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Activatable Near-Infrared Fluorescence Probe Facilitated High-contrast Lipophagic Imaging in Live Cells

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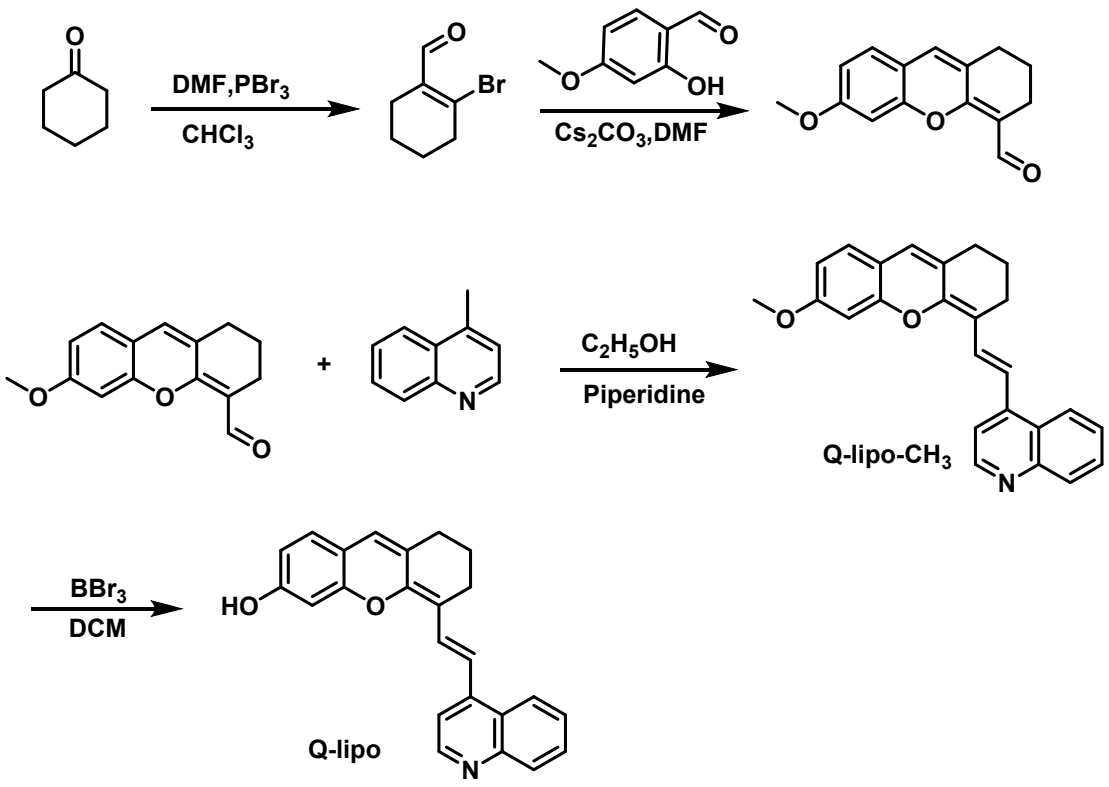
Scheme S1. Synthetic routes for the probe Q-lipo.



Chemicals and materials

Phosphorus tribromide, cesium carbonate, 4-methoxysalicylaldehyde, 4-methylquinoline and boron tribromide were purchased from Energy Chemical (Shanghai, China). Cyclohexanone, phosphorus oxychloride and organic solvents were of analytical grade and obtained from Sinopharm Chemical Reagent (Shanghai, China). All the organic solvents were dried over 4Å molecular sieves before use. HeLa cells (human cervical carcinoma cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). DMEM high glucose medium, penicillin, streptomycin and 10% heat-inactivated fetal bovine serum were purchased from Thermo Fisher (MA, USA). Hoechst 33342, Nile Red, Mito-Tracker Red and Lyso-Tracker Red were obtained from ThermoFisher Scientific. Hanks' Balanced Salt solution (HBSS) was purchased from Sigma. Ultrapure water with an electric resistance >18.25 MΩ obtained from a Millipore Milli-Q water purification system (Billerica, MA, USA) was used throughout the experiments. Thin-layer chromatography (TLC) was performed on silica gel aluminum sheets with an F-254 indicator. Column chromatography was conducted using 200-300 mesh SiO_2 (Qingdao Ocean Chemical Products).

Apparatus and Characterization

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance-III 400 instrument (Bruker) using tetramethylsilane (TMS) as an internal standard. Mass spectroscopy (MS) analysis was performed on LCQ advantage ion trap mass spectrometry (Thermo Fisher Scientific, Bremen, Germany). Fluorescence images of HeLa cells were obtained using an inverted fluorescence microscope (Nikon, Tokyo, Japan) with 60× objective lens. pH was determined using a Mettler-Toledo FE20 pH meter. UV-vis absorption spectra were recorded with a Shimadzu UV-2450 spectrophotometer with an interval of 2 nm. Fluorescence spectra were recorded on FS5 spectrofluorometer (Edinburgh, UK).

Synthesis of 2-bromocyclohex-1-ene-1-carbaldehyde

A mixture of chloroform (50ml) and dimethylformamide (DMF, 9 mL) was stirred in an ice bath for 10 min. Then phosphorus tribromide (9 mL) was slowly added to the mixture. After reaction for 45 min, cyclohexanone (4 mL) was added dropwise. After reaction, the mixture was poured into ice water, neutralized with saturated $NaHCO_3$,

and extracted with dichloromethane (DCM 50 mL × 3). The combined organic phase was washed with brine (50 mL × 2), and dried over Na_2SO_4. The mixture was filtered and the filtrate was concentrated under reduced pressure. The desired residue was purified by chromatography (petroleum ether : EA = 50 : 1) to yield 2-bromocyclohex-1-ene-1-carbaldehyde as a colorless liquid. ^{1}H NMR (400 MHz $CDCl_3$) δ (ppm):9.91 (s, 1H), 2.67 (s, 2H), 2.21 (d, J = 10 Hz 2H), 1.69-1.61 (m, 4H). ^{13}C NMR (100 MHz $CDCl_3$) δ (ppm): 193.56, 143.59, 135.2, 38.78, 24.96, 24.23, 21.05.

Synthesis of 6-methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde

A mixture of chloroform (50ml) and dimethylformamide (DMF, 9 mL) was stirred in an ice bath for 10 min. Then phosphorus tribromide (9 mL) was slowly added to the mixture. After reaction for 45 min, cyclohexanone (4 mL) was added dropwise. After reaction, the mixture was poured into ice water, neutralized with saturated $NaHCO_3$, and extracted with dichloromethane (DCM 50 mL × 3). The combined organic phase was washed with brine (50 mL × 2), and dried over Na_2SO_4. The mixture was filtered and the filtrate was concentrated under reduced pressure. The desired residue was purified by chromatography (petroleum ether : EA = 50 : 1) to yield 2-bromocyclohex-1-ene-1-carbaldehyde as a colorless liquid. ^{1}H NMR (400 MHz $CDCl_3$) δ (ppm):9.91 (s, 1H), 2.67 (s, 2H), 2.21 (d, J = 10 Hz 2H), 1.69-1.61 (m, 4H). ^{13}C NMR (100 MHz $CDCl_3$) δ (ppm): 193.56, 143.59, 135.2, 38.78, 24.96, 24.23, 21.05.

Synthesis of 6-methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde

Compound 2-bromocyclohex-1-ene-1-carbaldehyde was added to a mixture of cesium carbonate and 4-methoxysalicylaldehyde in DMF. After reacting for 12 h, the mixture was filtered and the filtrate was concentrated under reduced pressure. The desired residue was purified with chromatography (petroleum ether : EA =4 : 1) to yield 6-methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde as a yellow solid (1.2g, 70%). ^{1}H NMR (400 MHz $CDCl_3$) δ (ppm):10.32 (s, 1H), 7.08 (d, J = 8.28 Hz, 1H), 6.69-6.74 (m, 3H), 3.85 (s, 3H), 2.57 (t, J = 6.32 Hz, 2H), 2.45 (t, J = 6.2 Hz, 2H), 1.69-1.75 (m, 2H). ^{13}C NMR (100 MHz $CDCl_3$) δ (ppm): 187.59, 161.37, 160.77, 153.38, 127.45, 126.84, 126.58, 114.68, 112.58, 110.85, 100.49, 55.66, 29.94, 21.53, 20.42. MS (ESI) calcd for $C_{15}H_{14}O_3$ $[M+H]^+$ m/z 242.06, found 242.06.

Synthesis of Q-lipo-CH_3

6-Methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde (1.20 g, 5.50 mmol) and 4-methylquinoline (0.75 g，5.0 mmol) were dissolved in anhydrous ethanol, then the mixture was reacted at 80 °C under the protection of argon atmosphere for 12 h. The mixture was sequentially washed with brine, dried over Na_2SO_4 and filtered. The filtrate was concentrated by rotary evaporator, the residue was further purified by silica-gel column (eluent: DCM : methyl alcohol =10 : 1) to give Q-lipo-CH_3 as a red solid (0.90 g，60%). ^{1}H NMR (400 MHz $CDCl_3$) δ (ppm):8.87 (d, J = 4.8 Hz, 1H), 8.22 (d, J = 8.52 Hz, 1H), 8.11 (d, J = 8.52 Hz, 1H), 7.93 (d, J = 15.76 Hz, 1H), 7.66-7.73(m, 2H), 7.56 (t, J = 7.66 Hz, 1H), 7.14 (d, J = 15.8 Hz, 1H), 6.94 (d, J = 8.28 Hz, 1H), 6.62 (s, 1H), 6.54 (d, J = 8.2 Hz, 1H), 6.28 (s, 1H), 3.84 (s, 3H), 2.64 (d, J = 6.2 Hz, 2H), 2.54 (d, J = 6.2 Hz, 2H), 1.83-1.90(m, 2H). ^{13}C NMR (100 MHz $CDCl_3$) δ (ppm): 160.54, 154.13, 150.01, 149.24, 148.76, 144.08, 129.93, 129.84, 129.03, 127.82, 126.47, 126.35, 125.99, 123.40, 121.38, 117.58, 115.97, 115.49, 110.63, 109.26, 100.45, 55.55, 29.94, 24.98, 21.07. MS (ESI) calcd for $C_{25}H_{21}O_2N$ $[M+H]^+$ m/z 368.16, found 368.23.

