Electronic supplementary information

A Turn-On Fluorescent Probe Based on Hydroxylamine Oxidation for Detecting Ferric Ion Selectively in Living Cells

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1. Experimental Section

Instruments: Fluorescence spectra were obtained by FluoroMax-4 Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on NANO Drop 2000c UV-visible spectrophotometer (Thermo Fisher Scientific). ^{1}H NMR, ^{13}C NMR spectra were taken on a Bruker spectrometer. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). Determination of organic elements was obtained with Model PE-2400(II) element analyzer. The fluorescence images of cells were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×20, ×40).

Materials: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and HEPES were purchased from Sigma-Aldrich. Human breast adenocarcinoma cells (MCF-7) were obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). BOD-NHOH (0.10 mM) was prepared in DMSO and stored at 4 ^{o}C in darkness. All other reagents and chemicals were from commercial sources and of analytical reagent grade, and used without further purification. Solvents used for spectroscopic studies were purified and dried by standard procedures before use. The salts used in stock solutions of metal ions were NaCl, KCl, $CaCl_2$, $MgCl_2·6H_2O$, $ZnSO_4·7H_2O$, $PbCl_2$, $MnCl_2$, $HgCl_2$, $CoCl_2·2H_2O$, $NiCl_2·6H_2O$, $CdCl_2·2.5H_2O$, $FeCl_3·6H_2O$, $FeCl_2·4H_2O$, $AgNO_3$, $CuSO_4·5H_2O$, $Fe(ClO_4)_3$ and $NaClO_4$. The probe was oxidized by $Fe(ClO_4)_3$ in investigation of spectral properties, while $FeCl_3$ was used in cell experiments. Ultrapure water (Millipore, Bedford, MA, USA) was used throughout.

Absorption Analysis: Absorption spectra were obtained with 1.0-cm glass cells. The probe (DMSO, 0.1 mL, 0.1 mM) was added to a 10.0-mL color comparison tube. After dilution to 1.0 μM with 40 mM HEPES buffer, Fe^{3+} was added. The mixture was equilibrated for 5 min before measurement.

Fluorescence Analysis: Fluorescence spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The probe (DMSO, 0.1 mL, 0.1 mM) was added to a 10.0-mL color comparison tube. After dilution to 1.0 μM with 40 mM HEPES buffer, Fe^{3+} was added. The mixture was equilibrated for 5 min before measurement.

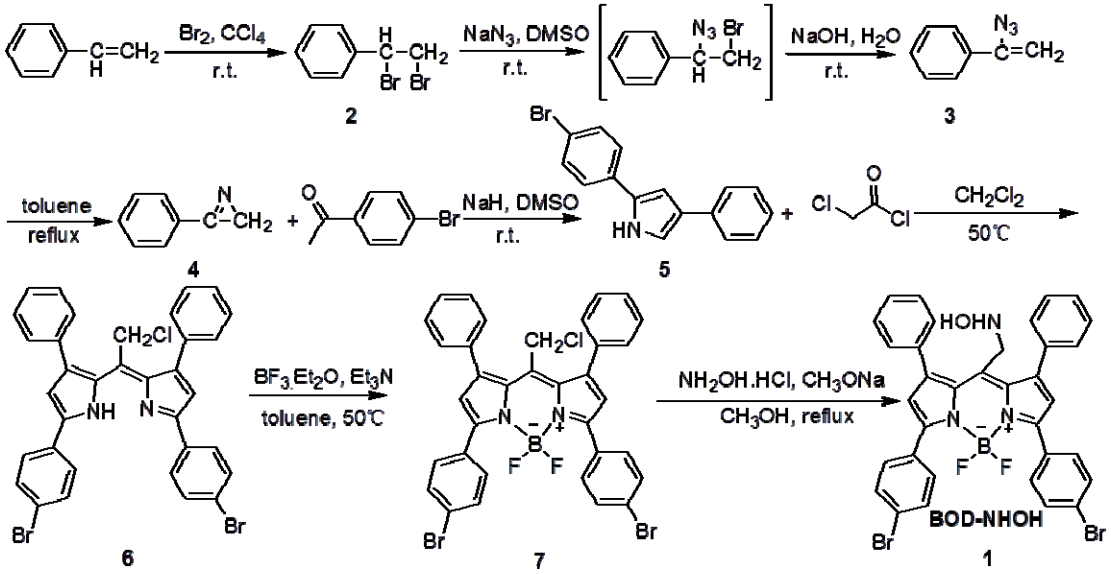
Confocal Imaging: Fluorescent images were acquired on an Olympus Fluo View FV1000 confocal laser-scanning microscope (Japan) with an objective lens (×20, ×40). The excitation wavelength was 559 nm. Cell imaging was carried out after being washed with physiological saline for three times.

