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Revealing the Dynamics of Mitochondrial Microenvironment during Apoptosis under Two-photon Fluorescence Lifetime Microscopy by a Cyclic Iridium (III) Complex

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1. Materials and Apparatus

All chemical reagents and organic solvents were purchased from Aladdin Industries and McLeans Reagent Network and dried and purified by standard methods. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained on Bruker AV 400 spectrometer (Germany), and the electrospray ionization (ESI) mass spectrum was recorded using an LTQ Orbitrap XL. UV absorption spectra were recorded on a UV-265 spectrophotometer. Fluorescence was measured on a Hitachi F-7000 spectrofluorometer and confocal imaging was performed using a Leica SP8.

2. Experimental Section

2.1 The density functional theory

Optimization was performed on B3LYP [LANL2DZ] without any symmetry restraints, and the TD-DFT {B3LYP[LANL2DZ]} calculations were carried out with the optimized structure. All calculations, including optimization and TD-DFT, were performed using the G09 software. Geometry optimization of singlet-singlet excitation energies were carried out with a basis set composed of 6-31G(d) for C N O Cl H atoms, and the LANL2DZ basis set for iridium atoms. The basis set was downloaded from the EMSL basis set library. The lowest 25 spin-allowed singlet-singlet transitions were taken into account in the calculation of the absorption spectra.

2.2 Synthesisand characterization of Mito-Ap

Ir-8OC-Cl: Add 8OC (0.27 g, 0.76 mmol) and $IrCl_3{\cdot}3H_2O$ (0.33 g.0.80 mmol) to a 100 mL Schreck vial, then add 15 mL ethylene glycol, avoid light, remove oxygen from the reactor and solvent by passing nitrogen, react for 15 min at 160°C, cool to ro om temperature, filter, wash twice with ethanol, dry and obtain0.51 g red powder yield: 70%. ^{1}H-NMR (400 MHz, d6- DMSO, ppm): δ = 8.86 - 8.37 (m, 4H), 7.94 (d, J=15.1, 4H), 7.46 (s, 2H), 4.20 (s, 2H), 1.76 (s, 2H), 1.19 (d, J=12.0, 10H), 0.83 (s, 3H). ESI-MS= $[M+H]^{+}$: cal:659.08 found:660.06.

Mito-Ap: To a 50 mL Shrek bottle, add Ir-8OC (0.18 g, 0.26 mmol), benzoquinoline (0.1 g, 0.52 mmol), ethylene glycol (10 mL), and nitrogen Protect from

light, heat to 180 °C, and react for 24 h. A clear red solution was obtained, saturated aqueous NH_4PF_6 solution (20 mL) was added, stirred at room temperature for 1 h, and an orange-yellow solid was obtained by suction filtration, which was purified by column chromatography. The chromatographic solution was dichloromethane: methanol (V/V=100:1). Obtained orange-yellow solid Mito-Ap 0.2 g, yield: 56.1%. ^{1}H NMR (400 MHz, d6- DMSO, ppm): δ 10.31 (d, J = 5.1 Hz, 1H), 8.94 (d, J = 8.1 Hz, 2H), 8.77 (d, J = 8.1 Hz, 2H), 8.60 (s, 1H), 8.41 (dd, J = 14.7, 8.2 Hz, 3H), 8.22 (d, J = 8.9 Hz, 1H), 7.92 (t, J = 7.8 Hz, 2H), 7.84 (d, J = 5.3 Hz, 1H), 7.46 (dd, J = 10.6, 5.3 Hz, 3H), 7.20 (m, 2H), 4.49 (t, J = 6.4 Hz, 2H), 1.95 (m, 2H), 1.58 (dd, J = 14.7, 7.0 Hz, 2H), 1.43 (m, 4H), 1.20 (t, J = 7.3 Hz, 4H), 0.95 (t, J = 6.9 Hz, 3H). ^{13}C-NMR (400 MHz, DMSO, ppm) δ=169.13, 158.49, 156.62, 155.65, 152.46, 150.86, 140.46, 140.12, 139.28, 138.99, 134.11, 129.80, 129.61, 129.48, 128.09, 127.83, 126.17, 123.99, 122.31, 111.71, 70.96, 60.29, 31.82, 29.27, 28.88, 25.99, 22.68, 14.55.ESI-MS: m/z: calcd for $C_{36}H_{35}ClIrN_4PF_6$: 912.17, found: 767.21 ($[M - PF_6^-]^+$).

2.3 Cell culture

Cell imaging was taken two days after Hep G2 culture. HepG2 (hepatocellular carcinoma of the liver) cells were purchased from Shanghai Biotechnology Co. Ltd. and the cells were provided by Shanghai Biotechnology Co. The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% FBS and 1% antibiotics (penicillin and streptomycin).at 37 degrees Celsius, maintained at 5% oxygen and 95% air. The cells were incubated with P9 for 30 min and photographed in a live cell workstation at a certain humidity, temperature, and pH to keep the cells in normal condition.

2.4 Co-localization assay

Hep G2 cells were cultured in glass-bottom dishes for two days, treated with 2 μM Mito-Ap for 10 min, and immediately imaged under a confocal microscope. For colocalization experiments, cells were first treated with 2 μM Mito-Ap （λ_{ex} = 840 nm and λ_{em} = 550-600 nm） for 10 min, and then co-stained for 10 min with 0.5 μM Mito-Tracker Deep Red (λ_{ex} = 633 nm and λ_{em} = 640-660 nm). Stained cells were then rinsed

with PBS and immediately imaged under the microscope.

2.5 Multiphoton fluorescence microscopy imaging

Before imaging, cells had been incubated in glass dishes for two days in advance. After well-adhered cells, the probe Mito-Ap was added and cells were incubated for 10 min. Cells were treated with 10 μM monensin and etoposide for 30 min, respectively, to increase mitochondrial viscosity and thus induce apoptosis. For the greater purpose, cells were washed three times with PBS. Cells were analyzed by confocal microscopy, and multiphoton fluorescence microscopy images by instrument Leica TCS SP8. Provided by DIVE FALCON, the device is equipped with a single-photon laser (405 nm) and a two-photon laser (adjustable output wavelength: 680−1080 nm, 80 MHz, 140 fs). Multiphoton Fluorescence Lifetime Imaging (FLIM) using Leica TCS SP8 Obtained with the 100x oil lens of DIVE FALCON. Confocal images by Leica LAS-X software for processing.