Synthesis of Q-lipo

Lipo-CH_3 (0.79 g，2.17mmol) was added in absolute DCM (10 mL) and stirred in an ice bath. Boron bromide (1.0 mL) was added dropwise and reacted for 8 h. Then, saturated sodium bicarbonate was added slowly to quench the excess boron bromide . The mixture was extracted with ethyl acetate (50 mL × 3). The organic phase was washed with brine (50 mL × 2), and dried over Na_2SO_4. Afterwards the mixture was filtered and the filtrate was concentrated under reduced pressure. The desired residue was purified with chromatography (methanol : DCM =1 : 5) to yield Q-lipo as a blue solid (0.61 g，80% yield). ^{1}H NMR (400 MHz, DMSO-d^6) δ (ppm): 9.94 (s, 1H), 8.83 (d, J = 5.32 Hz, 1H), 8.45 (d, J = 8.48 Hz, 1H), 8.87 (d, J = 4.8 Hz, 1H), 7.99-8.03(m, 2H), 7.89 (d, J = 4.92 Hz, 1H), 7.81 (t, J = 7.92 Hz, 1H), 7.66 (d, J = 16.0 Hz, 1H), 7.01-7.05(m, 1H), 6.62 (s, 1H), 6.55 (s, 1H), 6.49 (d, J = 8.32 Hz, 1H), 2.64 (t, J = 5.76 Hz, 2H), 1.95-2.03(m, 2H), 1.78 (t, J = 6.34 Hz, 2H). ^{13}C NMR (100 MHz DMF-d^7) δ (ppm): 160.62, 154.59, 154.37, 153.22, 141.87, 139.20, 137.48, 133.91, 128.58, 126.81, 125.68, 125.40, 121.22, 114.48, 114.23, 114.19, 112.36, 112.30, 102.49, 29.39, 24.77, 21.00. MS (ESI) calcd for $C_{25}H_{21}O_2N$ $[M+H]^+$ m/z 354.25, found 354.25. MS (ESI) calcd for $C_{25}H_{21}O_2N$ $[M+H]^+$ m/z 354.25, found 354.25.

In vitro Characterization of Q-lipo.

Glycerol solutions of different pH were obtained by mixing buffer solutions of different pH with glycerol. Q-lipo in different viscous solutions were prepared by adding the corresponding stock solution (1.0 mM Q-lipo in DMSO) to glycerol solutions of different buffers. The mixtures were shook for 3 min to eliminate air bubbles and stood at 25 ^{o}C for 30 min. Absorbance spectra were recorded with a UV spectrometer. Fluorescence spectra for Q-lipo were recorded with a fluorescence spectrometer using excitation wavelengths of 470 nm and 640 nm, respectively. For studying the effect of solvents on the emission spectra of the probes, stock solutions of probes were diluted with acetone, DCM, EA, ethanol (EtOH), PBS, THF and glycerol, respectively. Fluorescence spectra were recorded at room temperature after thorough mixing. The viscosity sensitivity of Q-lipo was investigated by measuring their fluorescence spectra in binary system of PBS-glycerol with different volume fractions of glycerol.

Cytotoxicity studies.

The cytotoxicity of Q-lipo against HeLa cells was studied using a WST-1 cell proliferation and cytotoxicity assay following the kit protocol. Briefly, cells were incubated with various concentrations of Q-lipo (0 - 30 μM) for 24 h, then WST-1 was added and incubated for another 4 h. Cell viability was determined by measuring the absorbance at 450 nm with a microplate reader.

Cell culture and fluorescence colocalization assay.

HeLa cells were grown in DMEM high glucose medium supplemented with 10% FBS (fetal bovine serum), penicillin (100 U/mL) and streptomycin (100 U/mL) in an atmosphere of 5% CO_2 at 37 °C. The cells were cultured on a 35-mm Petri dish with a 10-mm bottom well in a DMEM high glucose medium for 24 h and the dishes were washed with PBS for three times. For colocalization assay, the cultured HeLa cells were incubated with Q-lipo for 20 min, and stained with Hoechst 3342 (500 nm), Nile Red (0.5 μM), Mito-Tracker red (0.5 μM) and Lyso-Tracker Red (200 nM) for 30 min, respectively. Confocal images were acquired using the following conditions: Excitation wavelength for Q-lipo: 488 nm; Emission collection: 510 nm-580 nm; Excitation wavelength for Hoechst 3342: 405 nm; Emission collection: 420 nm-470 nm. Excitation wavelength for Mito-Tracker red: 559 nm; Emission collection: 580 nm-630 nm. Excitation wavelength for Lyso-Tracker Red: 559 nm; Emission

collection: 580 nm-630 nm. Excitation wavelength for Nile Red: 559 nm; Emission collection: 600 nm-680 nm.

Live cell fluorescence imaging of lipophagy with Q-lipo.

To investigate the ability of Q-lipo to image lipophagy, HeLa cells were treated by 10 μM Q-lipo in DMEM for 30 min. After washing with PBS for three times, the cells were cultured in the Hanks' Balanced Salt solution (HBSS) medium with low concentrations of glucose without fetal bovine serum (FBS) and amino acids. Fluorescence images were obtained at different time intervals with excitation wavelengths of 488 nm and 640 nm, respectively. For studying the subcellular distribution of Q-lipo stained lipid droplets upon lipophagy induction, colocalization assays were performed with LysoBlue Tracker to stain the lysosomes.

To investigate lipophagy in different conditions, the prepared cells were incubated in 10 μM Q-lipo in DMEM for 30 min. After washing with PBS for three times, the cells were incubated in different culture medium and fluorescence images were acquired at different time points. To investigate the effect of hypoxia on lipophagy, the cells were cultured in full DMEM supplemented with FBS in a hypoxia atmosphere. To investigate the effects of glucose-, glutamine- or both-depletion on lipophagy, the cells were cultured in DMEM without glucose-, glutamine- or both.

Flow Cytometry Analysis

HeLa cells were cultured at 1.0×10^7 cells/well in cell plates. Q-lipo was added and incubated for 30 min. The cells treated under different conditions were digested by trypsin. After washing, the cells were suspended in 0.5 mL.

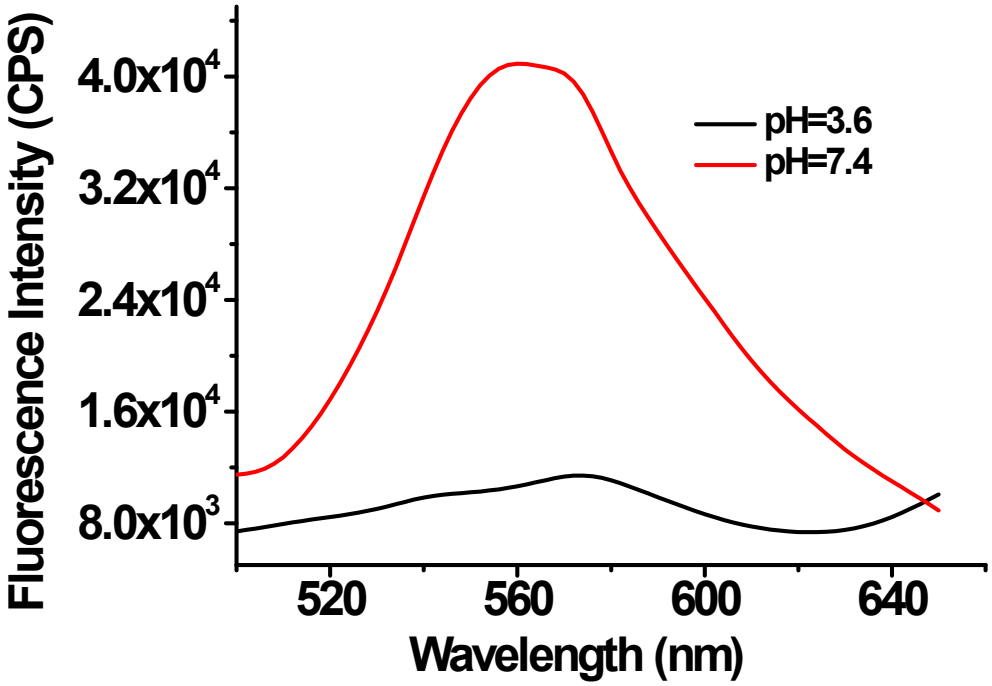


Figure S1. Fluorescence spectra for Q-lipo at pH 3.6 and 7.4, respectively.

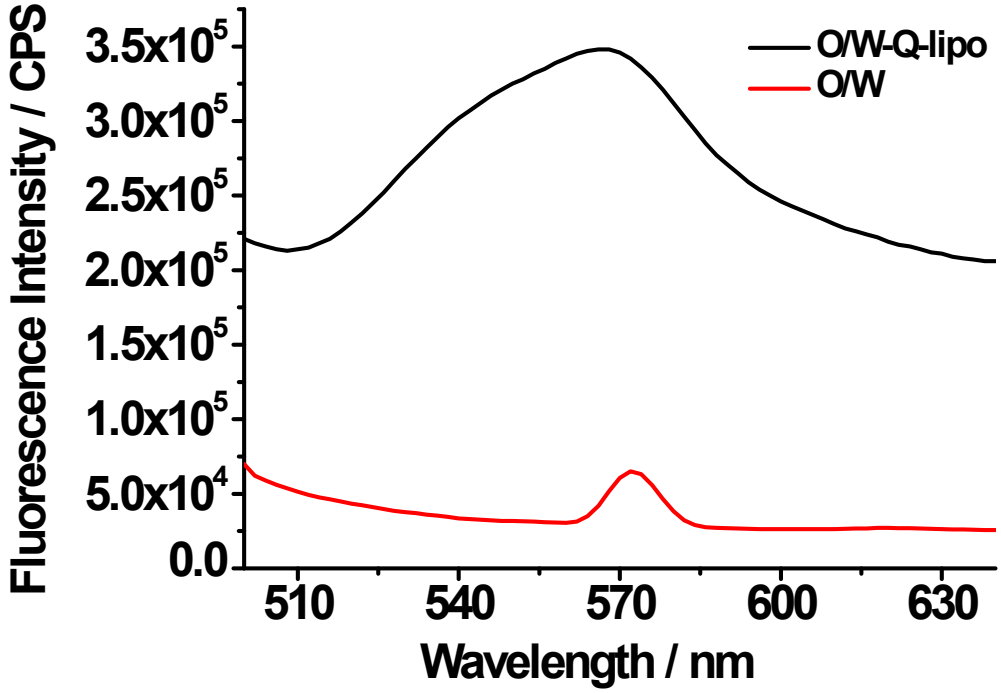


Figure S2. Fluorescence emission spectra of O/W emulsions (2 mM) with or without of Q-lipo (10 μM), respectively. The emission at ~570 nm for the O/W emulsion might be due to the impurities in the glycerol trioleate. The upshift of the fluorescence spectrum upon Q-lipo incubation was probably due to its enhanced fluorescence emission upon interaction with the O/W emulsions.

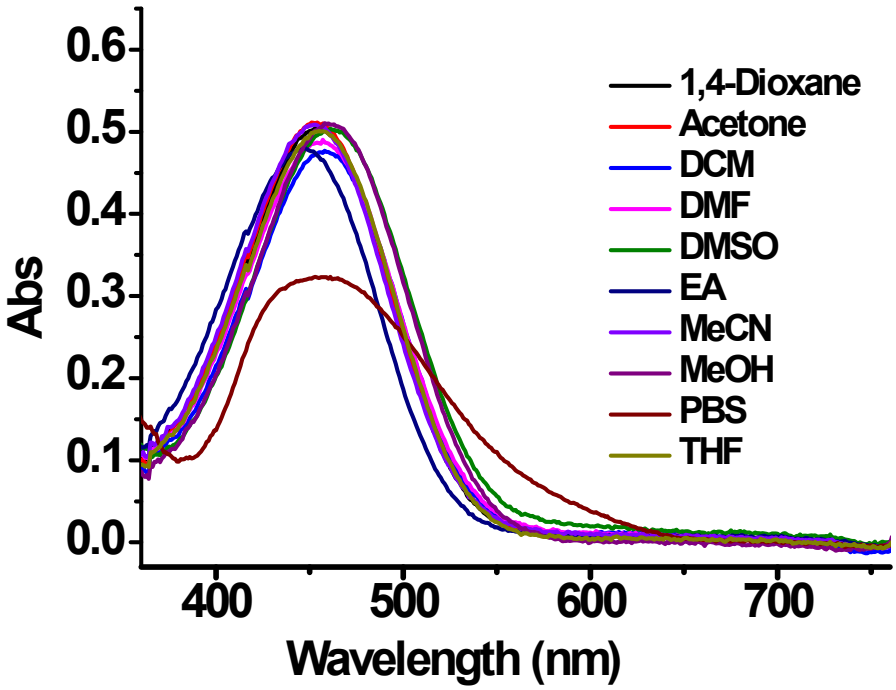


Figure S3. Absorption spectra for Q-lipo in different solvents.