Cell Culture: MCF-7 cells were obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum (FBS) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2.

2. Synthesis and Characterization of Compounds

The ^{1}H and ^{13}C chemical shifts are given in parts per million relative to those of Me_4Si, internal CD_3SOCD_3-D6 in the solvent.

Synthesis of the Probe:



Synthesis of 1-(1, 2-dibromoethyl) benzene (2): Bromine (8.0 g, 0.05 mol) in 30 mL CCl_4 was added slowly to a stirred and cooled (15-20 ^{o}C) solution of styrene (5.2 g, 0.05 mol) in 40 mL of CCl_4. After the addition was completed, the mixture was stirred for 2 h in 15-20 ^{o}C, and then the CCl_4 was removed *in vacuo*, remaining the residue of crystalline (12.2 g, 92.4%). ^{1}H NMR (500 MHz, CD_3SOCD_3-D6) δ (ppm): 3.99-4.11 (m, 2 H), 5.13-5.18 (m, 1 H), 7.30-7.39 (m, 5 H). LC-MS (API-ES): *m/z* $C_8H_8Br_2$, calcd 263.8972, found [M-Br^{+}]

182.9008. Elemental Analysis: Calc. for $C_8H_8Br_2$: C, 36.40; H, 3.05; Br, 60.54. Found: C, 36.39; H, 3.06, Br, 60.54%.

Synthesis of 2-phenyl-aza-methylcyclopropene (4): 1-(1,2-dibromoethyl) benzene (12.2 g, 46 mmol) was dissolved in 70 mL of dimethyl sulfoxide. A slow stream of N_2 was passed through the apparatus, sodium azide (4.9 g, 75 mmol) was slowly added into the solution and for 45 min afterward. The mixture became thick with precipitated azido bromide and was stirred for a further 13 h at 25 ^oC. The reaction mixture was treated with 2.0 g (50 mmol) of sodium hydroxide in 2.0 mL of deionized water. Stirring was continued at 25 ^oC for 24 h. The mixture was poured into 200 mL of 2% sodium bicarbonate aqueous solution and extracted with CH_2Cl_2. The extracts were washed with deionized water, and the CH_2Cl_2 was removed *in vacuo*, and evaporated to yield crude 1-azidostyrene as red oil. The oil was passed through a column of silicon dioxide using petroleum ether as an eluent. The eluent was removed *in vacuo* and the residual pale yellow oil was dissolved in 100 mL of toluene. The solution refluxed for 4 h. Removal of the solvent and distillation of the crude product, afforded 3.6 g (61%) of 2-phenyl-aza-methylcyclopropene.[1] ^1H NMR (500 MHz, CD_3SOCD_3-D6) δ (ppm): 1.70-1.74 (s, 2H), 7.65-7.73 (m, 3H), 7.87-7.93 (m, 2 H). LC-MS (API-ES): *m/z* C_8H_7N, calcd 117.0578, found [M^+] 117.0561. Elemental Analysis: Calc. for C_8H_7N: C, 82.02; H, 6.02; N, 11.96. Found: C, 82.02; H, 6.01; N, 11.97%.