2.6 Animal experiment

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Anhui University (serial number: 2021-042) based on the National Standard of China GB/T35892-2018 guidelines for Ethical Review of Experimental Animal Welfare. We have taken great efforts to reduce the number of animals used in these studies and also taken effort to reduce animal suffering from pain and discomfort.

HepG2 cells were cultured by adherence, and cells grown in log phase were mixed with serum-free Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA). The cell suspension (0.1 mL) was injected subcutaneously in the abdomen of nude mice. After 14 days, the tumor model was established, and the tumor was formed.

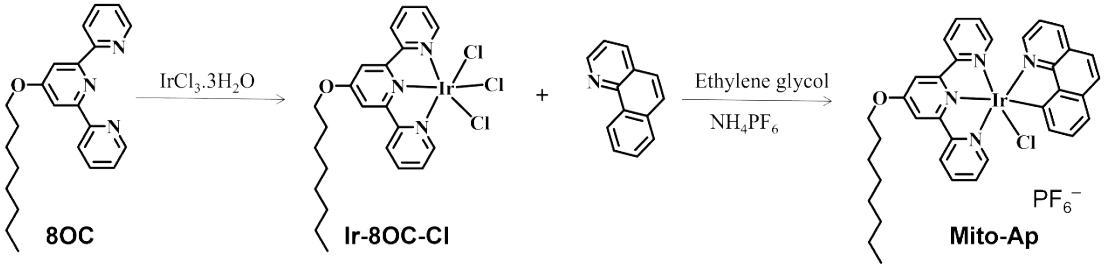

Fig. S1. Synthetic routes of Mito-Ap.

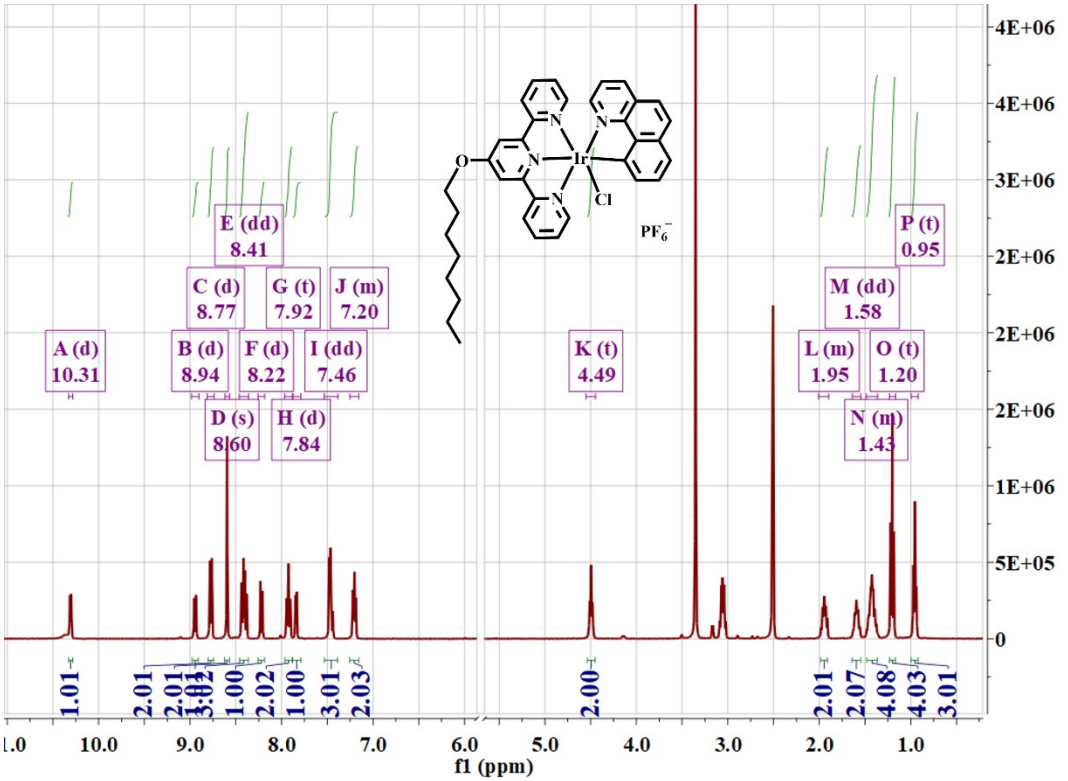

Fig. S2. ^{1}H NMR spectrum of Mito-Ap.

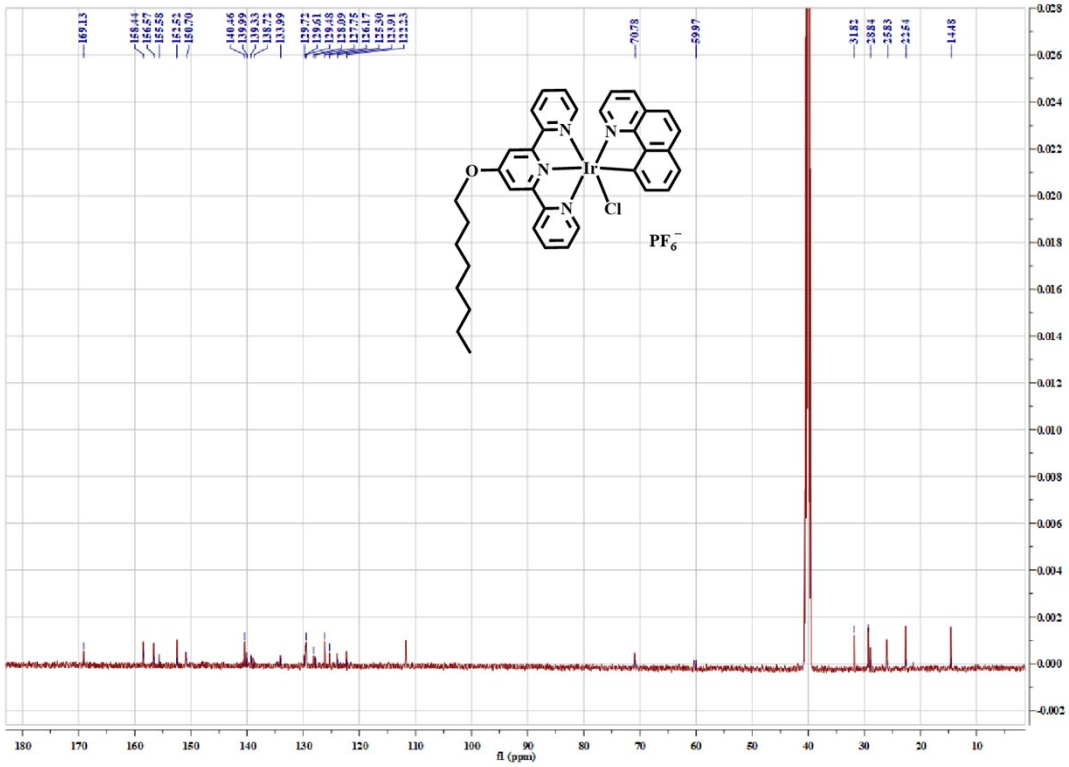

Fig. S3. ^{13}C NMR spectrum of Mito-Ap.

