Benzoxazole Functionalized Fluorescent Probe for Selective Fe^{3+} Detection and Intracellular Imaging in Living Cells

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CONTENTS

1. Material and methods

1.1. General methods

Elemental analyses were measured on a Vario EL Ⅲ analyzer. IR spectra were recorded on a Bruker Tensor 27 spectrometer. NMR spectra were performed on a Varian Inova-400MHz spectrometer. Mass spectra were performed with Bruker micrOTOF-Q II ESI-Q-TOF LC/MS/MS Spectroscopy. The absorbance spectra were performed on a Shimadzu UV-1700 spectrophotometer. Fluorescent spectra measurements measured were on a Hitachi F-4500 fluorescence spectrophotometer. Bioimaging of the sensors were performed on an Olympus FV1000 confocal microscopn and the excitation wavelength was set as 543 nm. The cell viability was determined at 490 nm absorbance using an ELx800 Absorbance Reader (BioTek Instruments, Inc.). The chemicals and reagents were all obtained from *Sigma-Aldrich Co. LLC.* All experiments were performed in compliance with the relevant laws and institutional guidelines of China and have been approved by *Xi'an University of Science and Technology (Xi'an, China)* and *Northwest University (Xi'an, China).* No experimentation with human subjects were included in this work. The mouse fibroblast L929 cells and Human Osteosarcoma MG-63 cells were purchased from the *Ministry of Education Key Laboratory of Synthetic and Natural Functional Molecule Chemistry, College of Chemistry & Materials Science, Northwest University.*

*1.2. Synthesis of the probe **Z1***

To a stirred solution of rhodamine B hydrochloride (0.4780 g, 0.001 mol) and 2-dichloroethane (5 mL), 1 mL of phosphorus oxychloride was added. The solution was refluxed for 8 h and concentrated by evaporation. The obtained crude acid chloride was dissolved in acetonitrile (2 mL). Then, a solution of 2-aminobenzoxazole (0.1340 g, 0.001 mol) and triethylamine (1 mL) in acetonitrile (50 mL) was added dropwise in 30 min. After refluxing for 4 h, the solvent was removed under reduced pressure to give a violet oil. Water was then added to the mixture, and the aqueous phase was extracted with dichloromethane (5 mL × 3). The organic layer was washed with water, dried over anhydrous $MgSO_4$, and filtered. Purification of the products

was by column chromatography on silica gel (Eluant: CH_2Cl_2:CH_3OH = 50:1, v:v) to get the probe as white crystal (0.3361 g, yield: 60.2%), Mp.: 236-238 °C. Anal. calcd. for $C_{35}H_{35}N_4O_3$: H, 6.30; C, 75.11; N, 10.01. Found: H, 6.28; C, 75.26; N, 10.00. IR (KBr, υ/cm^{-1}): 3082.1, 2970.2, 2929.7, 2895.0, 1703.1, 1633.6, 1614.4, 1587.3, 1550.7, 1515.9, 1467.8, 1446.4, 1429.3, 1338.5, 1286.5, 1269.1, 1222.9, 1168.8, 1122.5, 852.5, 811.9, 763.7, 703.9. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, *J* = 7.2 Hz, 1H), 7.54 (m, 3H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.16 (t, *J* = 7.6 Hz, 2H), 7.10 (t, *J* = 7.4 Hz, 1H), 6.53 (d, *J* = 8.8 Hz, 2H), 6.43 (s, 2H), 6.19 (dd, *J* = 6.4 Hz, *J* = 9.2 Hz, 2H), 3.30 (q, *J* = 10.6 Hz, 8H), 1.14 (t, *J* = 7.0 Hz, 12H).^{13}C NMR (101 MHz, $CDCl_3$) δ 168.81, 154.97, 152.97, 152.16, 148.96, 133.27, 129.40, 128.45, 128.33, 125.49, 125.15, 124.02, 123.44, 120.81, 119.88, 108.32, 105.64, 97.87, 69.14, 44.32, 12.59. Calc. for $[M + H]^+$ = 559.2709, found MS (ESI) m/z = 559.2749.

1.3. General procedure

Stock solutions of the probe (500 μmol·L^{-1}), metal ions (500 μmol·L^{-1}), other organotin compounds (500 μmol·L^{-1}, including butyltin trichloride, dibutyltin dichloride, tributyl tin chloride and tetrabutyltin) were all prepared in EtOH-H_2O (5:5, v/v) solution. The solutions of metal ions were prepared with hydrochloride salts of Li^+, Na^+, K^+, Ca^{2+}, Cd^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}, Cu^{2+}, Sn^{2+}, Fe^{2+}, Hg^{2+}, Fe^{3+}, Al^{3+}, and Cr^{3+} and the nitrate salt of Ag^+. Double distilled water was used throughout the experiment.

To a 25 mL volumetric tube, 5.0 mL 0.2 mol·L^{-1} PBS, 1.00 mL of 500 μmol·L^{-1} probe and different concentration of Fe^{3+} were added. The mixture was diluted to the mark with EtOH-H_2O (5:5, v/v) mixed solution. The Absorptions were recorded at 542 nm and the fluorescence intensities were recorded at 571 nm. The excitation and emission wavelength bandpasses were both set as 5.0 nm and the excitation wavelength was set at 530 nm.

1.4. Fluorescent imaging in living cells

Fluorescent imaging in living mouse fibroblast cells L929 cells and Human Osteosarcoma MG-63 cells were performed in the similar procedures. The cells were

cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, at 37 ºC in the humidified atmosphere with 5% CO_2 and 95% air. The cells were then cultured for 2 h until they plated on glass-bottomed dishes. The growth medium was then removed and the cells were washed with DMEM without FBS and incubated with 20 μmol·L^{-1} of the probe for 30 min at 37 ºC, washed three times with PBS and imaged. Then the cells were supplemented with 20 μmol·L^{-1} Fe^{3+} in the growth medium for 30 min at 37 °C and imaged.

1.5. In vitro cell viability assay

MCF-7 (1×10^6 to 5×10^6 cells) were seeded in 96-well plates and incubated in 150 μL of medium (RPMI Medium Modified, HyClone with 10% gibco FBS (Art. No. 10270) and 1% hydrostreptomycin (P/S), HyClone) added into each plate at 37 °C in the atmosphere of 5% CO_2 to allow the cells to attach. After incubated for 24 h, the medium was sucked out and 100 μL of fresh medium was added. A stock solution of 5 mM of the probe was prepared in DMSO (Sigma, Biological reagent level) and stored at -20 °C. The stock solution was diluted to the appropriate concentrations with cultured medium. The final concentration of DMSO was 2% (v/v). The same amount of DMSO was used as the vehicle control throughout this study. The solution of the probe in the cultured medium were serial diluted with final concentration of 100, 50, 25, 12.5, 6.25, 3.125 μmol/L and added to the microtiter plate and incubated for 24 h in the atmosphere of 5% CO_2 at 37 °C and then was sucked out the medium. MTT (3-(4,5-dimethy-1-thiazol-2-yl)-2,5-diphenyltetrazolium bromide)with 1 × PBS (HEART) was filtered with water-based 0.22 μm filter membrane to get the MTT solution in the concentration of 5 mg/mL. This solution was added to the previous medium in 10% proportion to form the MTT medium. 150 μL of fresh MTT medium was then added into each plate. After placed for 4 h in the atmosphere of 5% CO_2 at 37 °C, the MTT medium was sucked out and 150 μL of DMSO was added and placed on the shaking table for 10 min and then determined at 490 nm absorbance using an ELx800 Absorbance Reader. Experiments were performed in triplicate.