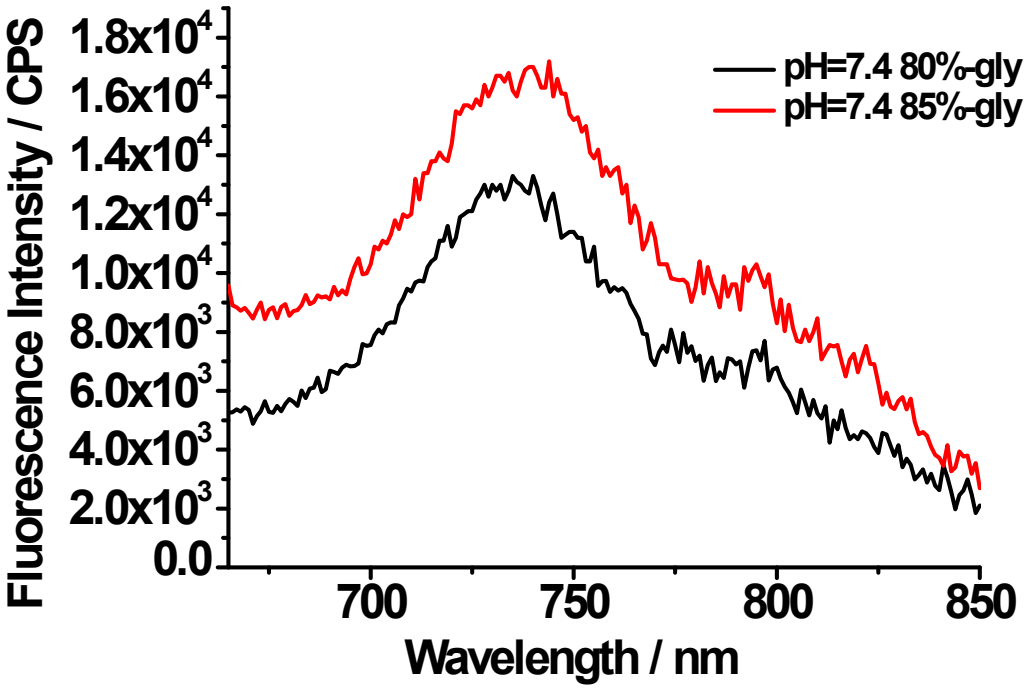


Figure S4. Fluorescence emission spectra for Q-lipo in 80% glycerol and 85% glycerol in pH 7.4 at 25°C.

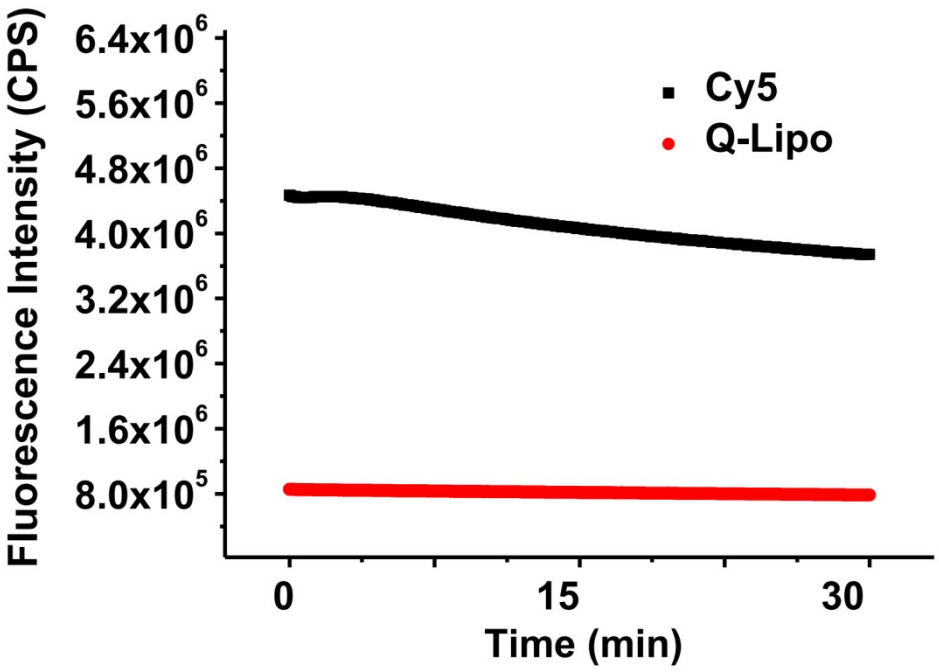


Figure S5. Photostability of Q-lipo using Cy5 as a reference.

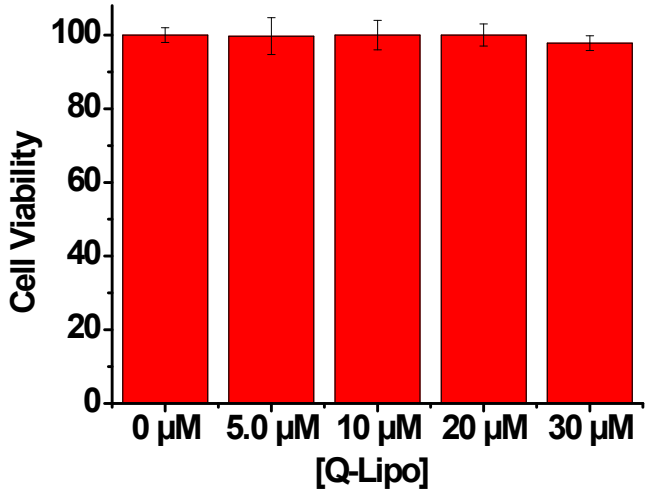


Figure S6. Cytotoxicity study for Q-lipo determined by WST-1 assay. HeLa cells were incubated with different concentrations of Q-lipo (0-30 μM) for 24 h. Data were presented as mean ± S.D. from three repetitive experiments.

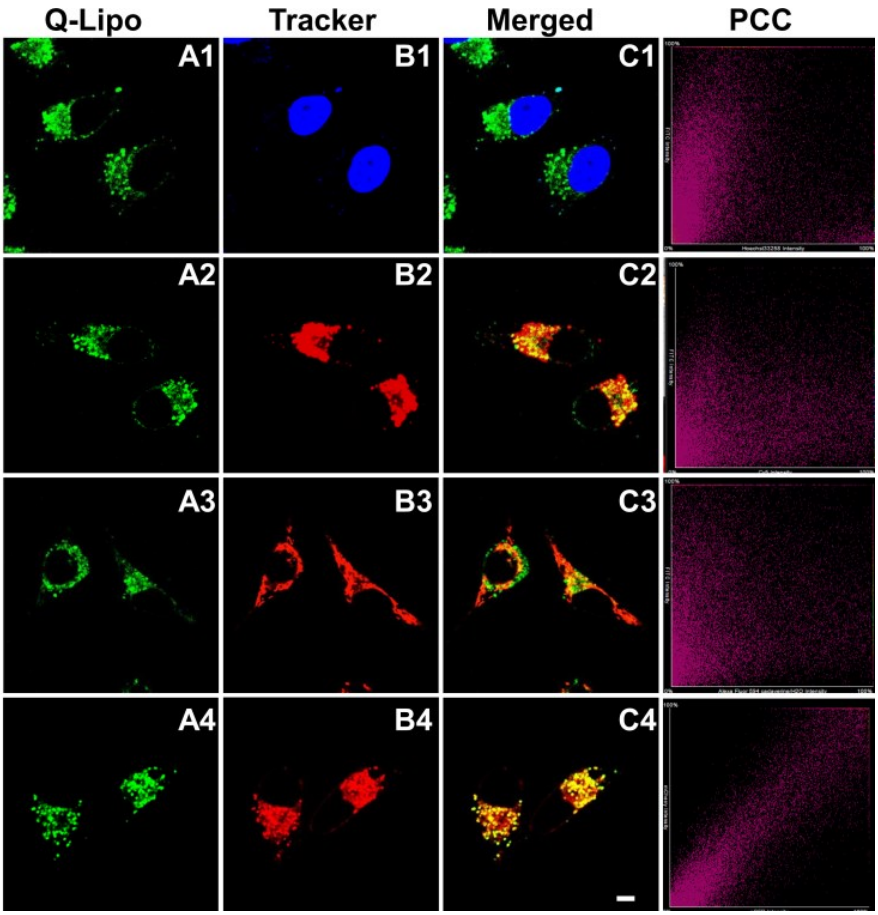


Figure S7. Colocalization assay of Q-lipo with Hoechst 33342 (A1-C1), Lyso-Tracker red (A2-C2), Mito-Tracker red (A3-C3) or Nile red (A4-C4), respectively.

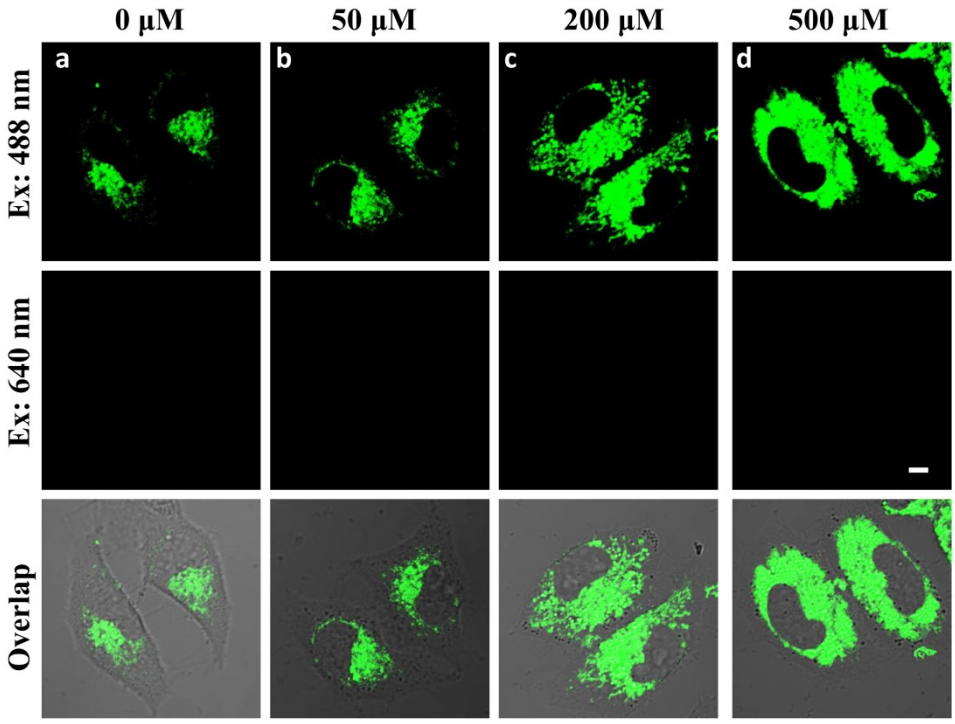


Figure S8. Fluorescence images of cells incubated with Q-lipo in different concentrations of oleic acid (a: 0 μM, b: 50 μM, c: 200 μM, d: 500 μM).

