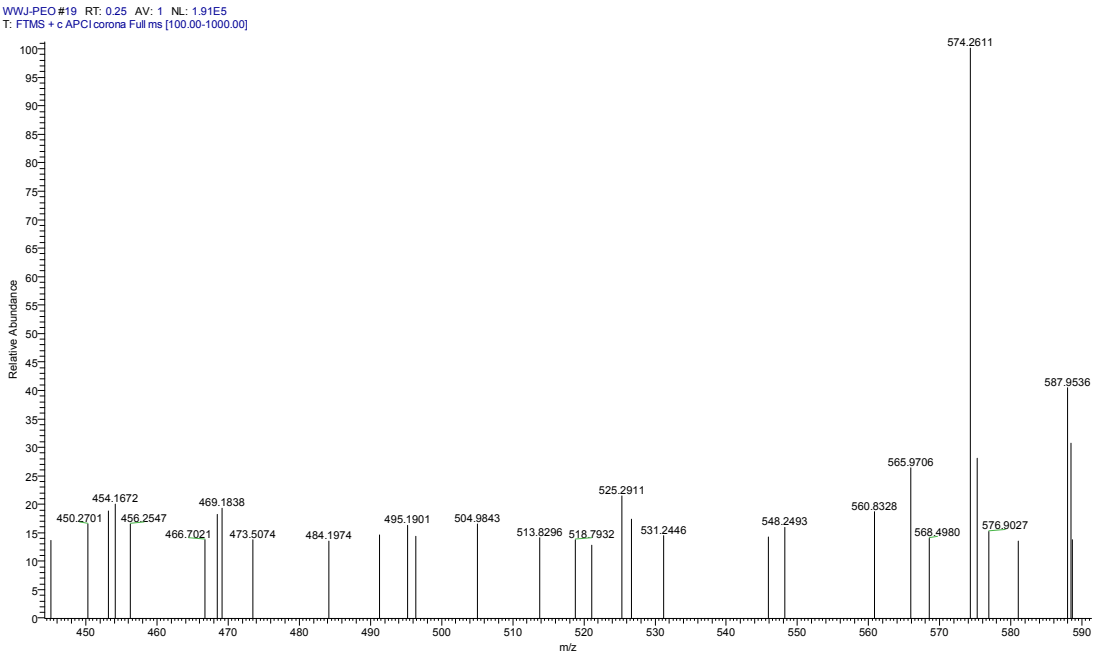


Fig. S15 ESI-TOF mass spectra of **Lyso-MCO**.

