*Supporting Information for*

# A FRET-based ratiometric two-photon fluorescent probe for Superoxide anion detecting and imaging in living cells and tissues

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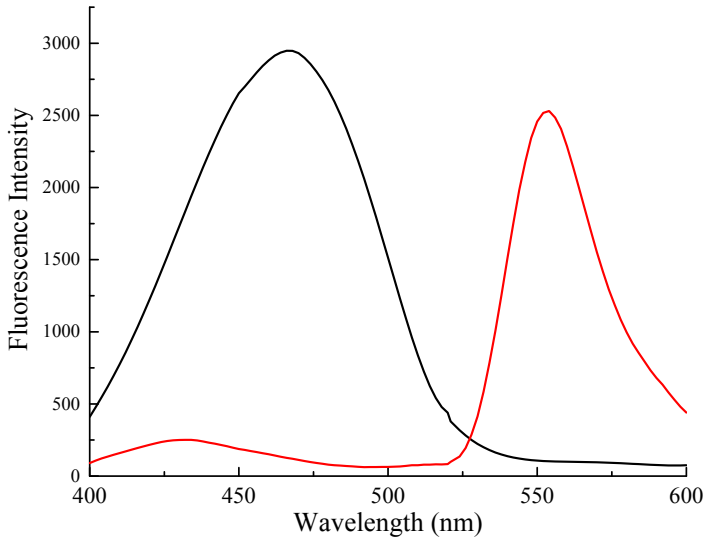



**Fig.S1** fluorescence spectra of **TFR-O** before (black line) and after (red line) reacted with $O_2^{\bullet-}$ (20 μM). $\lambda_{ex}$ = 740 nm

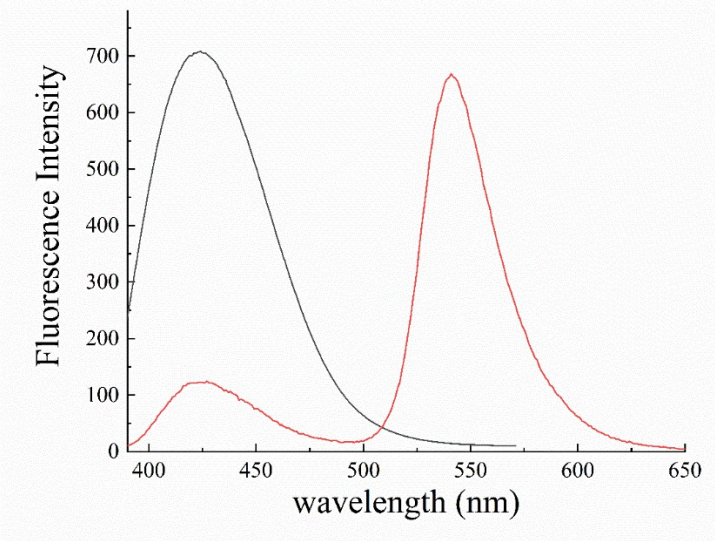



**Fig. S2.** Normalized emission spectra. The black and red lines represent donor derivative and **TFR-O** (10 μM) respectively, in PBS-DMSO (50/1, v/v, pH 7.4, 10 mM), as the respective fluorescence responses; $\lambda_{ex}$ = 370 nm. Donor derivative was synthesized by reported method.

**Energy Transfer Efficiency (ETE)** = [(fluorescence of donor-fluorescence of donor in cassette)/fluorescence of donor] × 100%[1-3].

