Electronic Supplementary Information

A Highly Selective Fluorescent Probe for Fe^{3+} in Living Cells: A Stress Induced Cell Based Model Study

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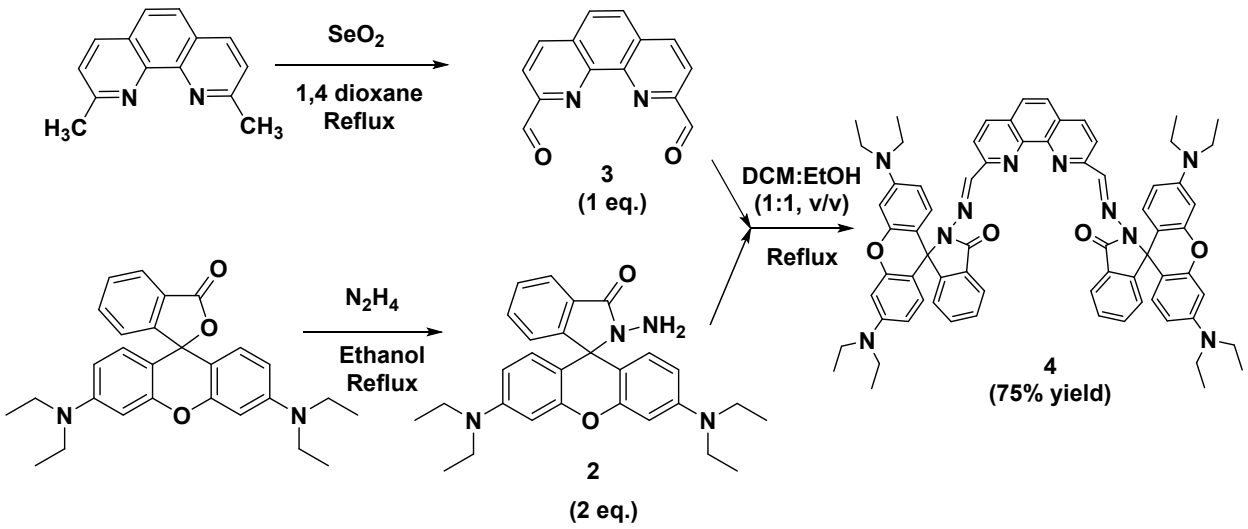
Procedure for sensing

UV-vis and fluorescence titrations were performed on 5.0 μM solution of ligand in H_2O/CH_3CN (7:3, v/v; buffered with 0.05 M tris-HCl, pH = 7.0; at 25 °C) mixture. Typically, aliquots of freshly prepared $M(ClO_4)_n$ (M = Pb^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, K^+, Na^+, Li^+, Fe^{2+}, Fe^{3+}, Al^{3+}, Ag^+, Zn^{2+} and Cd^{2+}; n = 1, 2 or 3) and tetrabutylammonium salts of anions (F^-, Cl^-, Br^-, I^-, CN^-, OAc^-, NO_3^-, $H_2PO_4^-$) standard solutions (10^{-1} M to 10^{-3} M) were added to record the UV-vis and fluorescence spectra. Hydrogen peroxide (H_2O_2) was delivered from 30% aqueous solution. In titration experiments, each time a 3 ml solution of ligand was filled in a quartz cuvette (path length, 1 cm) and spectra were recorded after the addition of appropriate analytes.

Synthetic routes and characteristic data



Synthesis of compound 3:

A mixture of 2,9-dimethyl-1,10-phenanthroline (0.100 g, 0.48 mmol) and selenium dioxide (0.266 g, 2.4 mmol) dissolved in 30 ml of 96% dioxane was heated under refluxing for 2 hr and then filtered the reaction mixture through celite while hot. The filterate was evaporated to give yellow crystals as product which was further recrystallized from acetone as pure yellow crystals with 70% yield; m.p. 231 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 8.30 (m, 4H, ArH), 8.80 (d, J = 6 Hz, 2H, ArH), 10.36 (s, 2H, -CH=O).

Synthesis of compound 4:

To compound **2** (0.115 g, 0.254 mmol) in a mixture of dichloromethane and ethanol (1:1, v/v) was added compound **3** (0.030 g, 0.127 mmol) at room temperature. The reaction mixture was then refluxed overnight. After that the solution was evaporated and the residue is obtaining crystallized from ethanol to give pure compound **4** in 75% yield; m.p. 250 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ = 1.16 (t, J = 7.5 Hz, 24H, CH_3), 3.32 (q, J = 8.33 Hz, 16 H, CH_2), 6.22 (d, J = 5.0 Hz, 4H, Ar-H), 6.50 (s, 4H, Ar-H), 6.57 (d, J = 10 Hz, 4H, Ar-H), 7.15 (d, J = 5.0 Hz, 2H, Ar-H), 7.48 (m, 4H, Ar-H), 7.68 (s, 2H, Ar-H), 8.02 (d, J = 10 Hz, 2H, Ar-H), 8.09 (d, J = 10 Hz, 2H, Ar-H), 8.29 (d, J = 10 Hz, 2H, Ar-H), 9.26 (s, 2H, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 125

MHz): δ = 12.62, 44.37, 66.17, 98.40, 106.02, 107.93, 120.38, 123.65, 123.85, 126.69, 127.60, 128.21, 128.62, 128.87, 133.44, 136.12, 145.25, 148.69, 148.96, 152.20, 153.28, 155.45, 164.99 ppm. ESI-MS (m/z) Calcd for $C_{70}H_{68}N_{10}O_4$ Calcd: 1112.54 (M); Found: 1135.5484 ($M+Na^+$).