Synthesis of 2-(4-bromophenyl)-4-phenyl-1H-pyrrole (5): *p*-Bromoacetophenone (4.0 g, 20 mmol), 2-phenyl-aza-methylcyclopropene (2.3 g, 15.8 mmol) and 60% sodium hydride (0.8 g, 20 mmol) were dissolved in 25 mL of DMSO, after which the mixture was stirred for 6 h at room temperature. It was then poured into 500 mL of ice water, and the pale yellow crystals were immediately separated out and filtered, which were repeatedly washed with water, and obtained 3.7 g (62.3%) pale yellow 2-(4-bromophenyl)-4-phenyl-1H- pyrrole.[2] ^1H NMR (500 MHz, CD_3SOCD_3-D6) δ (ppm): 7.01 (s, 1H), 7.10-7.15 (m, 2H), 7.30-7.35 (m, 5H); 7.55-7.65 (m, 4H), 11.50 (s, 1H, -NH). LC-MS (API-ES): *m/z* $C_{16}H_{12}BrN$, calcd 297.0153, found [M-H^+] 296.0245. Elemental Analysis: Calc. for $C_{16}H_{12}BrN$: C, 64.45; H, 4.06; Br, 26.80; N, 4.70 Found: C, 65.43; H, 4.07; Br, 26.80; N, 4.71%.

Synthesis of 8-chloromethyl-4,4-difluoro-1,7-diphenyl-3,5- di(1-bromophenyl)

-4-bora-3a,4a-diaza-*s*-indacene (BOD-CH_2Cl, 7): 0.91 g of chloro-acetyl chloride and 3.55 g of 2-(4-bromophenyl)-4-phenyl-1H-pyrrole were dissolved in 100 mL of CH_2Cl_2 purged with N_2. The mixture was stirred for 4 h at 50 ^oC. A blue-violet product could be observed when the solvent was evaporated, which was dissolved in 10 mL of CH_2Cl_2 without purification. To the solution added 190 mL of toluene was under N_2 atmosphere, and added 10 mL of CH_2Cl_2 and 5.4 mL triethylamine. The mixture was stirred for 30 min at room temperature, and 15 mL of BF_3-OEt_2 was added dropwise through constant pressure dropping funnel and for 15 min afterward. Next, the reaction was heated at 50 ^oC for 5-6 h. The mixture was extracted with CH_2Cl_2, and the organic layer was crude product of BOD-CH_2Cl. The crude product was washed with water and evaporated to dryness, and a deep red viscous product was observed. Purification by column chromatography on silica eluting with CH_2Cl_2/petroleum ether (5:4, v/v) gave the product BOD-CH_2Cl as a purple solid (0.15 g, 22.4%).[3] ^1H NMR (500 MHz, CD_3SOCD_3-D6) δ (ppm): 6.78-7.87 (m, 18H), 5.76 (s, 1H), 6.09 (s, 1H), 3.35-3.39 (t, 2H). LC-MS (API-ES): *m/z* $C_{34}H_{22}BBr_2ClF_2N_2$, calcd 701.9879, found [M-Cl^+] 667.0189 Elemental Analysis: Calc. for $C_{34}H_{22}BBr_2ClF_2N_2$: C, 58.12; H, 3.16; B, 1.54; Br, 22.74; Cl, 5.05; F, 5.41; N, 3.99 Found: C, 58.11; H, 3.16; B, 1.53; Br, 22.75; Cl, 5.04; F, 5.42; N, 4.00%.

