Electronic Supplementary Material (ESI) for Chemical Communications.

High-sensitivity fluorescence imaging of iron in plant tissues

Mi-Jeong Park, Hak-Sung Jung, Young-Jae Kim, Young-Ju Kwon, Jin-Kyu Lee* and Chung-Mo Park*

Department of Chemistry, Seoul National University, Seoul 151-747, Korea

E-mail: jinklee@snu.ac.kr, Tel: +82-2-879-2923, Fax: +82-2-882-1080

Plant Genomics and Breeding Institute, Seoul National University, Seoul 151-742, Korea

E-mail: cmpark@snu.ac.kr, Tel: +82-2-880-6643, Fax: +82-2-886-6697

[Contents]

Experimental procedures S2-S4

^{1}H-NMR spectrum of MPNBD S4

^{13}C-NMR spectrum of MPNBD S5

Mass analysis of MPNBD S6

Comparison of fluorescence responses of MPNBD in water and ethanol to various amounts of Fe^{3+} S7

Absorption spectra of MPNBD in the presence of different metal ions S8-S9

Fluorescence responses of MPNBD to various metal ions S10-S12

Calibration curve of MPNBD-Fe^{3+} in ethanol solution S13

Fluorescence response of MPNBD to various metal ions and to mixtures of Fe^{3+} and other metal ions. S14

Images of MPNBD-treated plants S15

Stability of MPNBD fluorescent probe S16

Effects of various metal ions on the MPNBD imaging of iron in plants S17

Procedures of different iron detection methods in plants S18

References S19

Experimental procedures

Synthesis of 7-(4-methylpiperazin-1-yl)-4-nitrobenzo-2-oxa-1,3-diazole (MPNBD) MPNBD was prepared following the procedure that has been described previously,[1] but with some modifications. 4-chloro-7-nitrobenzofurazan (0.5 g, 2.5 mmol) was dissolved in $CHCl_3$ (20 mL). 1-methylpiperazine (0.28 mL, 2.5 mmol) was added and stirred for 30 min at room temperature. The reaction mixture was concentrated on a rotary evaporator. The residue was purified by column chromatography (SiO_2, eluent: CH_2Cl_2/MeOH = 10/1) to afford 0.43 g of orange powder in 66% yield. ^{1}H- and ^{13}C-NMR spectra were measured on a Varian NMR System 500 MHz NMR Spectrometer (Varian, Palo Alto, CA). Data were acquired as follows; chemical shifts in ppm from tetramethylsilane as an internal standard in $CDCl_3$, integration, multiplicity (s = singlet, d = doublet, t = triplet). ^{1}H-NMR (500 MHz, $CDCl_3$): δ ppm 2.32 (s, 3H), 2.61 (t, 4H), 4.06 (t, 4H), 6.25 (d, 1H), 8.35(d, 1H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ ppm 145.18, 144.83, 144.78, 135.10, 123.54, 102.42, 54.55, 49.35, 45.84. HR-MS(FAB+) calculated for 264.1097 m/z, observed for 264.1097 m/z

Metal ion sensing by MPNBD A stock solution of MPNBD (14.81 μM) and metal solutions of $FeCl_3 \cdot 6H_2O$ (4.51 mM), $FeCl_2 \cdot 4H_2O$ (5.90 mM), $CuCl_2 \cdot 2H_2O$ (7.10 mM), $CrCl_3 \cdot 6H_2O$ (4.24 mM), $CaCl_2$ (6.53 mM), $MgCl_2 \cdot 6H_2O$ (6.70 mM), $MnCl_2 \cdot 4H_2O$ (3.48 mM), $NiCl_2$ (4.65 mM), and $ZnCl_2$ (4.81 mM) were prepared in ethanol. Metal solutions of KCl (4.60 mM) and NaCl (6.55 mM) were prepared in water. Two ml of the MPNBD stock solution and each of metal ion stock solutions were mixed. Ethanol was then added to each mixture to match the concentration of MPNBD in each mixture to 9.88 μM. The mixtures were analyzed by UV-Vis spectroscopy (Sinco, S-3100, Seoul, Korea) and fluorophotometry (JASCO, FP-6500, Tokyo, Japan) at room temperature. Light source for fluorophotometry was the 150W Xenon lamp with a diffraction grating to select the excitation light. The emission spectra were measured in the wavelength range from 490 to 750 nm under the excitation at 470 nm.

Plant materials and growth conditions *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used. The *Arabidopsis* plants were grown on 1/2 X Murashige & Skoog (MS) media containing 0.6% (w/v) agar (hereafter referred to as MS-agar plate) in growth chamber at 22^{o}C with white light illumination (120 μmol photons $m^{-2}s^{-1}$) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea) under long day conditions (16-hour light and 8-hour dark).

Staining of iron in *Arabidopsis* plants Four-day-old *Arabidopsis* plants grown on MS-agar plates were used. The Perls and Perls/DAB (3,3'-diaminobenzidine) staining procedures were conducted as described previously.[2,3] For the Perls staining, plants were vacuum infiltrated with a solution containing equal volumes of 4% (v/v) HCl and 4% (w/v) $K_4Fe(CN)_6$ for 15 minutes and subsequently incubated for 30 minutes in complete darkness at room temperature. For the DAB-intensified Perls staining, the Perls-stained plants were washed with distillated water, incubated in a methanol solution containing 0.01 M NaN_3 and 0.3% (v/v) H_2O_2 for 1 hour, and then washed with 0.1 M phosphate buffer (pH 7.4). For the DAB intensification, the plants were incubated for 10 to 30 minutes in 0.1 M phosphate buffer (pH 7.4) containing 0.0025% (w/v) DAB, 0.005% (v/v) H_2O_2, and 0.005% (w/v) $CoCl_2 \cdot 6H_2O$.

For fluorescence imaging of iron with MPNBD, four-day-old *Arabidopsis* plants were briefly washed with ethanol and vacuum infiltrated with a ethanol solution containing 50 μM MPNBD for 5 minutes. The plants were incubated in complete darkness for 10 minutes and washed with ethanol before fluorescence microscopy.

Fluorescence microscopy Plants stained by the Perls and Perls/DAB methods were analyzed using the Olympus BX51 microscope (Olympus, Tokyo, Japan). Images were acquired using the Olympus DP70 digital camera system and processed with the DPController and DP Manager softwares (Olympus). For fluorescence detection in the MPNBD-treated plants, the U-MWB2 fluorescence detection system (excitation filter BP460-490, dichronic mirror DM500, and barrier filter BA520IF) (Olympus) was used. Light was provided by a 100W High Pressure Mercury Burner (Olympus, BH2-RFL-T3). Fluorescence

images were taken with an exposure time of 250 ms at 40X magnification. High-magnification images were visualized using the Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany) with the following filter setup: excitation 488 nm, emission BP510-530 nm. Fluorescence images were analyzed using the ImageJ software (http://rsbweb.nih.gov/ij/), as described previously.[2,3]