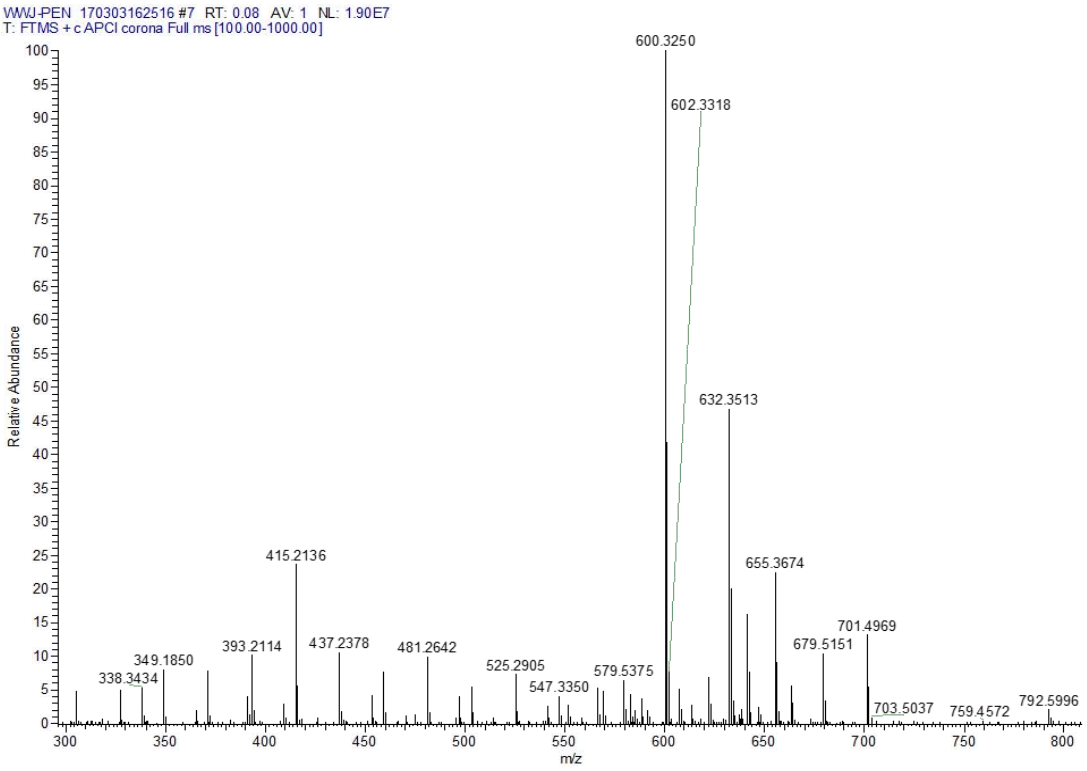


Fig. S16 ESI-TOF mass spectra of **Lyso-NCO**.

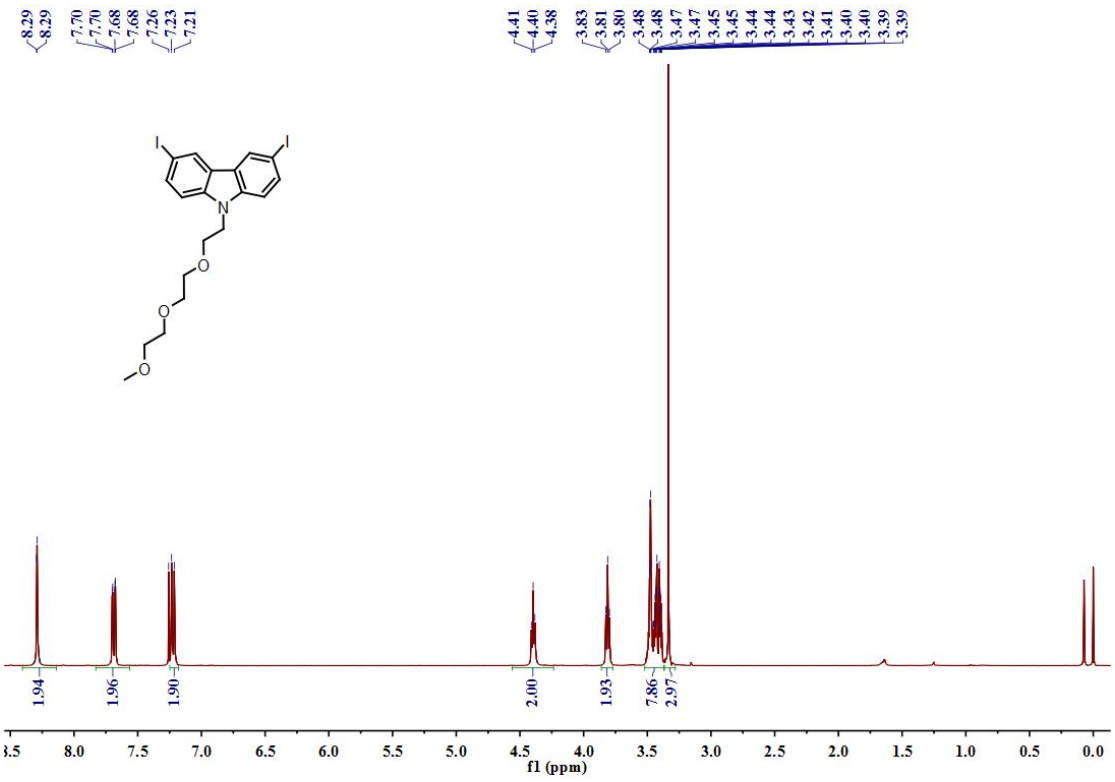


Fig. S17 ^{1}H NMR of compound **1** in $CDCl_3$.