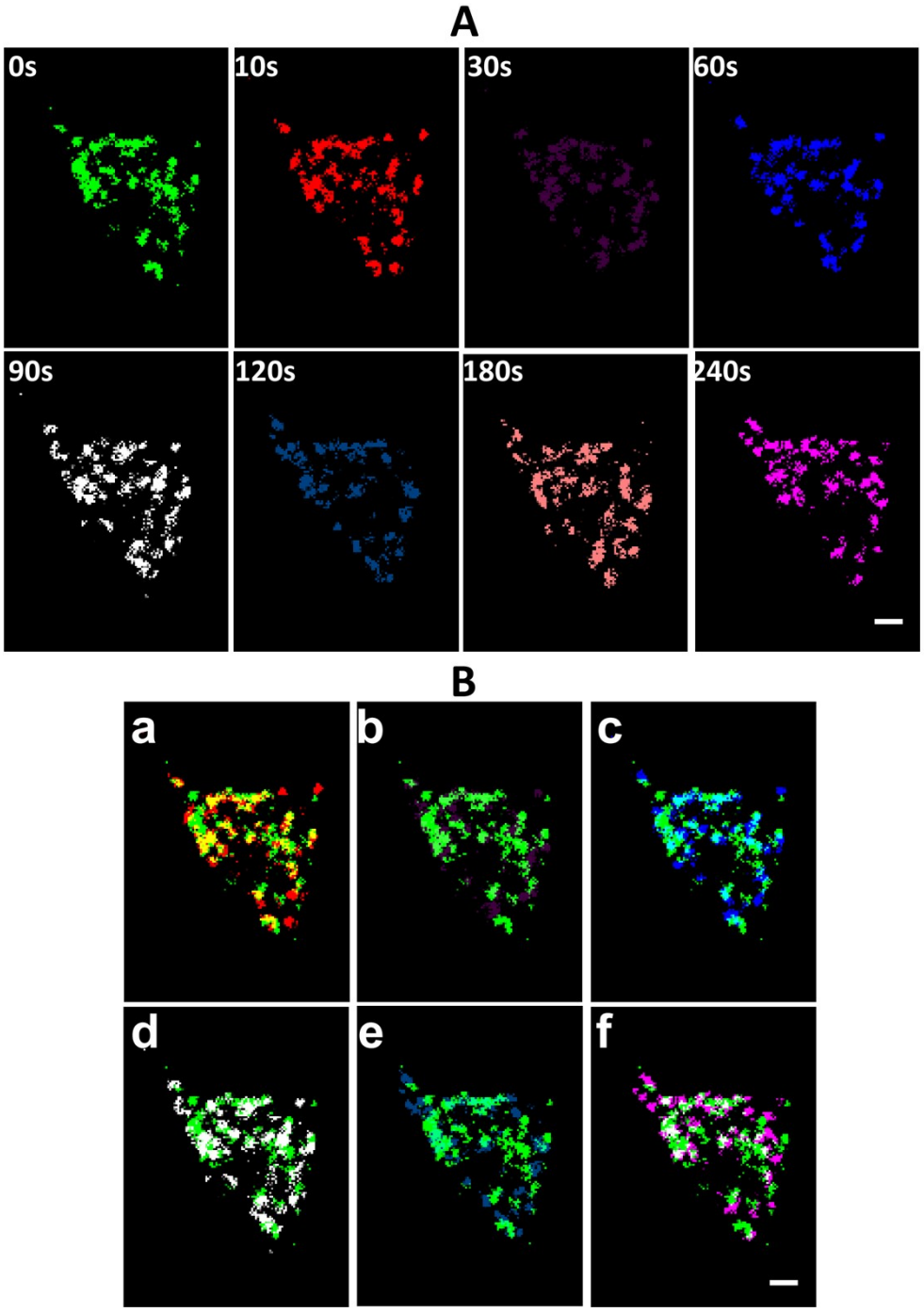


Figure S9. Fluorescence images of Hela cells stained with Q-lipo (10 μM). (A) Different pseudocolors are used to represent the fluorescence images at time points: 10 s, 30 s, 60 s, 90 s, 180 s and 240 s. (B) Merged images at two different time points: (a) 0 and 10 s. (b) 0 s and 30 s. (c) 0 s and 60 s. (d) 0 s and 90 s. (e) 0 s and 120 s. (f) 0 s and 240 s.

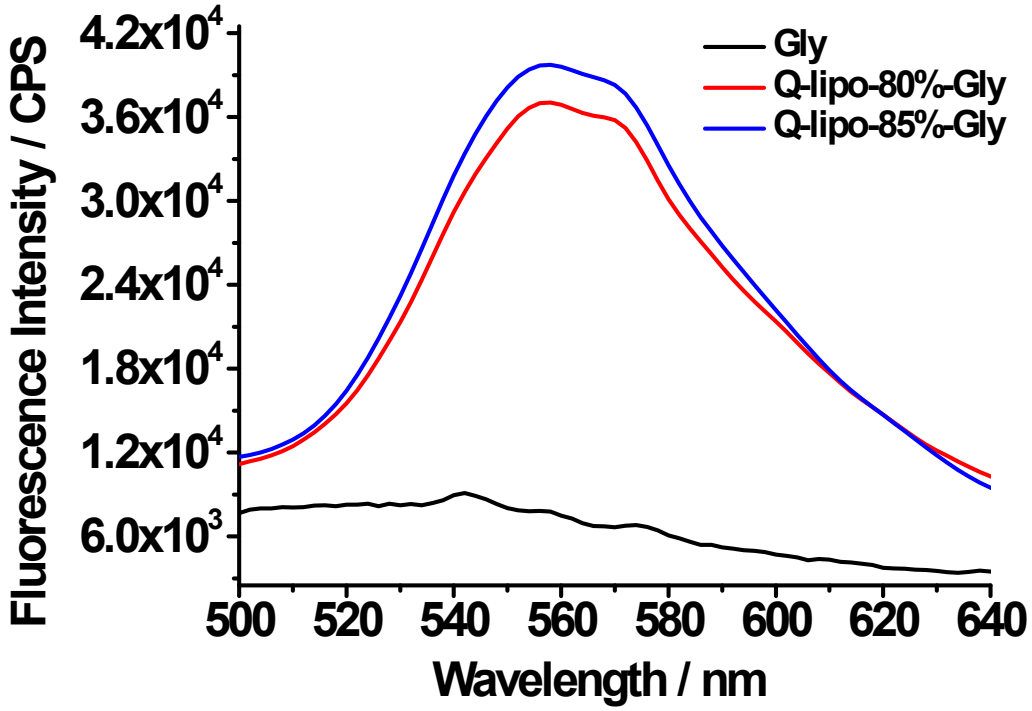


Figure S10. Fluorescence spectra of Q-lipo in 80% and 85% glycerol at 25°C. The slight upshift of the fluorescence spectra was probably due to the increased fluorescence emission.

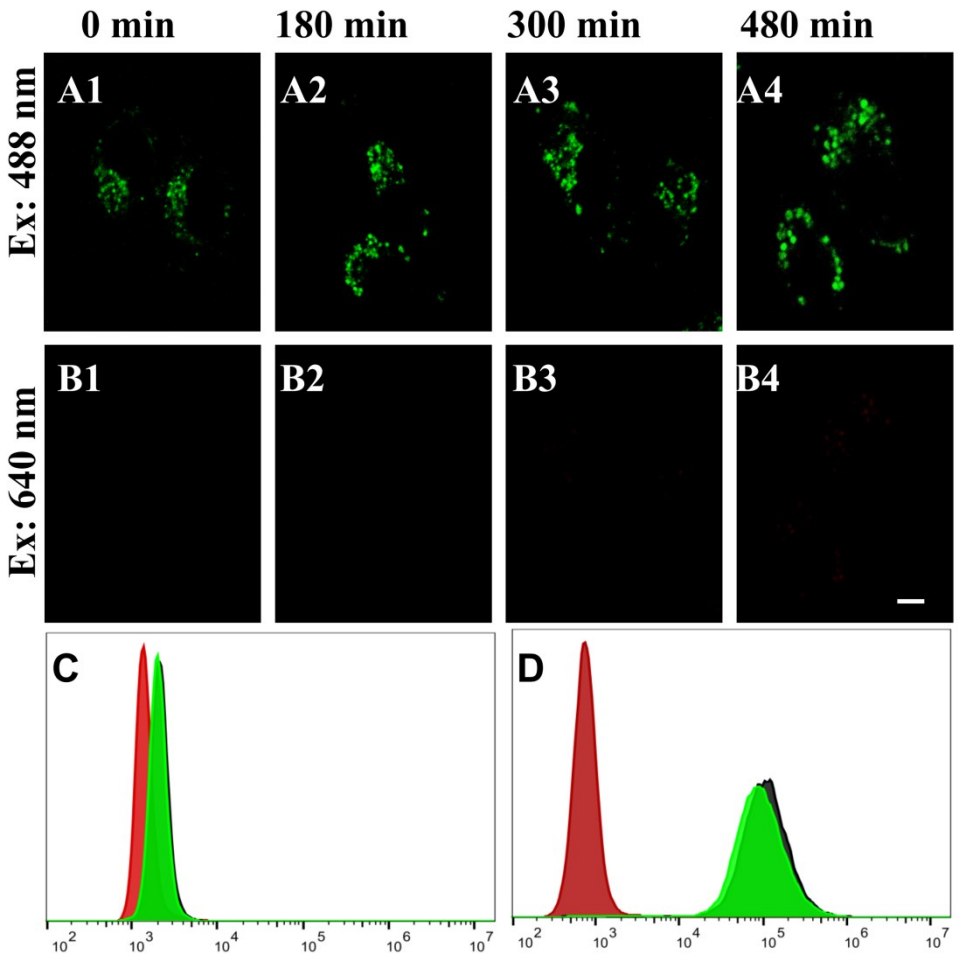


Figure S11. Fluorescence images of cells cultured in DMEM with FBS in hypoxia at 0 min, 180 min, 300 min and 480 min. (A1-A4, Ex: 488 nm; B1-B4, Ex: 640 nm). Flow cytometry analysis of cells cultured in DMEM with FBS in hypoxia at 0 min, 180 min, (C) Ex:488 nm, (D) Ex:640 nm. Red curves denoted control cells without treatment, black and green curves were cells incubated with Q-lipo and cultured in DMEM with FBS in hypoxia for 0 min and 180 min, respectively.