2. The solvent investigation of the probe.

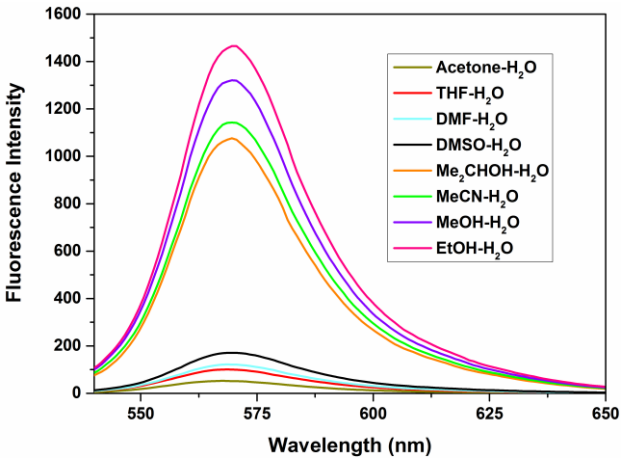

Fig. S1. Fluorescence intensity of the probe (20 μM) in presence of 1.0 equiv. of Fe^{3+} in different solutions with PBS buffer (0.2 M, pH 7.4), λ_{ex} = 530 nm.

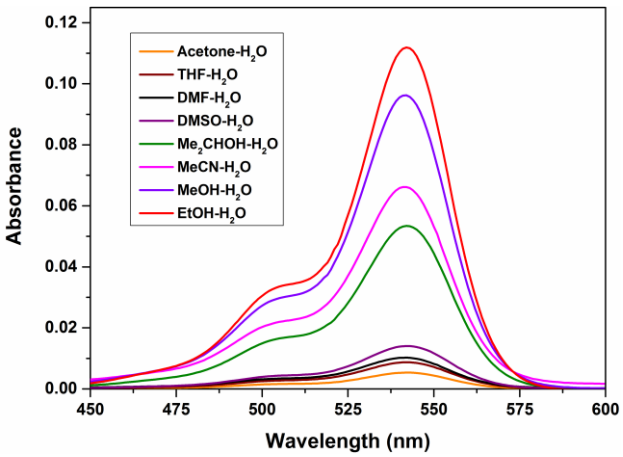

Fig. S2. Absorbance of the probe (20 μM) in the presence of 1.0 equiv. of Fe^{3+} in different solutions with PBS buffer (0.2 M, pH 7.4).

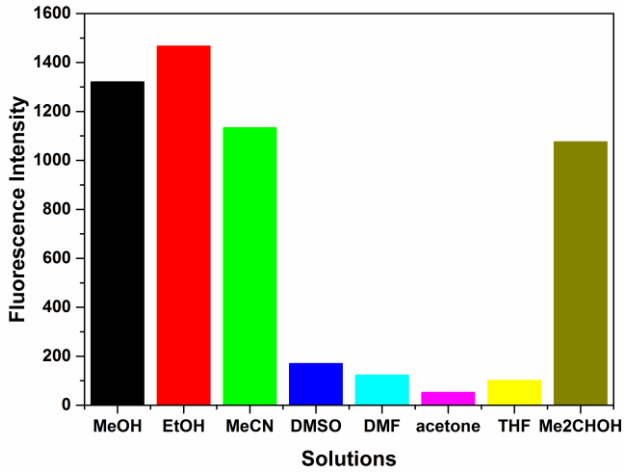

Fig. S3. Fluorescence intensity of the probe (20 μM) in presence of 1.0 equiv. of Fe^{3+} in different solutions with PBS buffer (0.2 M, pH 7.4), λ_{ex} = 530 nm.

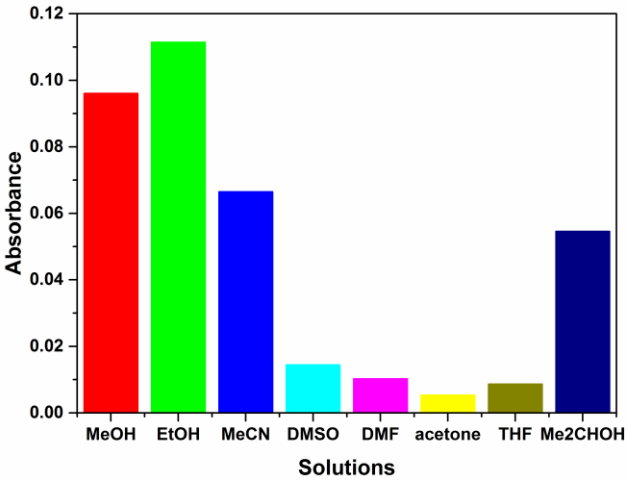

Fig. S4. Absorbance of the probe (20 μM) in the presence of 1.0 equiv. of Fe^{3+} in different solutions with PBS buffer (0.2 M, pH 7.4).

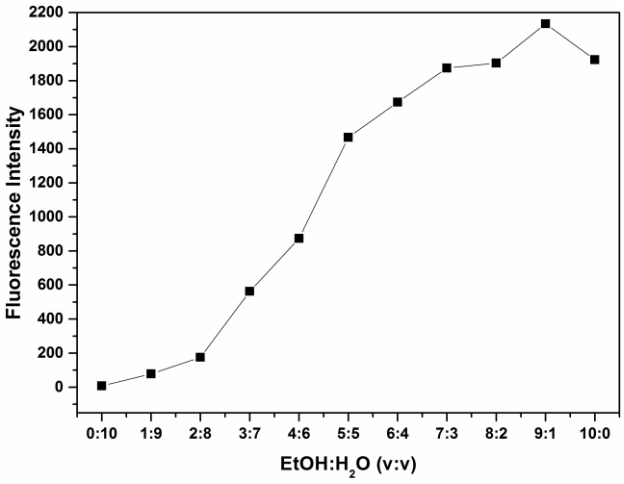

Fig. S5. Fluorescence intensity of the probe (20 μM) in presence of 1.0 equiv. of Fe^{3+} in different EtOH-H_2O solutions with PBS buffer (0.2 M, pH 7.4), λ_{ex} = 530 nm.