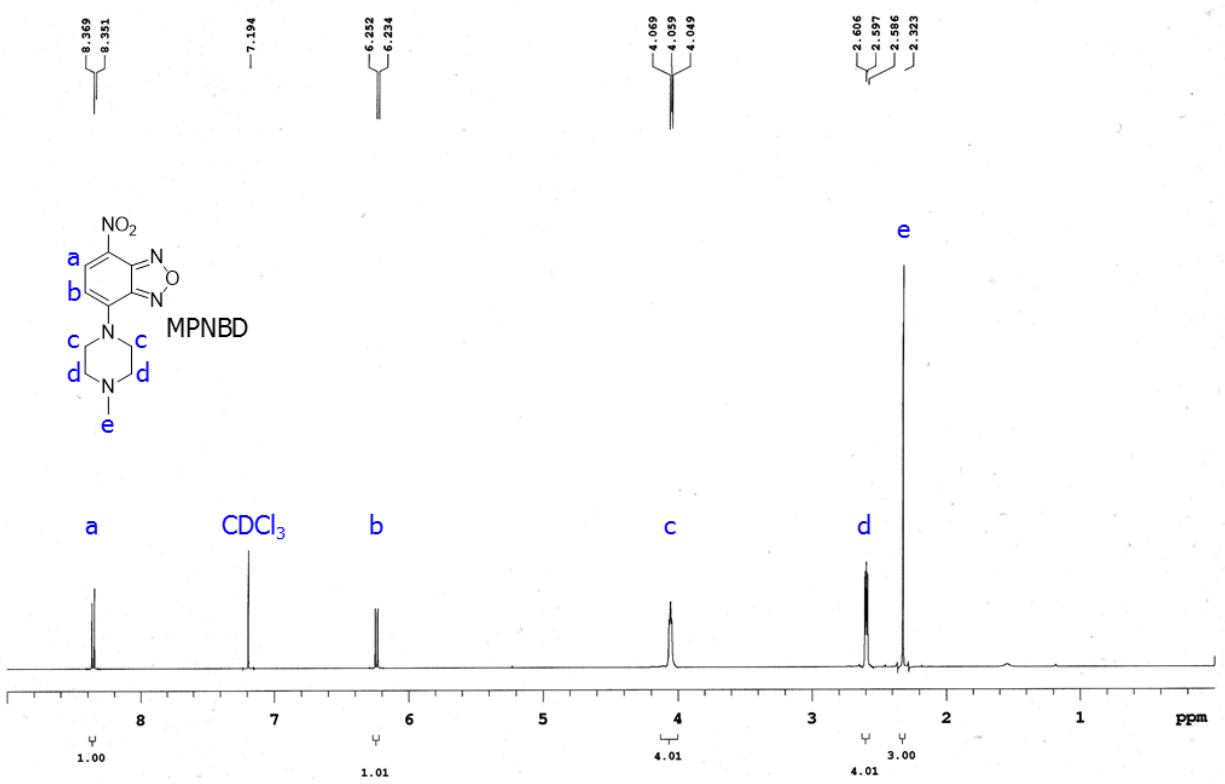


Figure S1. ^{1}H-NMR spectrum of MPNBD.

The spectrum were measured by the 500 MHz Varian NMR System (Varian, Palo Alto, CA) in $CDCl_3$, showing appropriate peaks consistent with the previously reported results in Ref 10 (Xiao and Qian, 2003). The integrated areas under the signal are reasonably proportional to the number of hydrogen atoms at each position; a : b : c : d : e = 1 : 1 : 4 : 4 : 3.

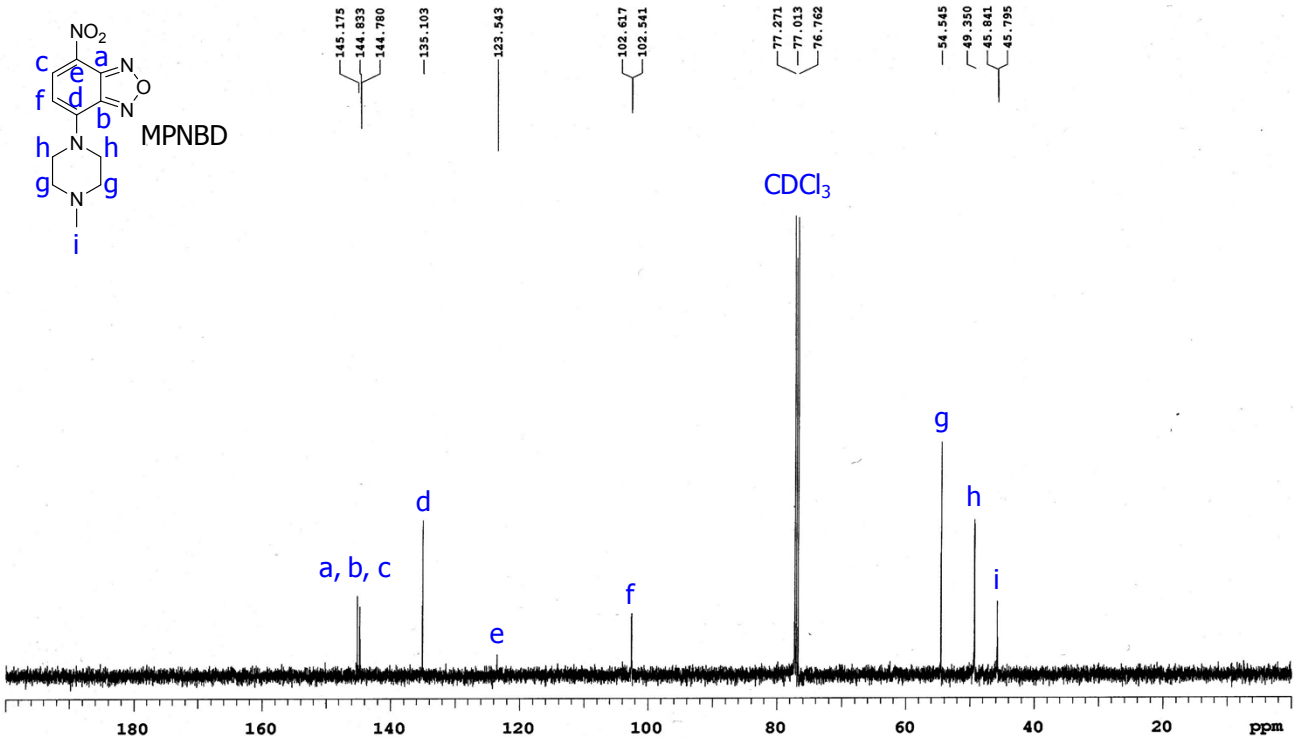
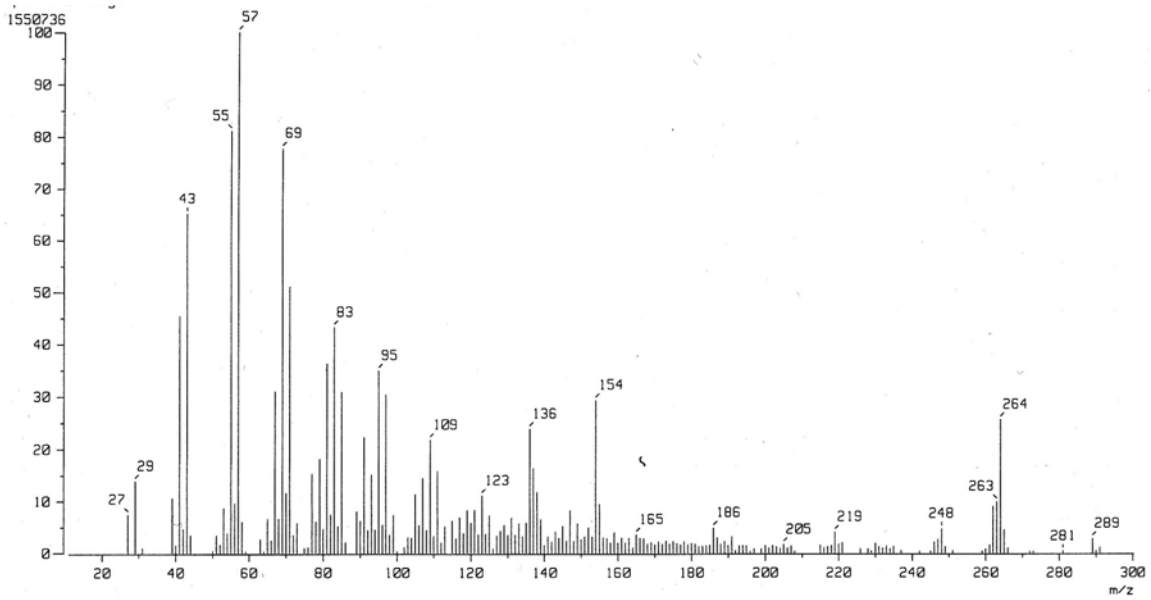