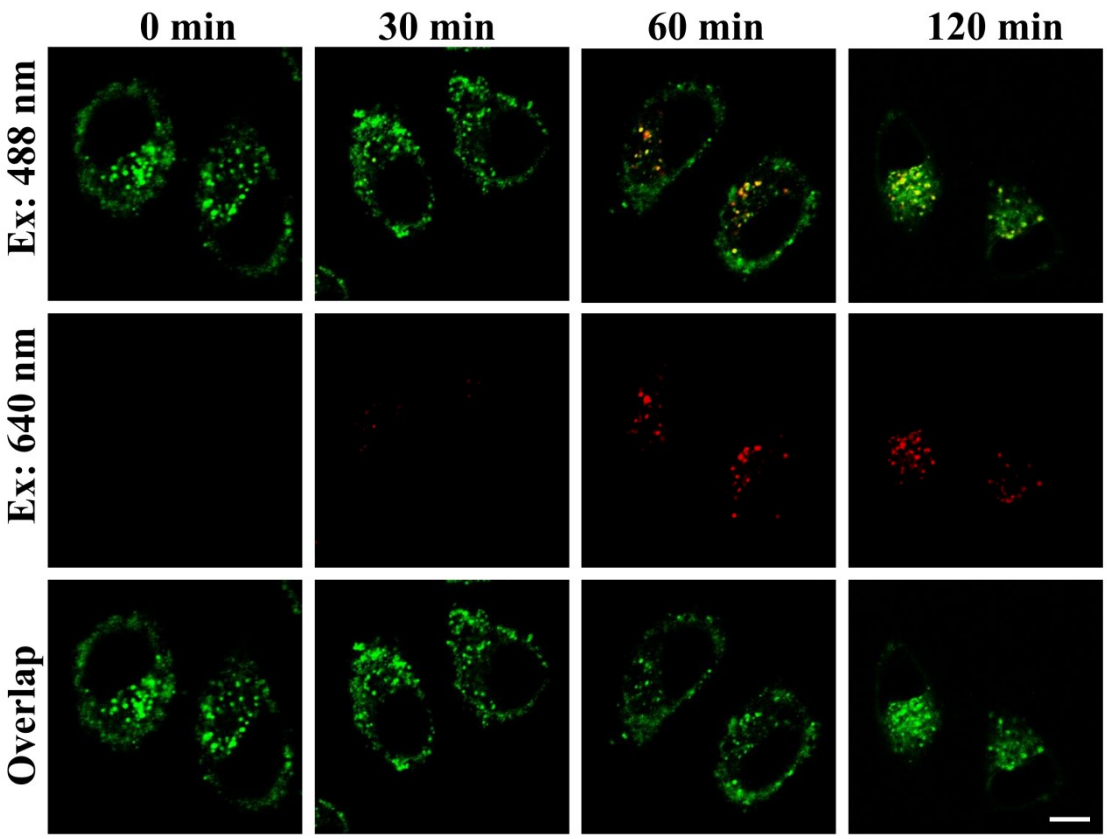


Figure S12. Fluorescence images of cells cultured in DMEM depleted of glucose at 0 min, 30 min, 60 min and 120 min.

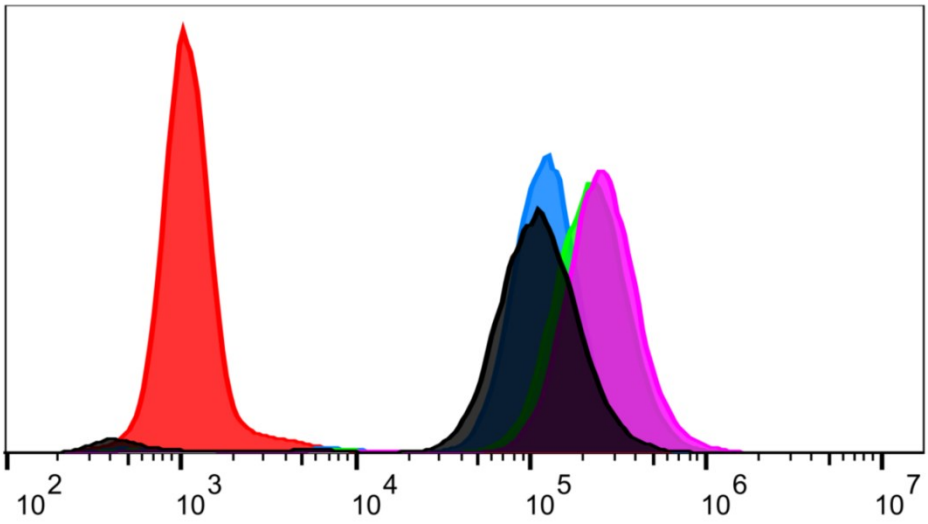


Figure S13. Flow cytometry analysis of cells cultured in DMEM depleted of glucose at 0 min, 30 min, 60 min and 120 min. Red curves denoted control cells without treatment, black, blue, green and purple curves were cells incubated with Q-lipo and cultured in DMEM depleted of glucose at 0 min, 30 min, 60 min and 120 min, respectively. Ex: 640 nm.

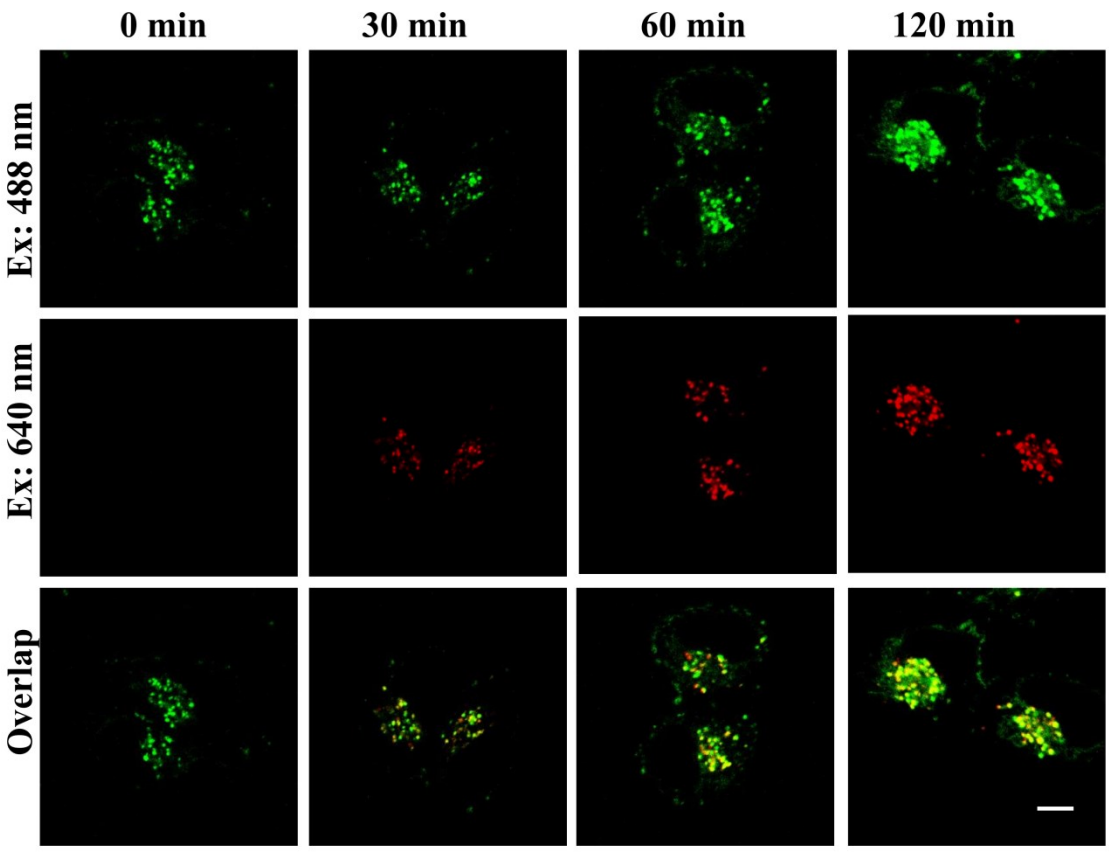


Figure S14. Fluorescence images of cells cultured in DMEM depleted of glutamine at 0 min, 30 min, 60 min and 120 min.

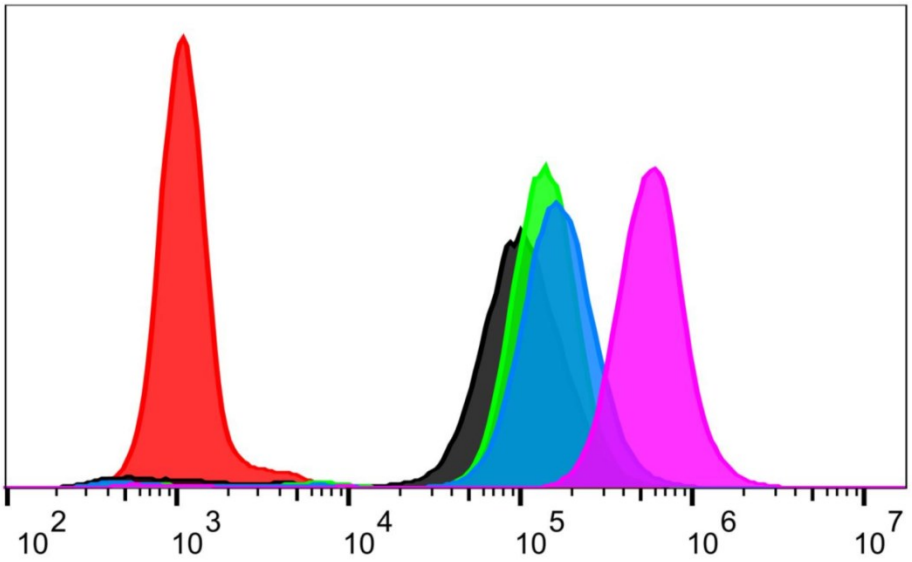


Figure S15. Flow cytometry analysis of cells cultured in DMEM depleted of glutamine at 0 min, 30 min, 60 min and 120 min. Red curves denoted control cells without treatment, black, green, blue and purple curves were cells incubated with Q-lipo and cultured in DMEM depleted of glutamine at 0 min, 30 min, 60 min and 120 min, respectively. Ex: 640 nm.

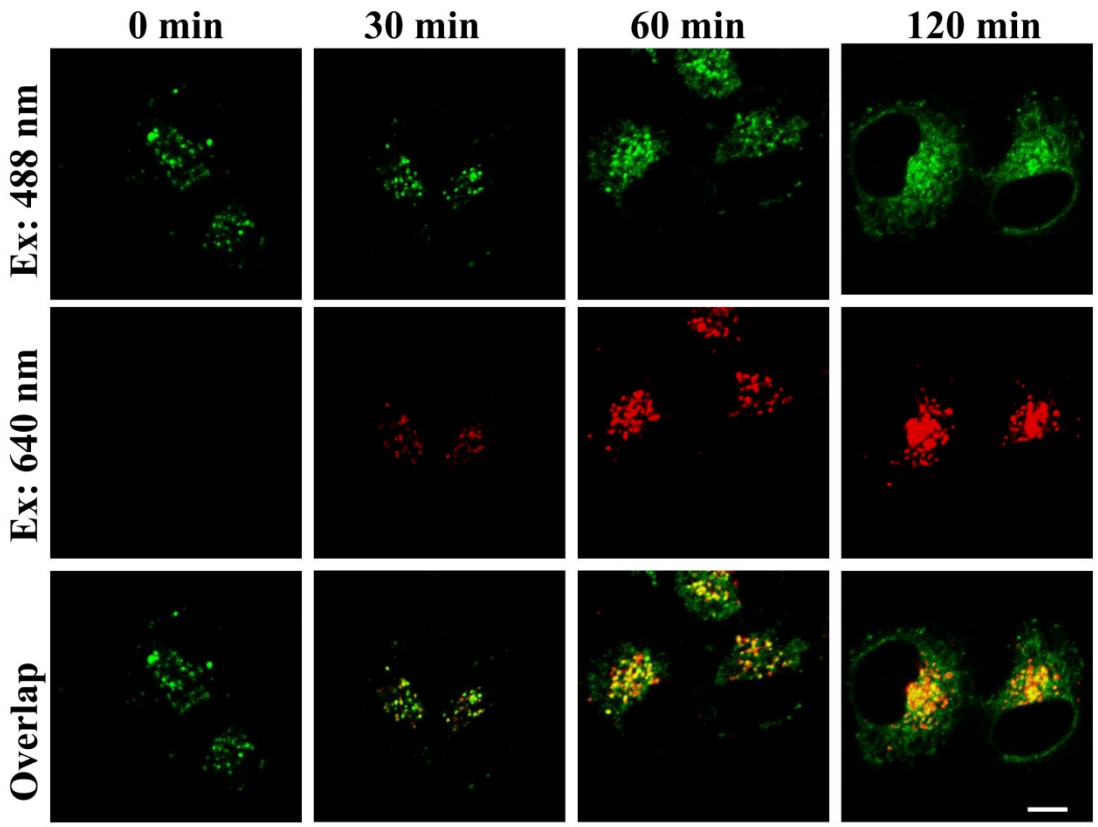


Figure S16. Fluorescence images of cells cultured in DMEM depleted of glutamine and glucose at 0 min, 30 min, 60 min and 120 min.