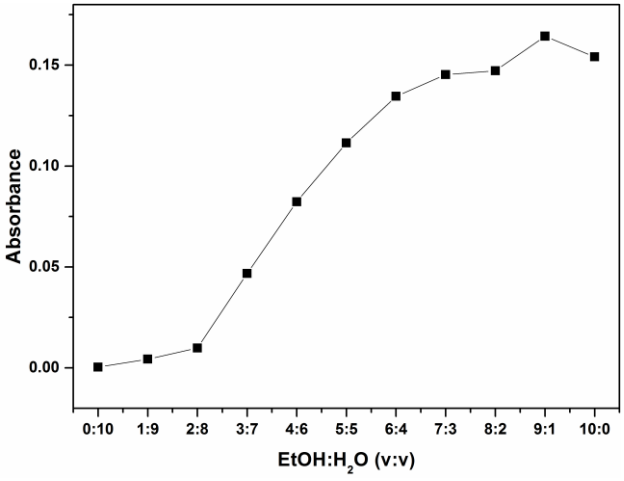

Fig. S6. Absorbance of the probe (20 μM) in the presence of 1.0 equiv. of Fe^{3+} in different EtOH-H_2O solutions with PBS buffer (0.2 M, pH 7.4).

3. Selective experiments of the probe.

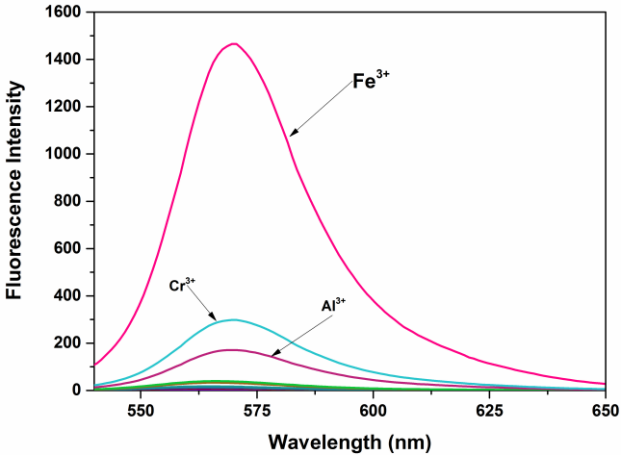

Fig. S7. Fluorescence spectra of the probe (20 μM) in EtOH-H_2O (5:5, v/v, PBS, pH 7.4) upon addition of various metal ions (20 μM), λ_{ex} = 530 nm.

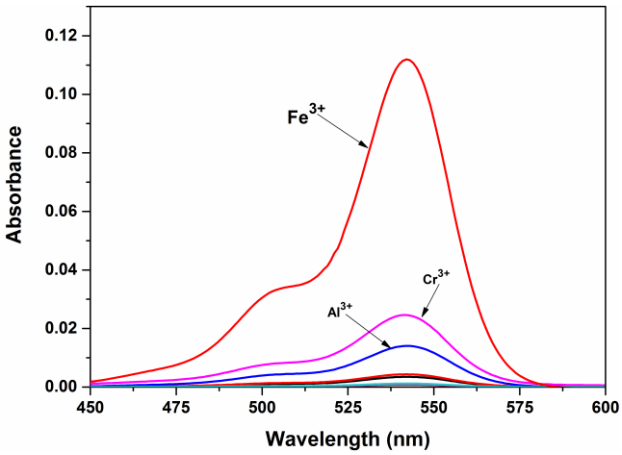

Fig. S8. Absorption spectra of the probe (20 μM) in EtOH-H_2O (5:5, v/v, PBS, pH 7.4) upon addition of various metal ions (20 μM).

4. Time-dependent responses of the probe.

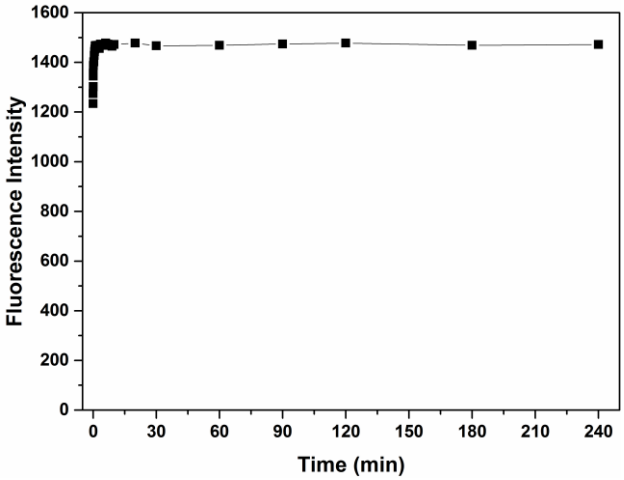

Fig. S9. Time-dependent fluorescent responses of the probe with Fe^{3+} in EtOH-H_2O (5:5, v/v, PBS, pH 7.4), λ_{ex} = 530 nm.

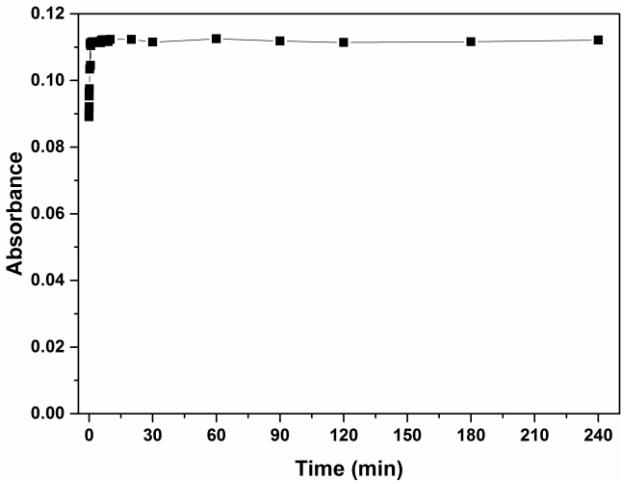

Fig. S10. Time-dependent absorption responses of the probe with Fe^{3+} in EtOH-H_2O (5:5, v/v, PBS, pH 7.4).

5. Linear relationship of the probe (20 μM) with Fe^{3+}.

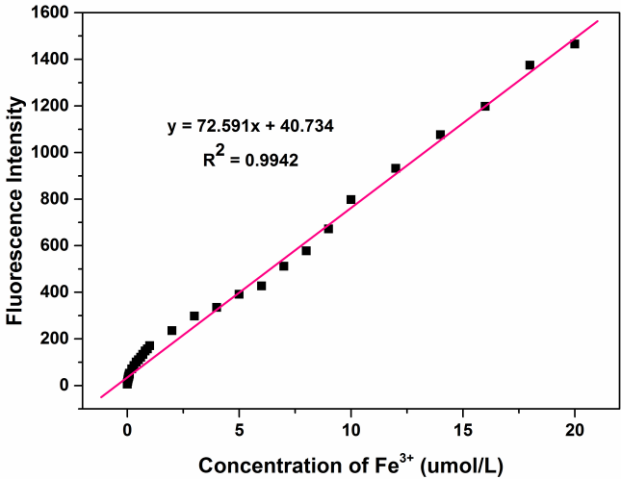

Fig. S11. Linear relationship of the fluorescence intensity of the probe (20 μM) in EtOH-H_2O (5:5, v/v, PBS, pH 7.4) as a function of Fe^{3+} concentration, λ_{ex} = 530 nm.