Figure S2. ^{13}C-NMR spectrum of MPNBD.

The spectrum were measured by the 500 MHz Varian NMR System in $CDCl_3$, exhibiting appropriate peaks (9 peaks) consistent with the previously reported results in Ref 10 (Xiao and Qian, 2003). The numbers at the peaks in the spectra indicate the numbers of different chemical environments where carbon atoms are located in the molecule (designated as a to i in the inset). Note that the peak intensity (or integrated area that is not shown in the spectra) is not simply proportional to the number of carbon atoms that gives rise to the signal because the nuclear overhauser effects from proton decoupling are not equal for all the carbons.

a



b

Observed m/z	Int%	Err[ppm / mmu]	U.S.	Composition
264.1096	100.0	-10.2 / -2.7	12.0	C 14 H 12 N 6
		-15.3 / -4.0	11.5	C 16 H 14 N 3 O
		+5.0 / +1.3	8.0	C 9 H 12 N 8 O 2
		-0.1 / +0.0	7.5	C 11 H 14 N 5 O 3
		-5.1 / -1.4	7.0	C 13 H 16 N 2 O 4
		+15.2 / +4.0	3.5	C 6 H 14 N 7 O 5
		+10.1 / +2.7	3.0	C 8 H 16 N 4 O 6
		+5.0 / +1.3	2.5	C 10 H 18 N O 7
		-17.2 / -4.5	-1.0	C 2 H 16 N 8 O 7
		+15.2 / +4.0	-2.0	C 7 H 20 O 10

Figure S3. Mass analysis of MPNBD.

(a) Fast atom bombardment-mass spectrometry (FAB-MS). (b) High resolution-mass spectrum (HR-MS) result of MPNBD. The mass of MBNBD was calculated for $C_{11}H_{14}N_5O_3$: 264.1096 and observed: 264.1096 by GC-MS using the JMS-700 MStation (JOEL, Tokyo, Japan).

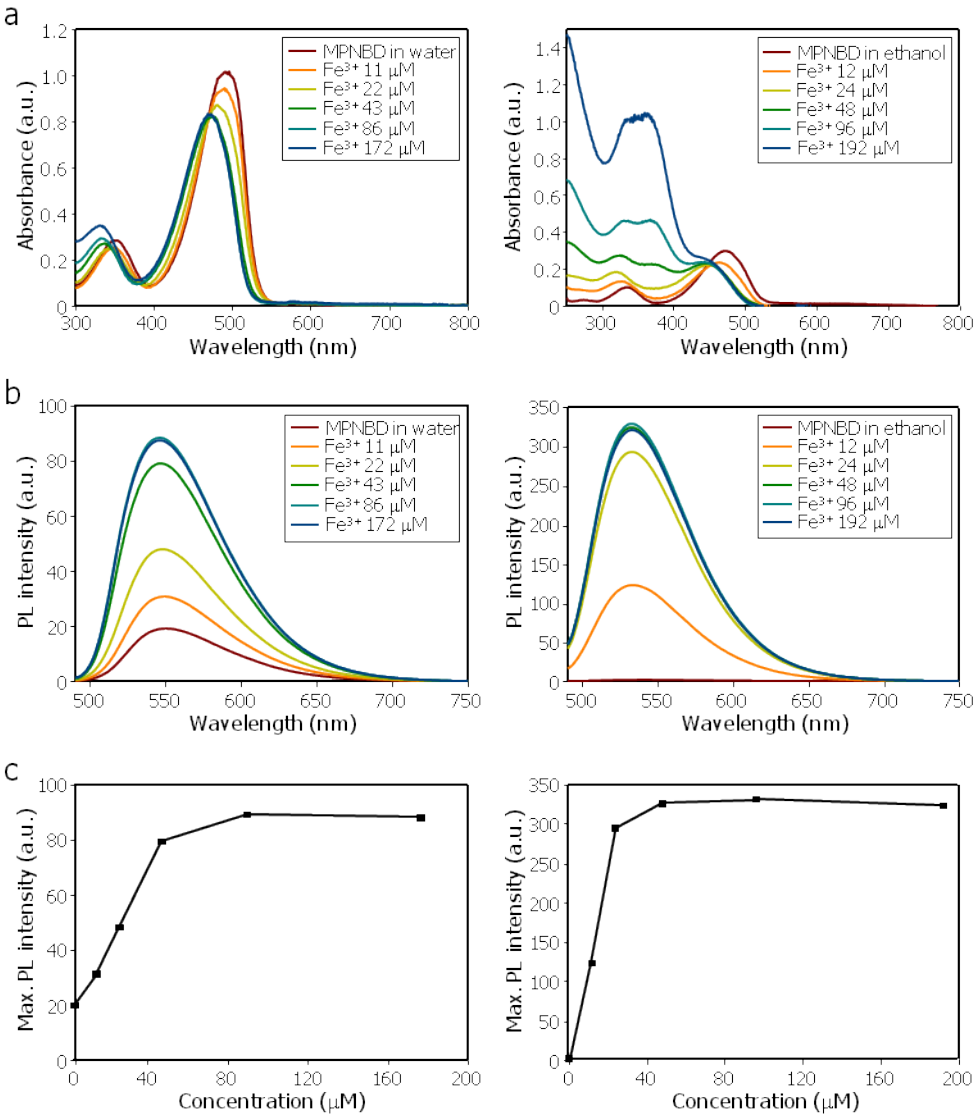


Figure S4. Comparison of fluorescence responses of MPNBD in water (42.2 μM) and ethanol (11.8 μM) to various amounts of Fe^{3+}.

(a) Changes in absorption spectra of MPNBD upon gradual addition of Fe^{3+} in water (left) and ethanol (right). (b) Changes in fluorescence spectra of MPNBD upon gradual addition of Fe^{3+} in water (left) and ethanol (right) under irradiation at 470 nm. (c) Fluorescence responses of MPNBD to different concentrations of Fe^{3+} in water (left) and ethanol (right). The excitation wavelength was 470 nm and the monitored maximum photoluminescence (PL) emission wavelengths were 548 nm (in water) and 533 nm (in ethanol), respectively, which are clearly shown in (b). a.u., arbitrary unit.