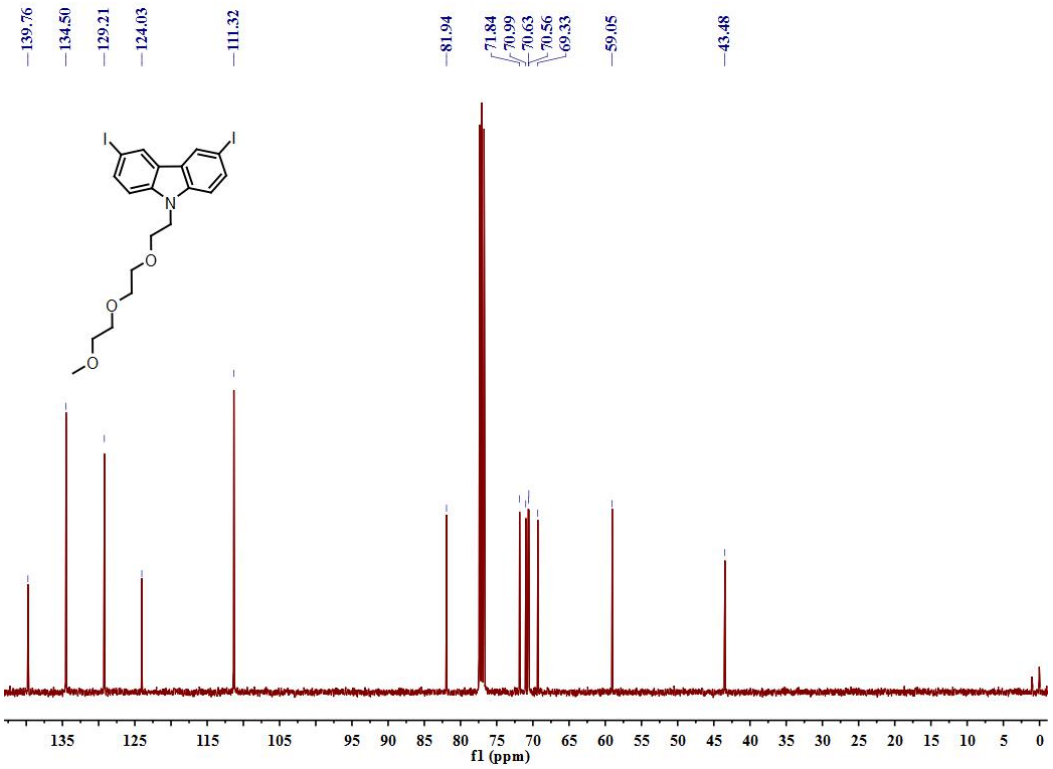


Fig. S18 ^{13}C NMR of compound **1** in $CDCl_3$.

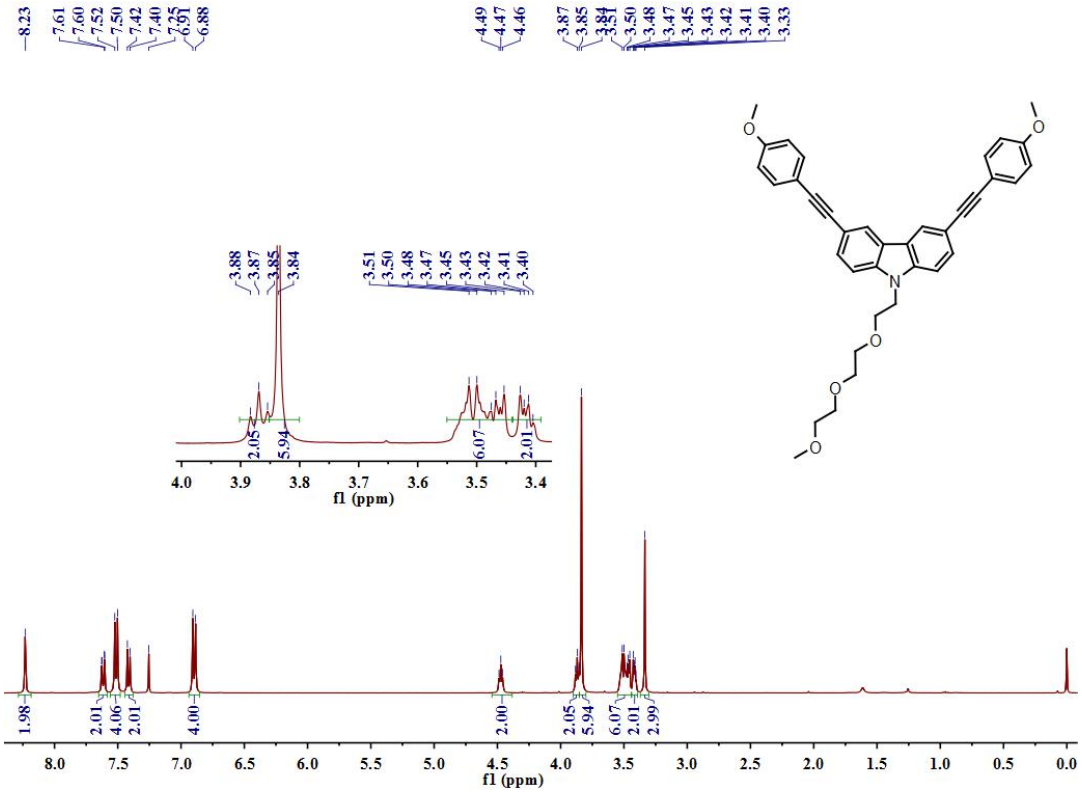


Fig. S19 ^{1}H NMR of **Lyso-MCO** in $CDCl_3$.