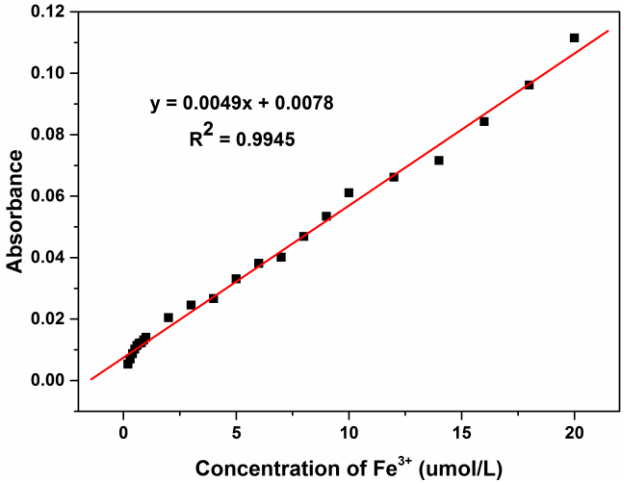

Fig. S12. Linear relationship of the absorbance of the probe (20 μM) in EtOH-H_2O (5:5, v/v, PBS, pH 7.4) as a function of Fe^{3+} concentration.

6. Job's plot of the probe with Fe^{3+}.

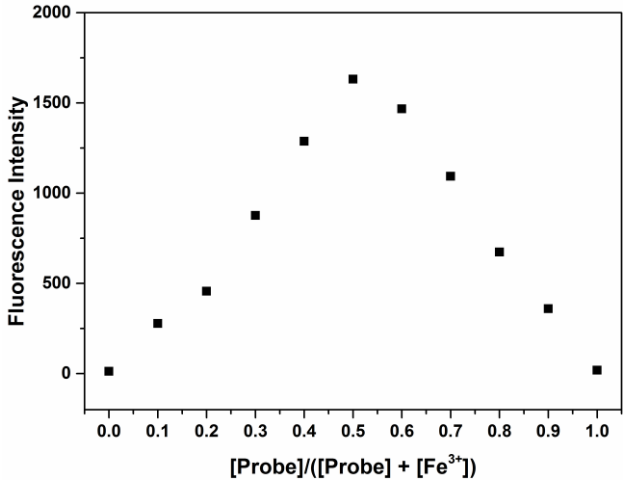

Fig. S13. Fluorescence spectrum for the Job's plot of the probe with Fe^{3+} in EtOH-H_2O (5:5, v/v, PBS, pH 7.4), λ_{ex} = 530 nm.

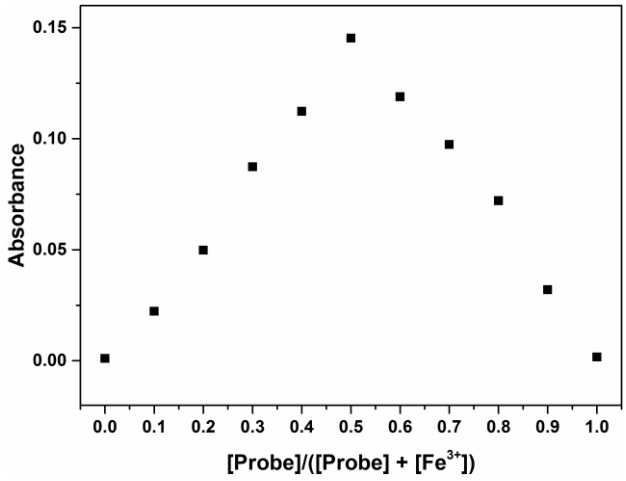

Fig. S14. Absorption spectrum for the Job's plot of the probe with Fe^{3+} in EtOH-H_2O (5:5, v/v, PBS, pH 7.4).

7. Reversible investigation of the probe for Fe^{3+}.

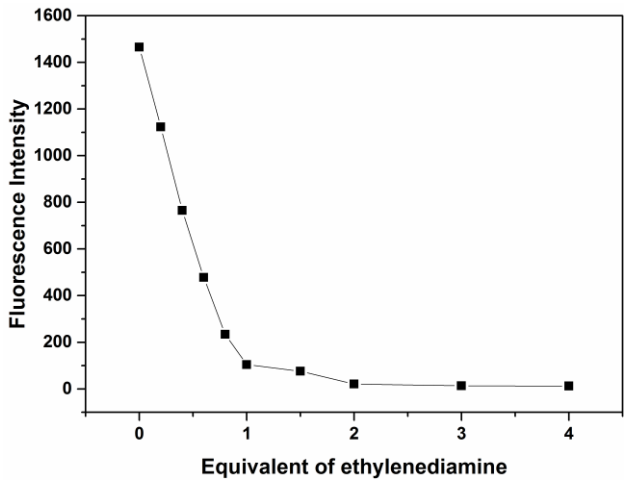

Fig. S15. Fluorescence intensity changes of the probe (20 μM) upon the addition of each equiv. of ethylenediamine with the presence of Fe^{3+} (20 μM) in EtOH-H_2O (5:5, v/v, PBS, pH 7.4), λ_{ex} = 530 nm.

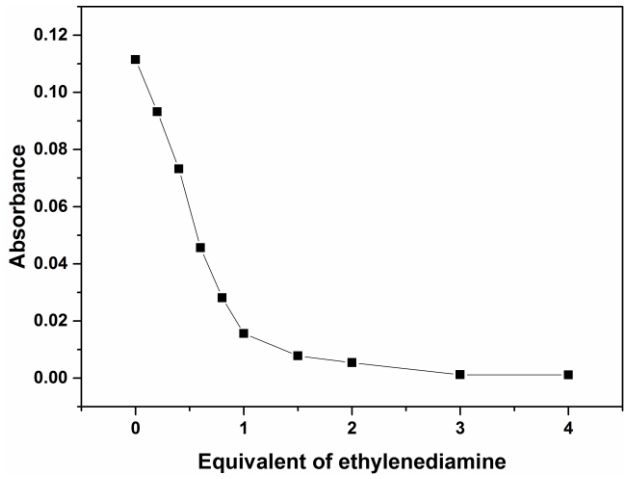

Fig. S16. Absorbance changes of the probe (20 μM) upon the addition of each equiv. of ethylenediamine with the presence of Fe^{3+} (20 μM) in EtOH-H_2O (5:5, v/v, PBS, pH 7.4).

