# Supporting Information

## Development of a fluorescent probe for detecting superoxide anions for monitoring the progression and treatment of acute spondylitis

# Experimental section

# 1. Experimental section

Synthesis of **ERO**

The synthesis route and predict response mechanism of **ERO** was shown in Fig. S1.

Compound 1 and 2 were prepared as described previously [1].

Dissolved compound 2 (290 mg) in 10 mL anhydrous dichloromethane in the 25 mL round bottom flask, added 70 μL of triethylamine, and then added trifluoromethylsulfonic anhydride (560 mg) at -76 ℃, reacted under nitrogen protection for 30 min, then transferred to room temperature for 2 h, vacuum spin dried the reaction solution, purified on a silica gel column with a mesh size of 200-300 mesh, and use dichloromethane as the eluent to obtain a yellow solid of 71 mg with a yield of 50%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 7.88 (d, $J$ = 8.8 Hz, 2H), 7.60 – 7.46 (m, 3H), 7.32 (d, $J$ = 16.2 Hz, 1H), 6.94 (s, 1H), 2.63 (s, 2H), 2.55 (s, 2H), 1.03 (s, 6H). $^{13}$C NMR (101 MHz, DMSO) δ 170.72, 155.56, 149.74, 137.30, 135.54, 132.00, 130.23, 124.19, 122.48, 114.13, 113.34, 77.84, 42.75, 32.15, 27.90. HRMS (ESI): m/z calculated for $C_{20}H_{17}F_3N_2O_3S$, 422.0912 $[M-H]^-$, found: 421.0834.

Cells cultural, biocompatibility and imaging

Raw 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin in a humidified atmosphere of 5/95 (v/v) of $CO_2$/air at 37 °C. For cell viability assay, cells were seeded on a 96-well plate at a density of $9 \times 10^3$ cells per well with culture media. After overnight culture, cells were treated with 0-40 μM **ERO** for 24 h. Then, reagents were removed, and cells were incubated with 100 μL DMSO for 10 min followed by MTT (20 μL, 5 mg/ml) solution for 4 h at 37 °C Absorbance was recorded using a microplate reader at 490 nm. The data were acquired from three independent tests.

Before confocal imaging, cells were grown on confocal microscope dishes and 10 μM **ERO** was added to cells for 30 min at 37°C. After washing with PBS (0.01M, pH 7.4), **ERO** was excited at

488 nm and the fluorescence was collected at 550-700 nm. In co location experiments, incubated at 37 ℃ for 15 min, Meantime, the organelle tracker including: ER tracker Green, (0.2 μM), LysoTracker Green (0.2 μM), and MitoTracker Green (0.2 μM), Lipid Tracker Green (0.2 μM), DAPI (0.2 μM), Golgi Tracker Green (0.2 μM) were added respectively. Then, the cells were washed and imaged on a confocal microscope. In the hypoxia experiment, $NaS_2O_3$ was treated with 1 μM for 1 hour to remove dissolved oxygen from the culture medium. After that, the cells were washed and replaced with sugar free culture medium. The cells were then placed in a hypoxia chamber and the degree of hypoxia was detected using an、 hypoxia indicator. The timing began when the oxygen concentration dropped to 1%.

Western blot analysis

Tris-glycine precast page gels were used for western blotting; while 8–20% gels were used to separate proteins with molecular weights of 10–180 kDa kDa. RAW 264.7cells were washed 3 times with ice cold PBS after they reached 100 confluence, and then, the cells were lysed in HEPES buffer for 30 min on ice. The concentrations of extracted cell proteins were measured with a BCA protein assay kit. Aliquots containing 40 μg of protein were electrophoresed in Tris-glycine precast page gels and transferred onto PVDF membranes. The PVDF membranes were blocked with Tris-buffered saline supplemented 5% milk and 0.1% Tween-20 for 2 h at room temperature. The PVDF membranes were incubated with primary antibodies diluted in blocking solution over night and then incubated with secondary antibodies. Finally, the results were captured by a Tannon 5200 work station, and the grey values were analysed by image analysis software (Alphalmager TM 2200 and GraphPad Prism 9).