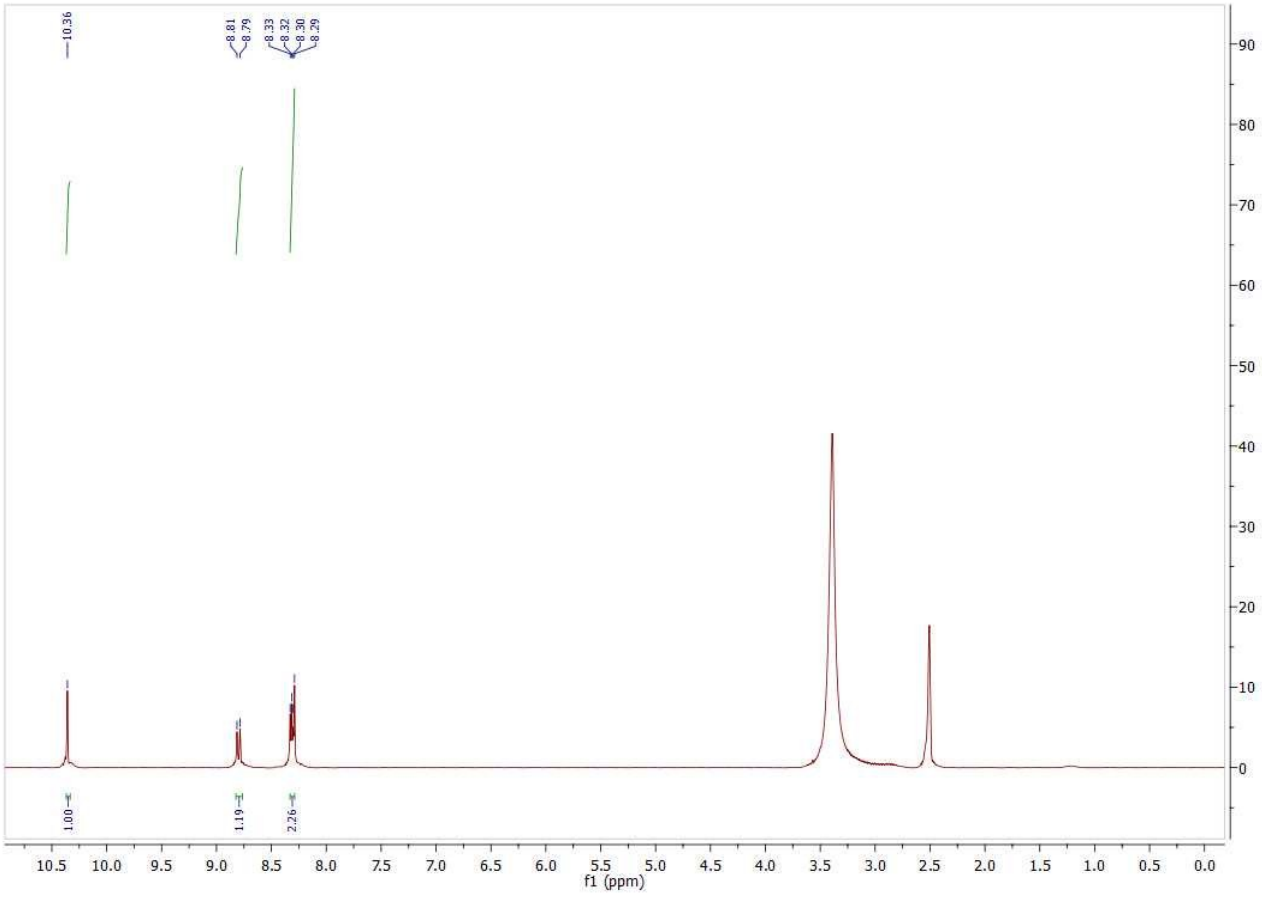


Figure S1: ^{1}H NMR spectrum of compound **3**

^{1}H NMR Spectrum of probe 4 in $CDCl_3$ (500 MHz)

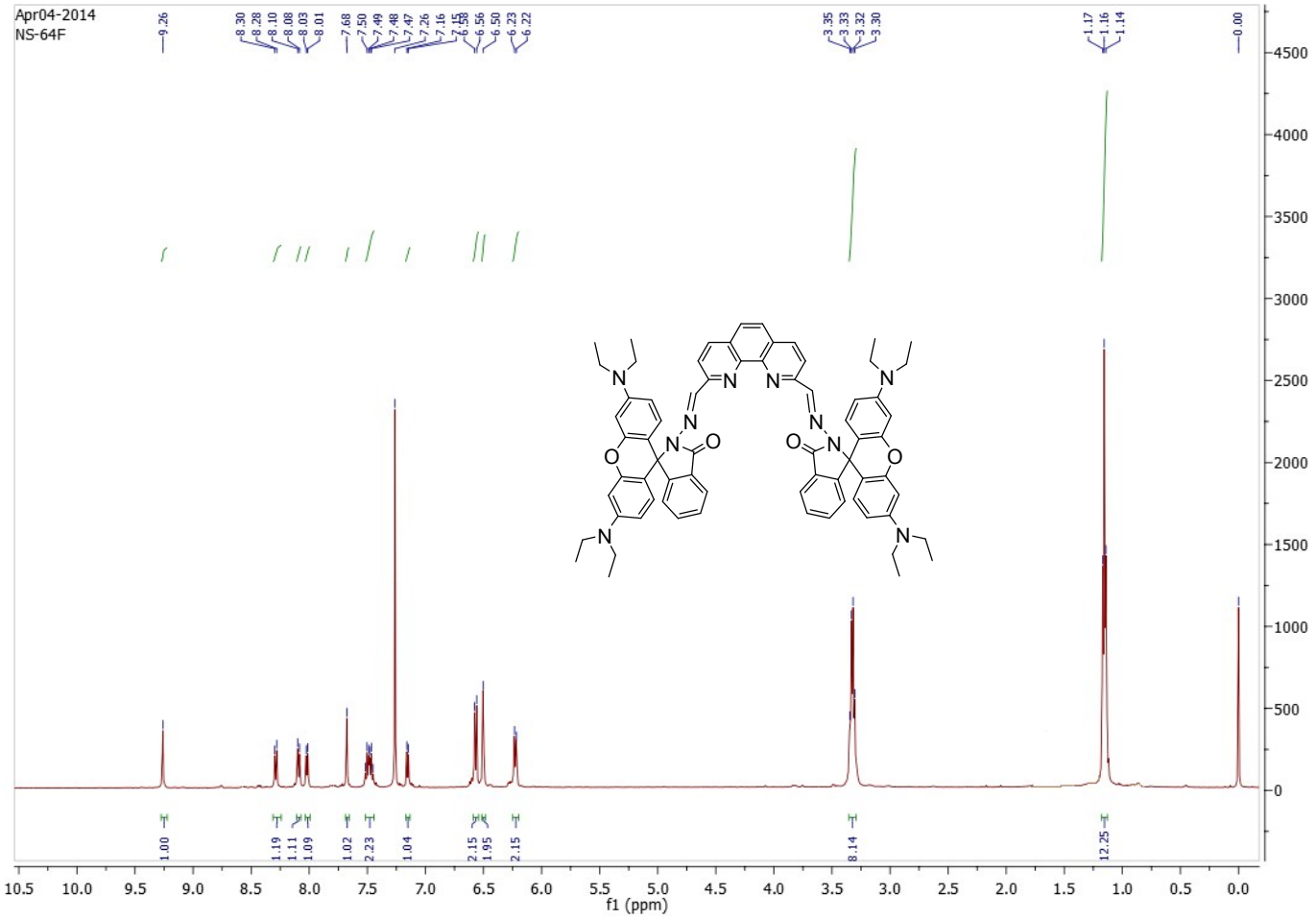


Figure S2: ^{1}H NMR spectrum of compound **4**

^{13}C NMR Spectrum of probe 4 in $CDCl_3$ (125 MHz)