For **TFR-O**, **ETE**= [(707.297- 122.14)/ 707.297] × 100%= 82.7%

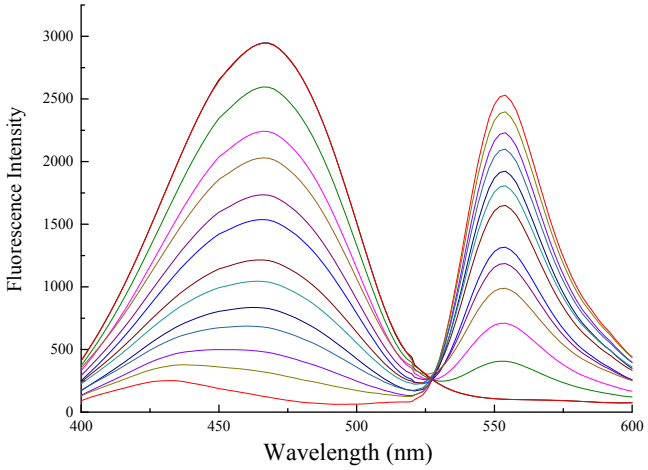



**Fig. S3** Fluorescence signal of TFR-O to $O_2^{\bullet-}$ with different concentrations (0−20 μM) in buffer solution.

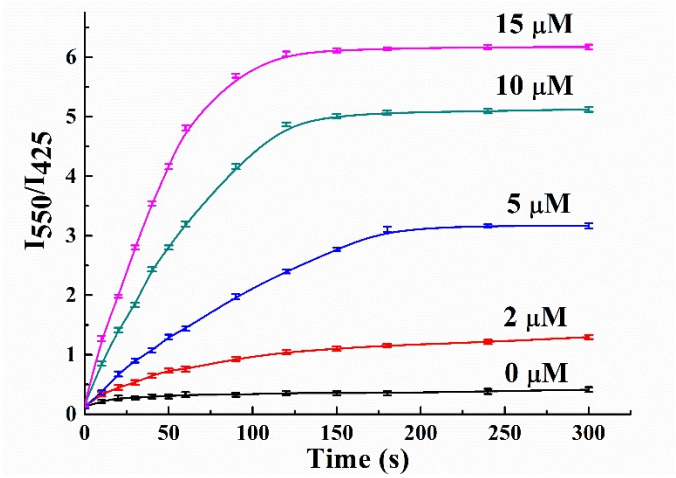



**Fig. S4** Real-time records for fluorescence ratio ($I_{550}$ /$I_{425}$) changes of **TFR-O** (10 μM) in the presence of different concentrations of $O_2^{\bullet-}$ (0, 2, 5, 10, and 15 μM). $\lambda_{ex}$ = 370 nm.

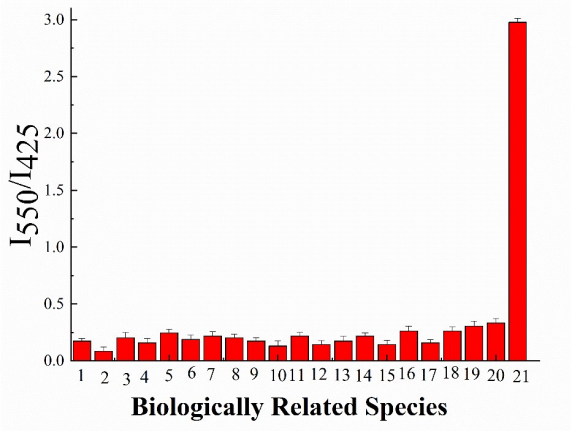



**Fig. S5** Fluorescence ratio ($I_{541}$ /$I_{448}$) changes of **TFR-O** (10 μM) to different biologically related substances. (0) blank, (1) NaCl, (2) KCl, (3) $CaCl_2$, (4) $MgCl_2$, (5) $ZnCl_2$, (6) $CuCl_2$, (7) $NaNO_2$, (8) $NaNO_3$, (9) $Fe^{2+}$, (10) $Fe^{3+}$, (11) $Mn^{2+}$, (12) glucose, (13) vitamin C, (14) $ONOO^-$,(15) $ClO^-$, (16) ·OH, (17) $^1O_2$, (18) $H_2O_2$, (19) NO, (20) HNO, (21) $O_2^{\bullet-}$ (5 μM)