Animal experiments

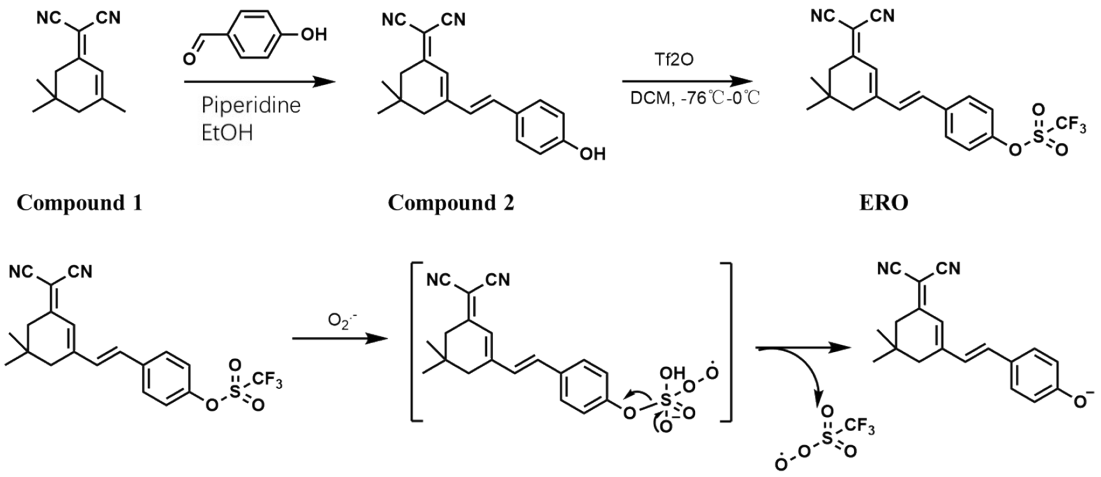
The wildtype (WT) zebrafish was provided by Nanjing EzeRinka Biotechnology Co., Ltd. The water temperature is maintained at 28℃ and the pH is kept between 7.0-7.4. The LPS (1 μg/mL) or resveratrol (20 μM) were added in water to treat zebrafish for corresponding and the probe was added to the water to allow the zebrafish to freely feed for 30 min.

5-weeks-old C57BL/6 mice were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). For the establishment of LPS-induced AS mice models, C57BL/6 mice were given with LPS (1 mg/kg) for 6 h through spinal injection administration. In treat group, intraperitoneal injected the resveratrol (50 mg/kg for 12 h) prior to LPS administration. The probe

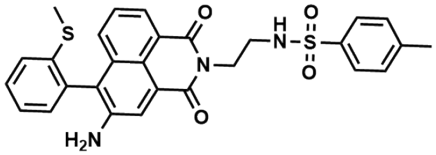
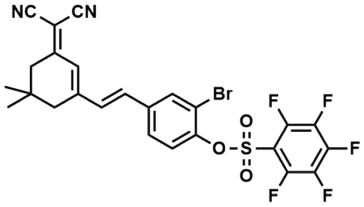
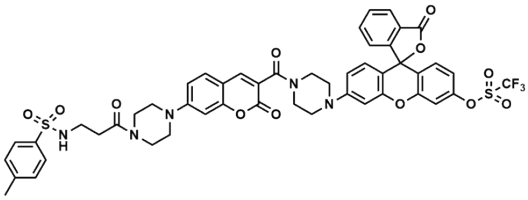
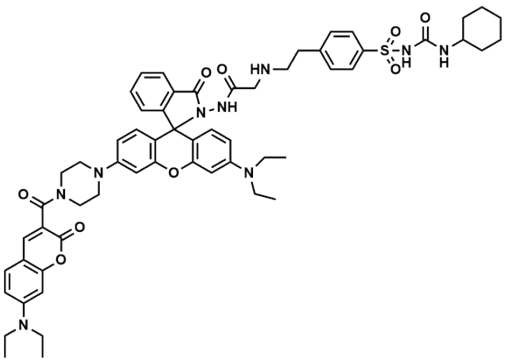
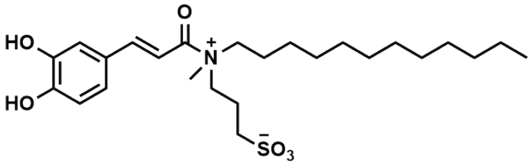
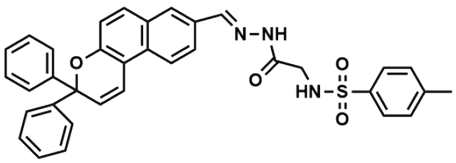
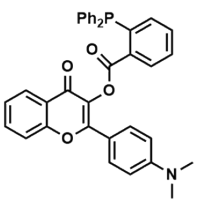
was incubated for 20 min through spinal injection before *in vivo* imaging.

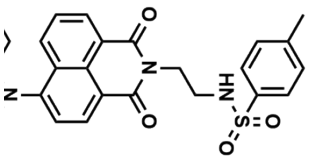
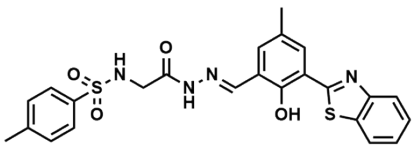
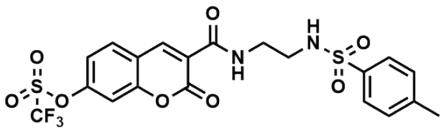
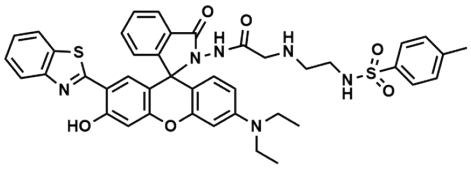
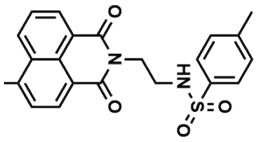
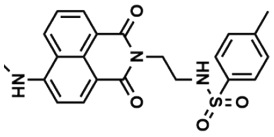
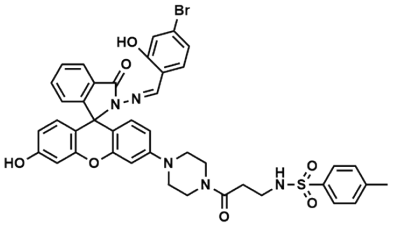
Instruments and materials.