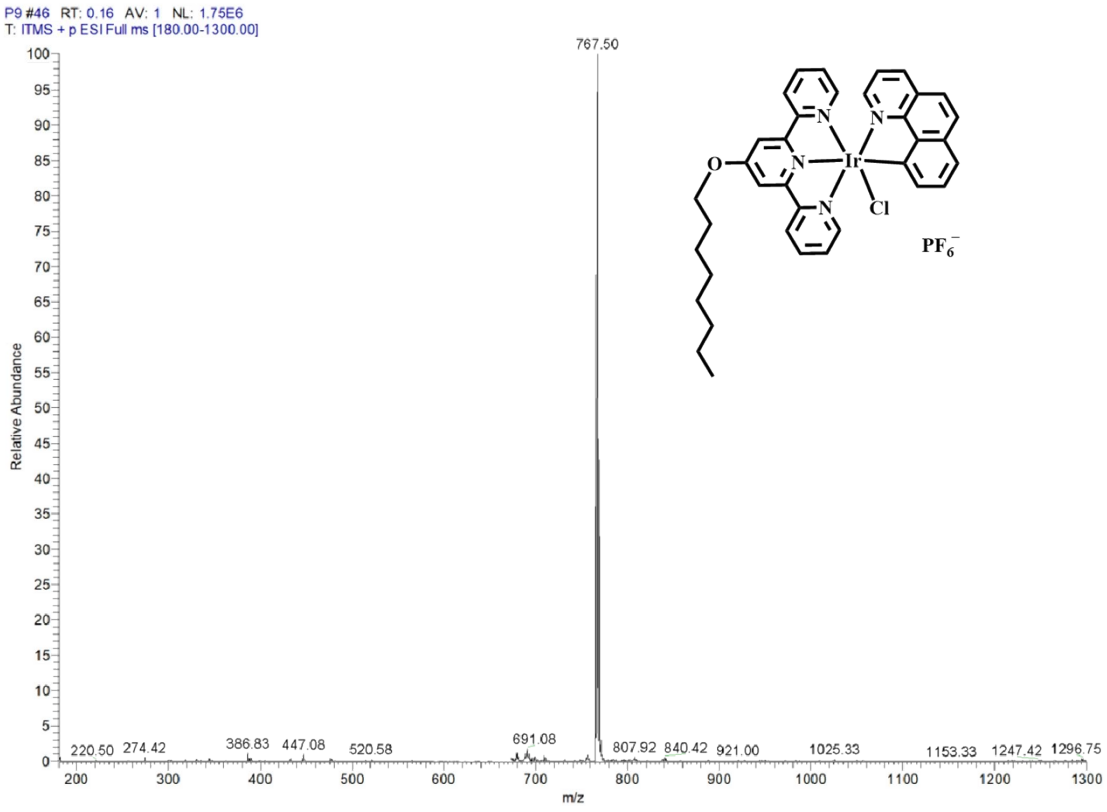

Fig. S4. MS spectrum of Mito-Ap.

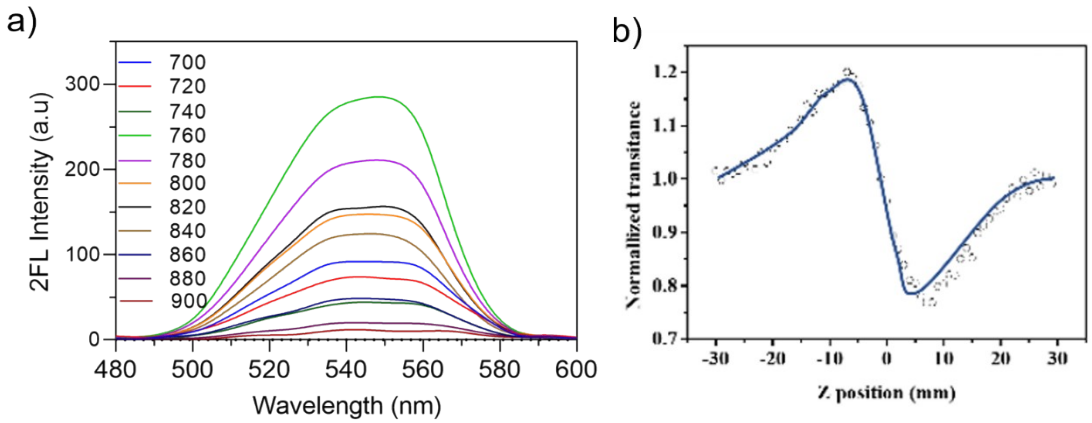

Fig. S5. Two-photon properties. (a) Two-photon fluorescence spectrum of Mito-Ap, (b) Closed-well (lower) Z-scan curve at 840 nm (c = 10 μM)

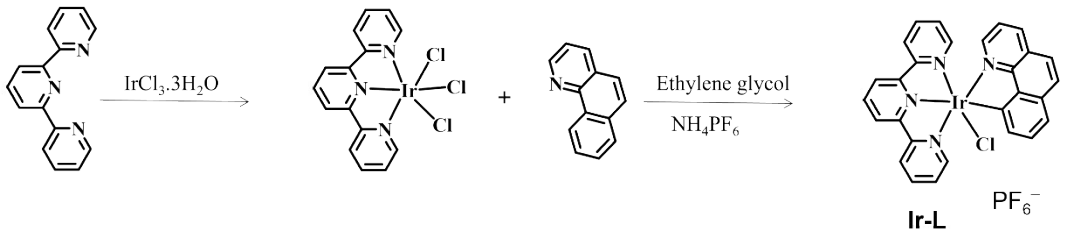

Fig. S6. Synthetic routes of Ir-L.