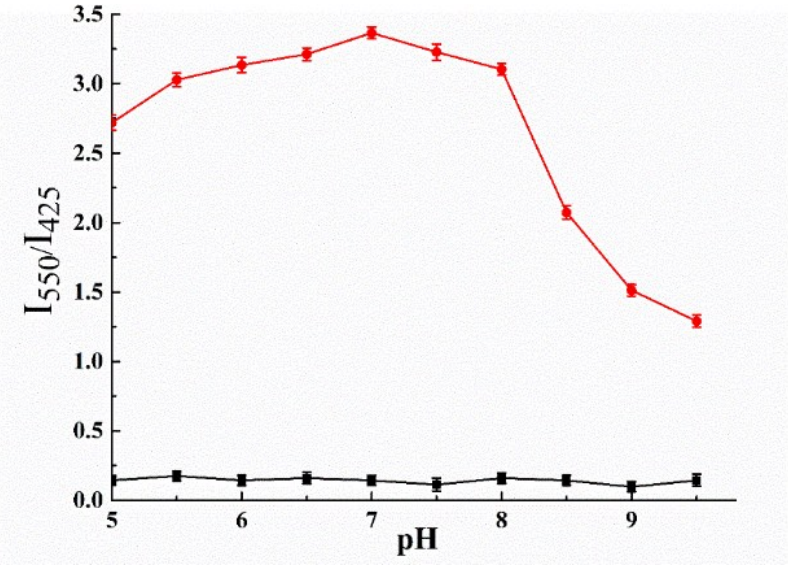



**Fig. S6.** The influence of pH on ratiometric fluorescence responses ($I_{550}$ /$I_{425}$) of **TFR-O** (10 μM) in PBS buffered/DMSO (50/1, v/v, pH 5.0-9.5, 10 mM), the pH were adjusted by NaOH (aq, 1 M) or HCl (aq, 1 M), $\lambda_{ex}$ = 370 nm. Ratiometric fluorescence responses are shown before (black line) and after (red line) the addition of $O_2^{\bullet-}$ (4 μM), respectively

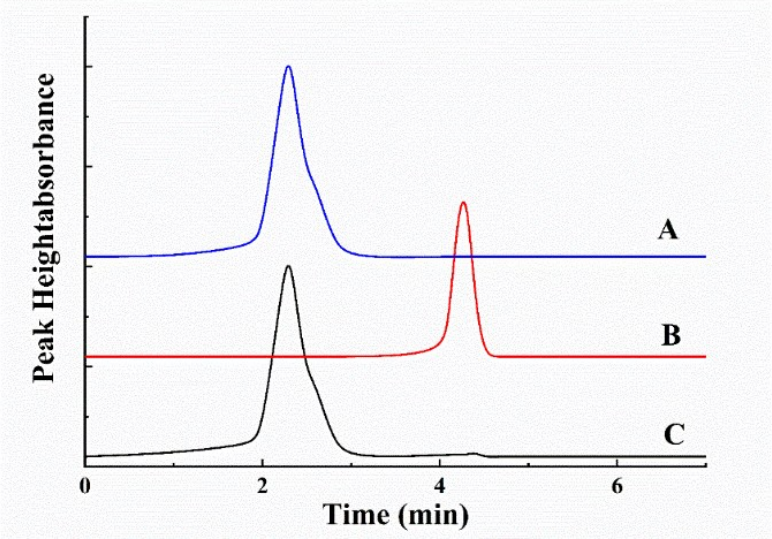



**Fig. S7.** Chromatograms of different reaction systems. A 100 μM **Np-Rhod**. B 100 μM probe **TFR-O**. C the reaction products of 100 μM probe **TFR-O** with $O_2^{\bullet-}$ (10 μM). Detection: UV-vis (425 nm) detector. Flow rate: 1mL/min. T: 20 °C. Injection volume: 100 μL. Mobile phase: acetonitrile–water, 4:1 (v/v).