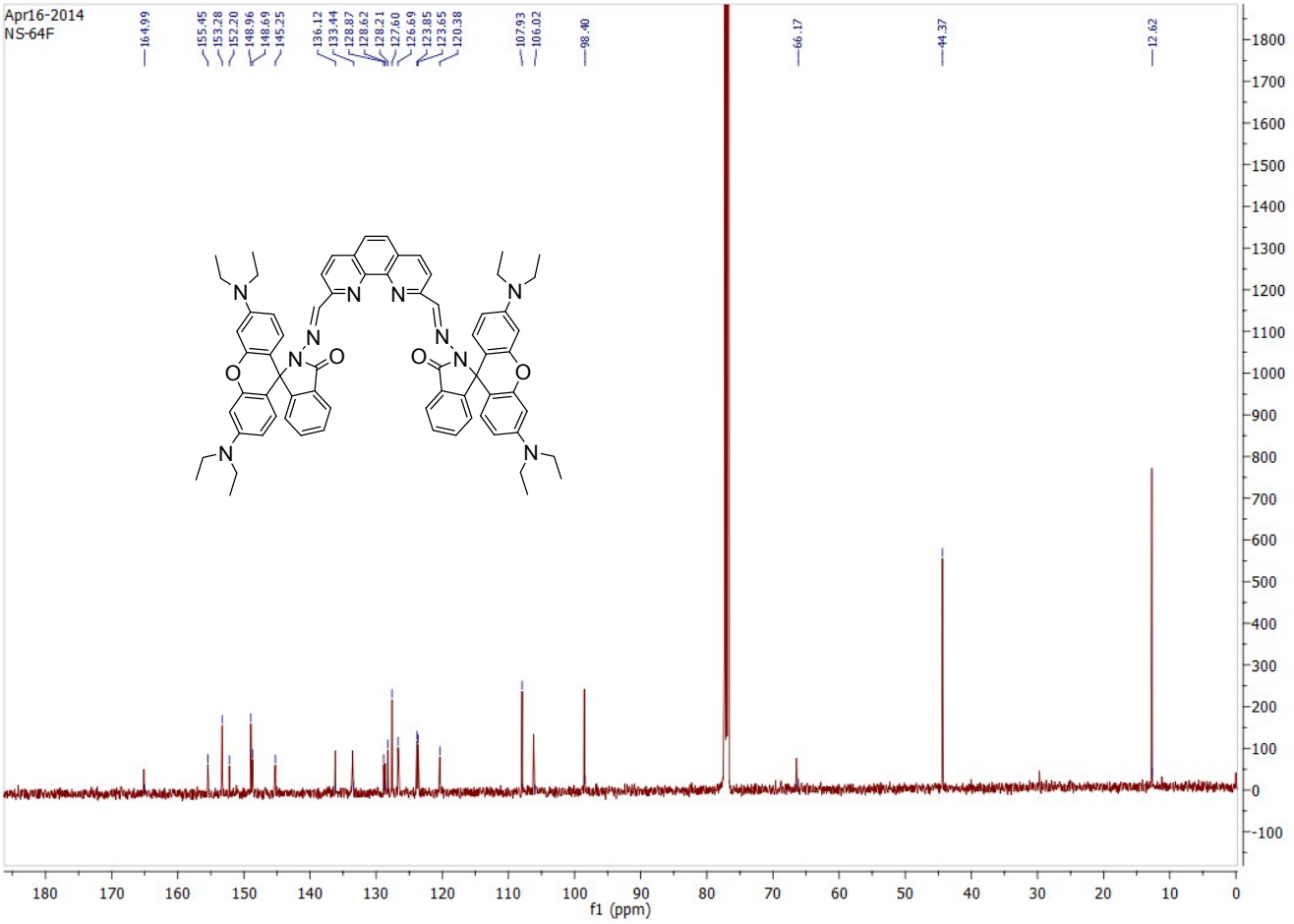


Figure S3: ^{13}C NMR spectrum of compound **4**

Mass Spectrum of probe 4

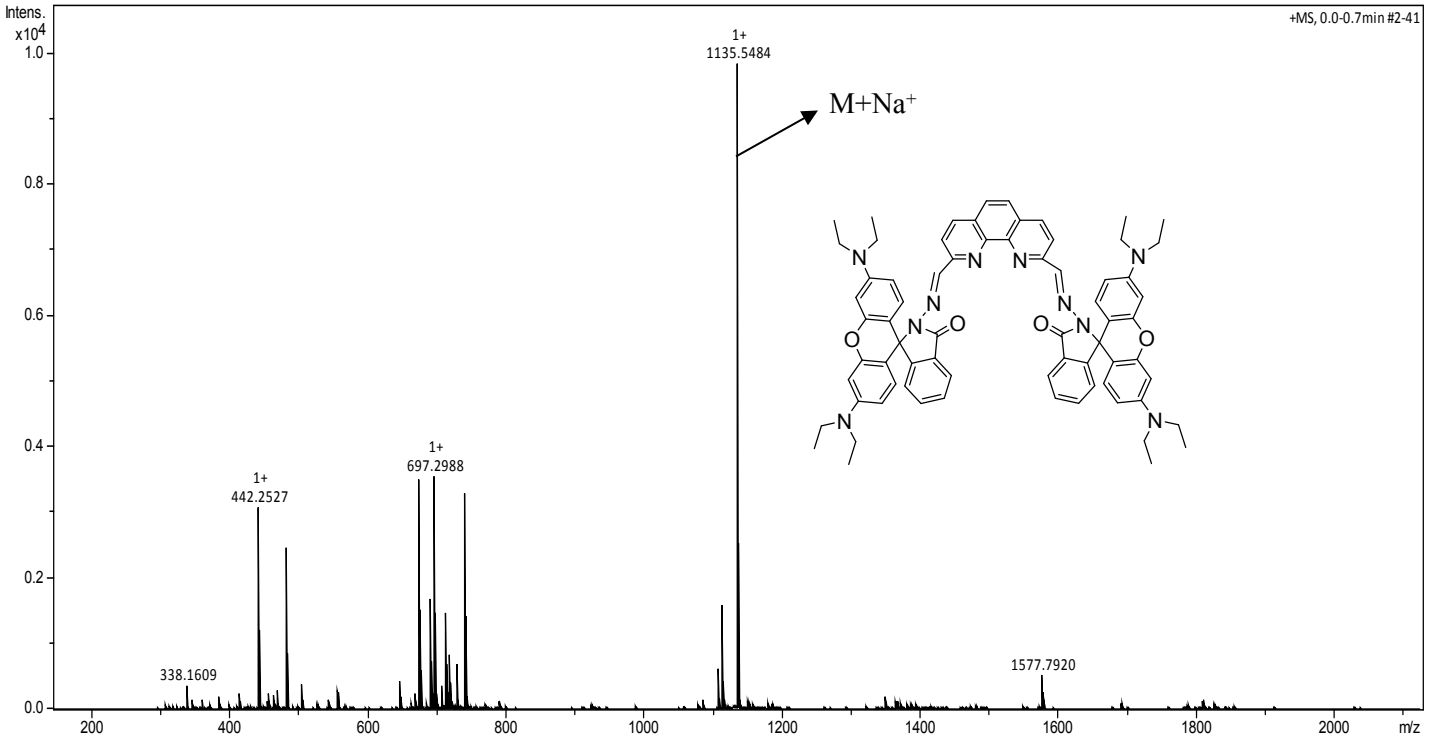


Figure S4: Mass spectrum of compound **4**

IR Spectrum of probe 4

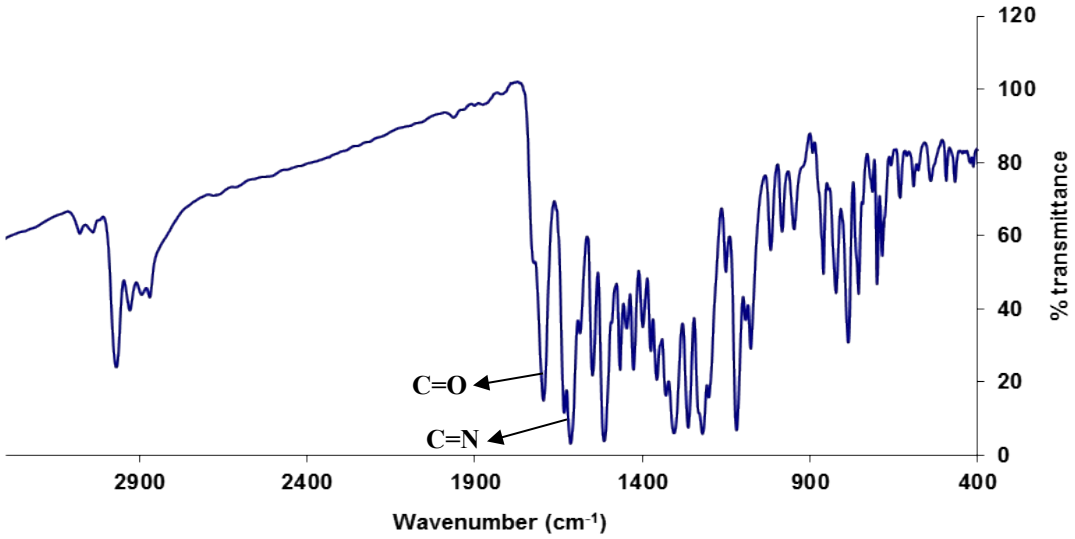


Figure S5: IR spectrum of compound **4**

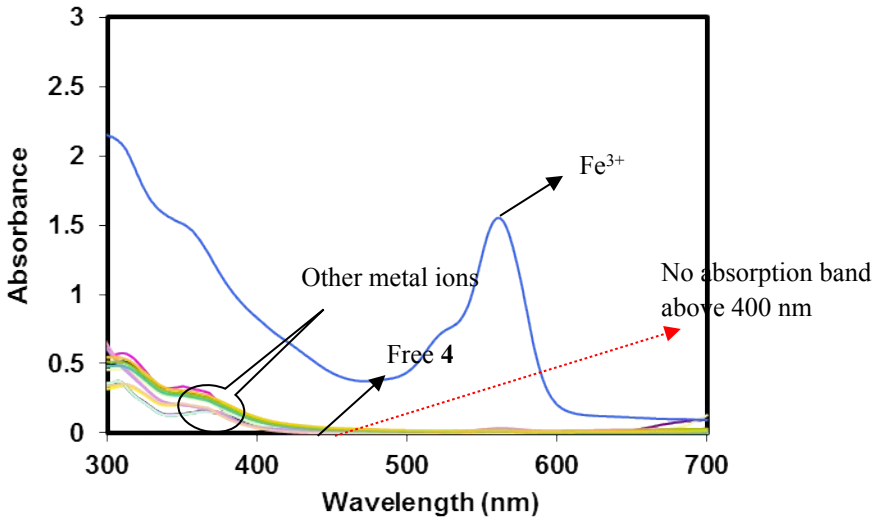


Figure S6. UV-vis spectrum of probe **4** upon the addition of different metal ions: Zn^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Na^{+}, Li^{+}, Al^{3+}, Cr^{3+}, Fe^{2+}, Fe^{3+}, Ag^{+}, Pb^{2+} (80 equiv each) in CH_3CN:H_2O (3:7, v/v) buffered with 0.05 M tris-HCl, pH = 7.0.

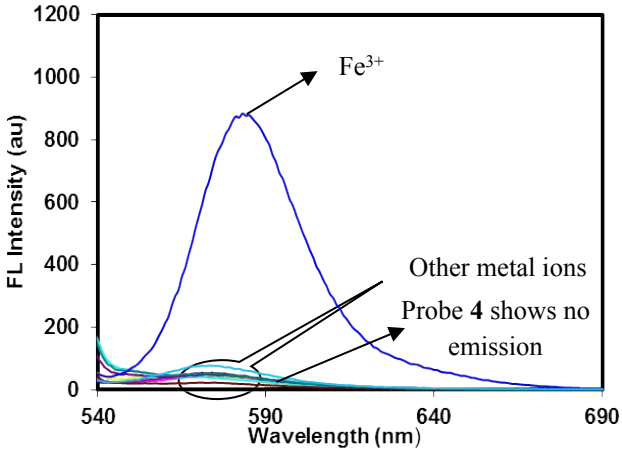


Figure S7. Fluorescence emission spectra of probe **4** (5 μM) in the presence of different metal ions: Fe^{3+} (80 equiv), Zn^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Na^{+}, Li^{+}, Al^{3+}, Cr^{3+}, Fe^{2+}, Ag^{+}, Pb^{2+} (200 equiv each) in CH_3CN:H_2O (3:7, v/v) buffered with 0.05 M tris-HCl, pH = 7.0; λ_{ex} = 510 nm.

Calculations for detection limit of receptor 4

