Electronic Supplementary Information

Mitochondria Targeted Near-Infrared Chemodosimeter for Upconversion Luminescence Bioimaging of Hypoxia

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Materials and methods

1. Materials and general instruments.

All chemicals were purchased from the commercial sources and used without further purification. NADPH was purchased from Roche. Rat liver microsomes, fetal bovine serum (FBS), Dulbecco's modified Eagle's Medium (DMEM), trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT), phosphate buffer (PBS), penicillin, streptomycin and DAPI were purchased from KeyGen Biotech (Nanjing, China). ^{1}H NMR spectra was collected on Bruker spectrometer at 300 MHz by using MeOD as the solvent. Mass spectroscopy data were measured with Waters Q-TOF MicroTM. The aimed products were purified by SepaBean machine with spherical C18 for reversed-phase separation chromatography and monitored by 600 nm detector.

2. Synthesis of NRh-O.

The preparation of NRh was obtained according to our previous literature [27]. As shown in Scheme S1, NRh-O was synthesized via the reported method [35]. NRh (100 mg, 0.146 mmol) was dissolved in CH_2Cl_2 (5 mL) and stirred in an ice bath. Then, $NaHCO_3$ (27 mg, 2.2 equiv.) and *m*-CPBA (77% w/w, 33 mg, 1.0 equiv.) were added to the solution, and the mixture was stirred at the room temperature for 2 h. Next, excess $NaHCO_3$ was added into the mixture. The solvent was removed and the crude product was purified by reversed-phase separation chromatography (C18, 65% CH_3OH/H_2O). The desired product NRh-O was obtained as a bluish violet solid in 5% overall yield.

^{1}H NMR (300 MHz, Methanol-d_4) δ 8.90 (d, J = 6.7 Hz, 1H), 8.75 (d, J = 7.8 Hz, 1H), 8.67 (d, J = 15.2 Hz, 1H), 8.36 (d, J = 8.9 Hz, 1H), 8.29 (d, J = 6.8 Hz, 1H), 8.22 – 8.09 (m, 1H), 7.97 – 7.83 (m, 3H), 7.53 – 7.46 (m, 2H), 7.39 (d, J = 15.3 Hz, 1H), 7.26 (dd, J = 8.7, 2.3 Hz, 1H), 7.20 – 7.08 (m, 1H), 6.88 (d, J = 8.6 Hz, 1H), 3.97 – 3.76 (m, 2H), 3.72 – 3.55 (m, 2H), 2.70 (t, J = 6.2 Hz, 2H), 2.53 – 2.26 (m, 2H), 1.93 – 1.70 (m, 4H), 1.67 (t, J = 7.2 Hz, 3H), 1.18 – 1.05 (m, 6H). MS (ESI): m/z = 573.2753 $[M]^+$; calcd. for $C_{37}H_{37}N_2O_3^+$ = 573.2748 $[M]^+$.

3. Assay with rat liver microsomes *in vitro*.

For the *N*-oxide reduction assays, cuvettes were gassed with nitrogen for 5 min to expel the air, and then gassed with nitrogen (×3) again to achieve anaerobic conditions. All the PBS buffer (100 mM, pH 7.4) and NADPH (50 μM) were pre-gassed with nitrogen for 30 min prior to use. PBS buffer (2 mL) and NRh-O (2 μL, 2 mM in DMSO) were mixed. Then, NADPH (50 μM) was added. Rat liver microsomes (0-150 μg/mL) were added to the cuvette. After mixing, fluorescence spectra were acquisited at the indicated time points at 37 °C. The FUCL spectra were acquired according to the following parameters: $\lambda_{ex/em}$ = 850/825 nm.

4. Cell culture and confocal microscope imaging.

Human malignant glioma cell line (U87MG) was brought from KeyGen Biotech (Nanjing, China). U87MG cells were cultured under humidified normoxic environment lat 37 ℃ and supplemented in DMEM medium with the condition (10% FBS and 1% penicillin/streptomycin).