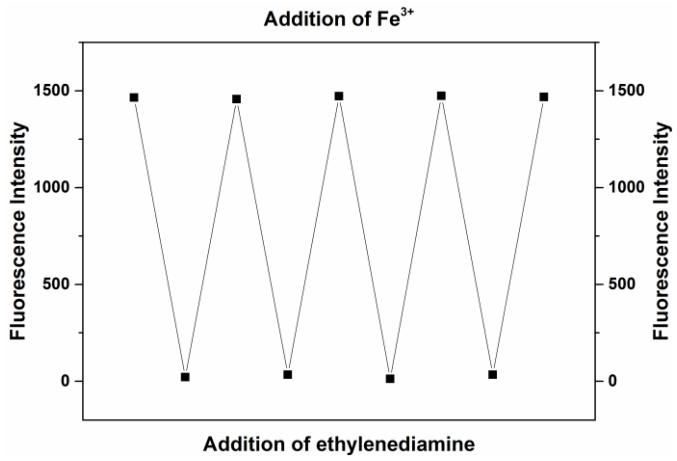

Fig. S17. Fluorescence intensity changes of the probe (20 μM) upon the addition of ethylenediamine and Fe^{3+} in EtOH-H_2O (5:5, v/v, PBS, pH 7.4), λ_{ex} = 530 nm.

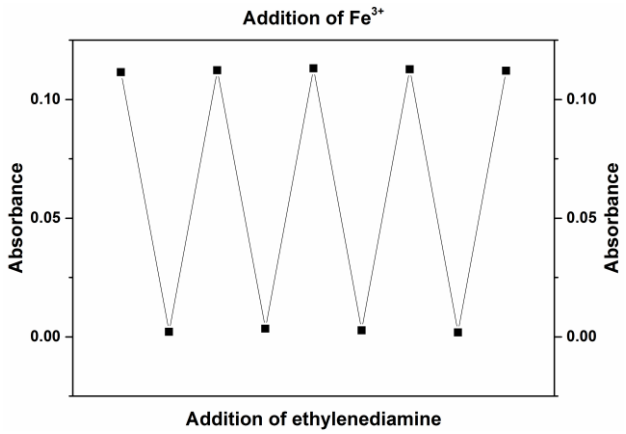

Fig. S18. Absorbance changes of the probe (20 μM) upon the addition of each equiv. of ethylenediamine and Fe^{3+} in EtOH-H_2O (5:5, v/v, PBS, pH 7.4).

8. Equations used for the calculation of association constant

The association constant was determined according to the references [1]. The probes bind with Fe^{3+} to form the complex in the 1:1 binding mode, the equilibrium can be described as follows:

$$R + Fe^{3+} \overset{K}{\rightleftharpoons} [RFe]^{3+} \quad (8\text{-}1)$$

Here, R and $[RFe]^{3+}$ denote the probe and the complex, respectively, and K denotes the association constant. The relative absorbance R is defined as the ratio of free R. $[R]_f$ is defined as the total amount of R. $[R]_t$ is defined as the absorbance in the EtOH-H_2O (5:5, v/v). It can be experimentally determined by measuring the absorbance values in the presence of different concentrations of Fe^{3+}.

$$\alpha = \frac{[R]_f}{[R]_t} = \frac{A_t - A}{A_t - A_0} \quad (8\text{-}2)$$

Here, A_0 and A_t are the limiting absorbance values for α=1 (in the absence of Fe^{3+}) and α=0 (the probe is completely complexed with Fe^{3+}), respectively. The relationship between the probe and the Sn^{4+} concentration can be represented as follows:

$$\frac{\alpha}{1 - \alpha} = \frac{1}{K[Fe^{3+}]} \quad (8\text{-}3)$$

It is apparent from Equation 8-3 that the relative absorbance α has a distinct functional relationship with the concentration of Fe^{3+} and the association constant K, which provides the basis for the detection of the K value. The experimental data were fitted to Equation 8-3 by adjusting the K value.

9. Equations used for the calculation of fluorescence quantum yield

Fluorescence quantum yields (Φ) were determined using optically matching solutions of rhodamine B (Φ_f = 0.97 in ethanol) as standards at an excitation wavelength of 530 nm and the quantum yields were calculated using Equation 9-1 according to the references [2]. Where Φ_u and Φ_s are the fluorescence quantum yields of the sample and standard, I_u and I_s are the integrated emission intensities of the corrected spectra for the sample and standard, A_u and A_s are the absorbance of the sample and standard at the excitation wavelength (530 nm in all cases), and η_u and η_s are the indices of refraction of the sample and standard solutions, respectively.

$$\Phi u = \Phi s \cdot \frac{Iu}{Is} \cdot \frac{As}{Au} \cdot \left(\frac{\eta u}{\eta s}\right)^2 \quad (9\text{-}1)$$

10. References

[1] (a) J. Bourson, J. Pouget and B.Valeur, *J. Phy. Chem.*, 1993, **97**, 4552-4557; (b) Y. Zhao, X. B. Zhang, Z. X. Han, L. Qiao, C. Y. Li, L. X. Jian, G. L. Shen and R. Q. Yu, *Anal. Chem.*, 2009, **81**, 7022-7030.

[2] (a) D. Y. Wu, W. Huang, C. Y. Duan, Z. H. Lin and Q. J. Meng, *Inorg. Chem.*, 2007, **46**, 1538-1540; (b) W. Huang, C. Song, C. He, G. Lv, X. Hu, X. Zhu and C. Duan, *Inorg. Chem.*, 2009, **48**, 5061-5072.