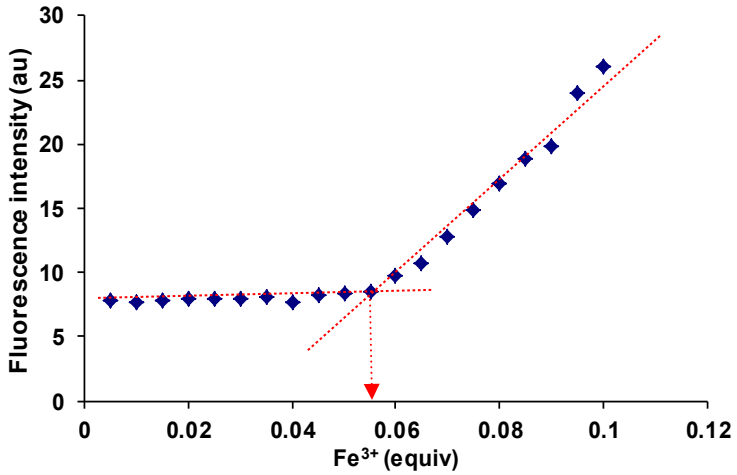


Figure S8. Figure showing the fluorescence intensity of **4** at 573 nm as a function of Fe^{3+} ions concentration.

To determine the detection limit of receptor **4**, fluorescence titration of **4** was carried out by adding aliquots of Fe^{3+} ions in minimum concentration and then we plotted fluorescence emission intensity at 580 nm as a function of Fe^{3+} ions concentration (equivalents). From this graph, the equivalents where we observed a large increase in fluorescence emission intensity multiplied by concentration of receptor **4** gave detection limit of receptor **4**.

Equation used for calculating detection limit (DL): The detection limit was then calculated by using the following equation:

$DL = C_L \times C_T$
C_L = Conc. of Ligand; C_T = Conc. of Titrant at which change observed.
Thus;
$DL = 5 \times 10^{-6} \times 0.055 = 27.5 \times 10^{-8}$ M.

Thus by using the above formula, detection limit (DL) was found to be 27.5×10^{-8} M i.e. probe **4** can detect Fe^{3+} in this minimum concentration.