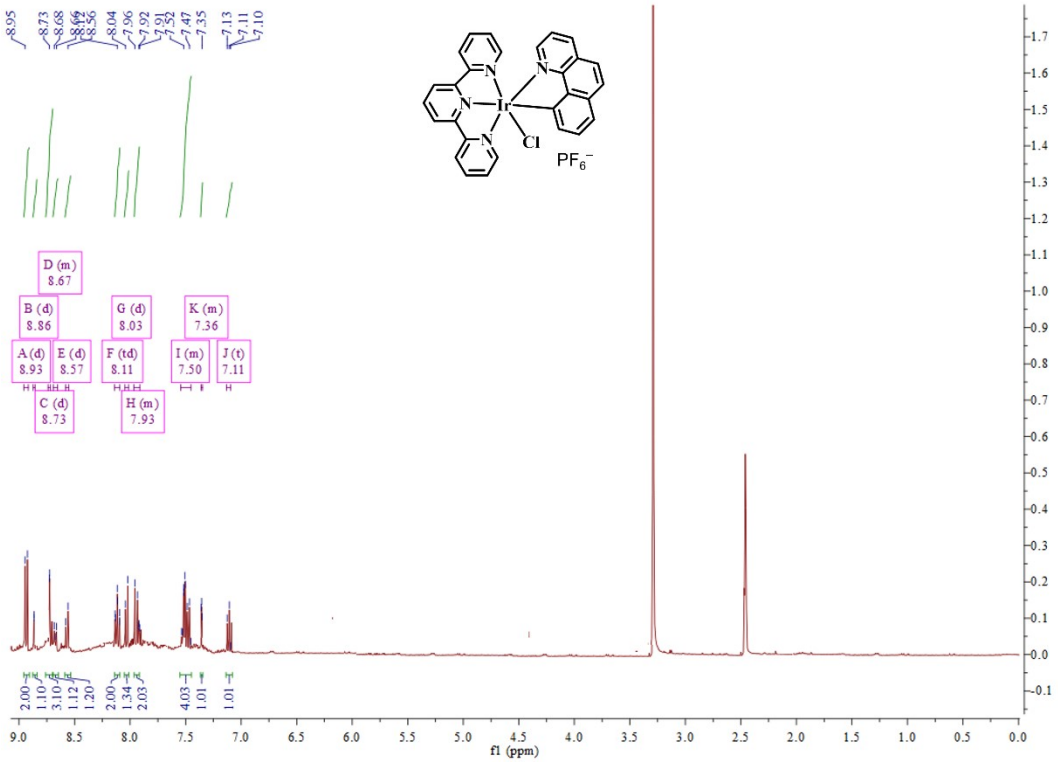

Fig. S7. ^{1}H NMR spectrum of Ir-L.

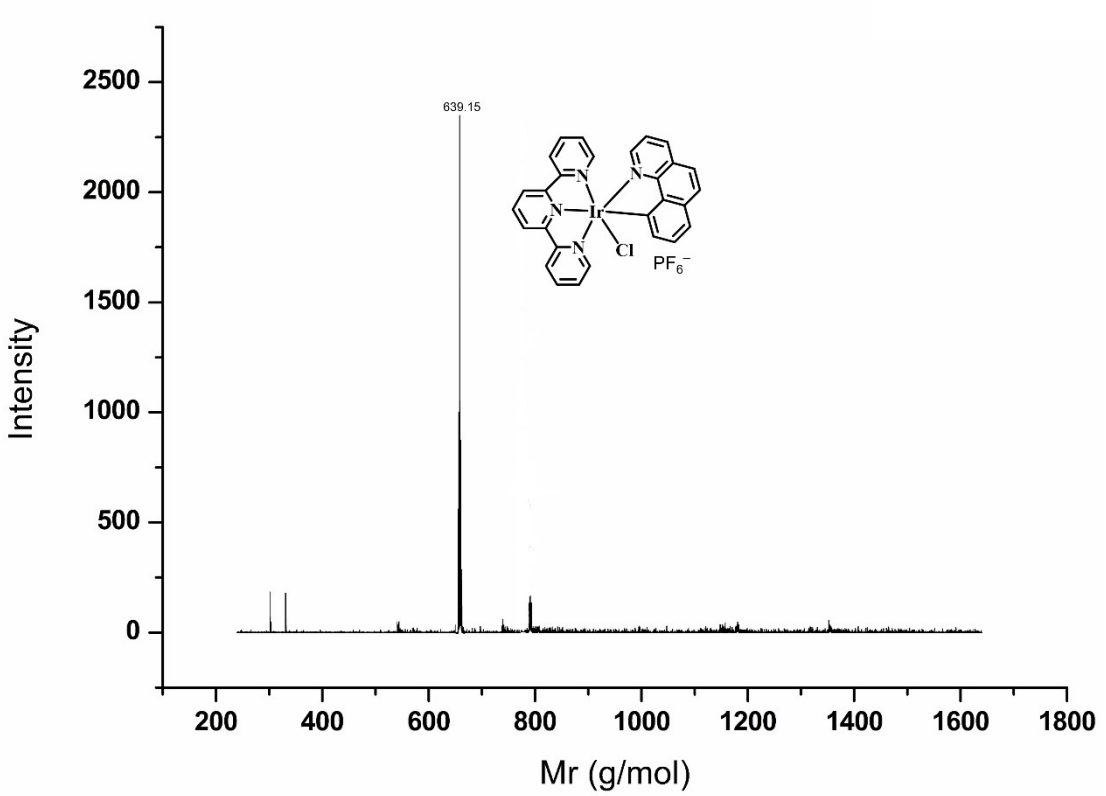

Fig. S8. The Maldi-tof-MS. of Ir-L.