Synthesis of 8- hydroxyethanamine-4,4-difluoro-1,7-diphenyl-3,5- di(1-bro mophenyl)-4-bora-3a,4a-diaza-s-indacene (BOD-NHOH, 1): 0.14 g BOD-CH_2Cl, 0.14 g hydroxylamine hydrochloride and 0.28 g anhydrous sodium methoxide were dissolved in 50 mL of methanol, and the mixture was stirred at reflux temperature for 24 h under N_2 atmosphere. After the reaction, anhydrous hydrogen chloride was bubbled. The solvent was evaporated, and the crude product of BOD-NHOH was purified by column chromatography over silica gel with pure CH_2Cl_2 to afford a brown powder of 0.06 g (42.9%).[4, 5, 6] ^1H NMR (500 MHz, CD_3SOCD_3-D6) δ(ppm): 10.09 (s, 1H), 8.03-6.96 (m, 18H), 6.70 (s, 1H), 6.60 (s, 1H), 4.25 (s, 1H), 3.62 (s, 1H), 2.36 (s, 2H). ^{13}C NMR (CD_3SOCD_3-D6, 100 MHz) δ (ppm): 132.84, 129.32, 129.08, 128.86, 128.68, 127.98, 126.72, 126.33, 125.78, 112.90, 108.23, 104.92, 42.85. LC-MS (API-ES): *m/z* $C_{34}H_{24}BBr_2F_2N_3O$, calcd 699.0327, found [M+H^+] 700.0402. Elemental Analysis: Calc. for

$C_{34}H_{25}BBr_2ClF_2N_3O$: C, 55.51; H, 3.43; B, 1.47; Br, 21.72; Cl, 4.82; F, 5.17; N, 5.71; O, 2.17. Found: C, 55.50; H, 3.42; B, 1.48; Br, 21.71; Cl, 4.83; F, 5.18; N, 5.70; O, 2.18%.

Characterization of 8-methyl-4,4-difluoro-1,7-diphen yl-3,5-di(1-bromophen yl)-4-bora-3a, 4a-diaza-s-indacene (BOD-CH_3): BOD-NHOH (50 mg, 70 μmol) was dissolved in 0.5 mL methanol. Subsequently, 10 equiv. of $Fe(ClO_4)_3$ was added. The mixture was equilibrated for 20 min. The mixture was concentrated to dryness under reduced pressure. The residue was purified by column chromatography over silica gel with pure CH_2Cl_2 to afford a brown powder of 0.41 mg (82%). LC-MS (API-ES): *m/z* $C_{34}H_{23}BBr_2F_2N_2$, calcd 668.0269, found [M+Na^+] 691.0164. Elemental Analysis: Calc. for $C_{34}H_{23}BBr_2F_2N_2$: C, 61.12; H, 3.47; B, 1.62; Br, 23.92; F, 5.69; N, 4.19. Found: C, 61.11; H, 3.48; B, 1.61; Br, 23.93; F, 5.68; N, 4.20%

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[3] M. Baruah, W. Qin, N. Basarić, W. M. De Borggraeve, N. Boens, *J. Org. Chem.*, 2005, **70**, 4152.

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[5] H. C. J. Ottenheijm, J. D. M. Herscheid, *Chem. Rev.*, 1986, **86**, 697.

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3. Effect of perchlorate ion

The probe was oxidized by iron triperchlorate in vitro experiments. To confirm verify whether there is fluorescence response to perchlorate ion, selectivity studies for perchlorate salts such as sodium perchlorate ($NaClO_4$), iron triperchlorate ($Fe(ClO_4)_3$) were carried out.