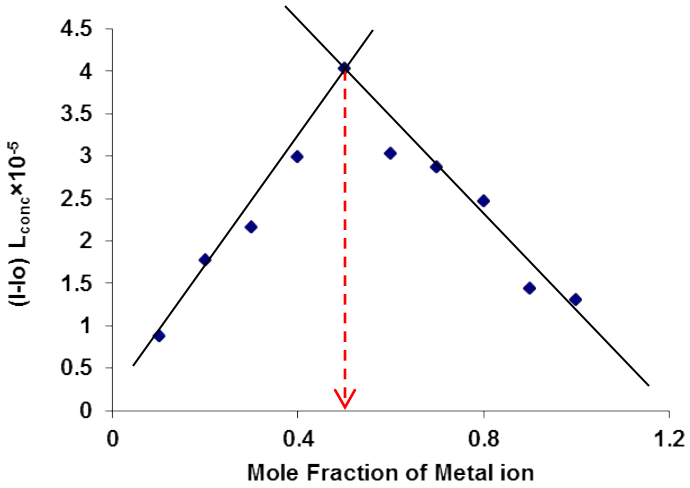


Figure S9. Job's plot for determining the stoichiometry (1:1) of receptor **4** and Fe^{3+} ions in H_2O/CH_3CN (7:3, v/v) buffered with 0.05 M tris-HCl, pH = 7.0.

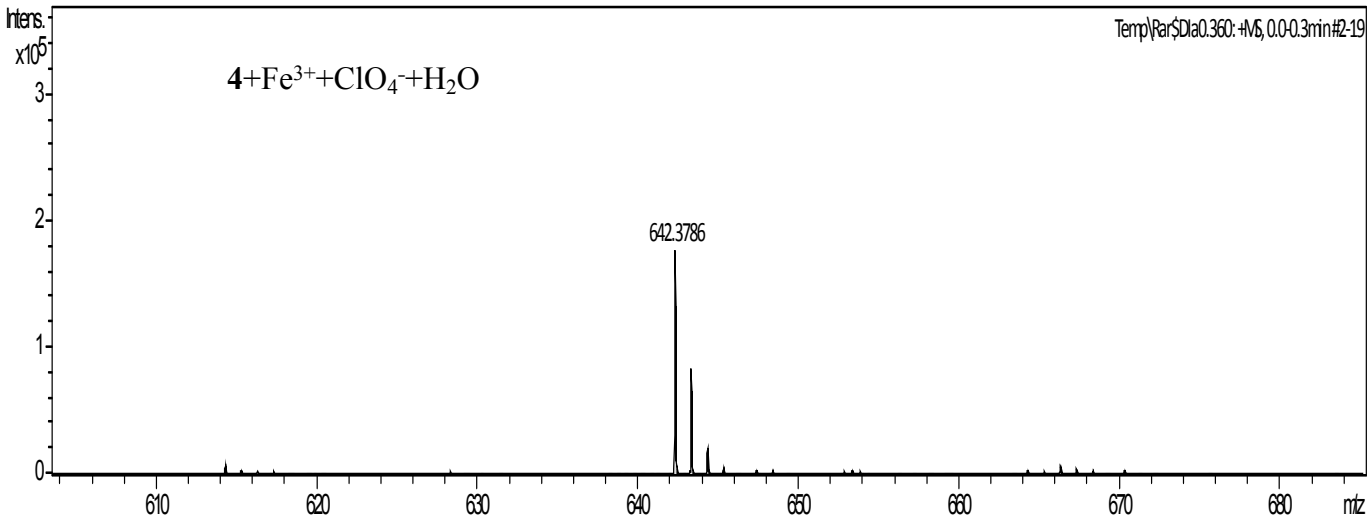


Figure S10: Mass spectrum of **4**-Fe^{3+} complex (indicating 1:1 binding)