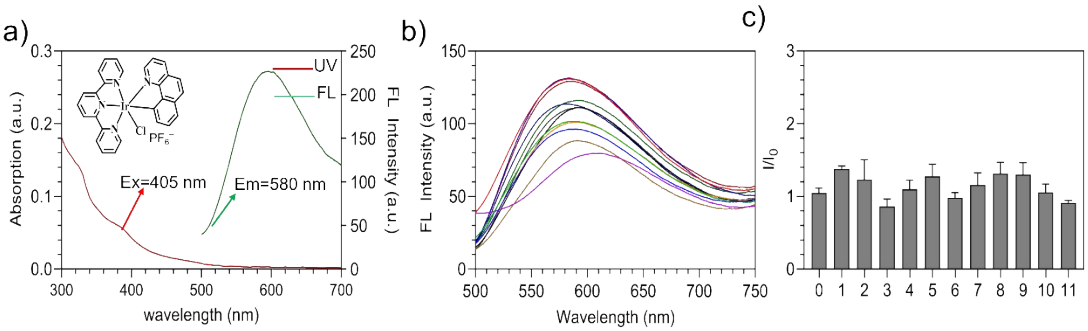

Fig. S9. In vitro selectivity of cyclometallic iridium complexes Ir-L without long alkyl chains. (a) UV-Vis absorption and emission spectra of Ir-L (10 μM) were measured in PBS. (b) Ir-L (10 μM) selectivity for various analytes: (1) blank, (2) Ca^{2+}, (3) Mg^{2+}, (4) cysteine, (5) glutathione, (6) liposome, (7) H_2O_2, (8) HClO, (9) RNA, (10) DNA, (11) glycerol. The sample concentration of samples 1-8 was 100 mM, and the sample concentration of samples 9-10 was 1 mg/mL. (c) Normalized graph of fluorescence intensity of different substances in (b). Error bars represent the average error of 3 measurements.

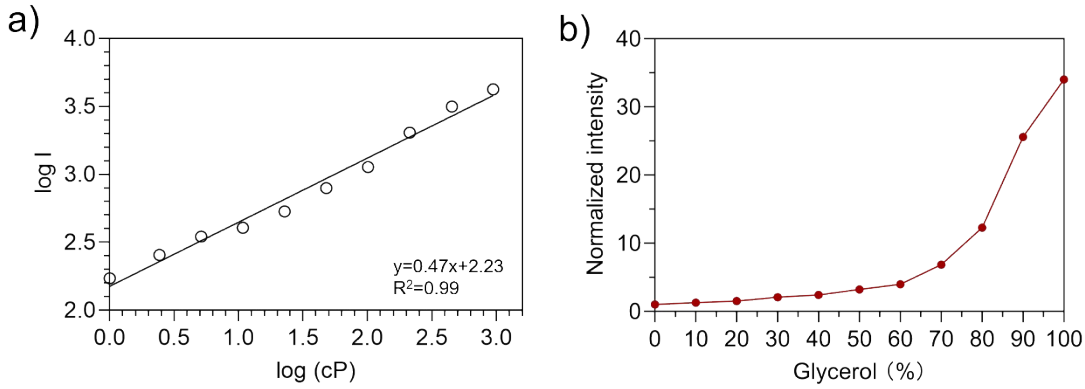

Fig. S10. Linear relationship between viscosity and fluorescence intensity under viscosity titration experiment.

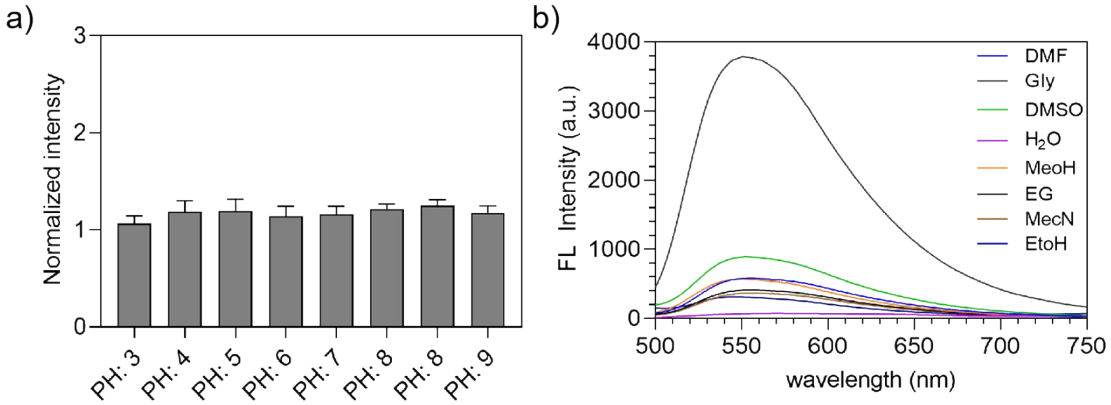

Fig. S11. (a) Normalized fluorescence intensity of Mito-Ap at different pH. (b) Fluorescence intensity of Mito-Ap in glycerol and different polar solvents.

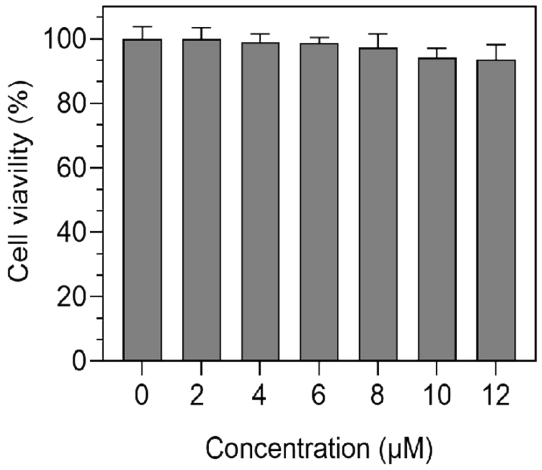

Fig. S12. MTT detection of HepG2 cells incubated with Mito-Ap at different concentrations.