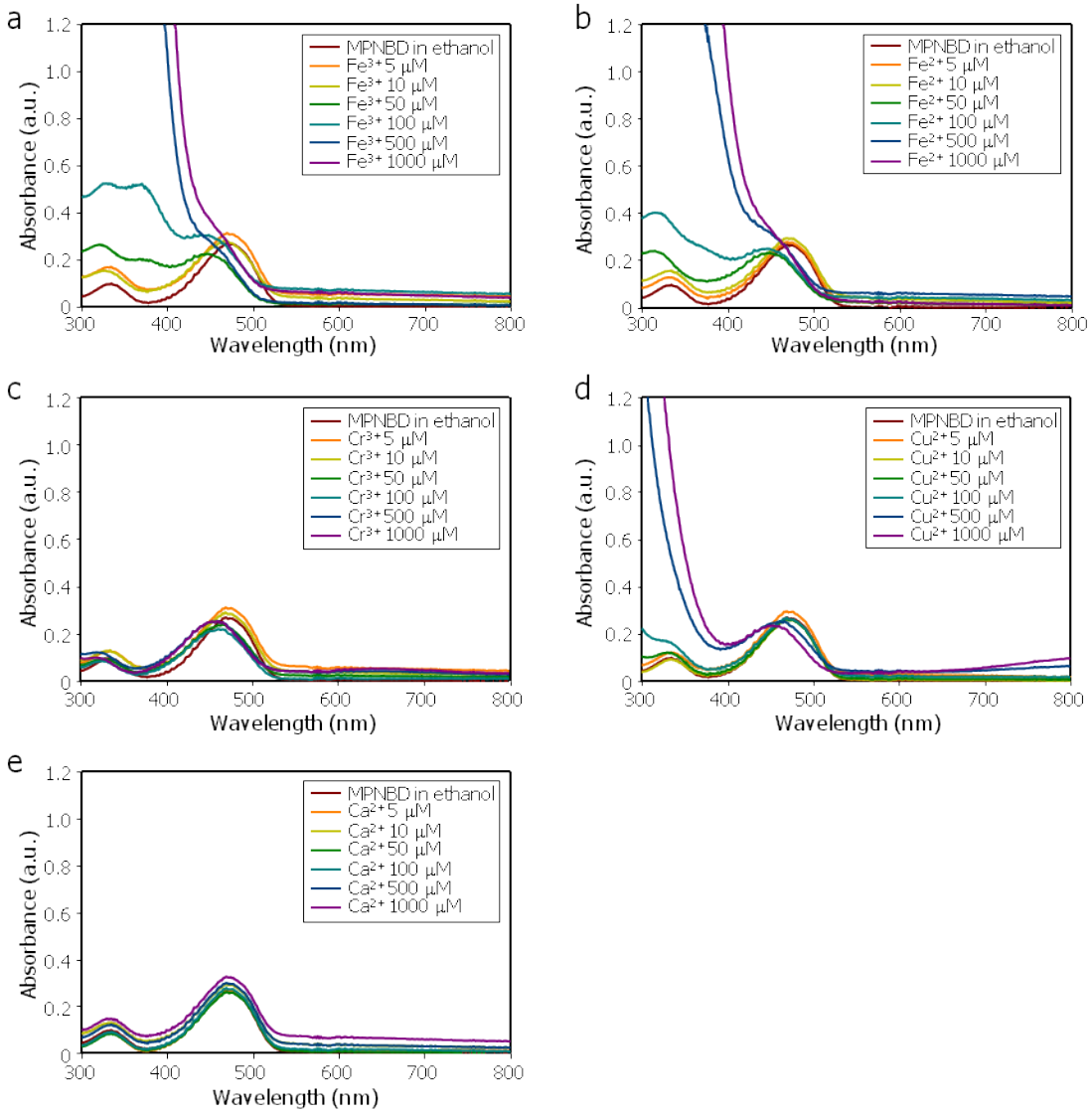


Figure S5. Absorption spectra of MPNBD (9.88 μM) in the presence of Fe^{3+}, Fe^{2+}, Cr^{3+}, Cu^{2+}, and Ca^{2+}. Changes of absorption spectra of MPNBD upon gradual addition (0 to 1000 μM) of Fe^{3+}(a), Fe^{2+}(b), Cr^{3+}(c), Cu^{2+}(d), and Ca^{2+}(e) in ethanol were examined. a.u., arbitrary unit.

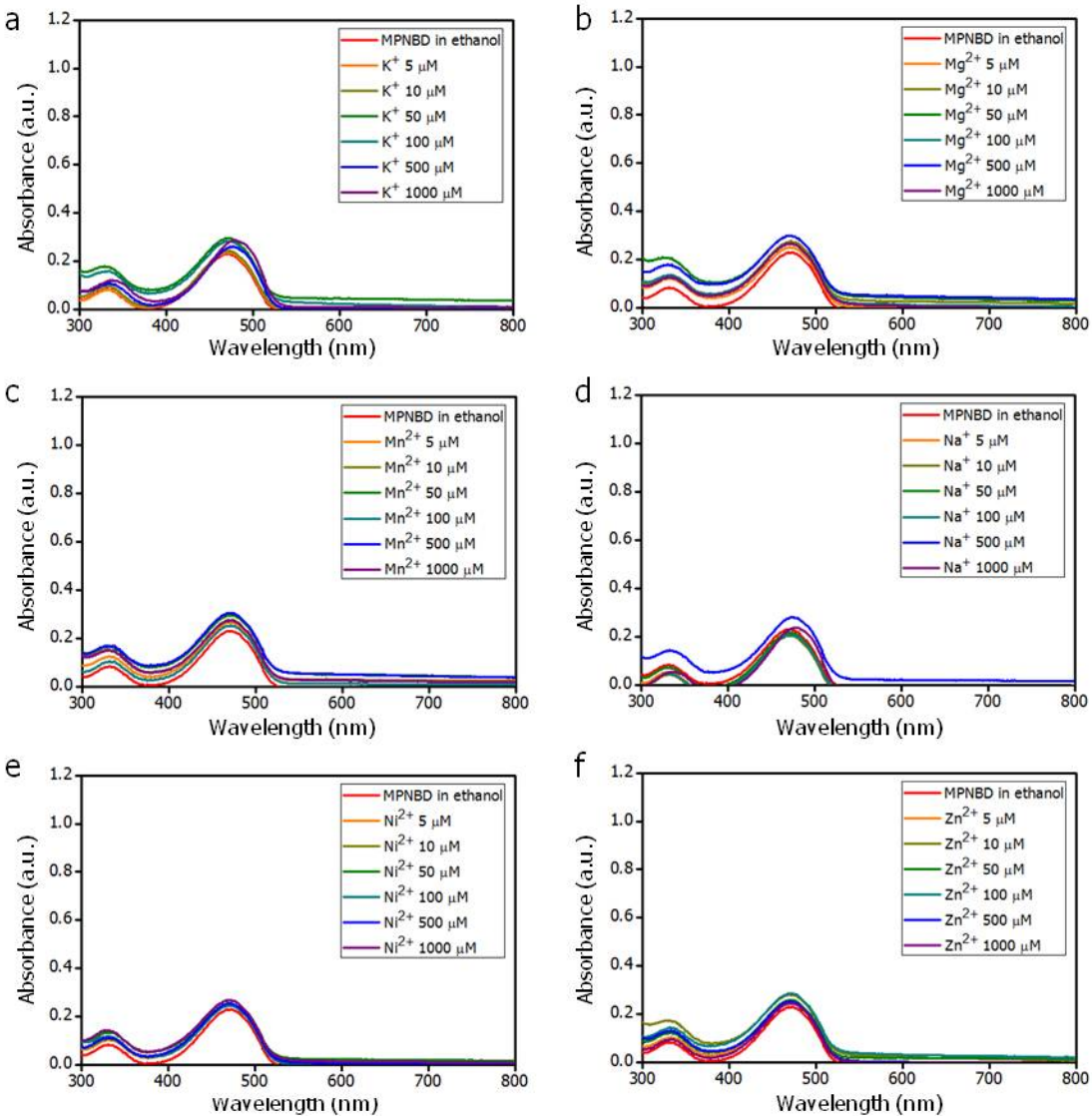


Figure S6. Absorption spectra of MPNBD (9.88 μM) in the presence of K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, and Zn^{2+}.