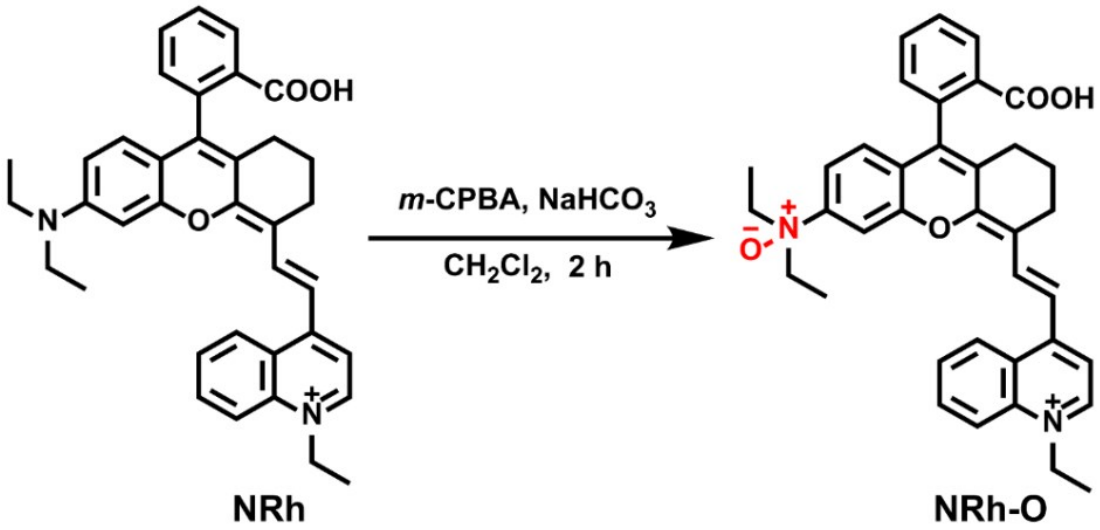
To evaluate cell cytotoxicity of NRh-O, U87MG cells (1×10^4/well) were pre-seeded in 96 well plates overnight. After replacing 200 μL fresh DMEM medium, the cells were incubated with different concentrations of NRh-O for 24 h. Then, the cell viabilities were measured via the standard MTT assay.

To further assess the applicability of NRh-O for monitoring hypoxia in cell, U87MG cells (5×10^4 /well) were planted into the confocal dishes. U87MG cells were respectively incubated under normoxia (20% O_2) or hypoxia condition (10% O_2 and 1% O_2) overnight and then treated with NRh-O (10 μM) in PBS buffer (containing 0.5% DMSO). After incubated for 30 min, the cells were washed with PBS (pH=7.4) for three times, stained with 4% paraformaldehyde and labeled with 4', 6-diamidino-2-phenylindole (DAPI) for 5 min. Under 10% oxygen concentration, after incubated with NRh-O for 30 min, the U87MG cells were washed and incubated with rhodamine 123 for another 5 min. Fluorescence images were collected by confocal laser scanning microscope (CLSM, Leica TCS SP5, Germany).

5. *In vivo* hypoxia imaging in murine tumor model.

Female Balb/c nude mice (5–6 weeks old) were purchased from Nanjing GemPharmatech Co., Ltd. and acclimated for a week. The Animal Care Guidelines of China Pharmaceutical University were followed by the overall animal experiments. U87MG cells (1×10^7) suspended in 100 μL PBS were grafted subcutaneously into the right rear thigh of each Balb/c nude mouse. Tumor assays were carried out when the tumor volumes reached to ~ 100 mm^3 after 20 – 30 days.

For *in vivo* imaging of hypoxia, the FUCL images of the mice after injecting NRh-O via subcutaneous injection (left) and intratumor injection (right) *in vivo* were recorded at different time points. Next, the probe NRh-O (500 μM in 100 μL PBS) was injected into U87MG-bearing mice via tail vein. The FUCL images of mice were collected at 0.5 h, 3 h, 6 h, 12 h and 24 h by a NIR imaging system with CCD camera (Princeton Instruments, USA, 810 ± 10 nm bandpass filter). After 6 h post-injection, the ex-vivo FUCL images of isolated organs (tumor, heart, liver, spleen, lung, kidney) were collected. To further verify whether the NRh-O can target to tumor region and return to NRh, the FUCL imaging of tumor frozen slices and three-dimensional (3D) microscope images were conducted in confocal microscopy.



Scheme S1. synthetic route of the probe NRh-O.