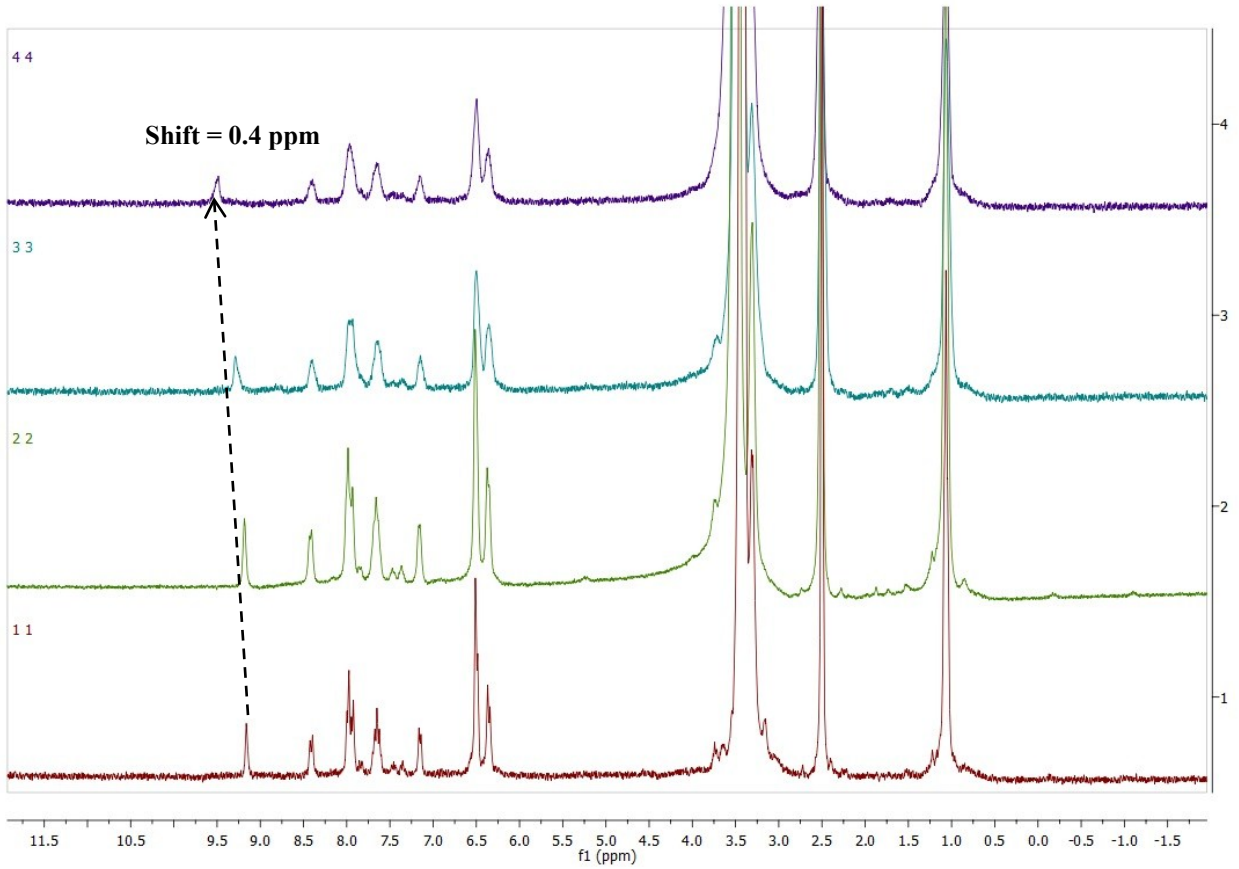


Figure S11a: ^{1}H NMR titration of **4** with Fe^{3+} ions

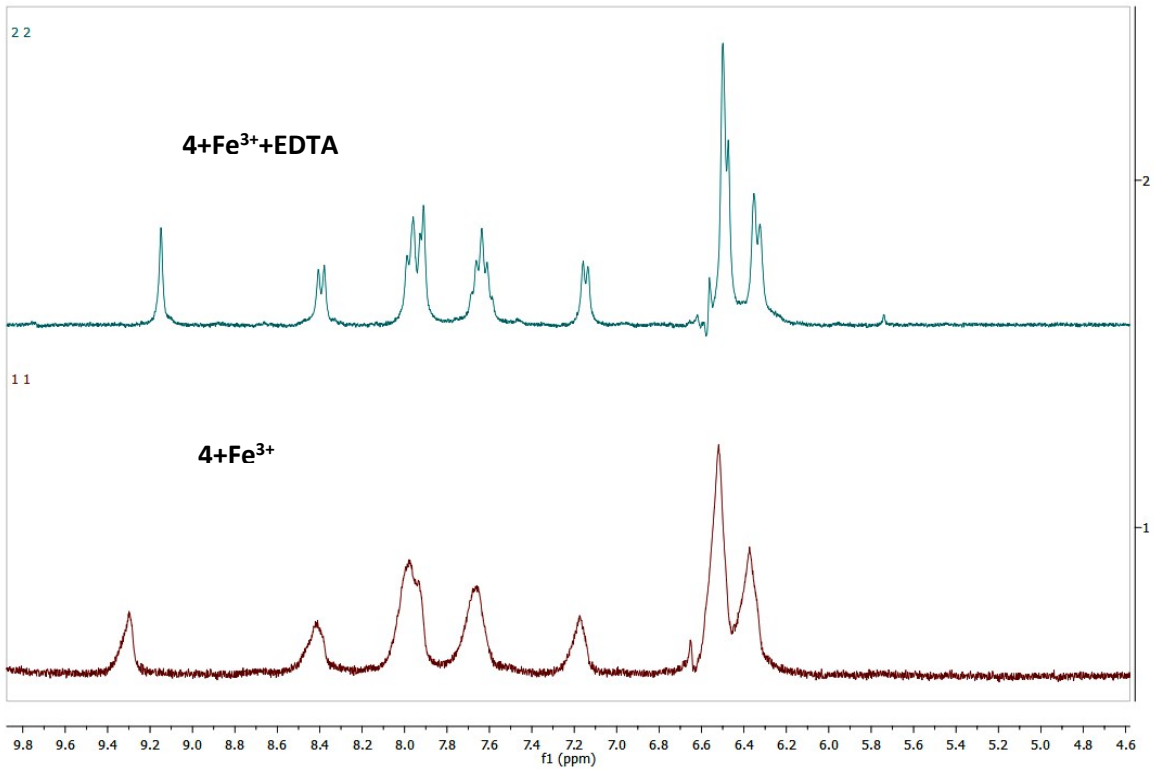


Figure S11b: ^{1}H NMR titration of **4** + Fe^{3+} with EDTA

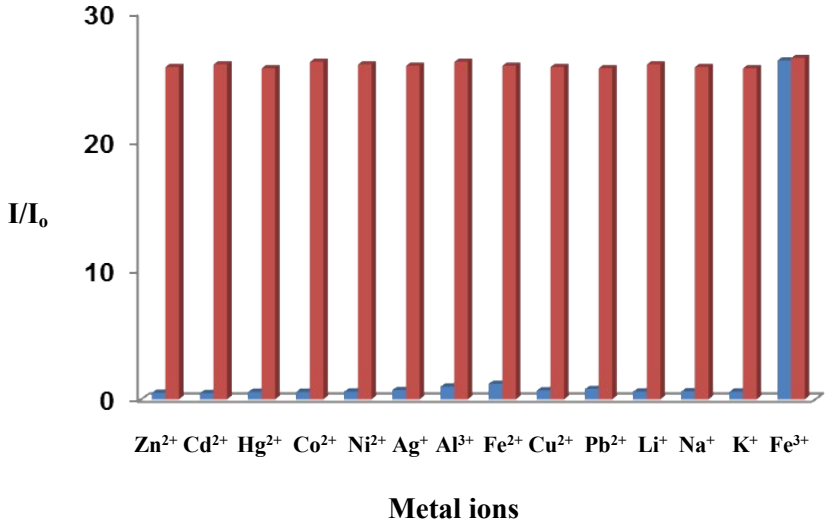


Figure S12. Fluorescence response of receptor **4** towards various metal ions: (a) Blue bars represent fluorescence selectivity (I/I_o) of **4** (5 μM) towards various metal ions; (b) Red bars represent competitive fluorescence selectivity (I/I_o) of **4** towards Fe^{3+} ions (80 equiv) in the presence of other metal ions (200 equiv each) in $CH_3CN:H_2O$ (3:7, v/v) buffered with 0.05 M tris-HCl, pH = 7.0; λ_{ex} = 510 nm.

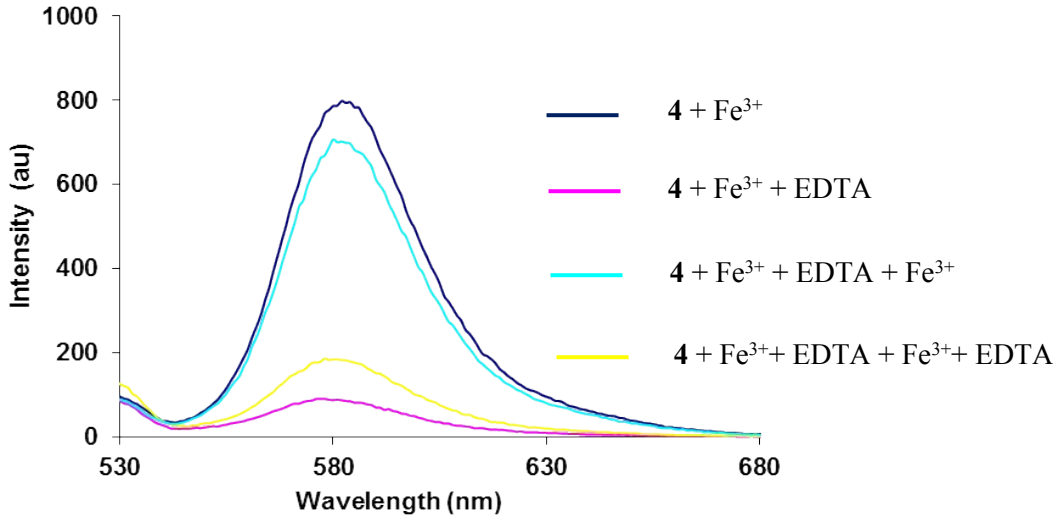


Figure S13: Fluorescence spectra showing reversibility of Fe^{3+} coordination to probe **4** by EDTA.