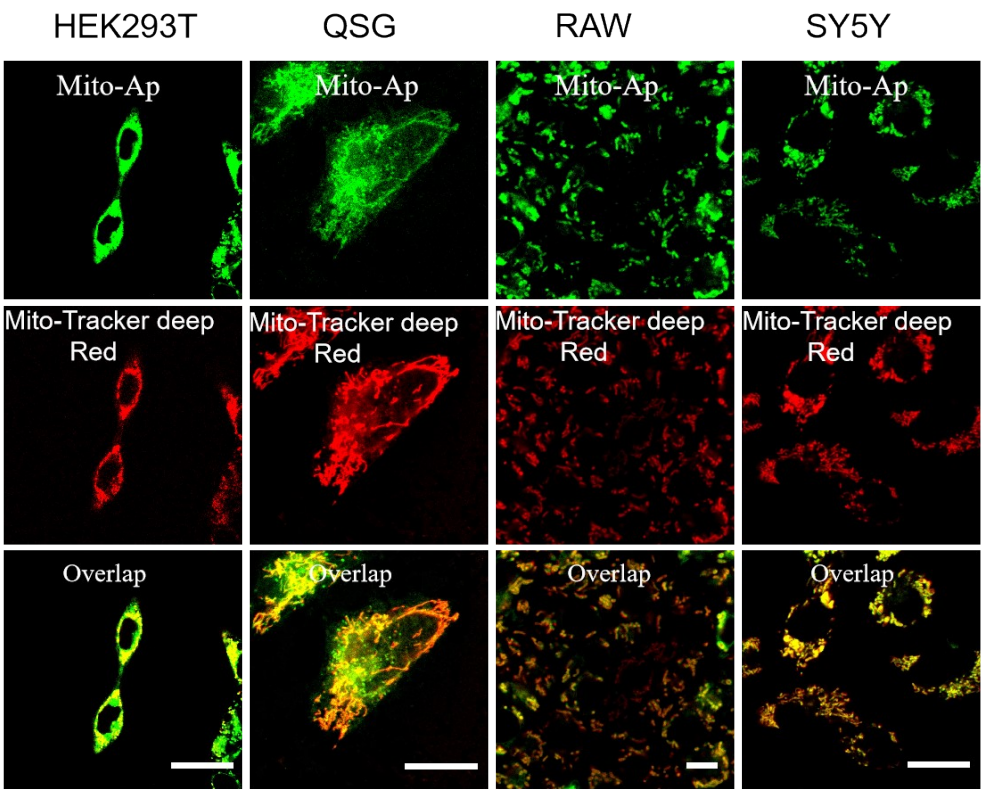

Fig. S13. Confocal imaging of Mito-Ap (2 μM, 10 min, λ_{ex} = 840 nm and λ_{em} = 550-600 nm) and Mito-Tacker deep Red (0.5 μM, 15 min λ_{ex} =633 nm and λ_{em} = 640-660 nm) in different cells. Scale Bar: 20 μm.

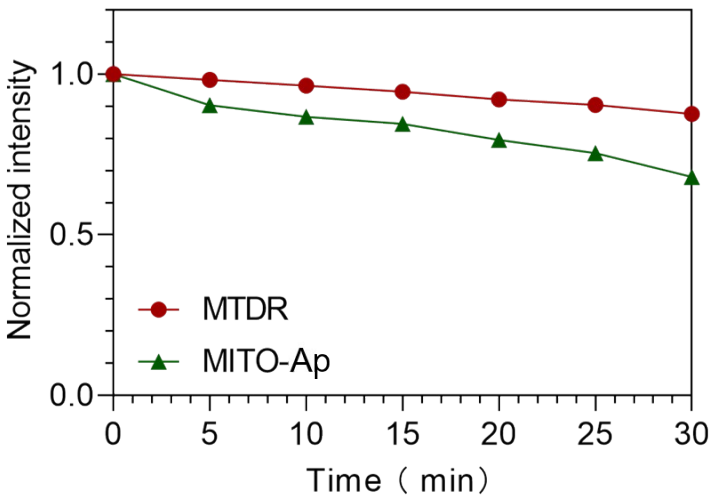

Fig. S14. Photostability experiments. Mito-Ap and commercial Mito-Tracker Deep Red were monitored for their cellular photostability by using fluorescence intensities at 840

nm and 633 nm.

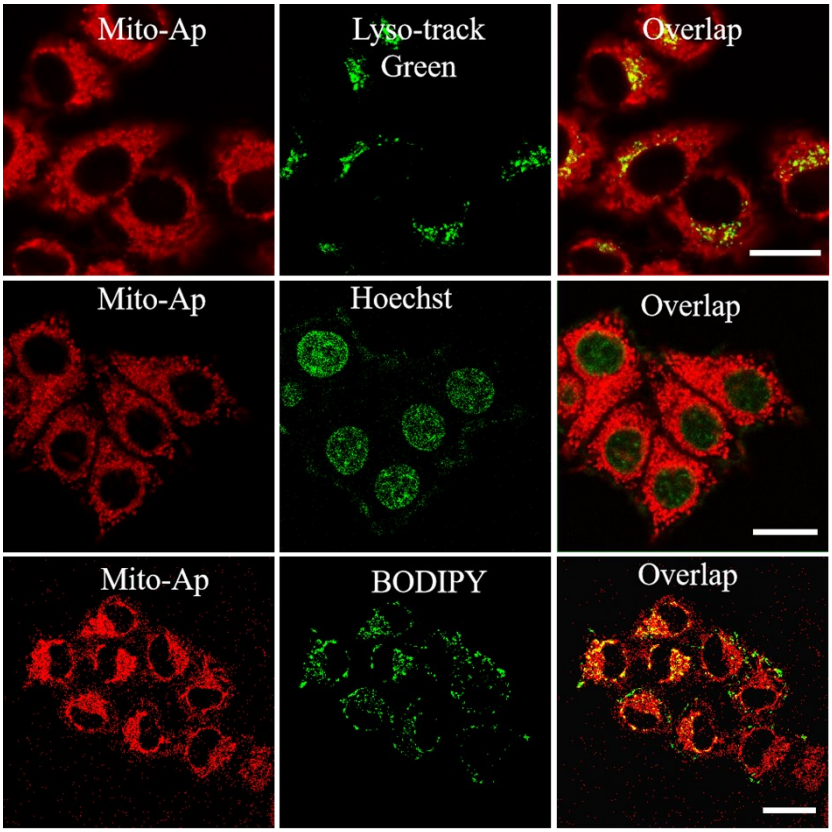

Fig. S15. Mito-Ap Co-localization with different organelles.Mito-Ap (2 μM, λ_{ex}: 840, λ_{em}: 550-600 nm) and Lyso-track Green (0.5 μM, λ_{ex}: 500nm; λ_{em}: 510-530 nm), Hoechst (1 μM, λ_{ex}:405nm,λ_{em}:450-500 nm) BODIPY (0.5 μM,λ_{ex}:493nm,λ_{em}:500-540 nm).Scale Bar = 20 μm.