11. IR, NMR and MS spectra.

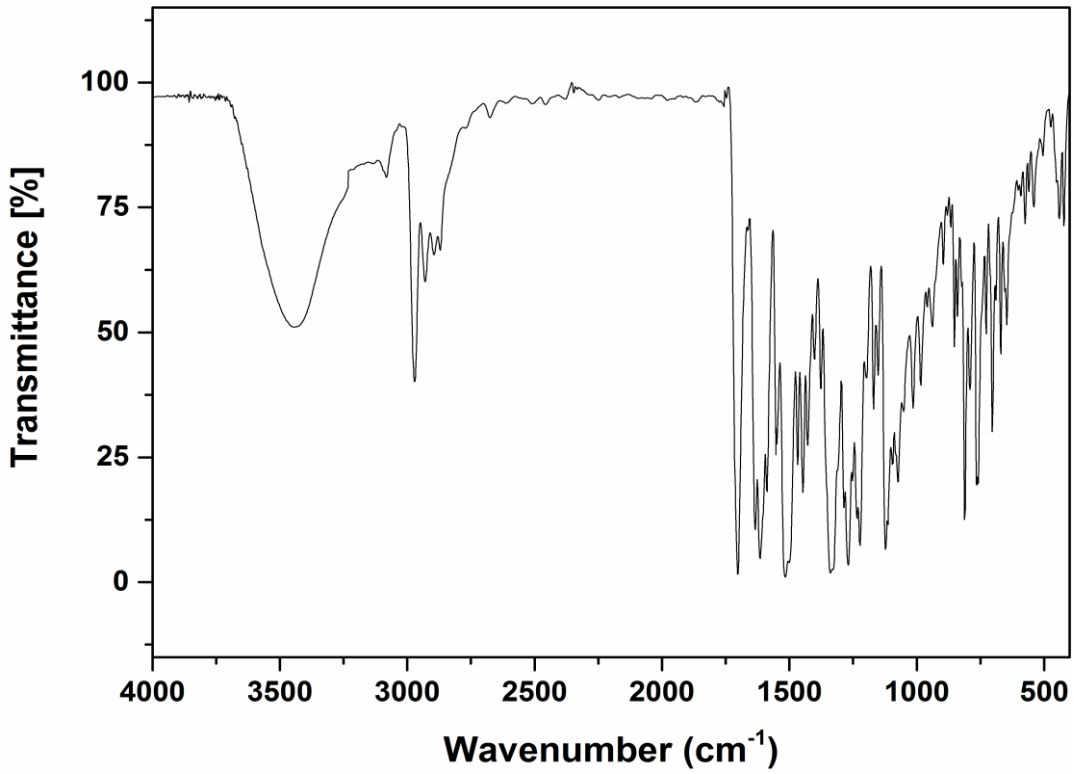

Fig. S19. IR spectrum of the probe in KBr disks.

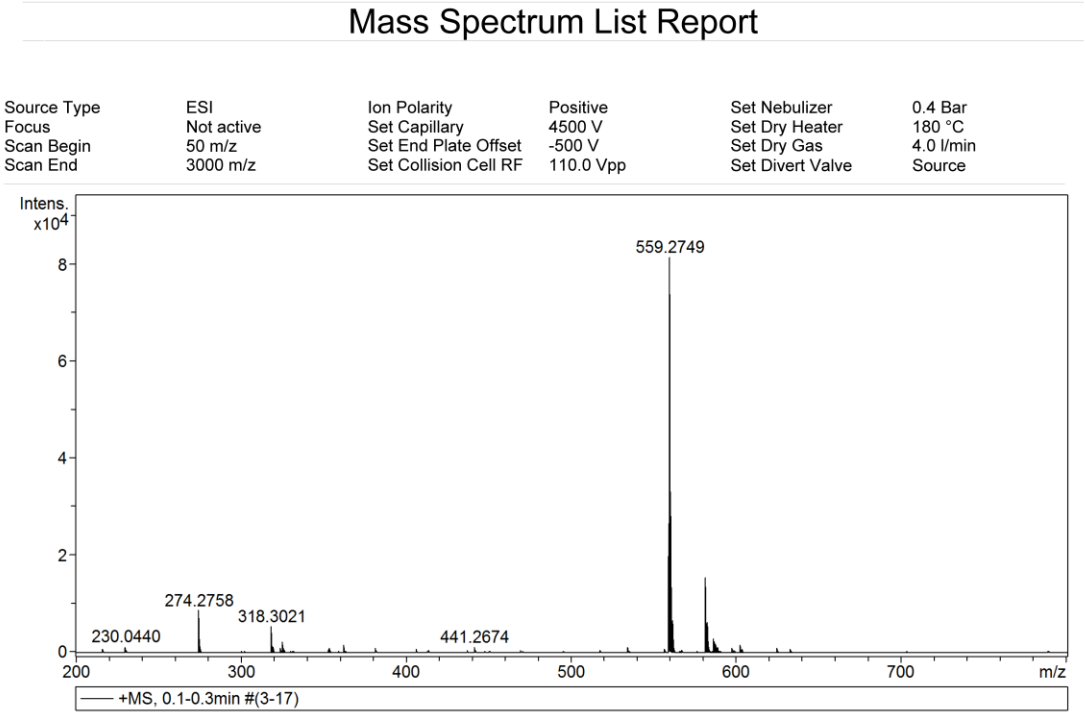

Fig. S20. Mass spectrum of the probe.

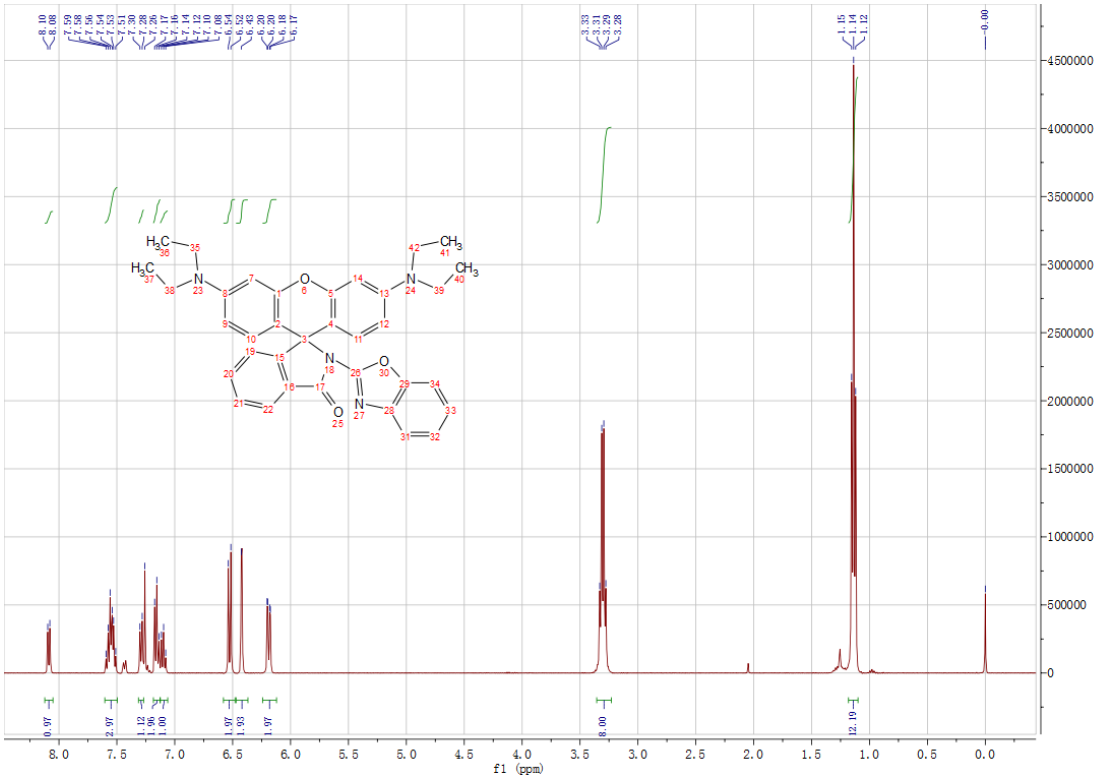

Fig. S21. ^{1}H NMR spectrum of the probe in $CDCl_3$.