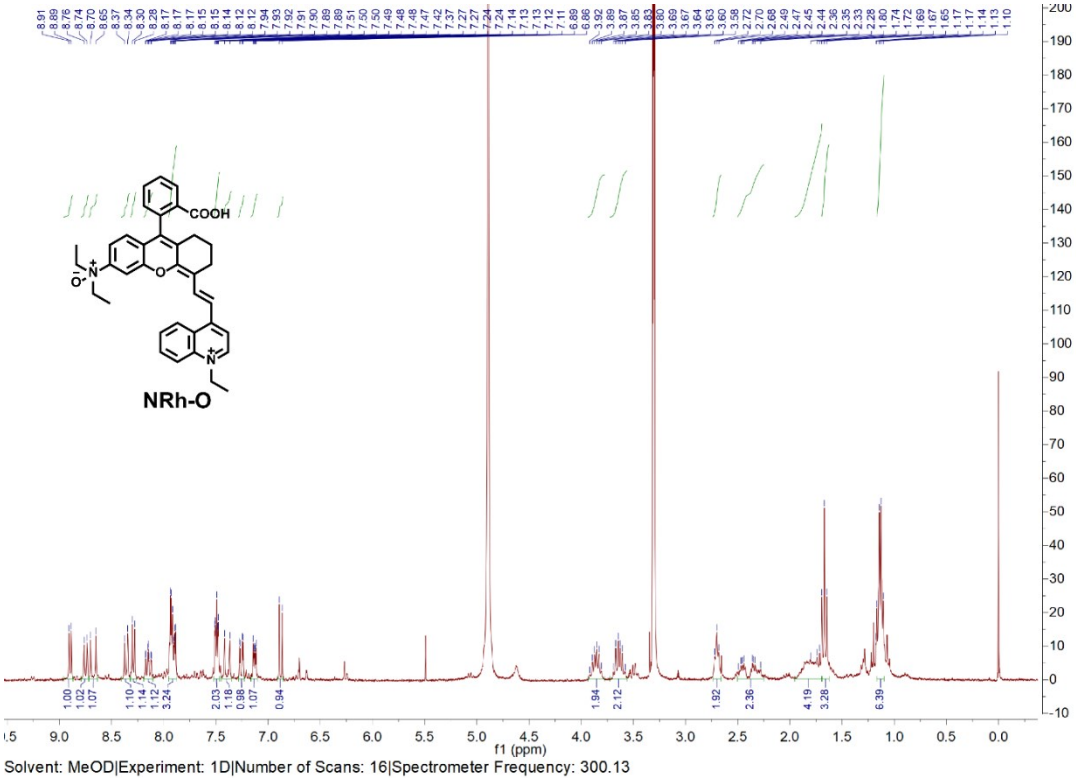


Figure S1. The ^{1}H NMR spectrum of compound NRh-O.

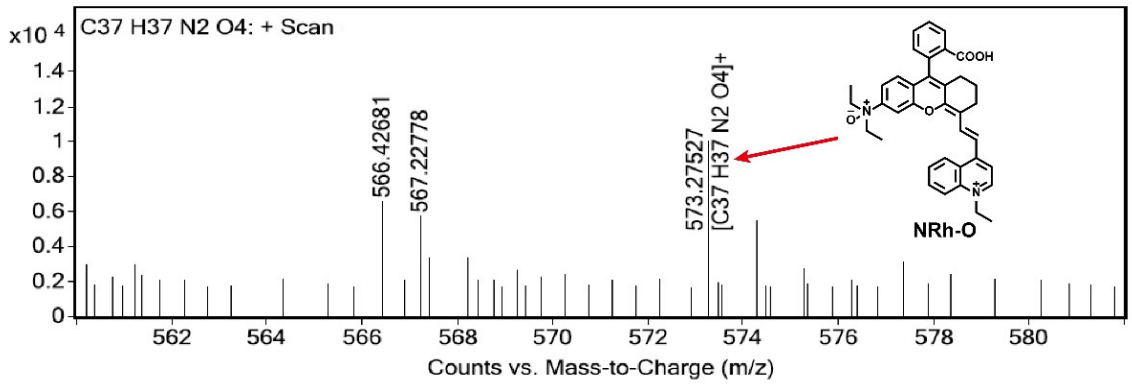
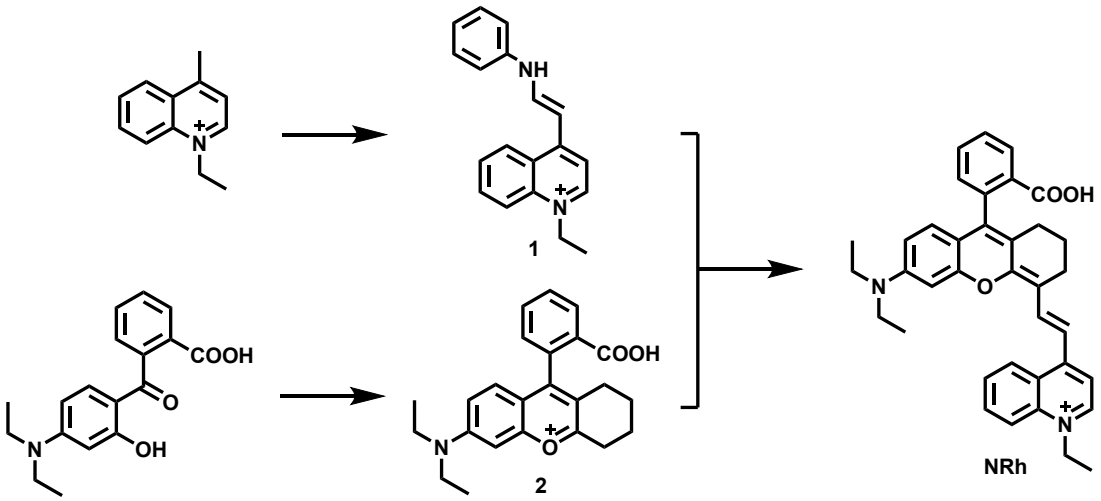


Figure S2. Mass spectra of compound NRh-O.



Scheme S2. Chemical structure and synthetic route of **NRh**.