Changes of absorption spectra of MPNBD upon gradual addition (0 to 1000 μM) of K^+(a), Mg^{2+} (b), Mn^{2+} (c), Na^+ (d), Ni^{2+}(e), Zn^{2+}(f) in ethanol were examined. a.u., arbitrary unit.

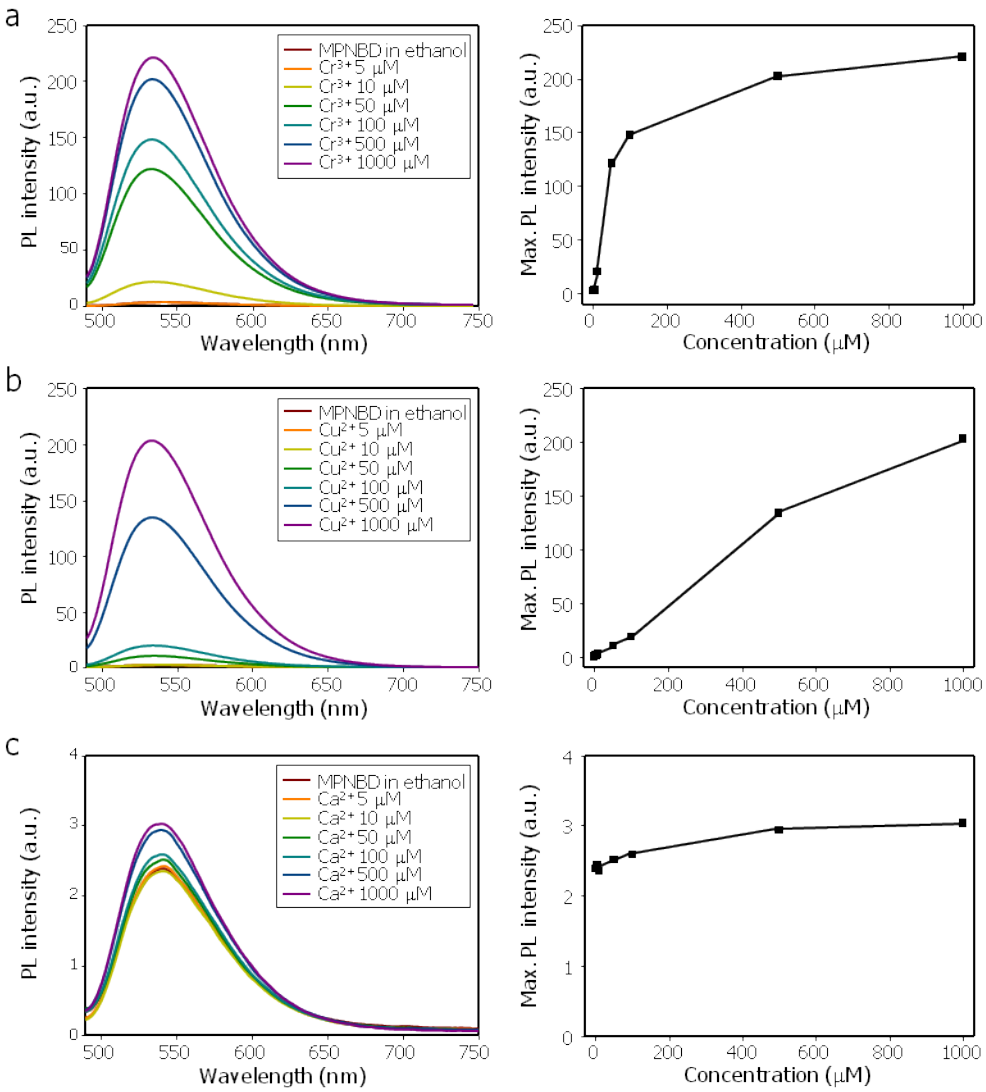
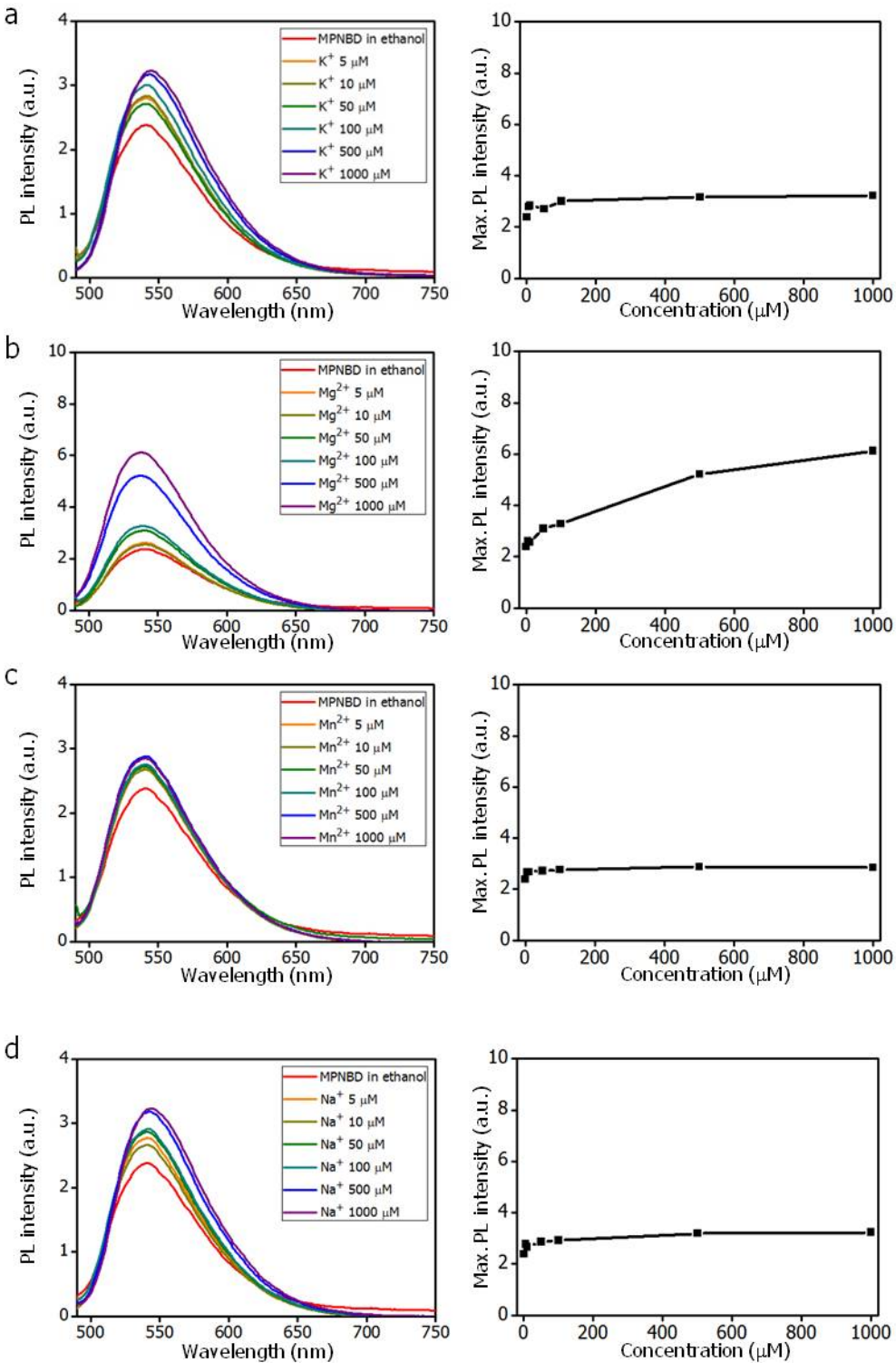