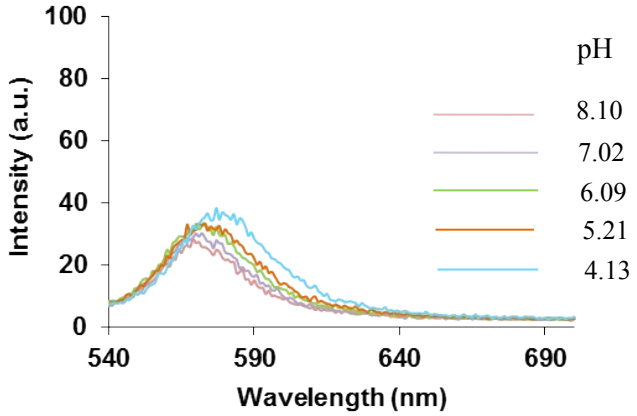


Figure S14: pH study (pH range 4.13-8.10) of probe **4** (5 μM) in CH_3CN:H_2O (3:7, v/v) buffered with 50 mM tris-HCl; λ_{ex} = 510 nm.

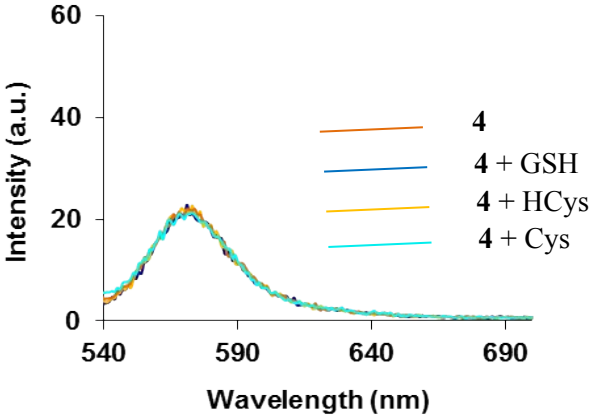


Figure S15: Fluorescence emission spectra of probe **4** (5 μM) in the presence of glutathione, homocysteine and cysteine (100 equiv. each) in CH_3CN:H_2O (3:7, v/v) buffered with 50 mM tris-HCl, pH = 7.0; λ_{ex} = 510 nm.

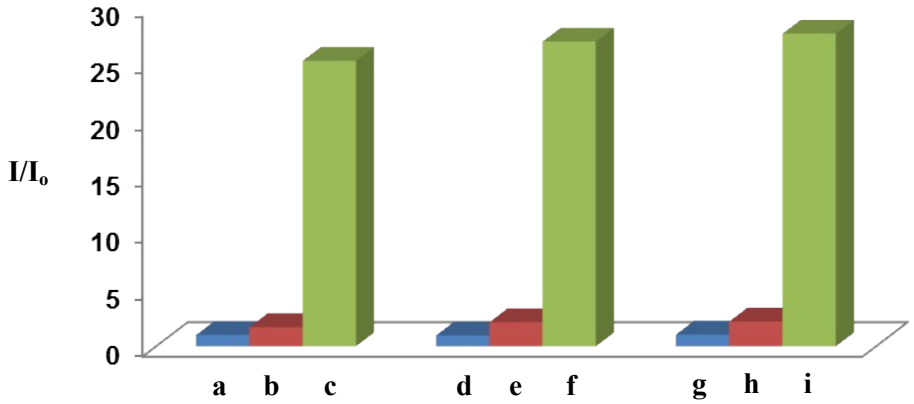


Figure S16: Bar diagram showing the fluorescence response of **4**+thiols in presence of Fe^{2+} and Fe^{3+}: (a) **4**+cysteine, (b) **4**+cysteine+Fe^{2+}, (c) **4**+cysteine+Fe^{3+}, (d) **4**+homocysteine, (e) **4**+homocysteine+Fe^{2+}, (f) **4**+homocysteine+Fe^{3+}, (g) **4**+glutathione, (h) **4**+glutathione+Fe^{2+}, (i) **4**+glutathione+Fe^{3+} in CH_3CN:H_2O (3:7, v/v) buffered with 50 mM tris-HCl, pH = 7.0; λ_{ex} = 510 nm.

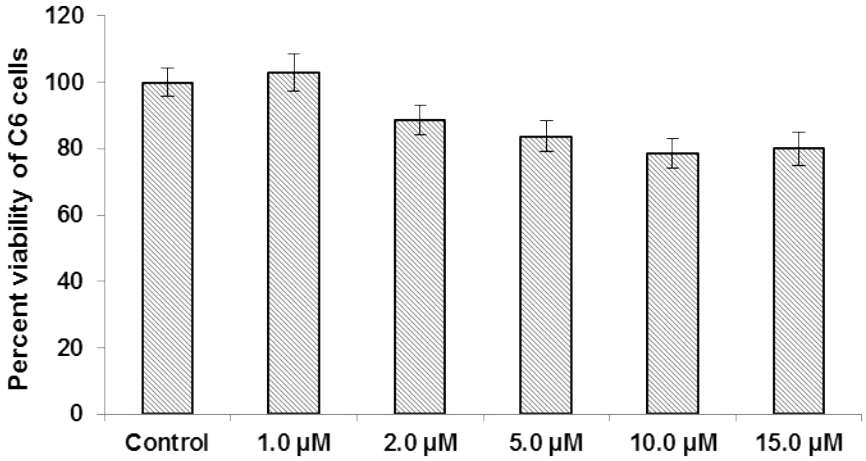


Figure S17: Viability of C6 cells is not influenced by lower doses of probe 4. C6 cells were treated with increasing concentrations of Probe **4** for 24 hours and percent cell viability was determined using MTT assay. The histograms represent the percent of viable C6 cells after treatment with 1.0 µM, 2.0 µM, 5.0 µM, 10.0 µM 15.0 µM of probe **4**, respectively. The viability of untreated cells (control) was taken as 100%. One-way analysis of variance (ANOVA) was applied.