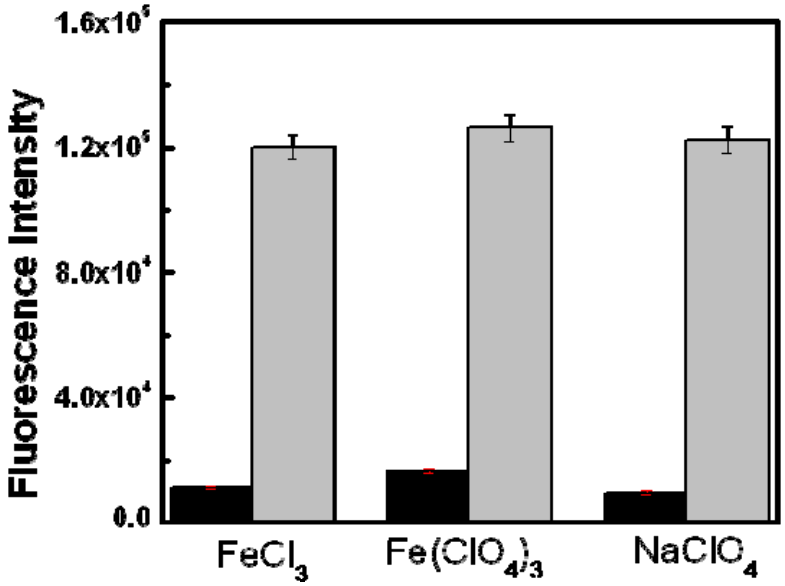

Fig. S1 Fluorescence responses (λ_{ex}=580 nm, λ_{em}= 610 nm) of 1.0 μM probe to perchlorate ions in 40 mM HEPES (pH 7.0): $FeCl_3$ (50.0 μM); $Fe(ClO_4)_3$ (0.2 mM); $NaClO_4$ (0.3 mM). In each group, the black bar represents the fluorescence intensity after addition of analytes, and the gray bar represents that after the subsequent addition of 50.0 μM Fe^{3+}.

4. Effect of pH Values

Standard fluorescence pH titrations were performed in the solution at probe concentration of 1.0 μM and Fe^{3+} concentration of 50 μM. As is shown in Figure S1, $\Delta F = F - F_0$, where F_0 is fluorescence intensity of the probe BOD-NHOH, and F is fluorescence intensity of BOD-CH_3.

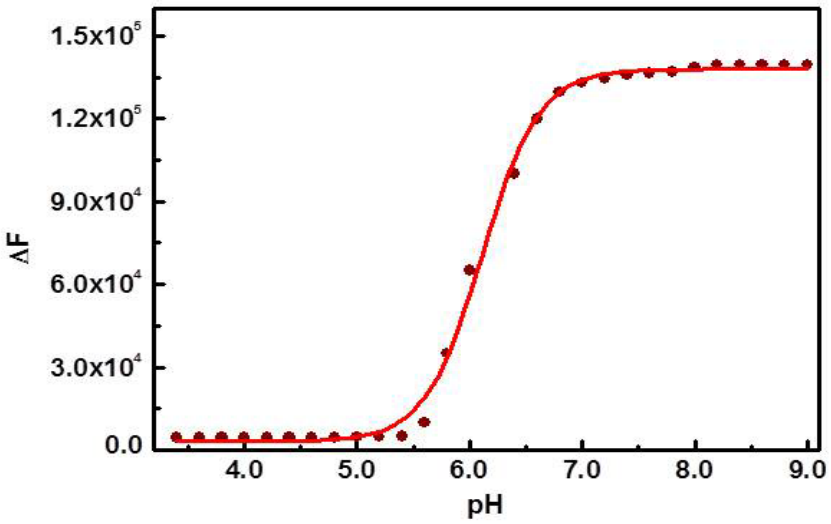

Fig. S2 The effect of pH value on the fluorescence intensity of probe (1.0 μM) in HEPES. pH

values: 4.0, 4.4, 4.8, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 9.0 (40 mM HEPES buffer solution).

5. Effect of hydrolysis of ferric ion

Indeed, for Fe^{3+}, generally there are complex hydrolysis phenomena in aqueous medium. The hydrolysis has been systematically investigated,[7] such as in the literature of "Hydrolysis of inorganic iron(III) salts". According to the above literature, in low Fe^{3+} concentration, it is universally existent in various hydrate forms; in high concentration, precipitate can be formed. On the other hand, the redox potential will become higher after being complexed, from 0.771 V going up to 0.900 V [8], i.e. $Fe^{3+} + e^- \rightleftharpoons Fe^{2+}$ $E^{\Theta}=0.771$ V ; $FeOH^{2+} + H^+ + e^- \rightleftharpoons Fe^{2+} + H_2O$ $E^{\Theta}=0.900$ V. This will facilitate our redox probe. So, the pH=7 condition is suitable for the probe.