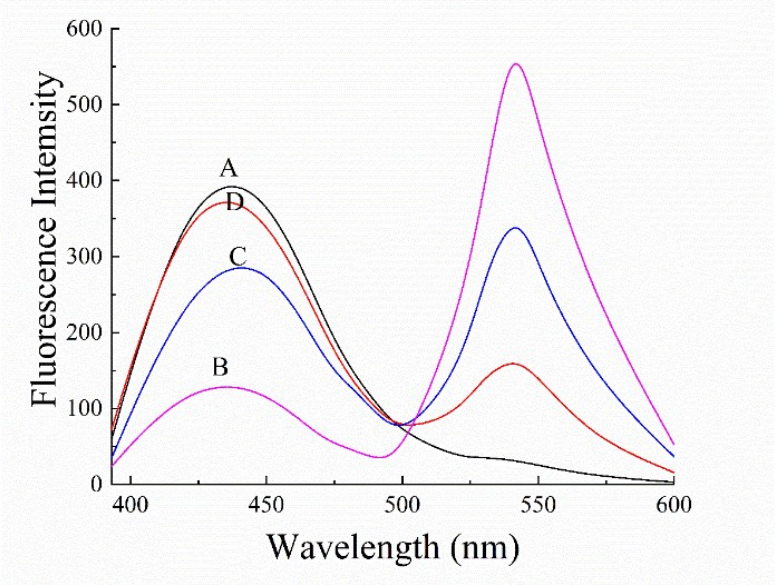



**Fig. S8.** Fluorescence emission spectra ($\lambda_{ex}$ = 370 nm) of different reaction systems. (A) **TFR-O** (10 μM) only; (B) the system (A) + $O_2^{\bullet-}$ (10 μM); (C) the system (B) + Tiron (10 μM); (D) the system (C) + Tiron (10 μM). All the reactions were performed in PBS buffered/DMSO (50/1, v/v, pH 7.4, 10 mM).

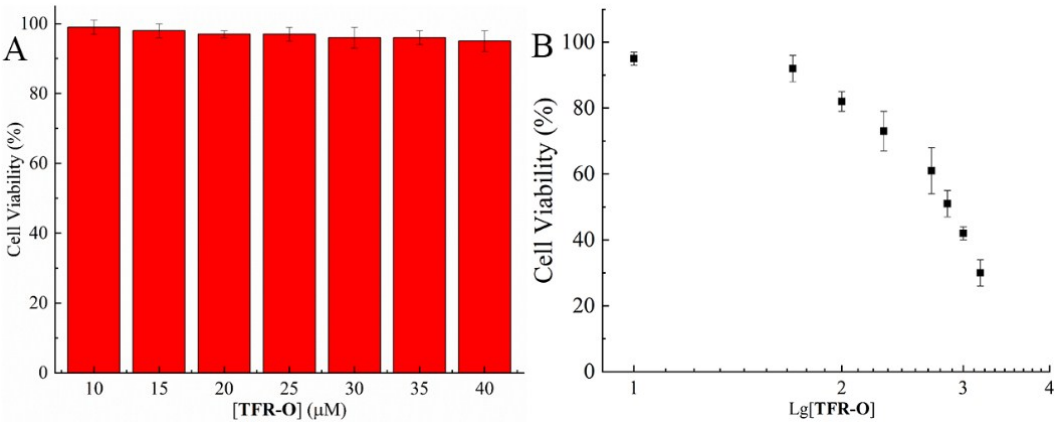



**Fig. S9.** Effects of **TFR-O** with varied concentrations on the viability of cells. (A) Effects of **TFR-O** at low concentrations (0-40 μM) on the viability of cells. (B) Effects of **TFR-O** at the concentrations from 10-1500 μM on the viability of cells. The viability of the cells without **TFR-O** is defined as 100%. The results are the mean ± standard deviation of five separate measurements.