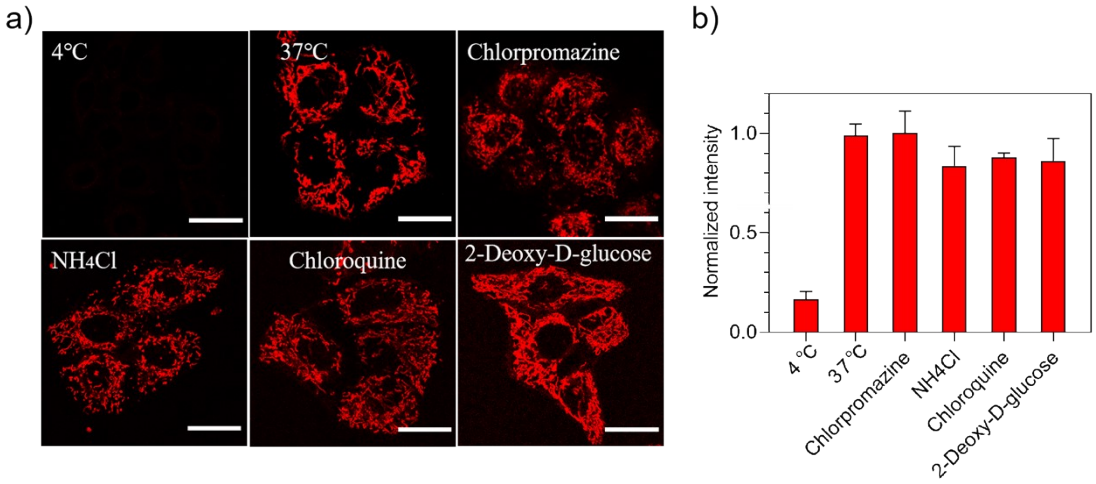

Fig. S16. Cellular uptake experiments. Chlorpromazine (10 μg/ml) Chloroquine (100

μM) NH4Cl (50 mM)　2-Deoxy-D-glucose (100 μM).Scale Bar = 20 μm.

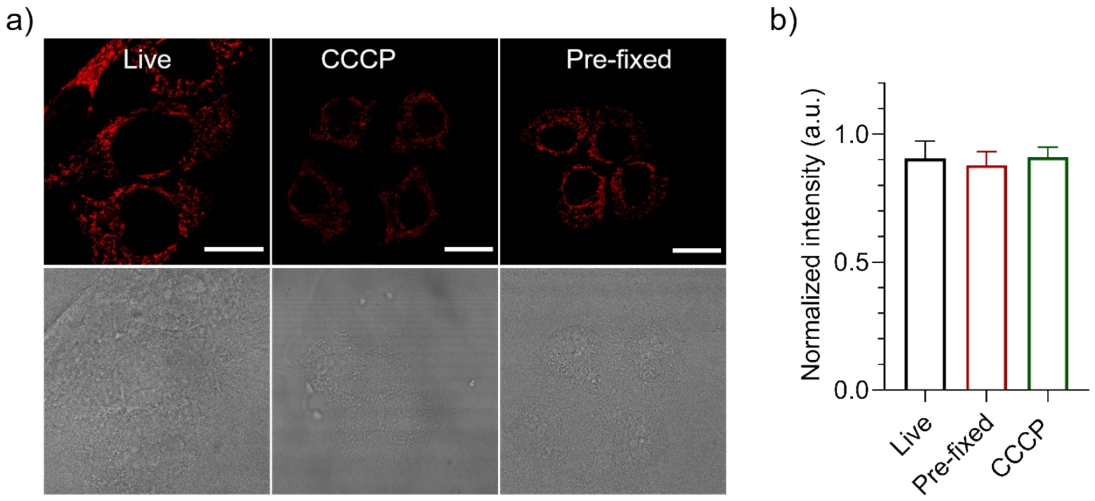

Fig. S17. (a) Mito-Ap images of live cells, fixed cells and CCCP-treated cells under two-photon fluorescence microscopy. (b) Figure (a) corresponds to the comparison of fluorescence intensity.Scale Bar = 20 μm.

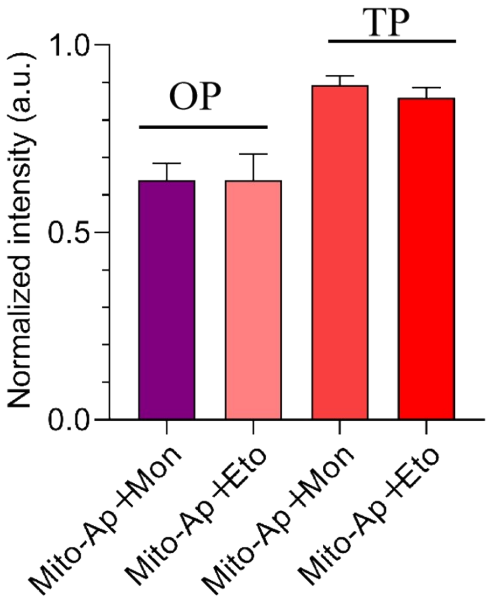

Fig. S18. Normalized fluorescence intensity of monensin and etoposide treated cells imaged in OP and TP mode.

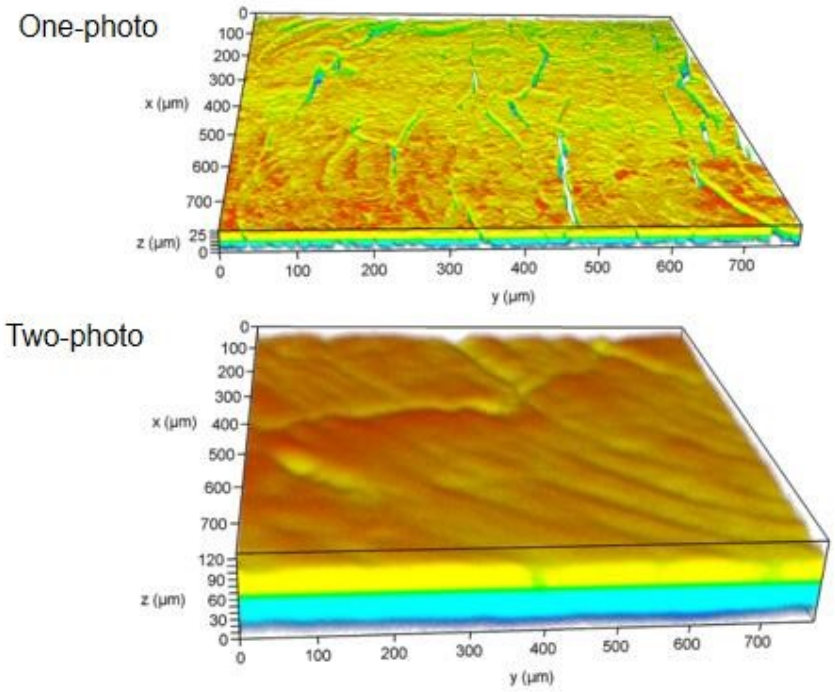

Fig. S19. One-photon (405-nm excitation) and two-photon (840 nm) 3D imaging of Mito-Ap in mouse liver tissue at a concentration of 100 μM for 30 min at room temperature.