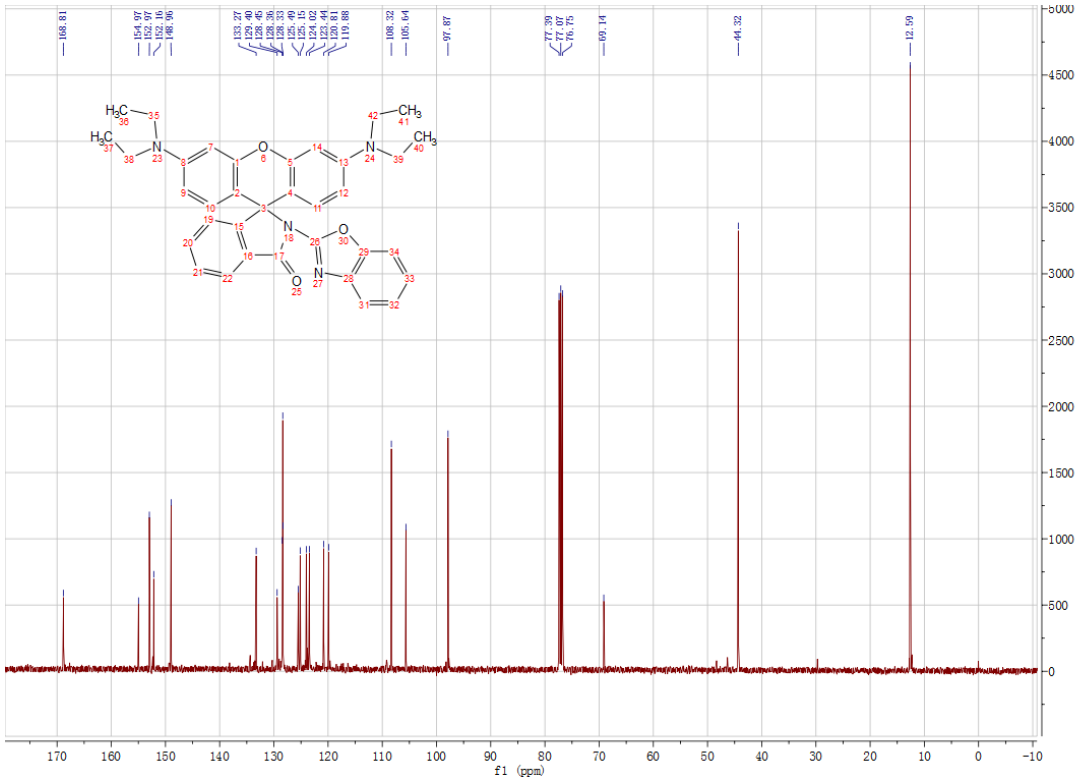

Fig. S22. ^{13}C NMR spectrum of the probe in $CDCl_3$.

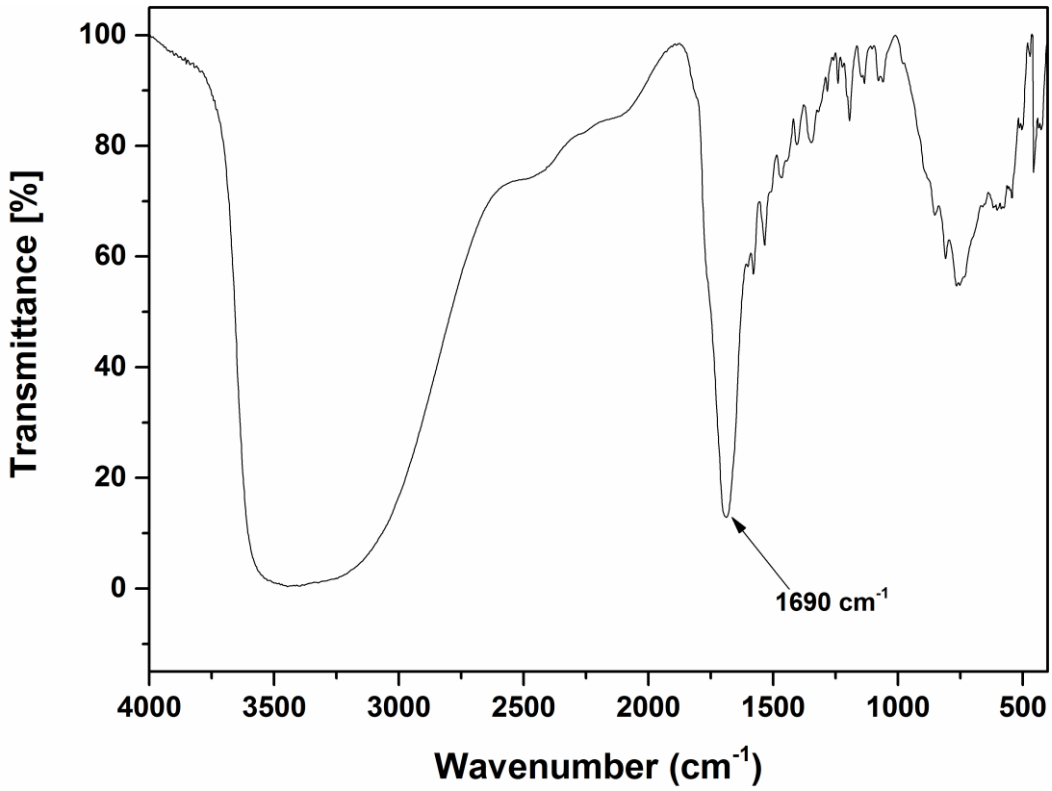

Fig. S23. IR spectrum of the probe with $FeCl_3$ in KBr disks.

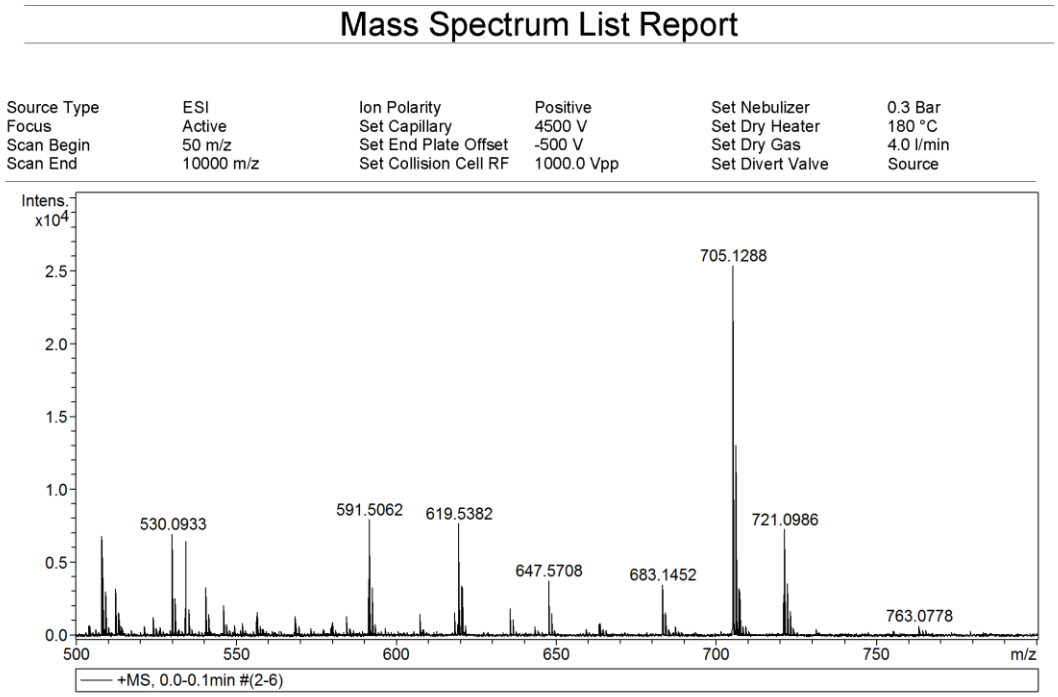

Fig. S24. Mass spectrum of the probe with $FeCl_3$.