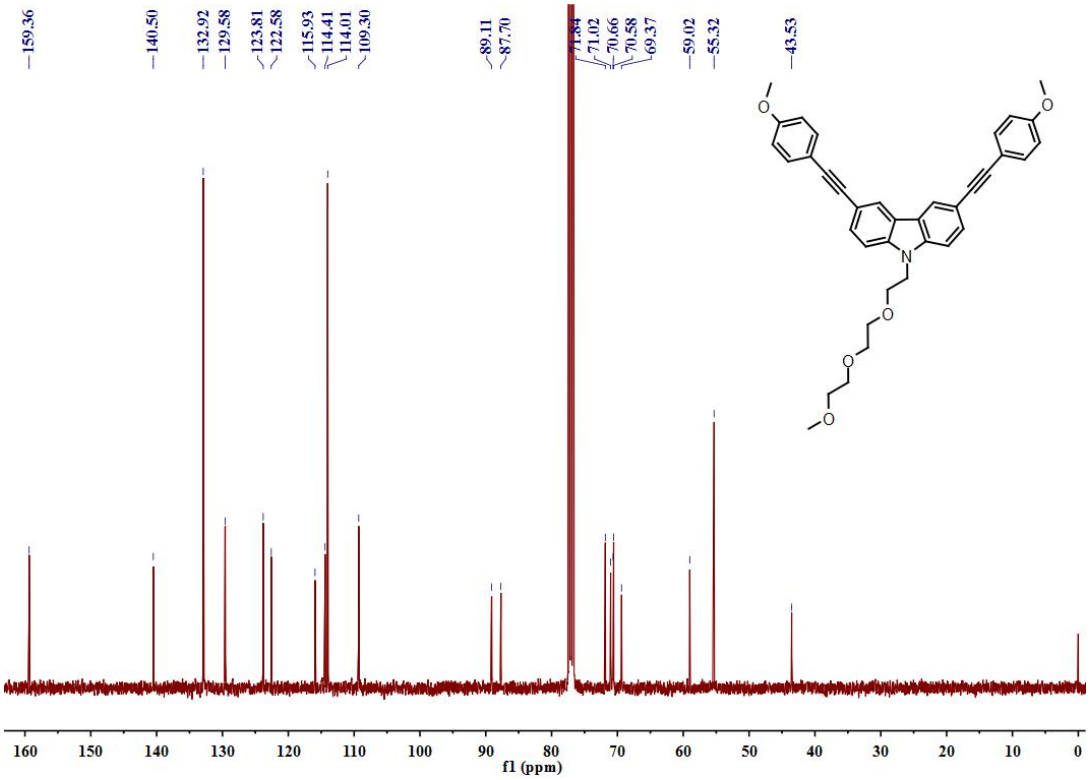


Fig. S20 ^{13}C NMR of **Lyso-MCO** in $CDCl_3$.