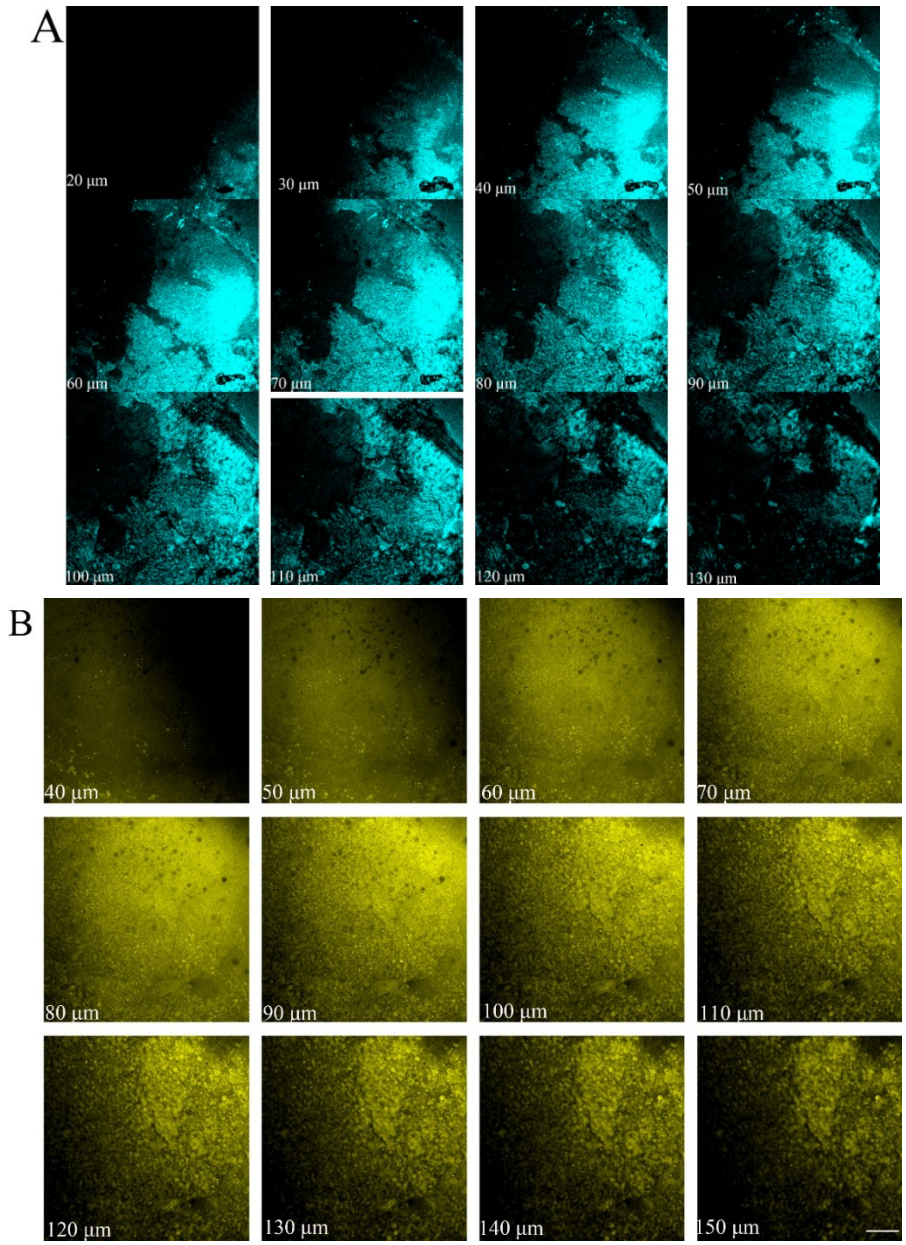



**Fig. S10.** (A) Two-photon fluorescence images of a fresh rat liver slice incubated with **TFR-O** (10 μM) only at the depths of approximately 20-160 μm; (b) Two-photon fluorescence images of a fresh rat liver slice pretreated with **TFR-O** (10 μM) and then with PMA at the depth of approximately 0-152 μm. Excitation at 740 nm, Scale bar = 200 μm, cyan channel: $\lambda_{em}$= 420-480 nm; yellow channel $\lambda_{em}$=490-600 nm