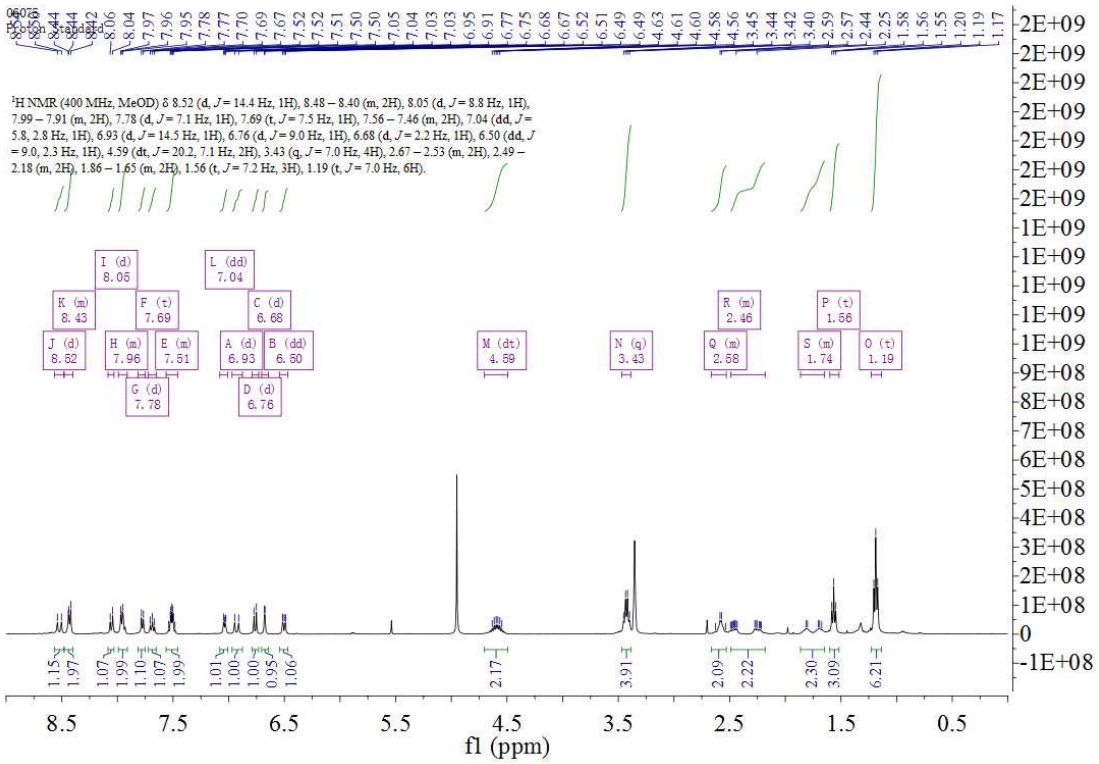


Figure S3. The ^{1}H NMR spectrum of compound **NRh**.

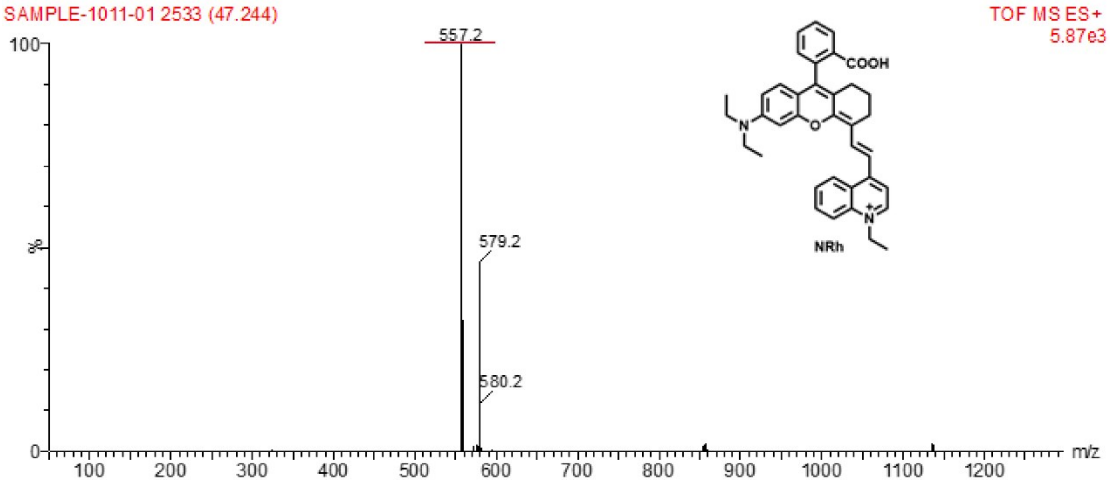


Figure S4. Mass spectra of compound **NRh**.