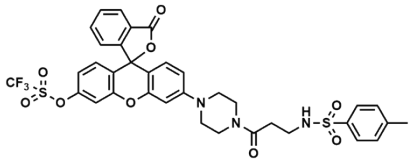
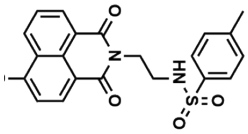
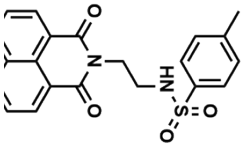
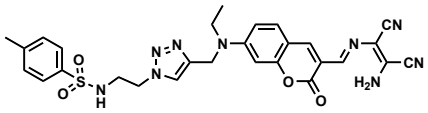
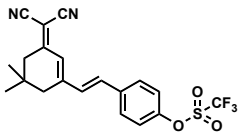
All chemicals and solvents are commercially available and used without further purification. TLC plate was used to monitor the reaction and detected by an ultraviolet lamp (254 nm or 365 nm). Compounds were purified by a silica chromatography column using the indicated solvent mixture. $^{1}H$ and $^{13}C$ NMR spectra were recorded on AVANCE NEO 500. High-resolution mass spectra (HRMS) were obtained by UPLC-G2-XS-Qtof (Waters). Fluorescence spectra were collected with a Hitachi F-7100 fluorescence spectrophotometer. Absorption spectra were determined on Techcomp UV2600-vis spectrometer using a 1 cm optical path length cell. Fluorescence imaging was conducted with confocal laser scanning microscopy (CLSM, Zeiss LMS880). The in vivo imaging was carried out using the FUSION FX imaging system (Vilber Lourmat).




**Scheme S1.** The synthesis route and predict response mechanism of **ERO**

| Structure | ER co-localization coefficient | Test ROS | Model | Ref |
|---|---|---|---|---|
|  | 0.90 | HOBr | Tunicamycin induced cell ER stress | [2] |
|  | 0.90 | $ONOO^-$ | Acute lung injury | [3] |
|  | 0.93 | $O_2^{\cdot-}$ | Depression | [4] |
|  | 0.95 | HClO | Atherosclerosis | [5] |
|  | 0.86 | $O_2^{\cdot-}$ | Hepatic ischemia-reperfusion injury | [6] |
|  | - | HClO | LPS-treated zebrafish | [7] |
|  | 0.94 | HNO | Parkinson's Disease | [8] |

| | | | | |
|---|---|---|---|---|
| | 0.93 | $ONOO^-$ | Cecum ligation and puncture | [9] |
|  | | | | |
|  | 0.92 | HClO | LPS-treated zebrafish | [10] |
|  | 0.89 | $O_2^{\cdot-}$ | - | [11] |
|  | 0.95 | HClO | - | [12] |
|  | 0.81 | HClO | Tunicamycin induced cell ER stress | [13] |
|  | 0.89 | $^1O_2$ | Brain injury | [14] |
|  | 0.89 | HClO | Diabetes | [15] |

| | | | | |
|---|---|---|---|---|
|  | 0.92 | $O_2^{\cdot-}$ | - | [16] |
|  | 0.94 | HClO | Tunicamycin induced cell ER stress | [17] |
|  | 0.94 | HClO | Sleep deprivation | [18] |
|  | 0.96 | HClO | - | [19] |
|  | 0.97 | $O_2^{\cdot-}$ | Acute spondylitis | This work |

**Scheme S2.** Fluorescent probes targeting ER for sensing ROS in the past five years.

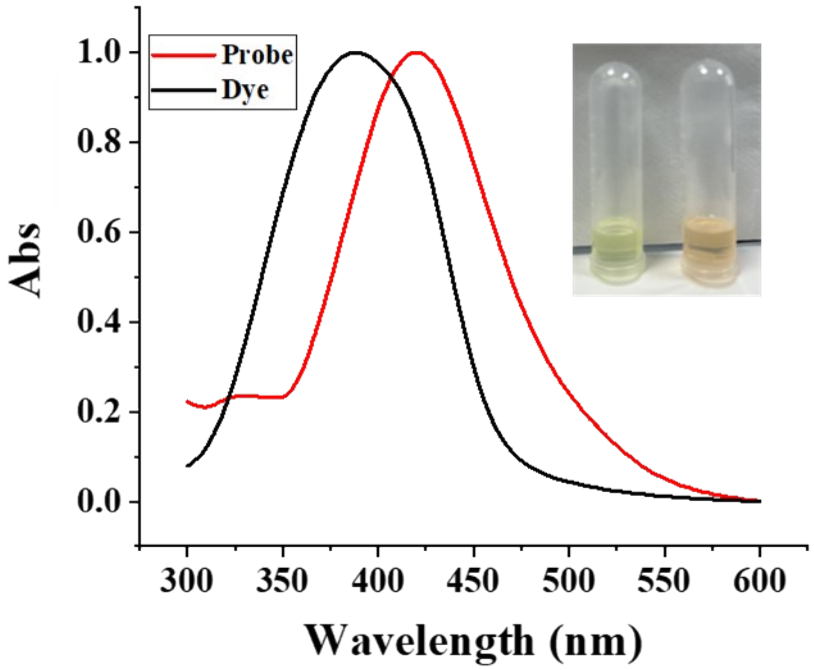



**Fig. S1.** Absorption of the probe **ERO** and fluorescent report dye compound 2, insert: left: the probe **ERO**, right: compound 2.