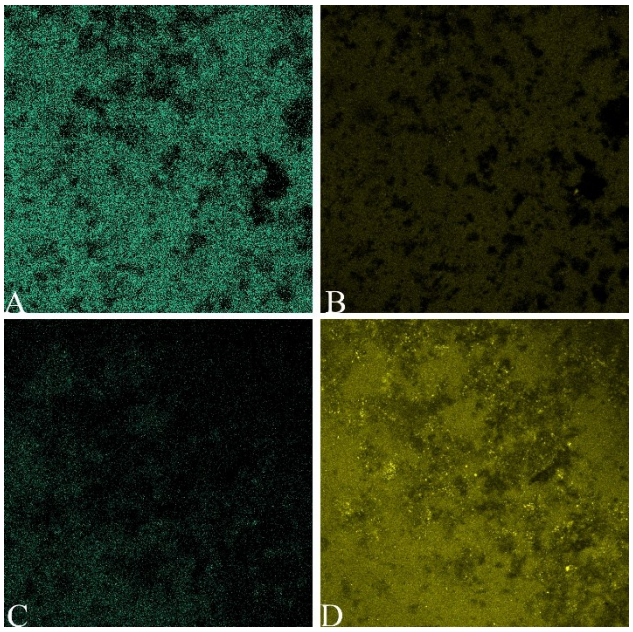
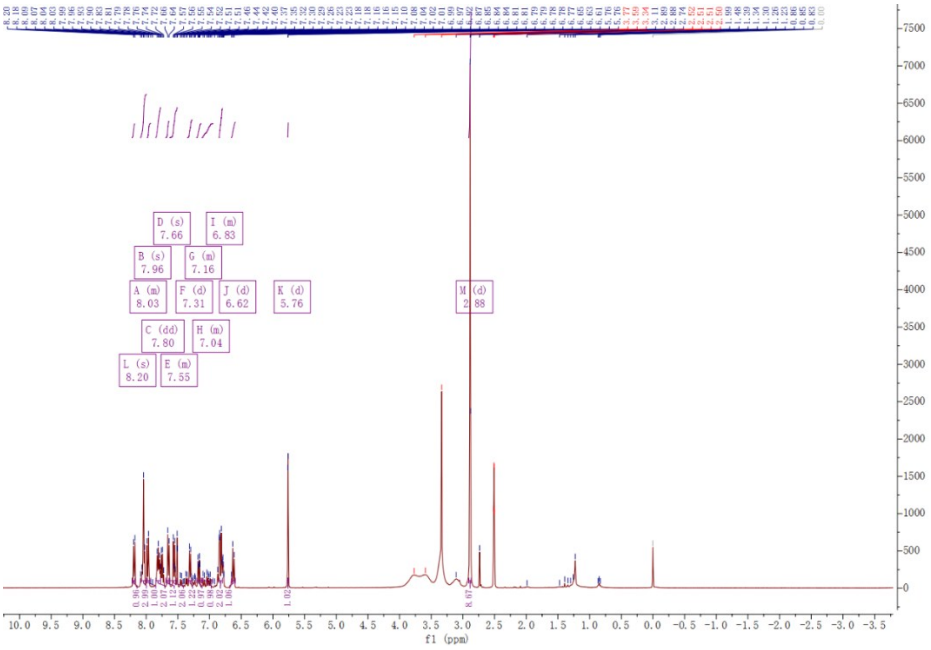
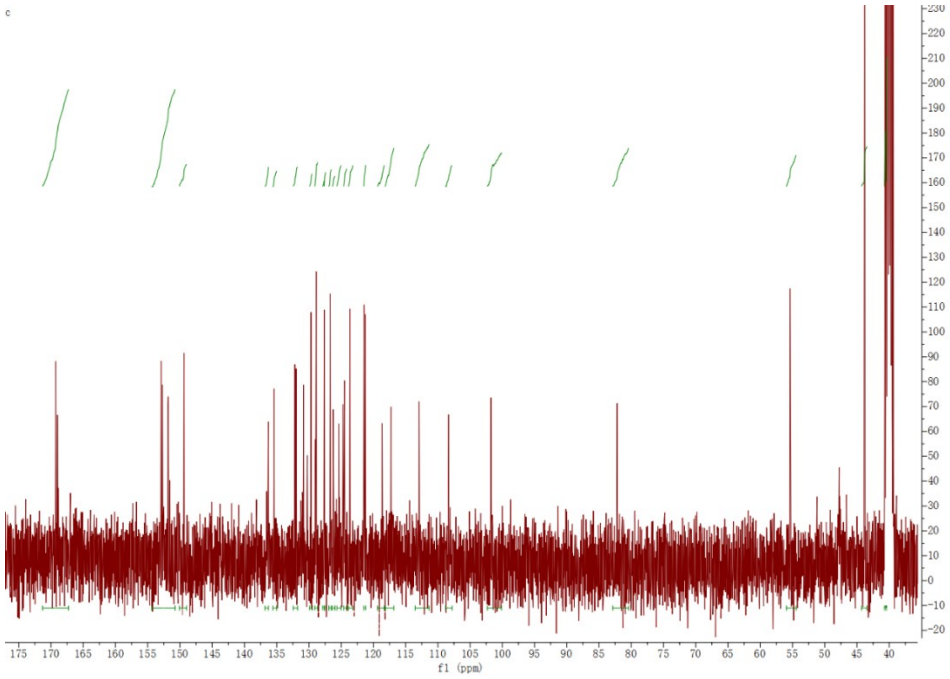