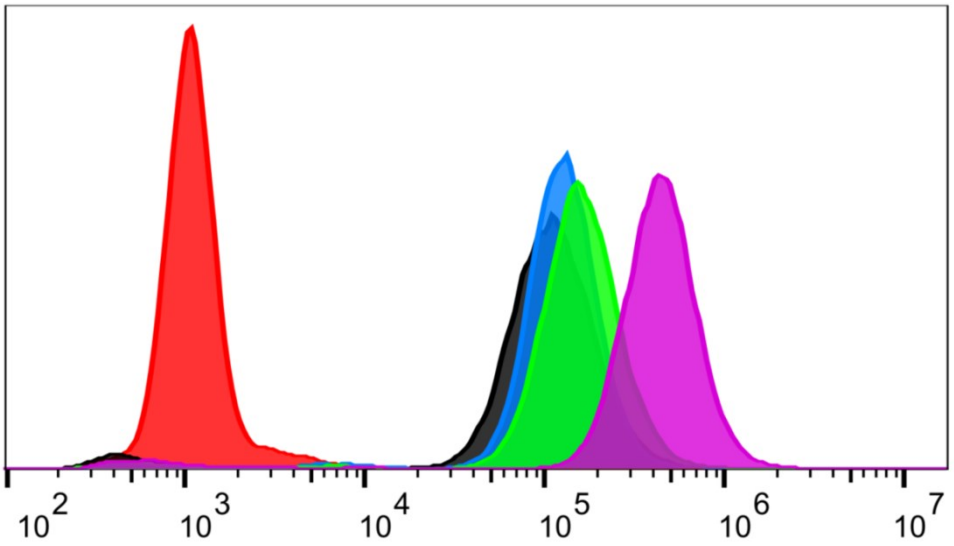


Figure S17. Flow cytometry analysis of cells cultured in DMEM depleted of glucose and glutamine at 0 min, 30 min, 60 min and 120 min. Red curves denoted control cells without treatment, black, blue, green and purple curves were cells incubated with Q-lipo and cultured in DMEM depleted of glucose and glutamine at 0 min, 30 min, 60 min and 120 min, respectively. Ex: 640 nm.

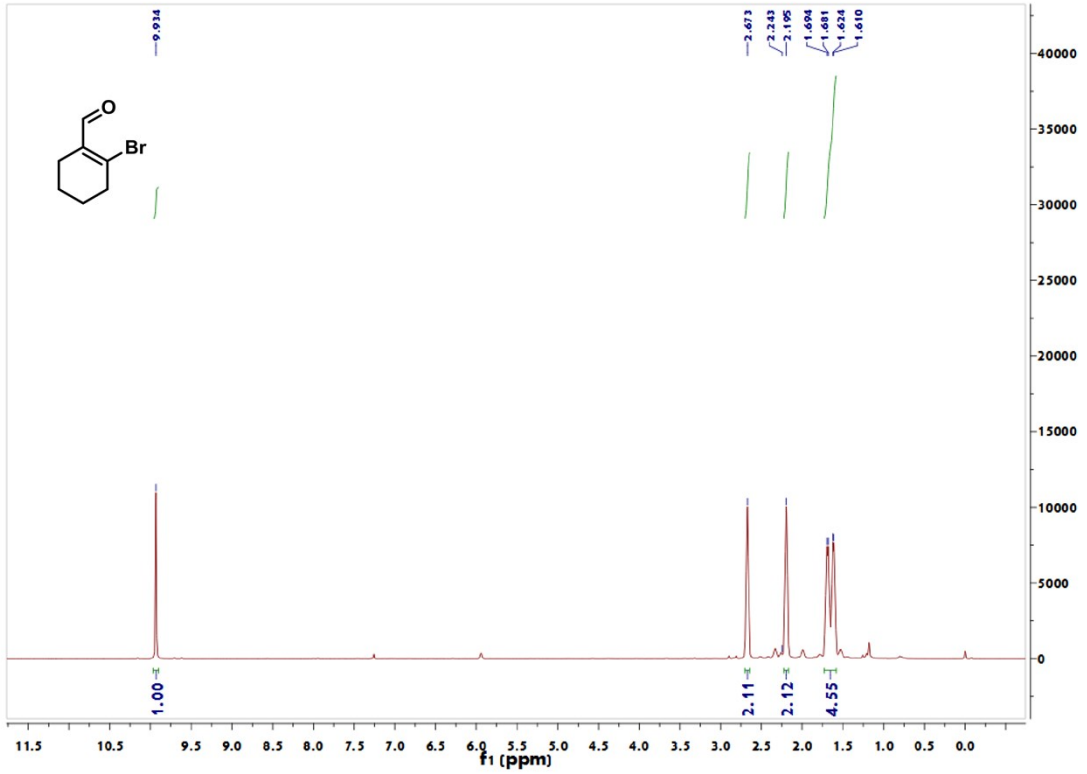


Figure S18. [1]H-NMR spectrum of 2-bromocyclohex-1-ene-1-carbaldehyde.

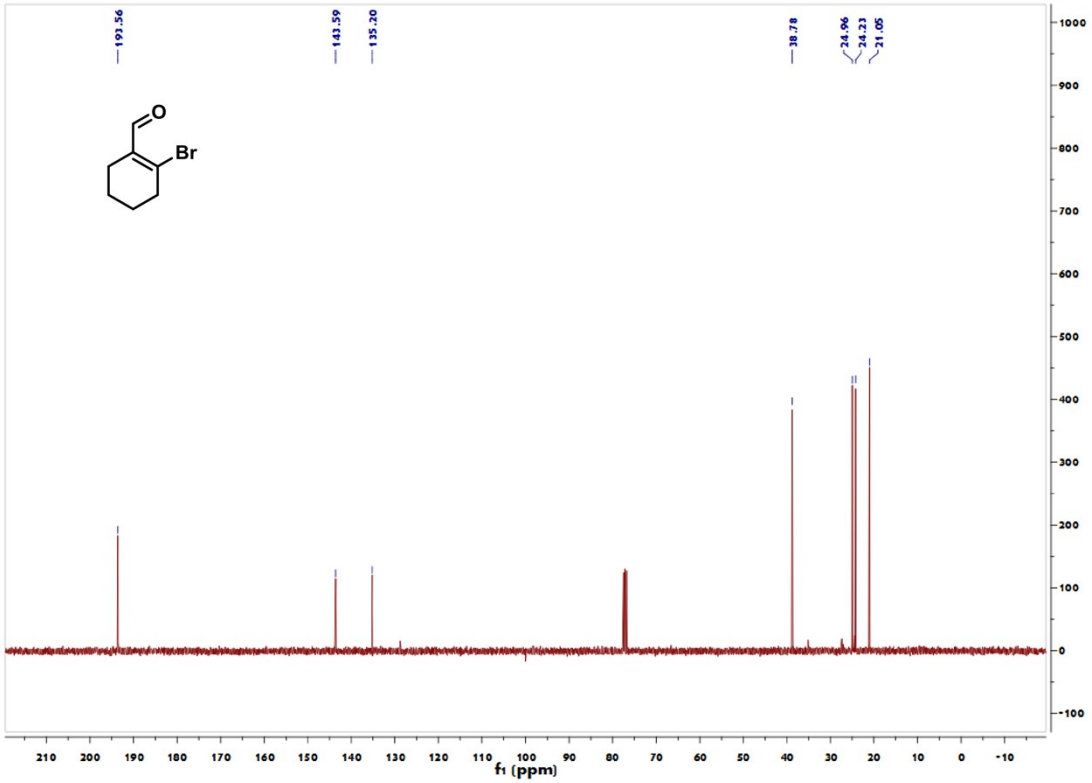


Figure S19. [13]C-NMR spectrum of 2-bromocyclohex-1-ene-1-carbaldehyde.

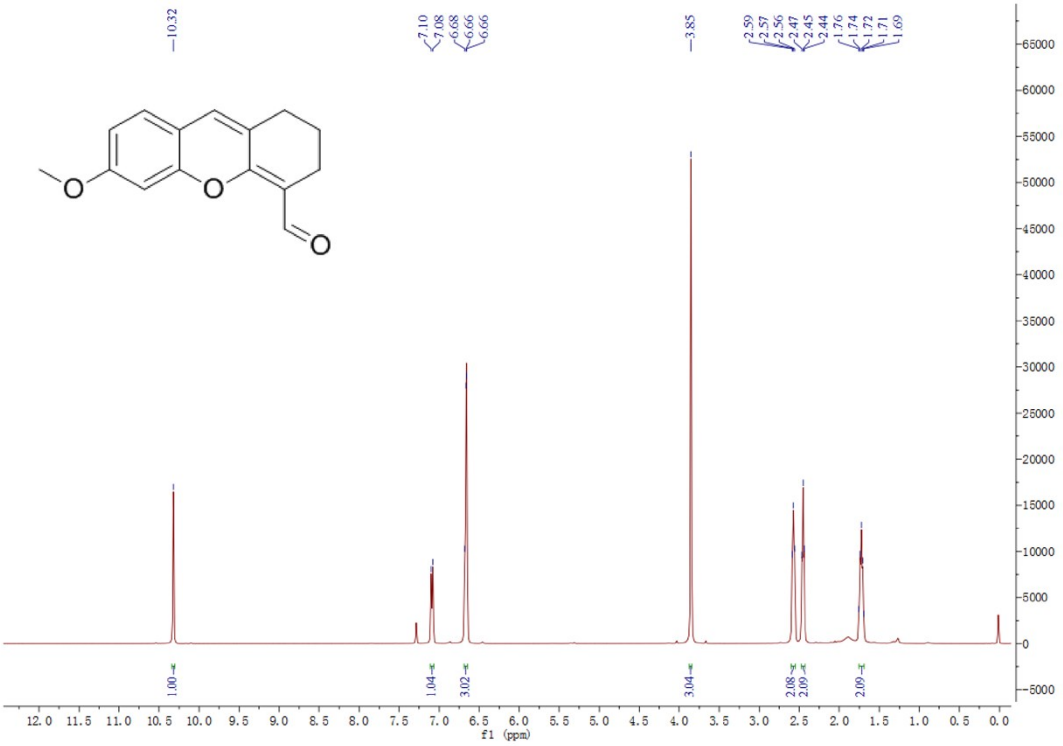


Figure S20. ^{1}H-NMR spectrum of 6-methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde.