Figure S7. Fluorescence responses of MPNBD to Cr^{3}, Cu^{2+}, and Ca^{2+}.

Changes of fluorescence spectra and maximum emission intensity of MPNBD upon addition (0 to 1000 μM) of Cr^{3+}(a), Cu^{2+}(b) and Ca^{2+}(c) in ethanol were examined. The excitation wavelength was 470 nm and the monitored maximum photoluminescence (PL) emission wavelengths were 534 nm (Cr^{3+}, Cu^{2+}) and 540 nm (Ca^{2+}), respectively. a.u., arbitrary unit.


a
PL intensity (a.u.)
Wavelength (nm)
MPNBD in ethanol
K+ 5 μM
K+ 10 μM
K+ 50 μM
K+ 100 μM
K+ 500 μM
K+ 1000 μM
Max. PL intensity (a.u.)
Concentration (μM)
b
PL intensity (a.u.)
Wavelength (nm)
MPNBD in ethanol
Mg2+ 5 μM
Mg2+ 10 μM
Mg2+ 50 μM
Mg2+ 100 μM
Mg2+ 500 μM
Mg2+ 1000 μM
Max. PL intensity (a.u.)
Concentration (μM)
c
PL intensity (a.u.)
Wavelength (nm)
MPNBD in ethanol
Mn2+ 5 μM
Mn2+ 10 μM
Mn2+ 50 μM
Mn2+ 100 μM
Mn2+ 500 μM
Mn2+ 1000 μM
Max. PL intensity (a.u.)
Concentration (μM)
d
PL intensity (a.u.)
Wavelength (nm)
MPNBD in ethanol
Na+ 5 μM
Na+ 10 μM
Na+ 50 μM
Na+ 100 μM
Na+ 500 μM
Na+ 1000 μM
Max. PL intensity (a.u.)
Concentration (μM)

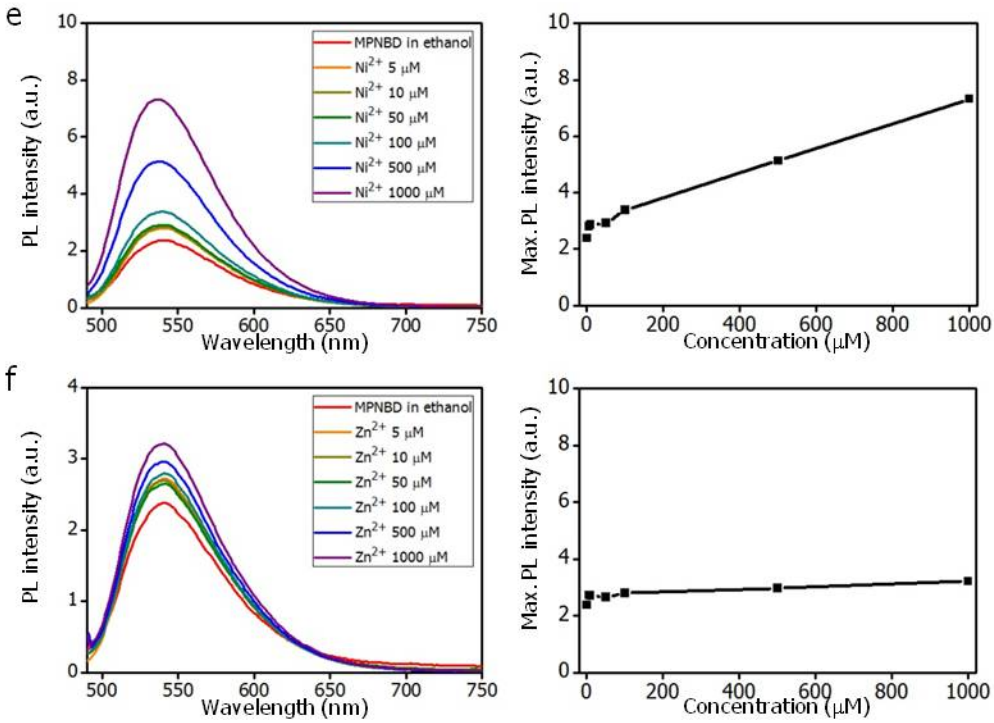


Figure S8. Fluorescence responses of MPNBD to K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, and Zn^{2+}.

Changes of fluorescence spectra and maximum emission intensities of MPNBD upon gradual addition (0 to 1000 μM) of K^+(a), Mg^{2+}(b), Mn^{2+}(c), Na^+(d), Ni^{2+}(e), and Zn^{2+}(f) in ethanol were examined. The excitation wavelength was 470 nm, and the monitored maximum photoluminescence (PL) emission wavelength was 541 nm. a.u., arbitrary unit.

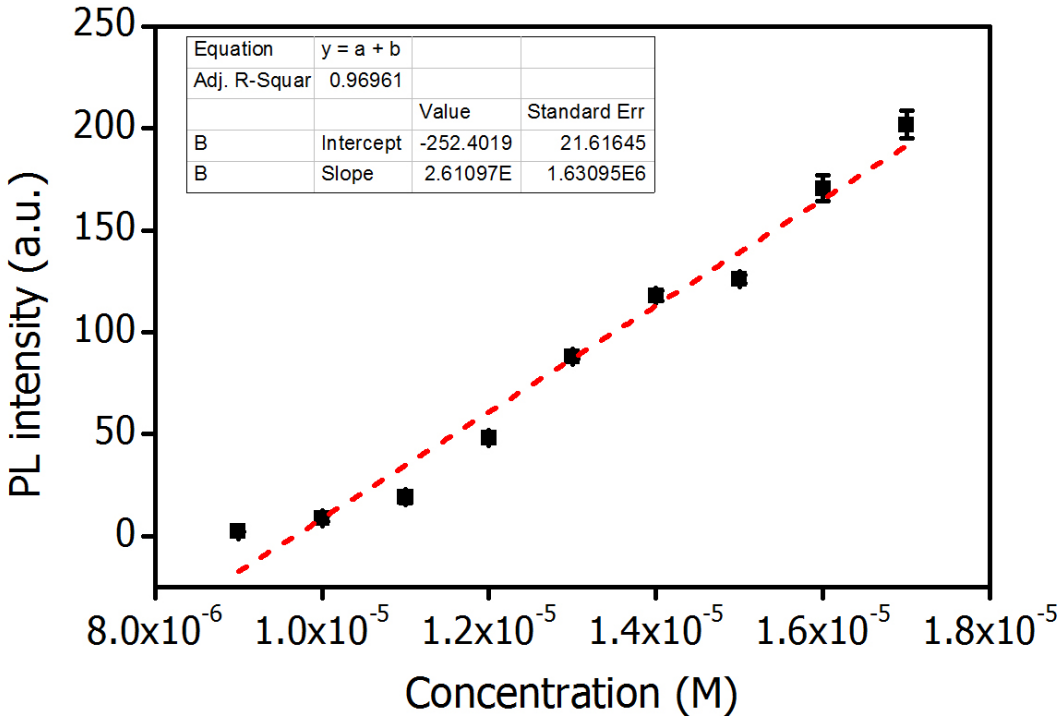


Figure S9. Calibration curve of MPNBD-Fe^{3+} in ethanol solution.