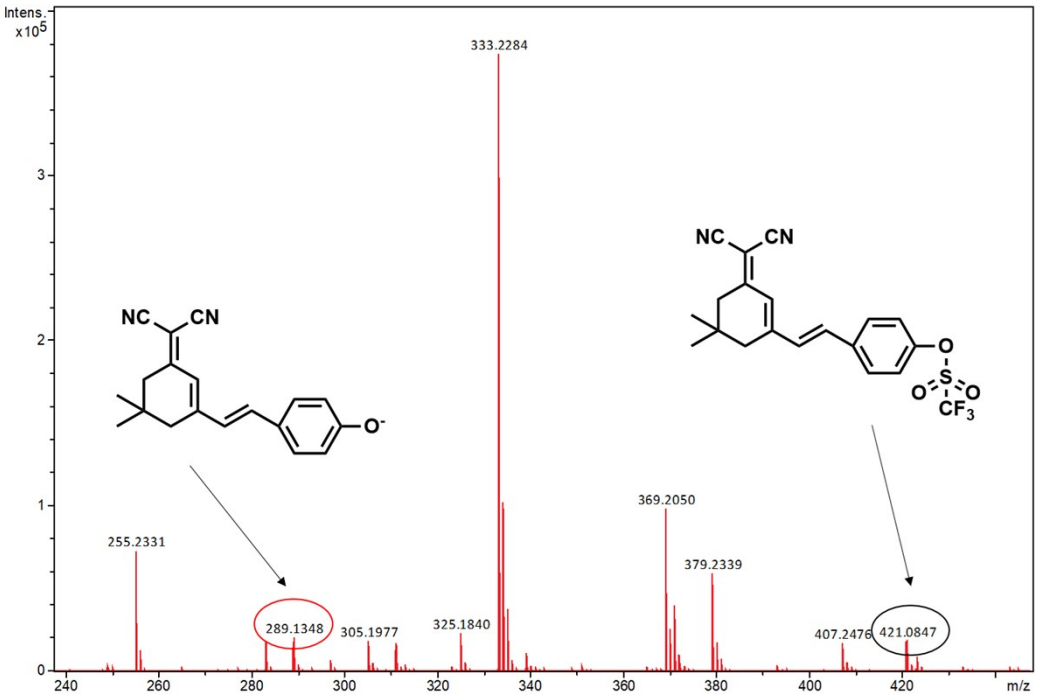



**Fig. S2** Mass spectra of the probe **ERO** reacted with $O_2^{\bullet-}$.

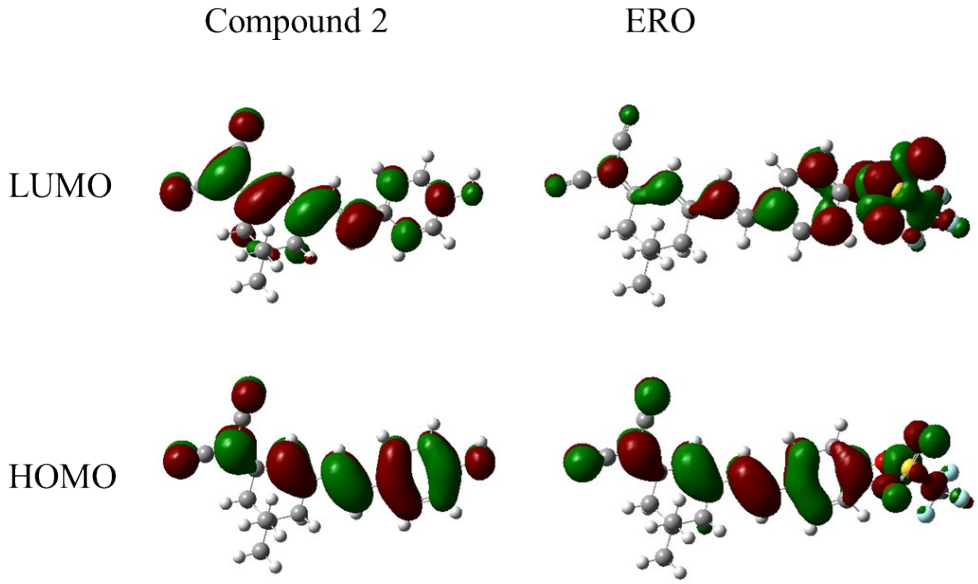



**Fig. S3** The HOMO/LUMO frontier molecular orbital calculated by TDDFT showing the S1→S0 transition of compound 2 and the probe **ERO**.