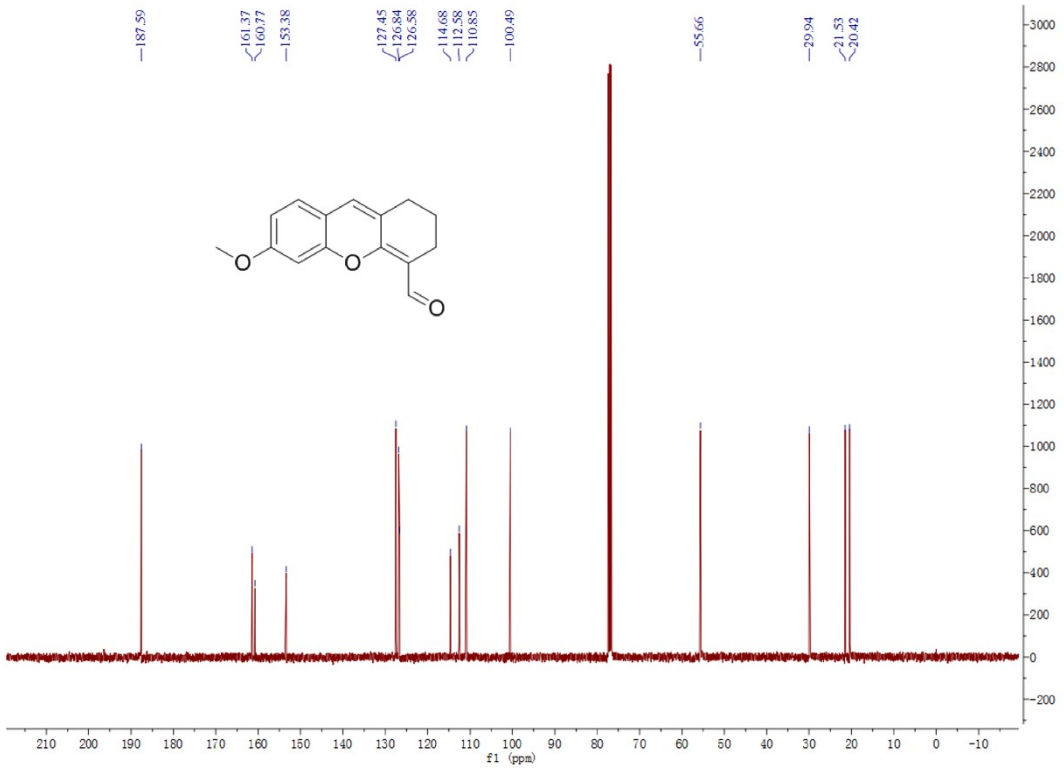


Figure S21. ^{13}C-NMR spectrum of 6-methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde.

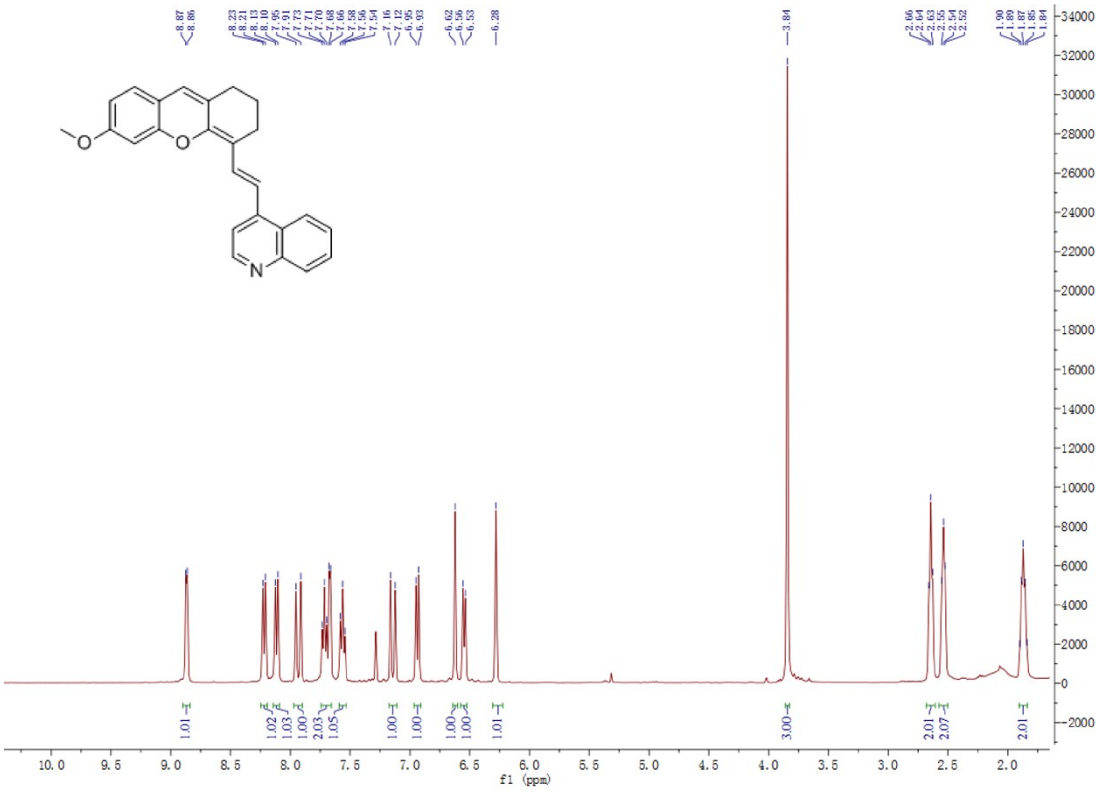


Figure S22. ^{1}H-NMR spectrum of Q-lipo-CH_3.

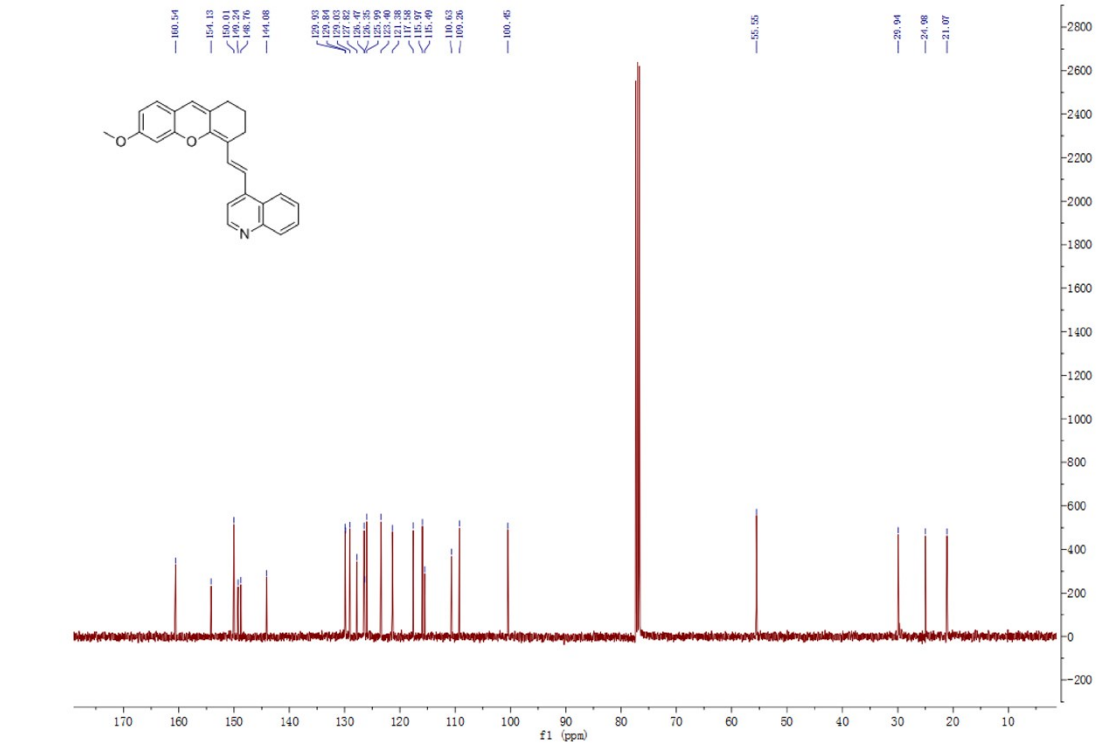


Figure S23. ^{13}C-NMR spectrum of Q-lipo-CH_3.

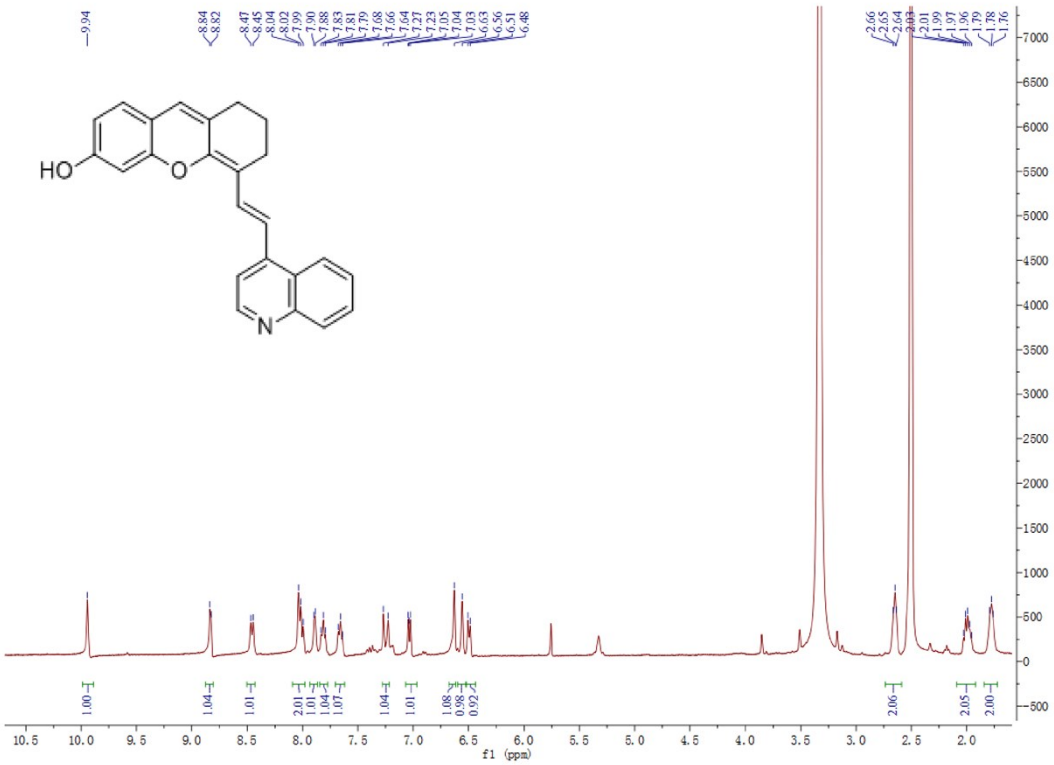


Figure S24. ^{1}H-NMR spectrum of Q-lipo.