The excitation wavelength was 470 nm, and the monitored maximum photoluminescence (PL) emission wavelength was 533 nm. The detection limit (DL) of Fe^{3+} ions using MPNBD was determined from the following equation: $DL = K \times SD/S$, where K=3; SD is the standard deviation of the blank solution; S is the slope of the calibration curve. $DL = K \times SD/S = 3 \times 0.07032/2.61 \times 10^7 = 8.08 \times 10^{-6}$ M. The PL intensity was measured 3 times and averaged. Vertical bars indicate the standard error of the mean.

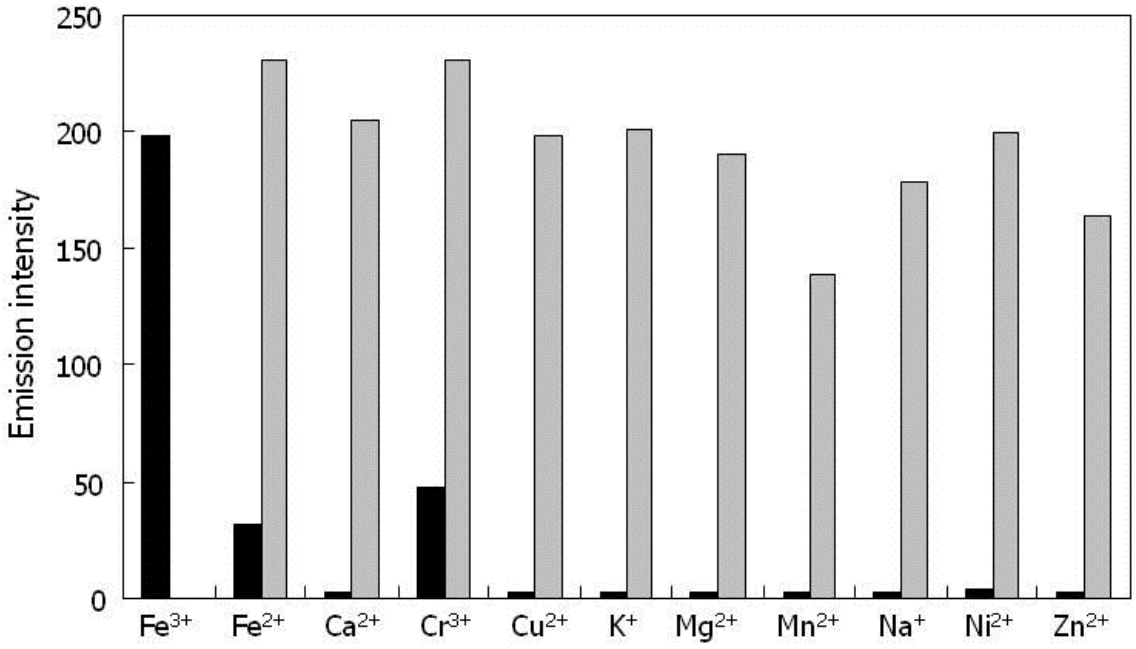


Figure S10. Fluorescence response of MPNBD to various metal ions and to mixtures of Fe^{3+} and other metal ions.

Fluorescence responses of MPNBD to Fe^{3+} (20 μM) or 20 μM of other metal ions (black bars) and to mixtures of Fe^{3+} (20 μM) and 20 μM of other metal ions (gray bars) in ethanol solution were compared.

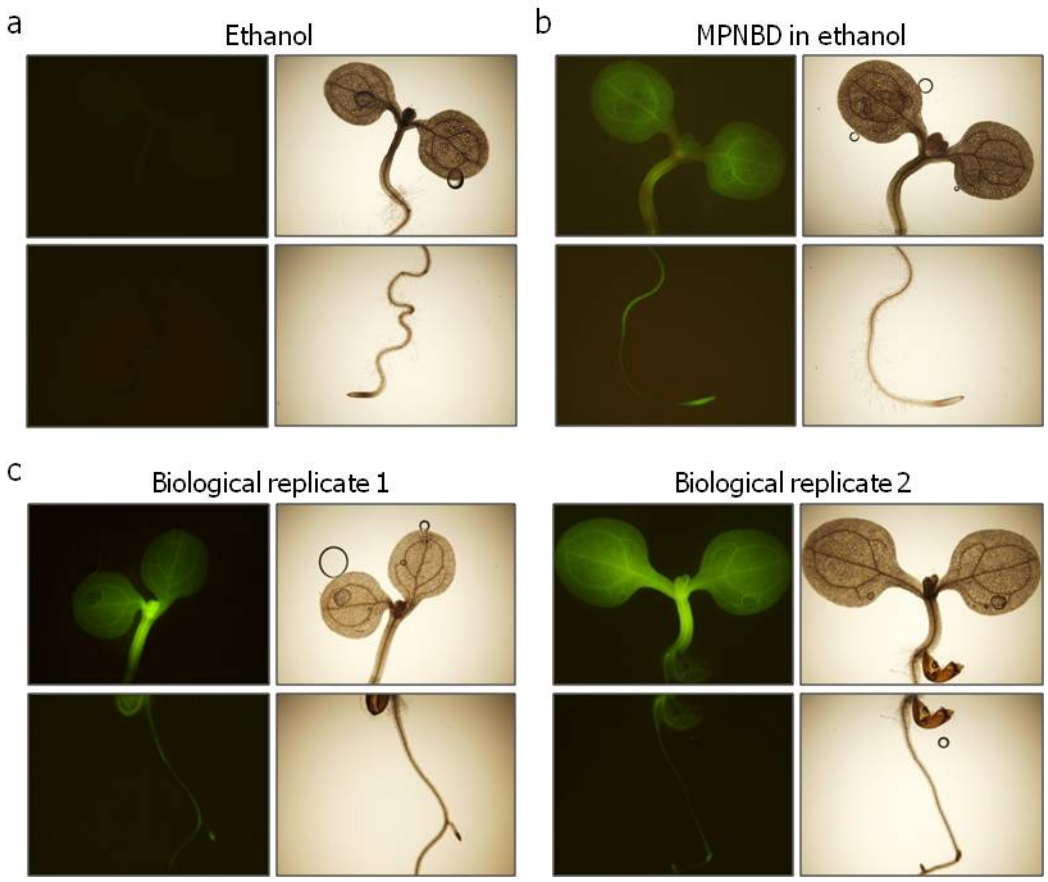


Figure S11. Images of MPNBD-treated plants.