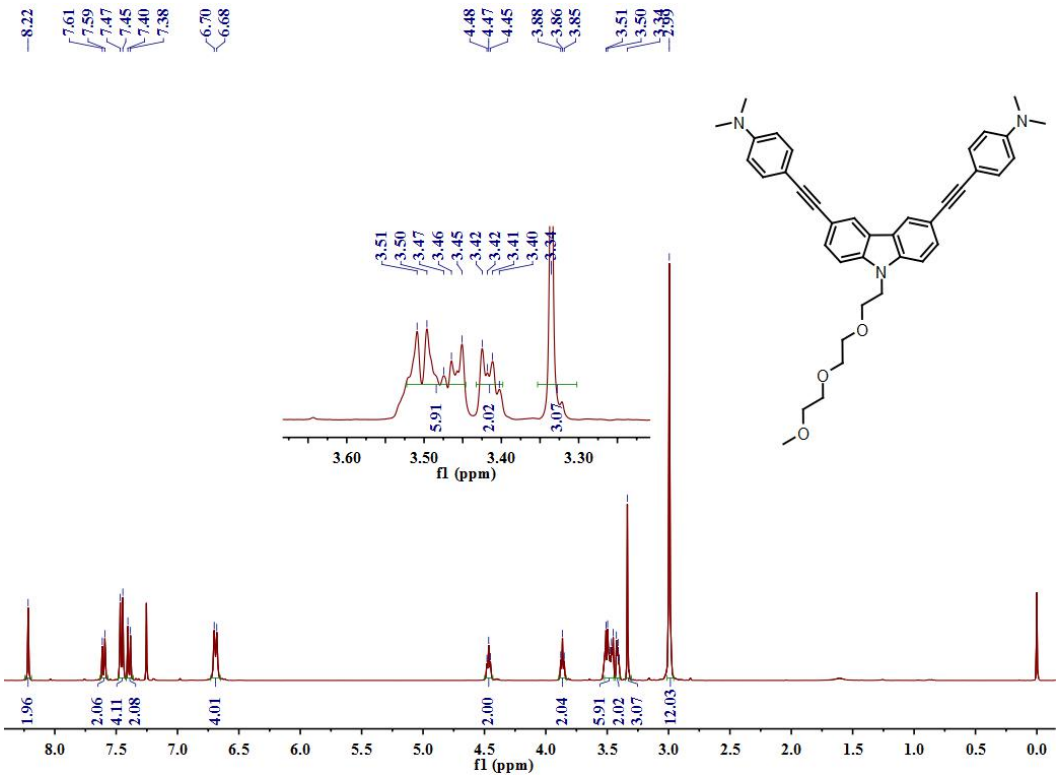


Fig. S21 1H NMR of **Lyso-NCO** in $CDCl_3$.

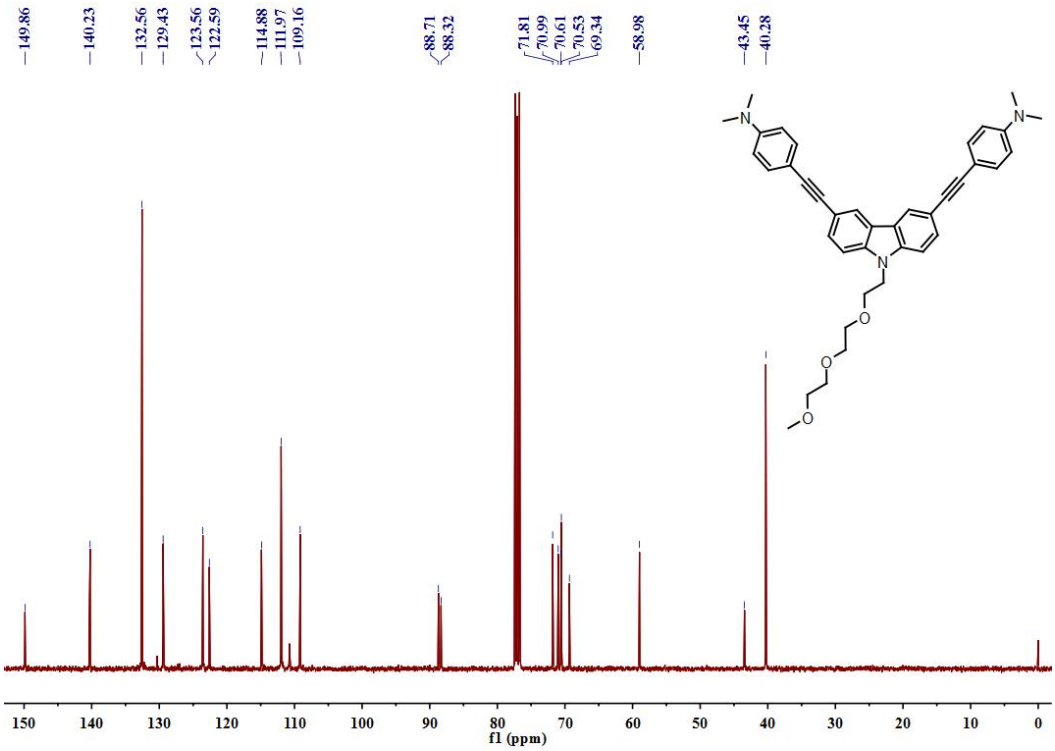


Fig. S22 ^{13}C NMR of **Lyso-NCO** in $CDCl_3$.