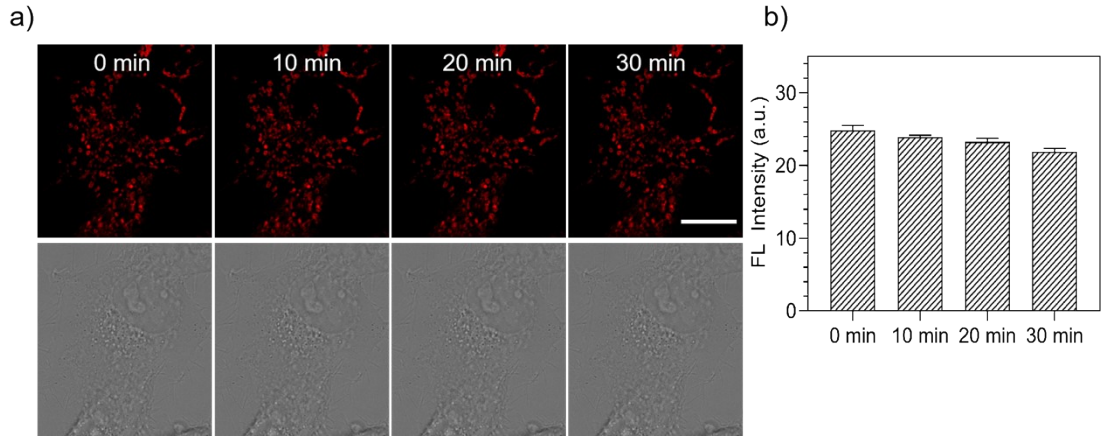

Fig. S20. (a) Confocal imaging of Mito-Ap without any treatment at different periods in HepG2. （b) The fluorescence intensity of Mito-Ap at different times in Figure (a).Scale Bar = 20 μm.

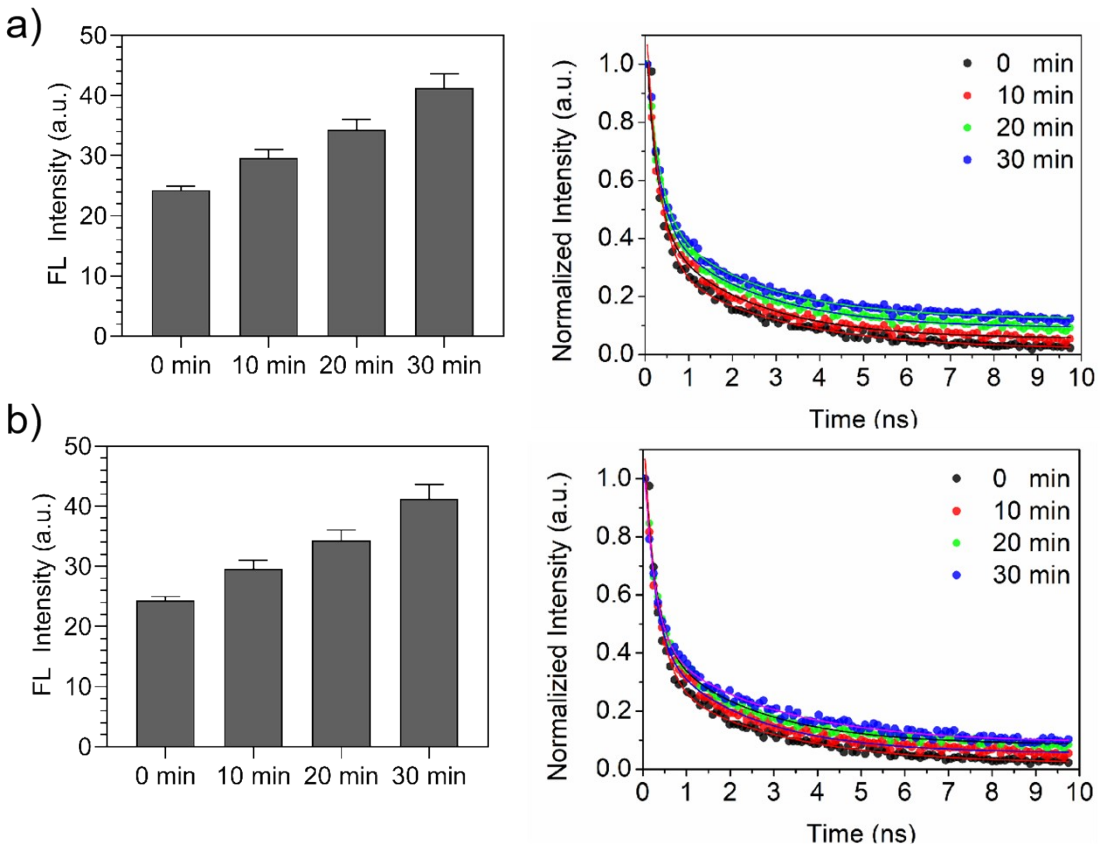

Fig. S21. (a) Fluorescence intensity and lifetime changes after different times of monensin treatment. (b) Fluorescence intensity and lifetime changes after etoposide treatment at different times.

Table S1 Two-photon properties data of Mito-Ap in DMSO (c = 1.0 × 10^{-3} mol/L).

Complex	λ/nm	β (cm GW^{-1})	σ(GM)	γ (cm^2/W)	Re ($\chi^{(3)}$) (esu)	Im ($\chi^{(3)}$) (esu)	$\chi^{(3)}$ (esu)
Mito-Ap	800	0.014	268	6.09×10^{-15}	2.54×10^{-13}	3.09×10^{-8}	3.09×10^{-8}