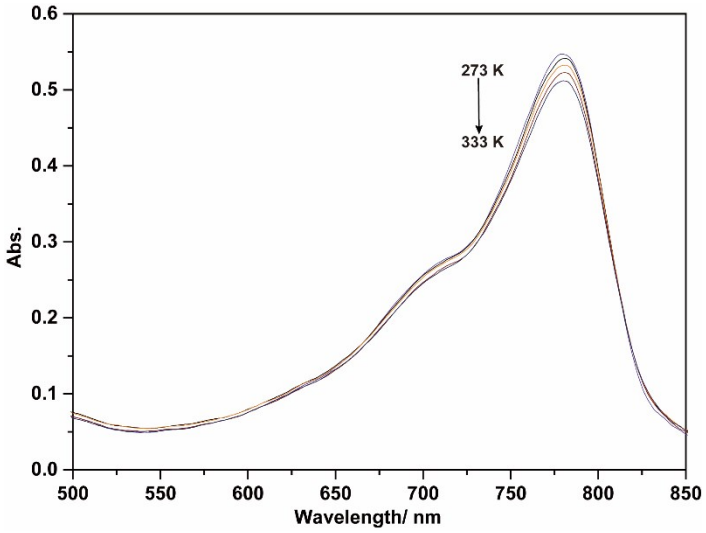


Figure S5. The absorption of NRh in ethanol at different temperatures (273 K-333 K).

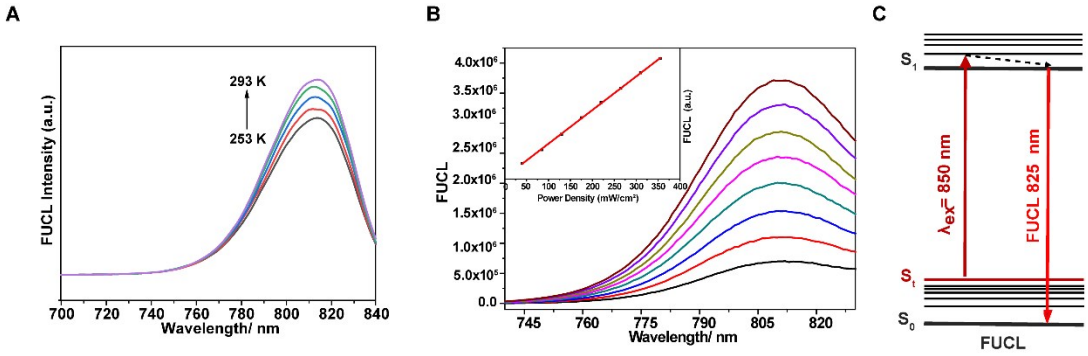


Figure S6. (A) FUCL emission intensity as a function of temperature (253 K-293K, Ex = 850 nm). (B) FUCL emission spectra of NRh in ethanol at different power density. (Insert: FUCL emission intensity as a function of power density (Ex = 850 nm). (C) Illustration of the mechanism of FUCL

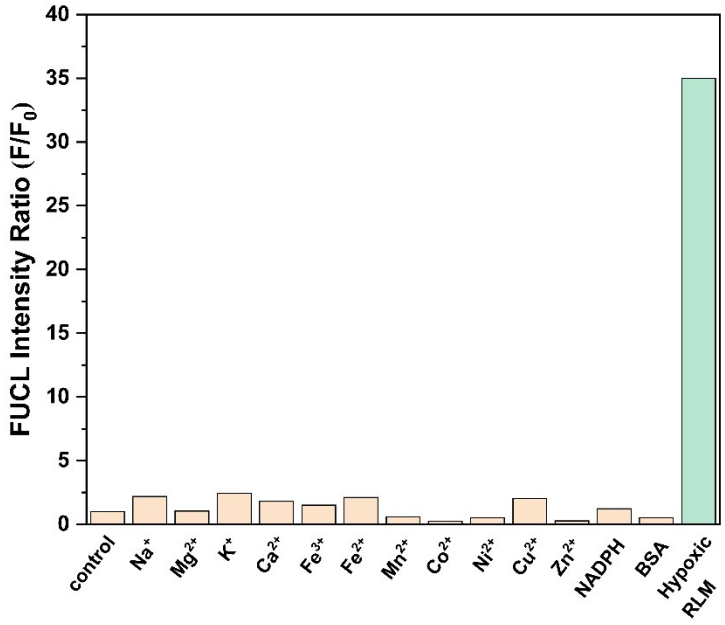


Figure S7. FUCL intensity ratio of NRh-O at λ_{em} = 825 nm for different analytes.