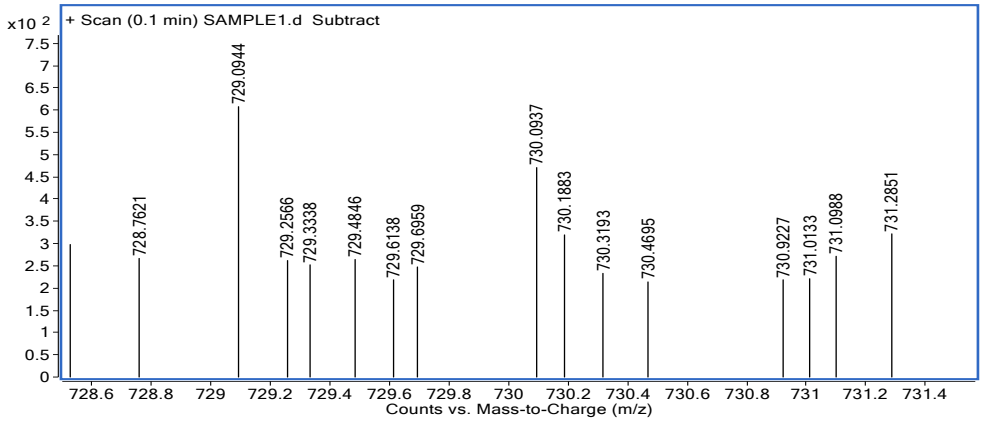

**Fig. S11** one-photon imaging of mice liver sample treated with **TFR-O** treatment with DPBS only (a and b), and PMA (c and d). Excitation at 405 nm, cyan channel: $\lambda_{em}$= 420-480 nm; yellow channel $\lambda_{em}$=490-600 nm