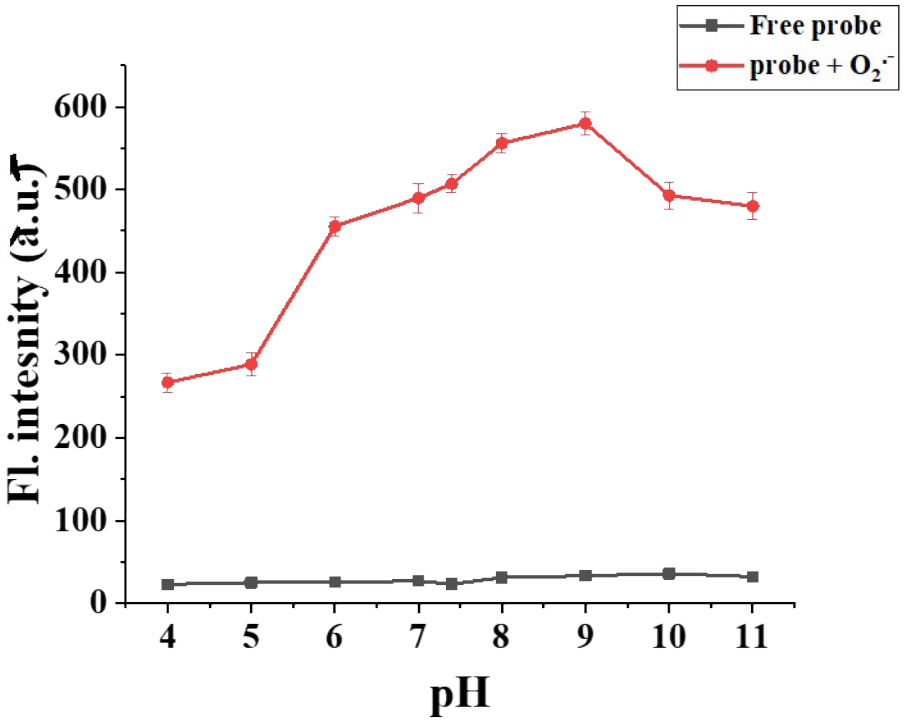



**Fig. S4** pH dependent response of the probe **ERO** to $O_2^{\cdot -}$.

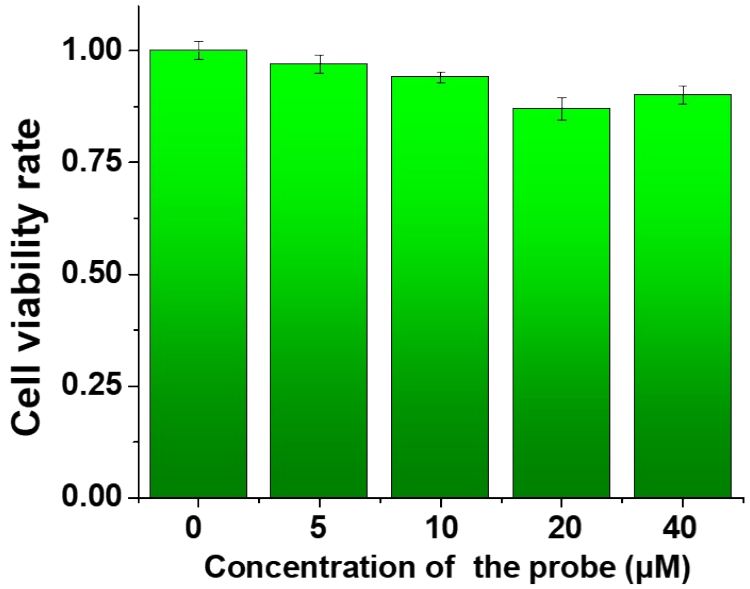



**Fig. S5** Cytotoxicity of the probe **ERO** by MTT assay. (n = 3)

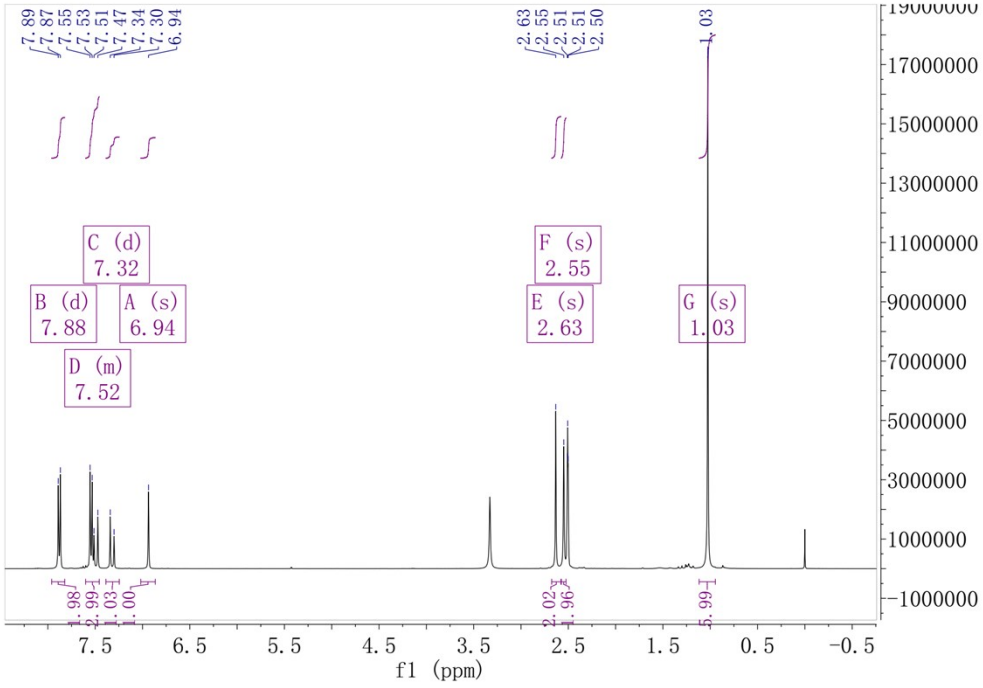



**Fig. S6.** $^{1}$H NMR of the probe **ERO**

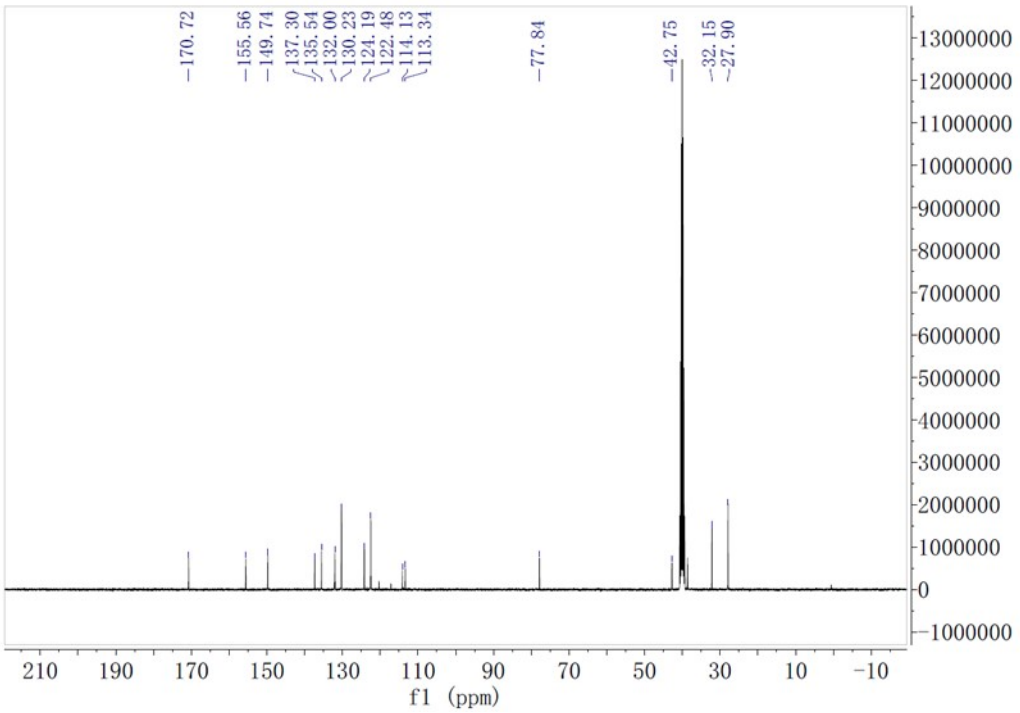



**Fig. S7.** $^{13}$C NMR of the probe **ERO**

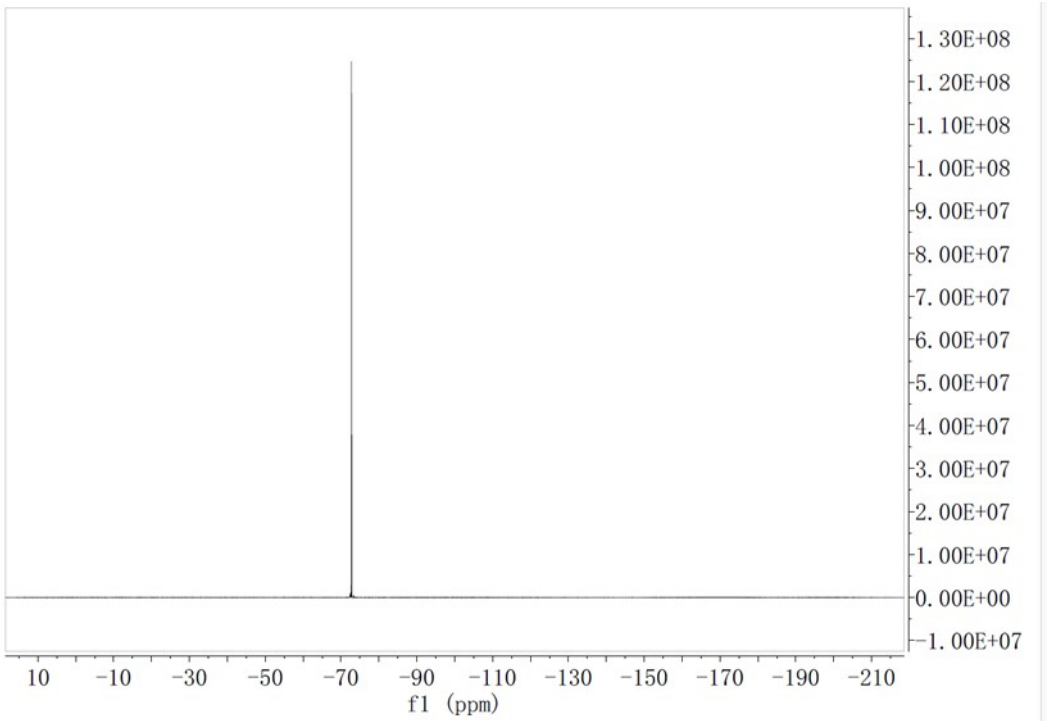



**Fig. S8.** $^{19}$F NMR of the probe **ERO**

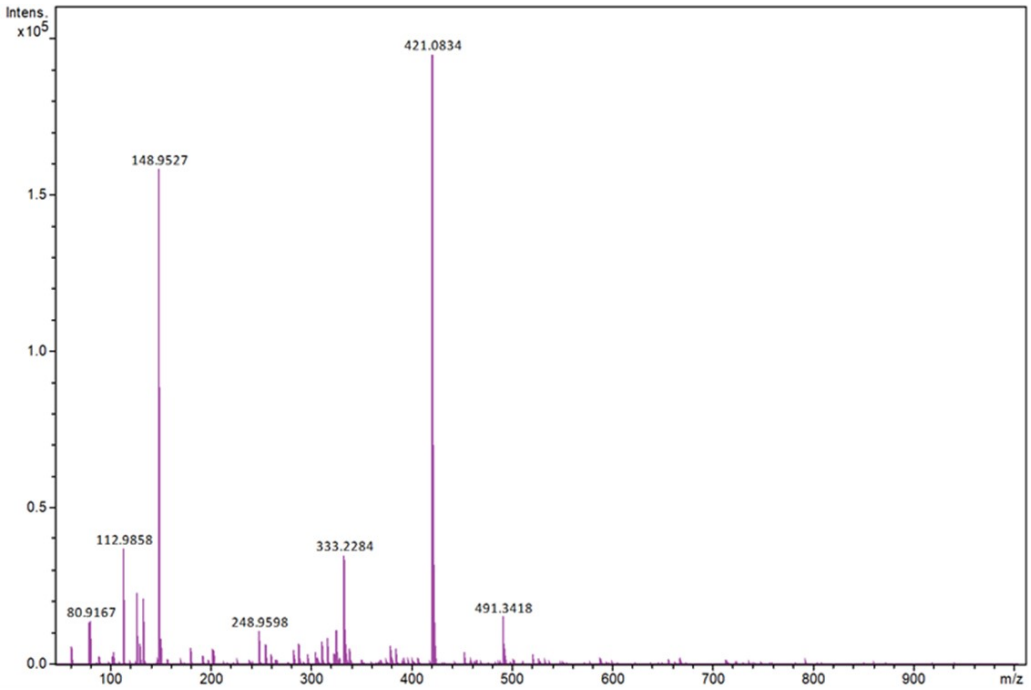



**Fig. S9.** HRMS of the probe **ERO**

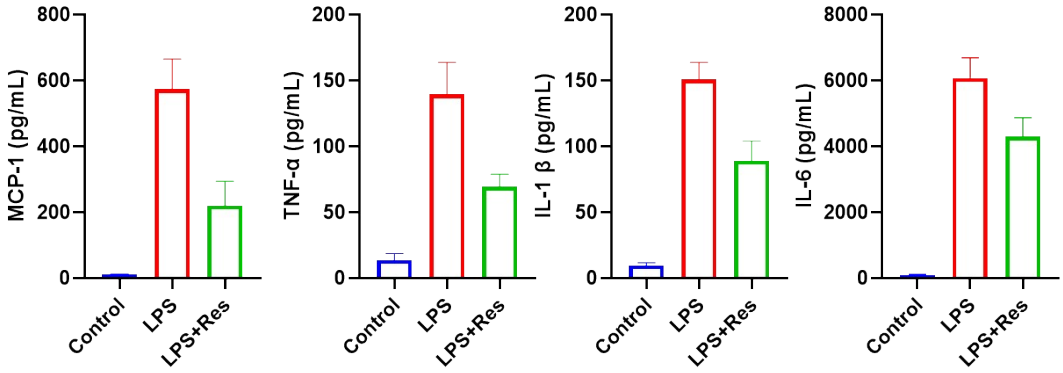



**Fig. S10.** Serum levels of MCP-1, TNF-α, IL-1β, and IL-6.

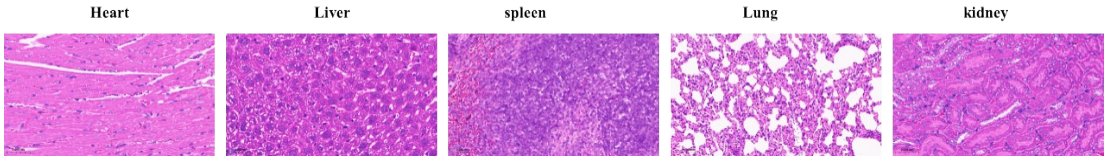