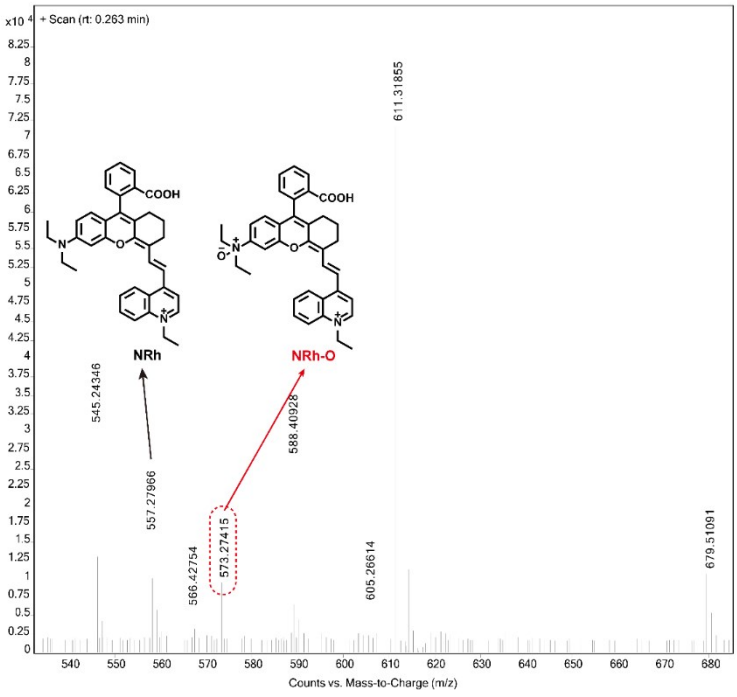


Figure S8. Mass spectra of reaction mixture NRh and NRh-O.

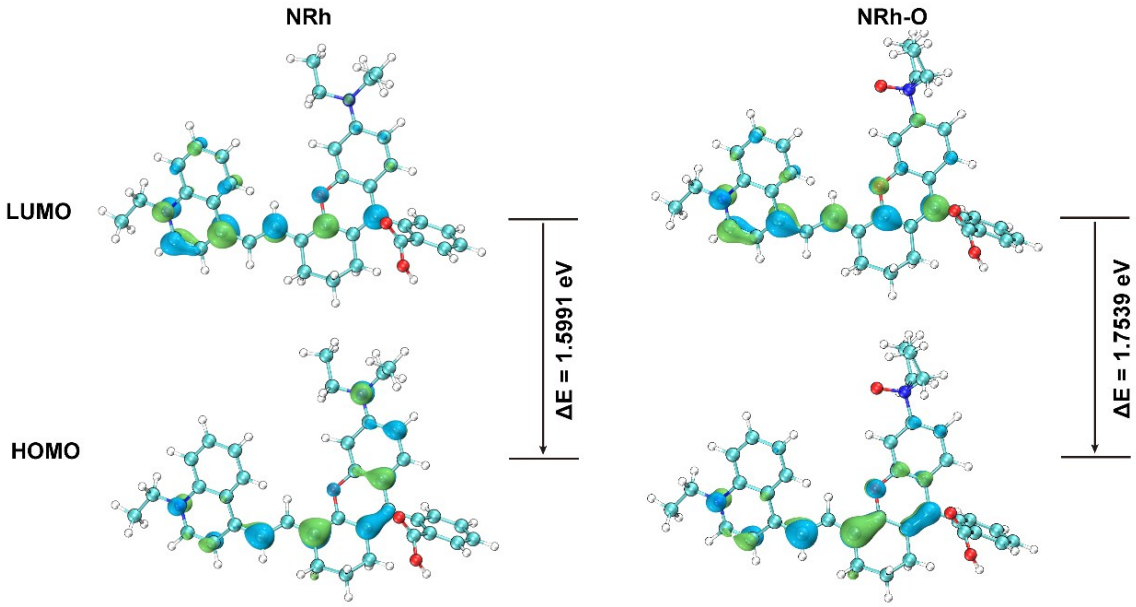


Figure S9. The major contributing orbitals HOMO and LUMO for the excited-state NRh and NRh-O.

Table S1. The percentage of major contributions HOMO→LUMO for the ground-state excited-state NRh and NRh-O.

Experiments / Molecules	The Major contributions of HOMO →LUMO calculated with TD-DFT/B3LYP/6-311G(d)	
	ground state	excited state
NRh	99.69%	99.58%
NRh-O	99.70%	99.80%

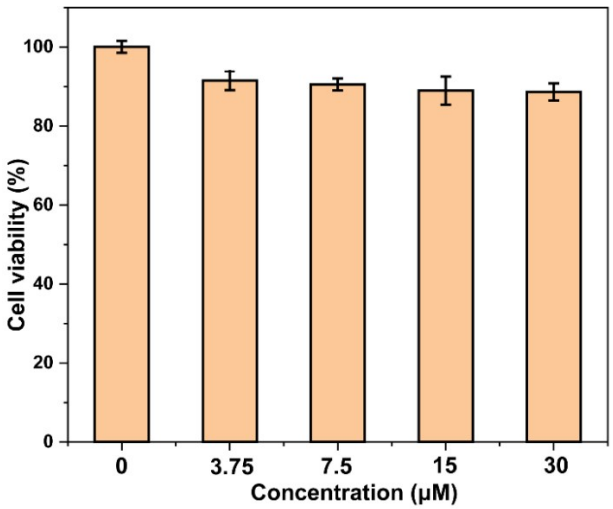


Figure S10. The MTT assay of NRh-O in U87MG cells.