## Mass Spectra, $^{1}$H NMR and $^{13}$C NMR

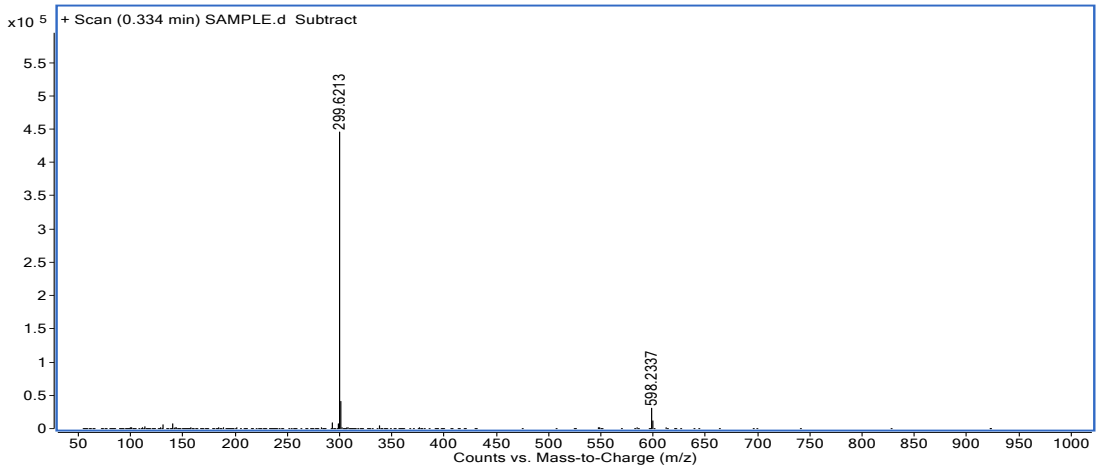
The Mass spectra and NMR of **TFR-O**

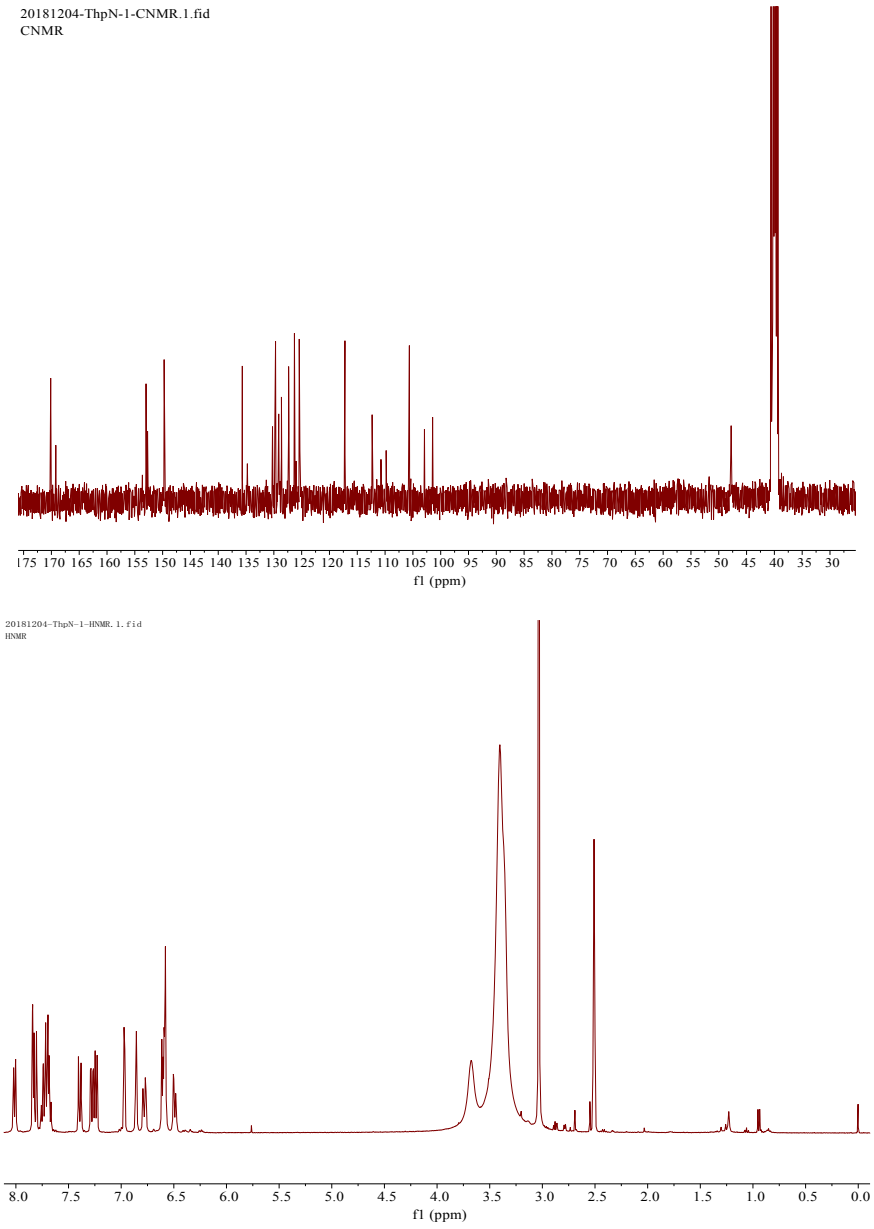





The Mass spectra and NMR of **Np-Rhod**






## References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3. L. Zhou, X. Zhang, Y. Lv, C. Yang, D. Lu, Y. Wu, Z. Chen, Q. Liu and W. Tan, *Anal. Chem.*, 2015, **87**, 5626-5631.