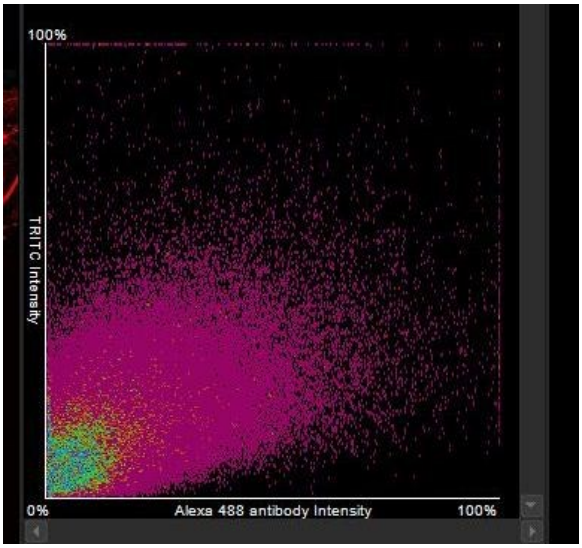


Figure S18: Colocalization plot of probe **4** + LysoTracker Green

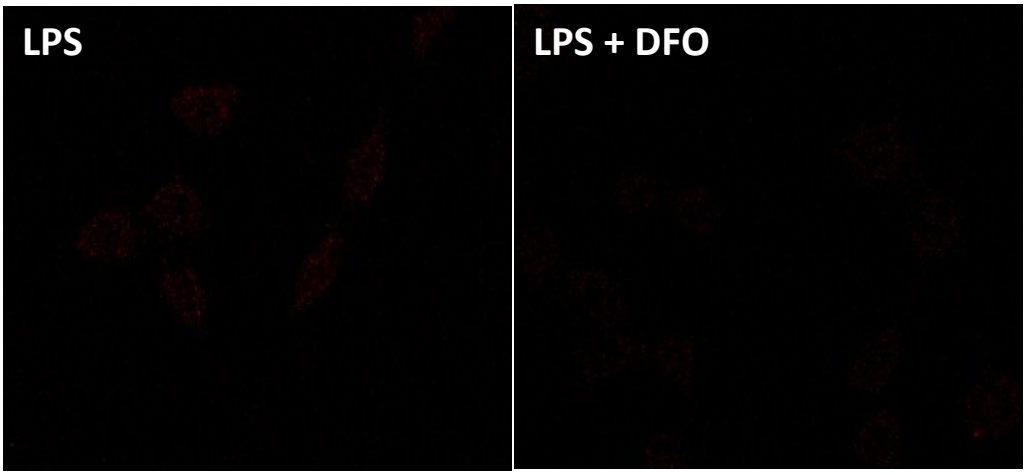


Figure S19: Confocal microscopy images of C6 cells were stained with 10.0 μM probe **4**. (A) Cells stained with 10.0 μM probe **4** for 20 min and then incubated with LPS for 3 hrs. (B) Cells stained with 10.0 μM probe **4** for 20 min followed by incubated with LPS for 3 hrs and then treated with DFO 50 μM.

Table: Comparison of probe 4 with previous reports on Fe^{3+} sensors in literature

S. No	Journal	Solvent system	Detection limit	Fenton reaction	Endogenous detection	Monitoring Stress induced detection of Fe^{3+}	Organelle Specific detection
1	**Present Manuscript**	**H_2O/CH_3CN**	**27.5×10^{-8} M**	**Yes**	**Yes**	**Yes**	**Yes**
2	Angew. Chem. Int. Ed. 2010, 49, 4576	in water at pH 7	10^{-7} M	No	No	No	No
3	Chem. Commun., 2015, 51, 5001	Triple distilled water TDW/DMSO, 9: 1, v/v) at pH 7.0–7.4	9.1×10^{-9} M	Yes	No	No	No
4	ACS Appl. Mater. Interfaces 2016, 8, 7440−7448	in HEPES buffer solution (10 mM, pH 7.4)	0.1 nM (Turn-Off)	No	No	No	Yes
5	Biosensors and Bioelectronics 2014, 58 219–225	aqueous solutions	90 nM	No	No	No	No
6	J. Mater. Chem. A, 2015, 3, 542	------	0.1 μM.	No	No	No	No
7	Chem. Commun., 2014, 50, 4631	Tris-HCl buffer (20 mM, pH 7.20) containing 50% methanol (v/v)	5.8×10^{-7} M.	No	No	No	No
8	ACS Appl. Mater. Interfaces 2014, 6, 18408−18412	H_2O-CH_3CN (9:1 v/v)	1.3×10^{-7} M	No	No	No	No

9	Scientific Reports | 6:23558 | DOI: 10.1038/srep23558	----	----	No	No	No	No
10	ACS Appl. Mater. Interfaces 2015, 7, 23958−23966	20 mM Tris-HCl buffer (ethanol-H_2O, 1:1 v/v; pH 7.0)	0.02 μM	No	No	No	No
11	Inorg. Chem. 2014, 53, 2144−2151	THF/H_2O (99:1, 0.01 M HEPES buffer, pH = 7.4).	4.8 μM	No	No	No	No

Procedure of cell imaging[1]

Cell culture and treatments

C6 glioma cell lines were obtained from NCCS, Pune, India. The cells were maintained in DMEM medium supplemented with 1X PSN (GIBCO), 10% FBS (Biological Industries) at 37 °C in humid environment containing 5% CO_2. For fluorescence detection, cells were seeded on 18 mm coverslips in 24 well plates.

Treatment and Fluorescence Detection in the Cells

For the purpose of this study, six groups were chosen as follow for the fluorescence detection:

(I) Group in which C6 glioma cells treated with probe **4** (10 μM) only,

(II) Cells were treated with probe **4** (10 μM) and then exposed to Fe^{3+} (20 μM) for 30 min.

(III) Cells were treated with Fe^{3+} (20 .0 μM) for 30 min, then with DFO (30 μM) for 40 min and finally exposed to probe **4** (10.0 μM) for 20 min.

(IV) Cells were treated with probe **4** (10 μM) and then exposed to H_2O_2 (50 μM).

(V) Exogenous Fe^{3+} detection from Fenton reaction: Cells were incubated with probe **4** (10 μM) for 20 min, then with Fe^{2+} (20 μM) for 30 min and finally exposed to H_2O_2 (50 μM).

(VI) Cells were pre-treated with DFO and then incubated with H_2O_2 (50 μM) and then with probe **4**.

Endogenous detection: Cells were treated with 5.0 μg/ml LPS for 3 hrs and then with probe **4** for 20 min.

Live cell imaging:

C6 cells were seeded in the confocal dish and then incubated for 24 h at 37 °C temperature under 5% CO_2. Probe **4** (10.0 μM) was then added into the cells with a 20 min incubation period and after that ascorbic acid (AA) (1.0 mM) was added into the cells (60 min incubation) then exposed to LPS (5 μg/ml) for 3 hrs. Finally, cells were lively monitored for next 3 hrs using an AIR Nikon Laser Confocal Microscope using bench top incubator maintained at 5% CO_2 and 37 °C. Fluorescent images were captured at different time intervals: 0, 60, 120 and 180 min. λ_{ex} = 543 nm and λ_{em} = 560±20 nm.

References

1. Y.-Q. Sun, J. Liu, H. Zhang, Y. Huo, X. Lv, Y. Shi and W. Guo, *J. Am. Chem. Soc.,* 2014, **136**, 12520–12523.

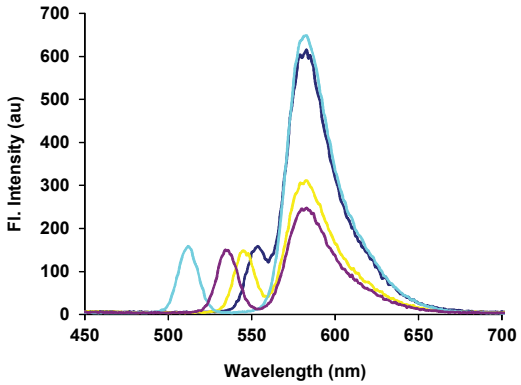


Figure S20: Fluorescence spectra of probe **4** +Fe^{3+} ions in various excitation wavelengths.

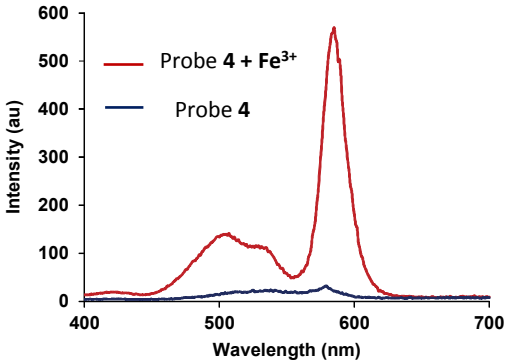


Figure S21: Fluorescence excitation spectra of receptor **4** and **4**-Fe^{3+} complex.