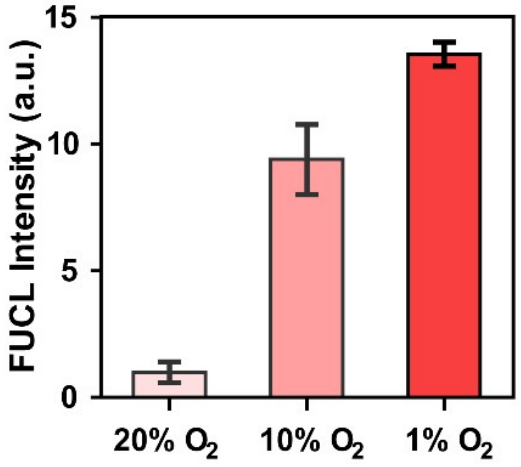


Figure S11. Semi-quantification of the FUCL intensities in U87MG cells after treated with NRh-O of the corresponding images.

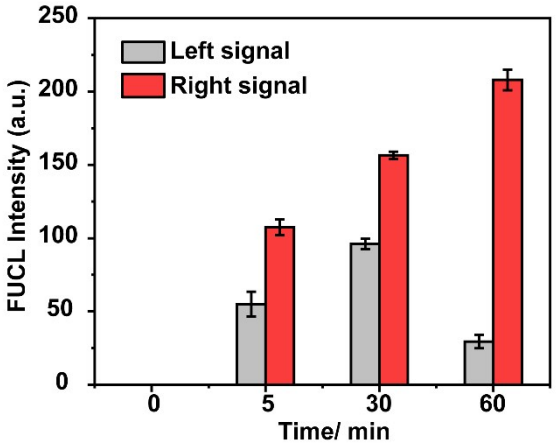


Figure S12. The FUCL intensity of NRh-O in subcutaneous region (left) and intratumor region (right) at different time points. The FUCL signal was collected at λ_{em} = 810 ± 10 nm under excitation with 850 nm laser.

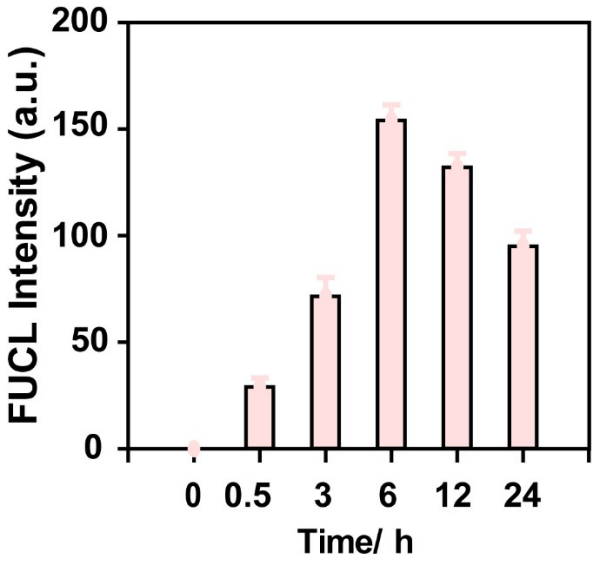


Figure S13. Semi-quantified FUCL intensities of NRh-O in tumor region at different time points. The FUCL signal was collected at λ_{em} = 810 ± 10 nm under excitation with 850 nm laser.

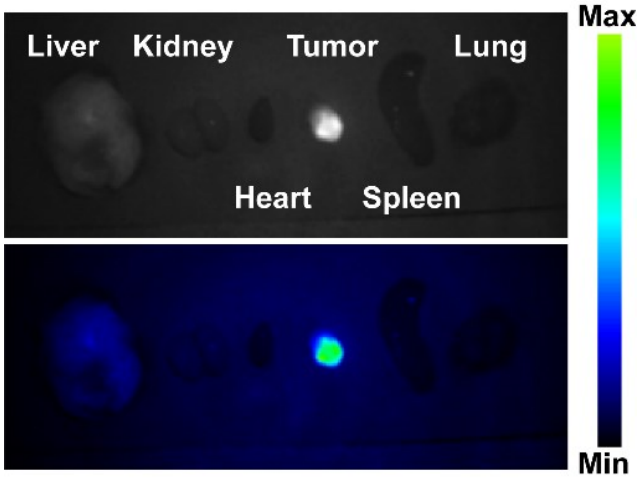


Figure S14. The ex-vivo FUCL images of isolated organs at 6 h post-injection of NRh-O.