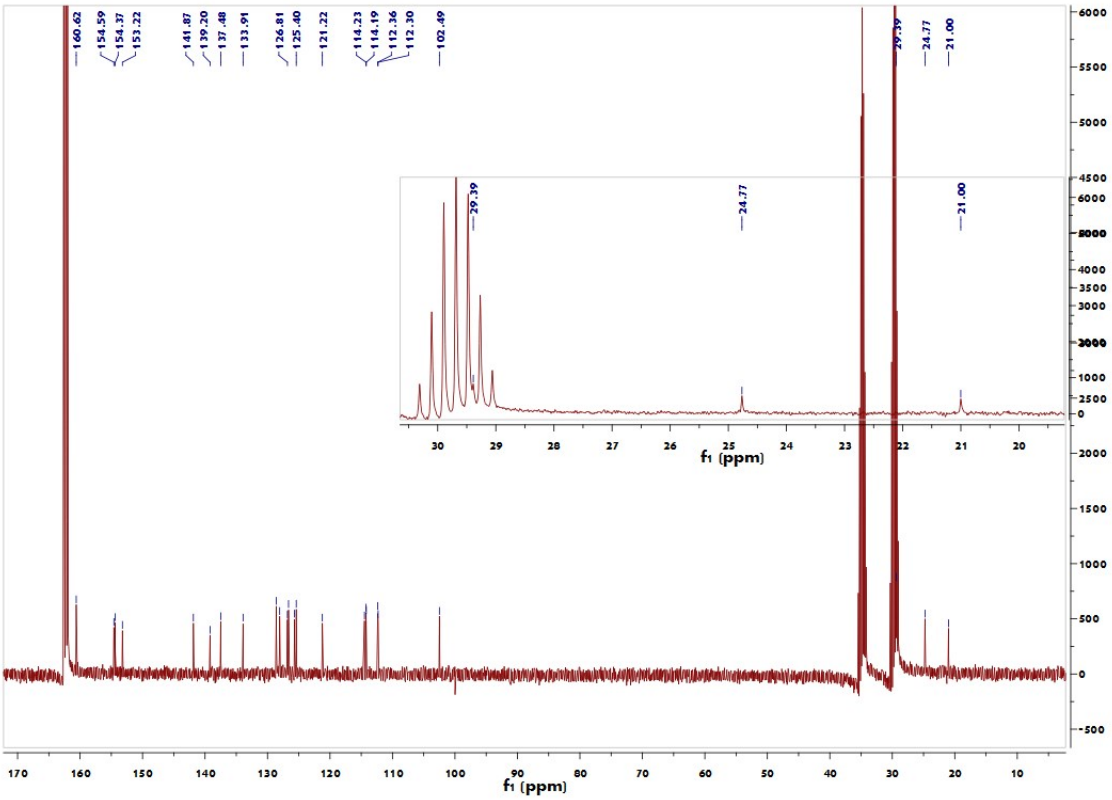


Figure S25. ^{13}C-NMR spectrum of Q-lipo.

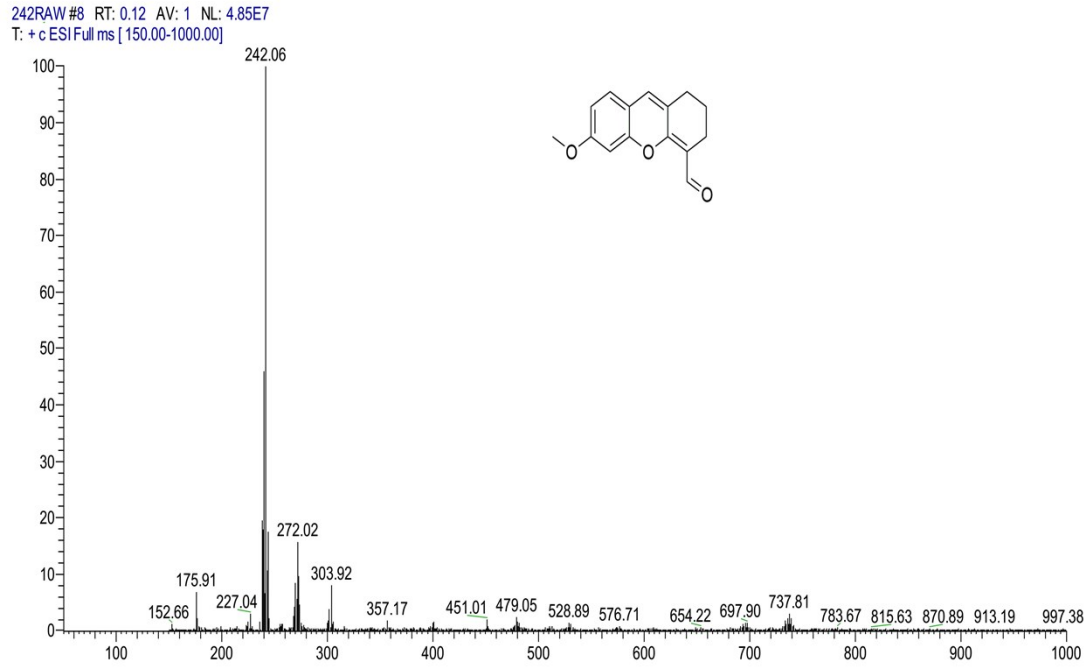


Figure S26. ESI-MS spectrum of 6-methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde.

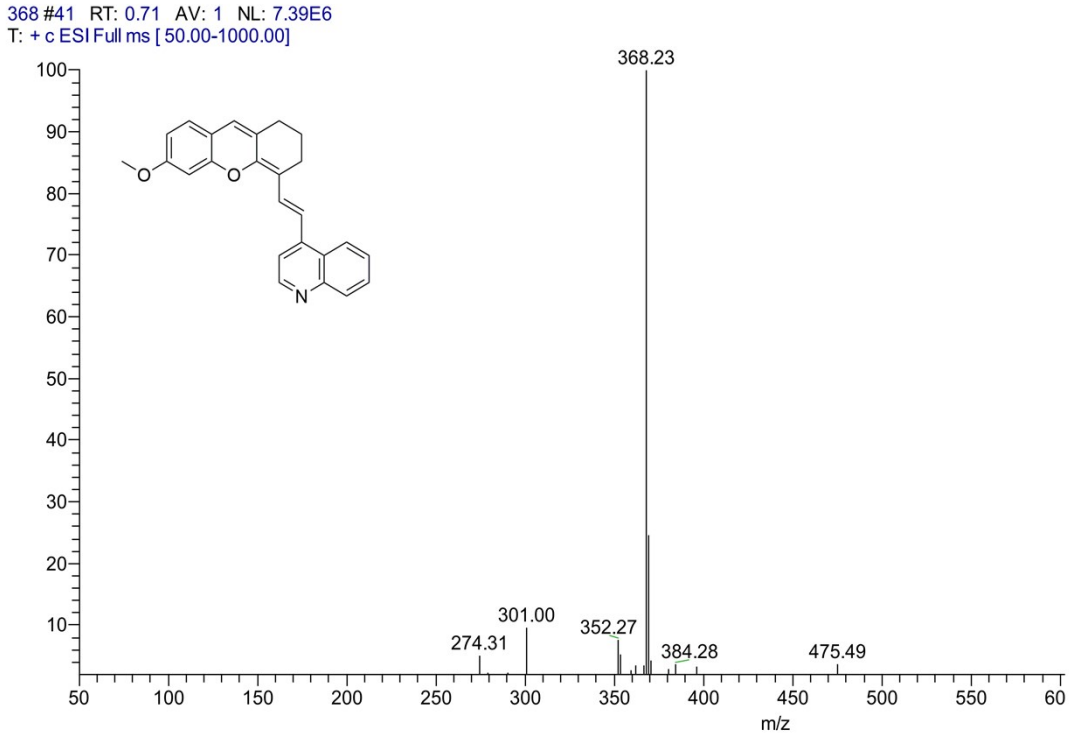


Figure S27. ESI-MS spectrum of Q-lipo-CH_3.

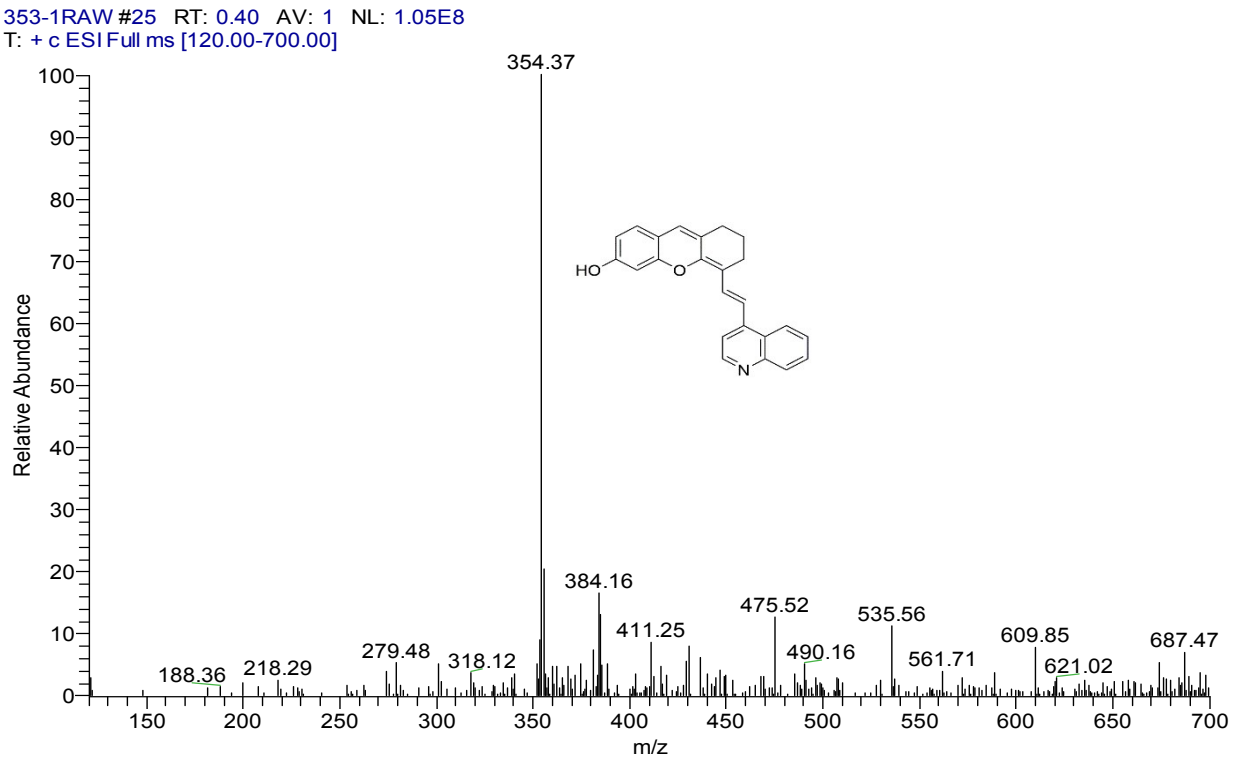


Figure S28. ESI-MS spectrum of Q-lipo.