**Fig. S11.** HE staining of each organ.

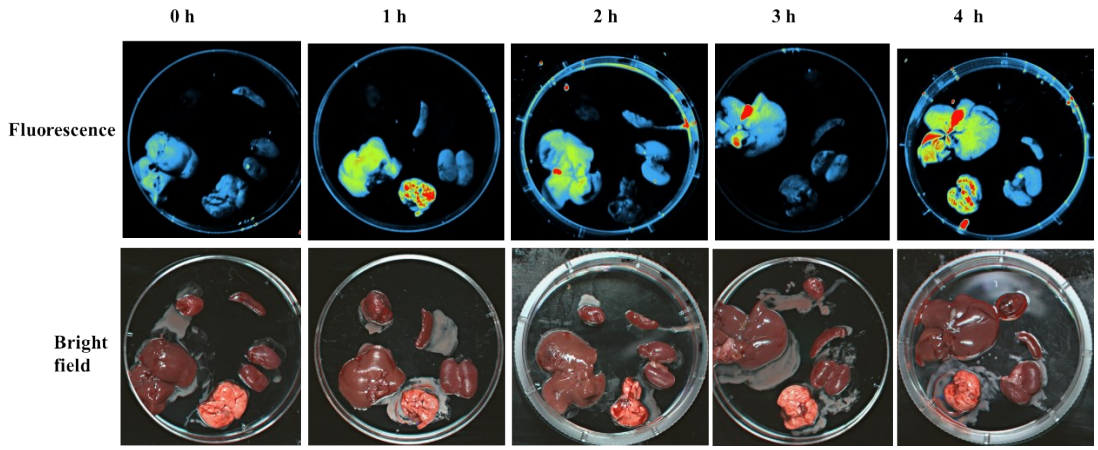



**Fig. S12.** Image of mice organ after injection of probe ERO (10 μM) in different time.


1. W. Shu, S. P. Zang, C. Wang, M. X. Gao, J. Jing and X. L. Zhang, *Anal Chem*, 2020, **92**, 9982-9988.
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3. K. Yang, Y. Liu, M. Deng, P. P. Wang, D. Cheng, S. J. Li and L. W. He, *Anal Chim Acta*, 2024, **1286**.
4. B. L. Dong, J. X. Wang, M. Wang, Q. X. Chen, X. Q. Kong, J. Chang, X. B. Li, T. Yue and Y. Wang, *Talanta*, 2024, **268**.
5. L. J. Gui, J. Yan, J. Y. Zhao, S. Y. Wang, Y. Y. Ji, J. Liu, J. S. Wu, K. Yuan, H. Liu, D. W. Deng and Z. W. Yuan, *Biosens Bioelectron*, 2023, **240**.