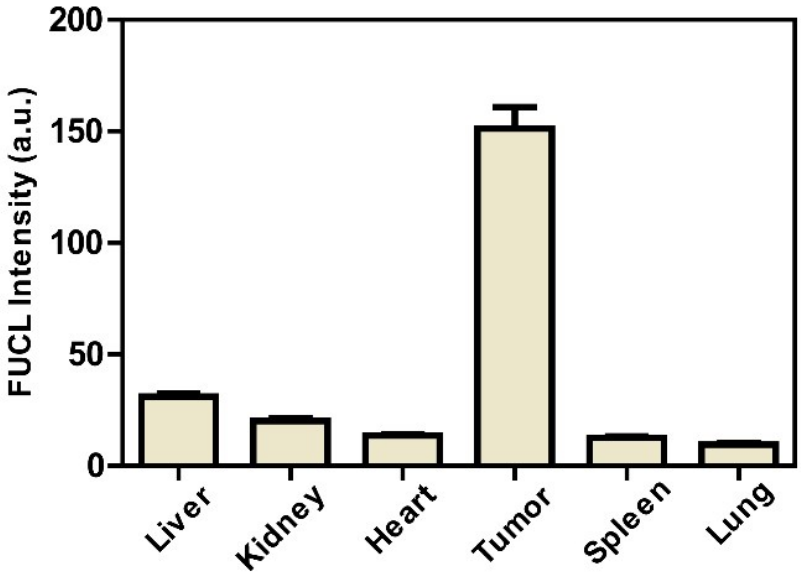


Figure S15. Semi-quantification of the FUCL intensities in major organs after treated with NRh-O at 6 h post- injection.

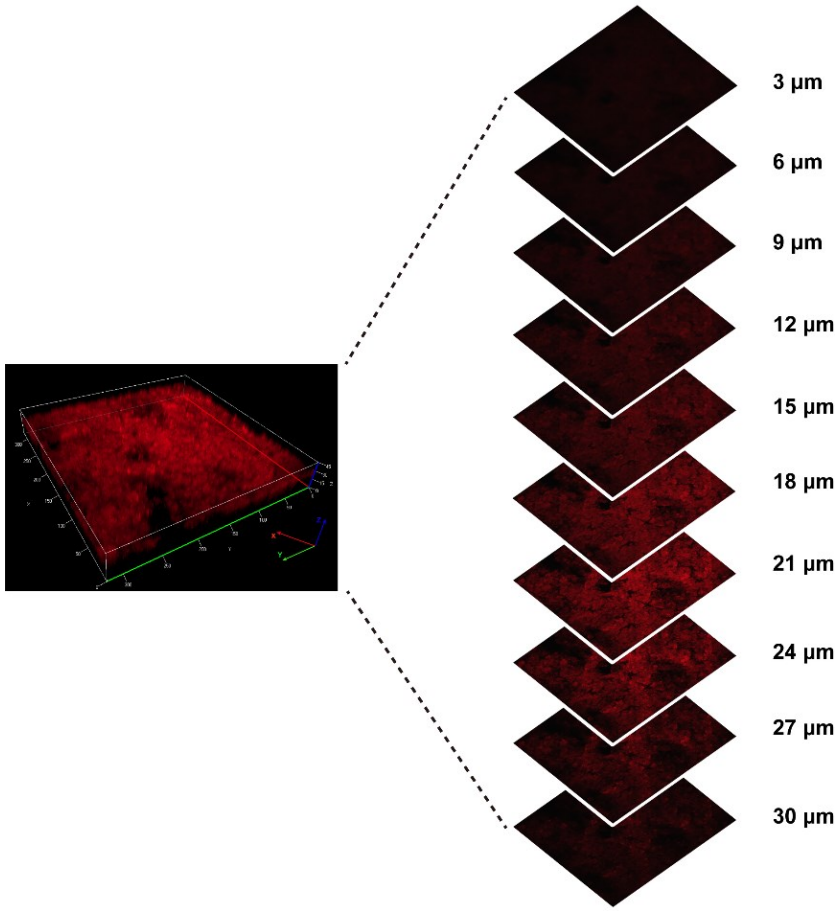


Figure S16. Three-dimensional (3D) microscope image of tumor region after vein injecting NRh-O at 6 h. (Left) Two-dimensional (2D) FUCL imaging of the frozen slices of U87MG tumor 6 h after vein injection of NRh-O. (Right)

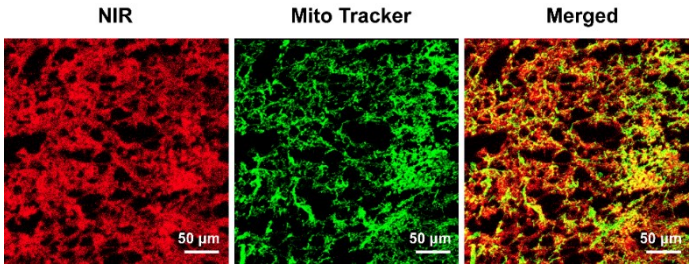


Figure S17. Confocal microscopic images from NRh-O colocalized to mitochondria in the frozen slices of U87MG tumor. Scale bar =50 µm.