Four-day-old whole *Arabidopsis* seedlings grown on ½ X Murashige & Skoog (MS) media containing 0.6% (w/v) agar (hereafter referred to as MS-agar plates) were treated with either ethanol (a) or 50 μM MPNBD in ethanol (b). The seedlings were visualized by optical and fluorescence microscopy. Fluorescence images are shown in the left panels and optical microscope images are shown in the right panels. Note that no fluorescent signals were detected in the ethanol-treated plants. To evaluate the reproducibility of the MPNBD-mediated fluorescent signals in plants, two additional plants (biological replicates 1 and 2) were treated with MPNBD independently under the identical conditions (c).

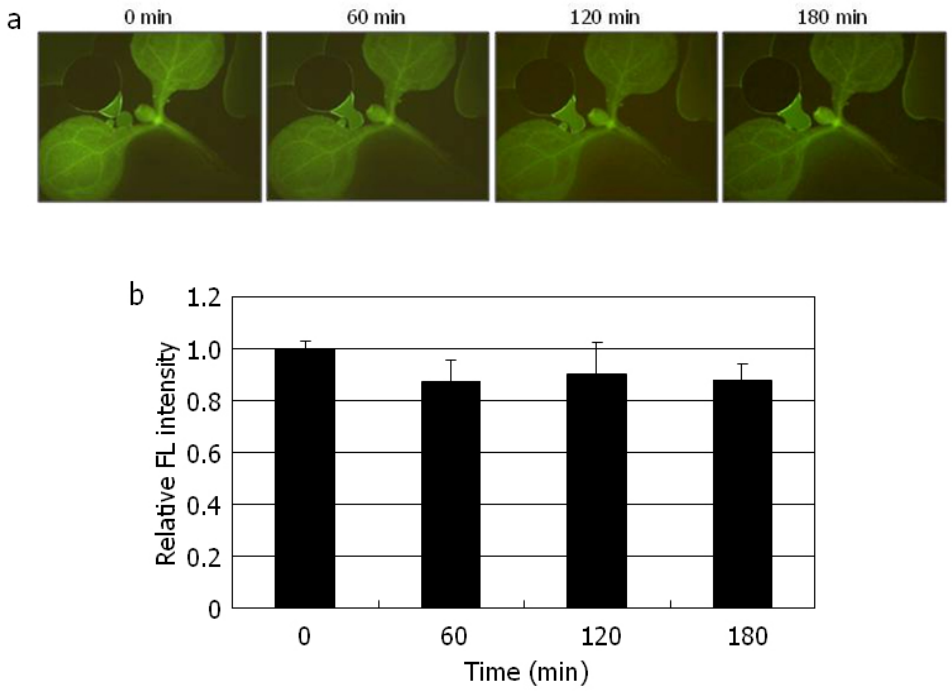


Figure S12. Stability of MPNBD fluorescent probe.

Four-day-old whole *Arabidopsis* seedlings grown on MS-agar plates were treated with 50 μM MPNBD in ethanol, as described in **Figure S11**. (a) Fluorescence images obtained at the indicated time points after MPNBD treatments. (b) Quantification of the data in (a). The fluorescence (FL) intensity was measured using the ImageJ software (http://rsbweb.nih.gov/ij/), as described previously.[2,3] Note that the fluorescent signals do not significantly diminish for up to 3 h.

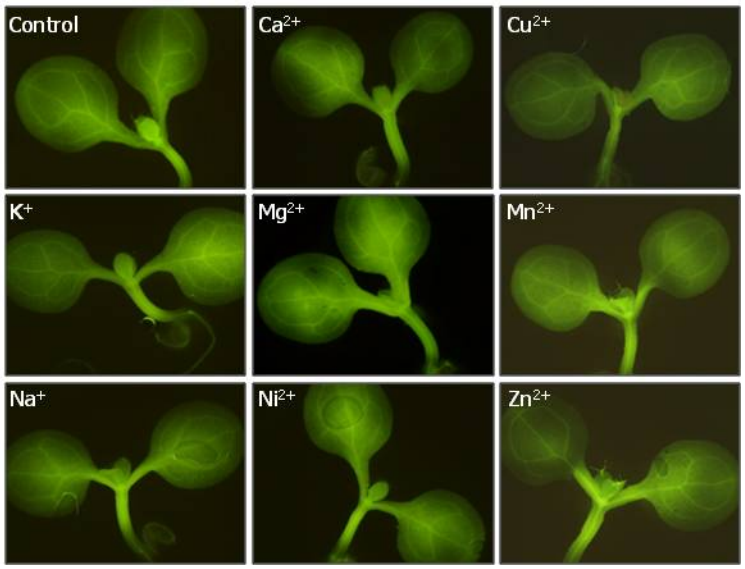


Figure S13. Effects of various metal ions on the MPNBD imaging of iron in plants.

Four-day-old whole *Arabidopsis* seedlings grown on MS-agar plates were treated with 500 μM of each metal ion for 12 h and treated with 50 μM MPNBD in ethanol, as described in **Figure S11**. Note that no significant changes in fluorescent signals were detected in the presence of other metal ions.

Table S1. Procedures of different iron detection methods in plants.

Perls	**Perls/DAB**	**MPNBD**
1. Vacuum infiltration for 15 min in 4% HCl/4% $K_4Fe(CN)_6$. 2. Incubation at room temperature for 30 min. 3. Wash with deionized water.	1. Vacuum infiltration for 15 min in 4% HCl/4% $K_4Fe(CN)_6$. 2. Incubation at room temperature for 30 min. 3. Wash with deionized water. 4. Incubation for 1 h in 0.01M NaN_3 / 0.3% H_2O_2 in methanol. 5. Wash with 0.1 M phosphate buffer (pH 7.4). 6. Incubation for 30 min in 0.025% DAB, 0.005% H_2O_2, 0.005% $CoCl_2$ in 0.1M phosphate buffer (pH 7.4). 7. Wash with deionized water.	1. Wash with ethanol. 2. Vacuum infiltration for 5 min in 50 μM MBNBD in ethanol. 3. Incubation at room temperature for 10 min. 4. Wash with ethanol.
~ 1 h	~ 3h	< 20 min
Poor staining in the leaves Low sensitivity (Detection limit : 375 μM)	Moderate sensitivity (Detection limit : 100 μM) Longer time	High resolution High sensitivity (Detection limit : 8.08 μM) Fast and cheap
Ref S4 (Roschztterardtz *et al*., 2009)	Ref S4 (Roschztterardtz *et al*., 2009) Ref S5 (Meguro *et al*., 2007)	This work

References

S1 Y. Xiao and X. Qian, *Tetrahedron Lett*., 2003, **44**, 2087.

S2 M.R. Mancuso, R. Davis, S.M. Norberg, S. O'Brien, B. Sennino, T. Nakahara, V.J. Yao, T. Inai, P. Brooks, B. Freimark, D.R. Shalinsky, D.D. Hu-Lowe and D.M. McDonald, *J. Clin*. *Invest*., **116**, 2610.

S3 T. Hirayama, K. Okuda and H. Nagasawa, *Chem*. *Sci*., 2013, **4**, 1250.

S4 H. Roschzttardtz, G. Conéjéro, C. Curie and S. Mari, *Plant Physiol*., 2009, **151**, 1329.

S5 R. Meguro, Y. Asano, S. Odagiri, C. Li, H. Iwatsuki and K. Shoumura, *Arch*. *Histol*. *Cytol*., 2007, **70**, 1.