6. W. Zhang, W. J. Fan, X. Wang, P. Li, W. Zhang, H. Wang and B. Tang, *Anal Chem*, 2023, **95**, 8367-8375.
7. Y. M. Tian, G. B. Liu, W. N. Wu, X. L. Zhao, X. F. Han, Y. C. Fan, Y. Wang and Z. H. Xu, *Spectrochim Acta A*, 2023, **296**.
8. H. Kang, W. Shu, J. Yu, Y. P. Wang, X. L. Zhang, R. B. Zhang, J. Jing and X. L. Zhang, *Anal Chem*, 2023, **95**, 6295-6302.
9. Z. X. Zhan, L. Chai, H. H. Yang, Y. C. Dai, Z. L. Wei, D. N. Wang and Y. Lv, *Anal Chem*, 2023, **95**, 5585-5593.
10. J. L. Yan, L. Zhang, W. N. Wu, Y. Wang and Z. H. Xu, *Bioorg Chem*, 2023, **131**.
11. H. Wei, Y. Wang, Q. X. Chen, Y. R. Sun, T. Yue and B. L. Dong, *J Fluoresc*, 2023, **33**, 509-515.
12. C. Jiang, X. R. Xu and C. Yao, *Spectrochim Acta A*, 2022, **273**.
13. Y. J. Tang, S. He, X. F. Guo and H. Wang, *Analyst*, 2021, **146**, 7740-7747.
14. H. Huang, B. Y. Chen, L. F. Li, Y. Wang, Z. F. Shen, Y. G. Wang and X. Li, *Talanta*, 2022, **237**.
15. W. Y. Zhang, W. H. Song and W. Y. Lin, *J Mater Chem B*, 2021, **9**, 7381-7385.
16. Y. R. Lu, R. F. Wang, Y. R. Sun, M. G. Tian and B. L. Dong, *Talanta*, 2021, **225**.
17. S. P. Zang, X. X. Kong, J. Cui, S. Su, W. Shu, J. Jing and X. L. Zhang, *J Mater Chem B*, 2020, **8**, 2660-2665.
18. Q. N. Xia, X. Y. Wang, Y. N. Liu, Z. F. Shen, Z. G. Ge, H. Huang, X. Li and Y. G. Wang, *Spectrochim Acta A*, 2020, **229**.
19. J. T. Hou, H. S. Kim, C. Duan, M. S. Ji, S. Wang, L. T. Zeng, W. X. Ren and J. S. Kim, *Chem Commun*, 2019, **55**, 2533-2536.