12. MTT assay results of the probe.

Table S1. MTT assay results, calculated inhibition ratio and IC_{50} value of the probe for MCF-7 cell.

[**Z1**]/μM	1	2	3	Average	Inhibition ratio	IC_{50}/μM
3.125	0.549	0.594	0.497	0.5467	-0.1758	>100
6.25	0.467	0.578	0.574	0.5397	-0.1608	
12.5	0.437	0.393	0.357	0.3957	0.1490	
25	0.346	0.33	0.335	0.3370	0.2751	
50	0.331	0.337	0.294	0.3207	0.3103	
100	0.279	0.266	0.285	0.2767	0.4049	

13. Comparison of recent reported Fe^{3+} probes.

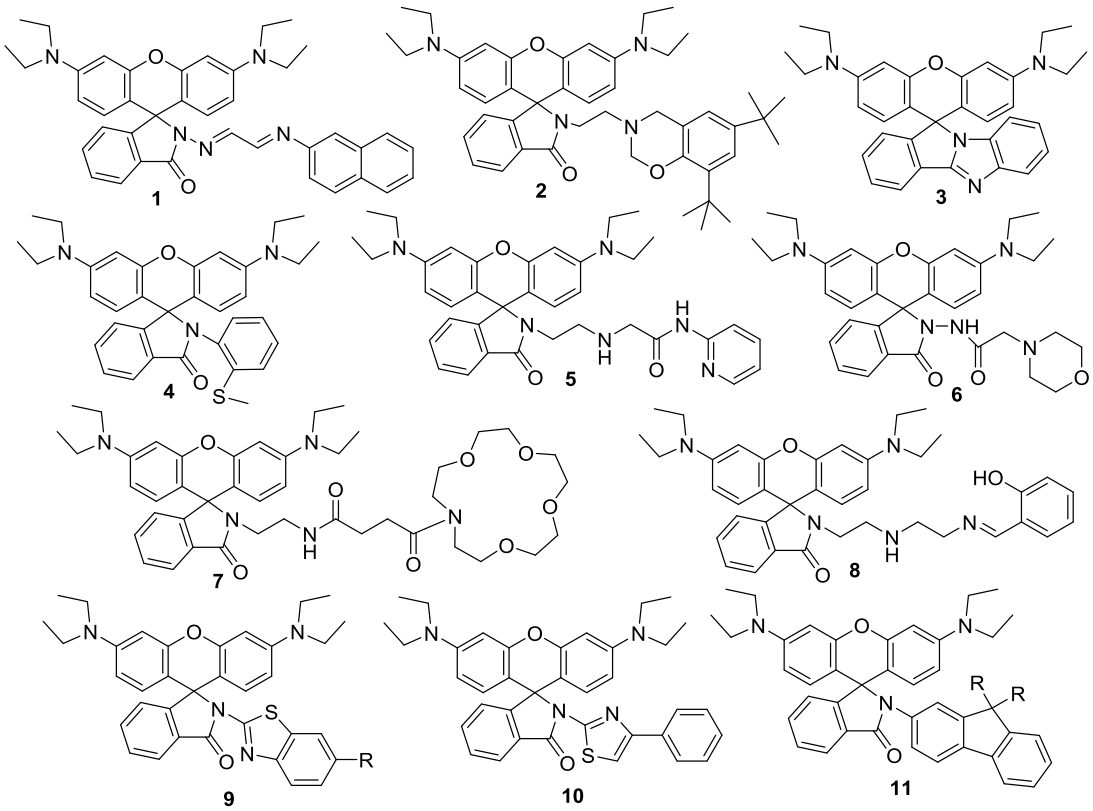

Fig. S25. Structures of recent reported Fe^{3+} probes.

Table S2. Comparative results of selected rhodamine probes for recognizing Fe^{3+}

Probe	Solution (v : v)	Selectivity	Limit (μM)	Quantum yield	Formation constant (M^{-1})	Biological application
1	H_2O–MeCN (7 : 3)	Fe^{3+} mainly Cr^{3+}, Al^{3+} weakly	0.27	0.24	0.25×10^4	Not Done
2	H_2O–MeCN (3 : 7)	Fe^{3+} mainly	0.11	0.57	1.5×10^4	HepG2 cell imaging
3	Tris-HCl buffer	Fe^{3+} mainly Cu^{2+} slightly	2.74	Not determined	Not determined	MGC-803 cell imaging
4	H_2O–MeOH (1 : 1)	Fe^{3+} mainly	0.36	0.36	1.15×10^5	HepG2 cell imaging
5	H_2O–MeOH (1 : 1)	Fe^{3+} mainly Hg^{2+}, Cr^{3+} weakly	0.057	Not determined	0.67×10^4	A549 cell imaging
6	H_2O–MeOH (2 : 8)	Fe^{3+} mainly, Cr^{3+}, Al^{3+} obviously	1900	Not determined	4.3×10^4	Not Done
7	H_2O–MeOH (1 : 1)	Fe^{3+} mainly, Cr^{3+}, Rh^{3+}, Sn^{2+} weakly	0.396	Not determined	2.46×10^4	L-02 & PC12 cell imaging
8	H_2O–EtOH (1 : 2)	Fe^{3+} mainly Cr^{3+} obviously	0.54	0.01	2.98×10^3	*Bacillus sp.* cell imaging
9	H_2O–EtOH (4.5 : 5.5)	Fe^{3+} mainly	5.0	0.34, 0.52	4.52×10^4	HeLa cell imaging
10	MeOH	Fe^{3+} mainly	5.0	0.34	6.56×10^4	Not Done
11	H_2O–EtOH (1 : 1)	Fe^{3+} mainly	0.9	0.32, 0.74	5.11×10^6	L929 cell imaging
Z1	**H_2O–EtOH (1 : 1)**	**Fe^{3+} mainly Cr^{3+}, Al^{3+} slightly**	**0.003**	**0.86**	**5.45×10^4**	**MG-63 & L929 cell imaging cytotoxicity test**