The present test media of pH 7.0 with satisfactory imaging results is a very good validation for living cells screen and imaging of the developed probe. The living MCF-7 cells exhibited exciting observations (Fig. 3 and 5) during the experimenting.

According to the response above, whether the Fe^{3+} is free cation or hydrate, the developed turn-on fluorescent probe will be effective. The probe based on redox, far stronger than complexation, precipitation, or neutralization, has demonstrated its significant advantages that hydroxylamine can be easily oxidized by Fe^{3+} while other commonly coexistent metal ions almost have no interference.

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6. MTT Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: Cytotoxicity in vitro was measured by using the methyl thiazolyl tetrazolium (MTT) assay in MCF-7 cells. Cells were seeded into 96-well cell culture plate at 4000 /well, cultured at 37 ^{o}C and 5% CO_2 for 48 h, and then different concentrations of chemosensor BOD-NHOH (0, 10^{-3},

10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M) were added to the wells. The cells were then incubated for 48 h at 37 ^{o}C under 5% CO_2. Subsequently, 20 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 ^{o}C under 5% CO_2. Cells were lysed in triple liquid (10% SDS, 0.012 M HCl, 5% isopropanol), and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (Tecan, Austria).

Calculation of IC_{50} values was done according to Huber and Koella.[9] The following formula was used to calculate the inhibition of cell growth: Cell viability (%) = (mean of Abs. value of treatment group / mean Abs. value of control) • 100%.

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7. Fluorescence Response of the Probe to Excess Fe^{3+}

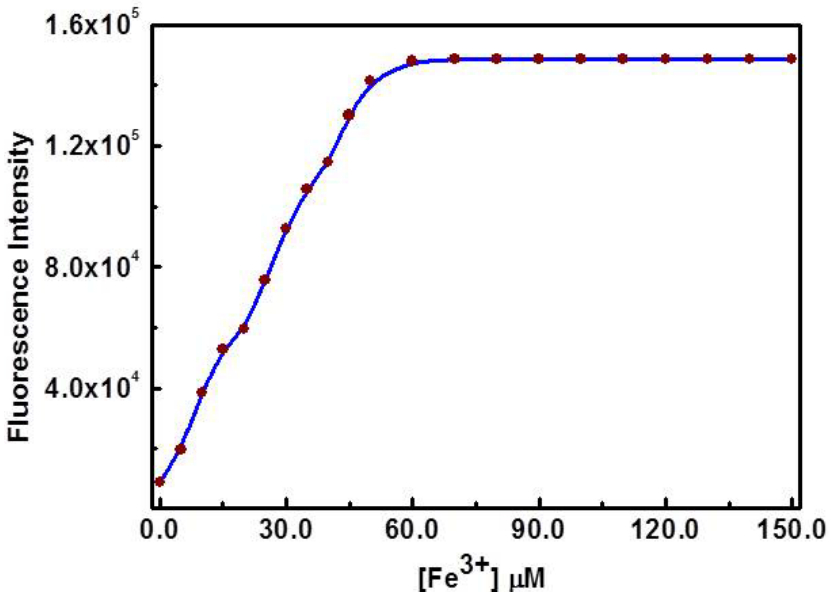

Fig. S3 Fluorescence response of BOD-NHOH (1.0 μM) towards Fe^{3+}: 0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 60.0, 70.0, 80.0, 90.0, 100.0, 110.0, 120.0, 130.0, 140.0, 150.0 μM. The fluorescence intensity was acquired in 40 mM HEPES (pH 7.0), with emission at 615 nm.

8. Bright-Field Images of Fig. 3

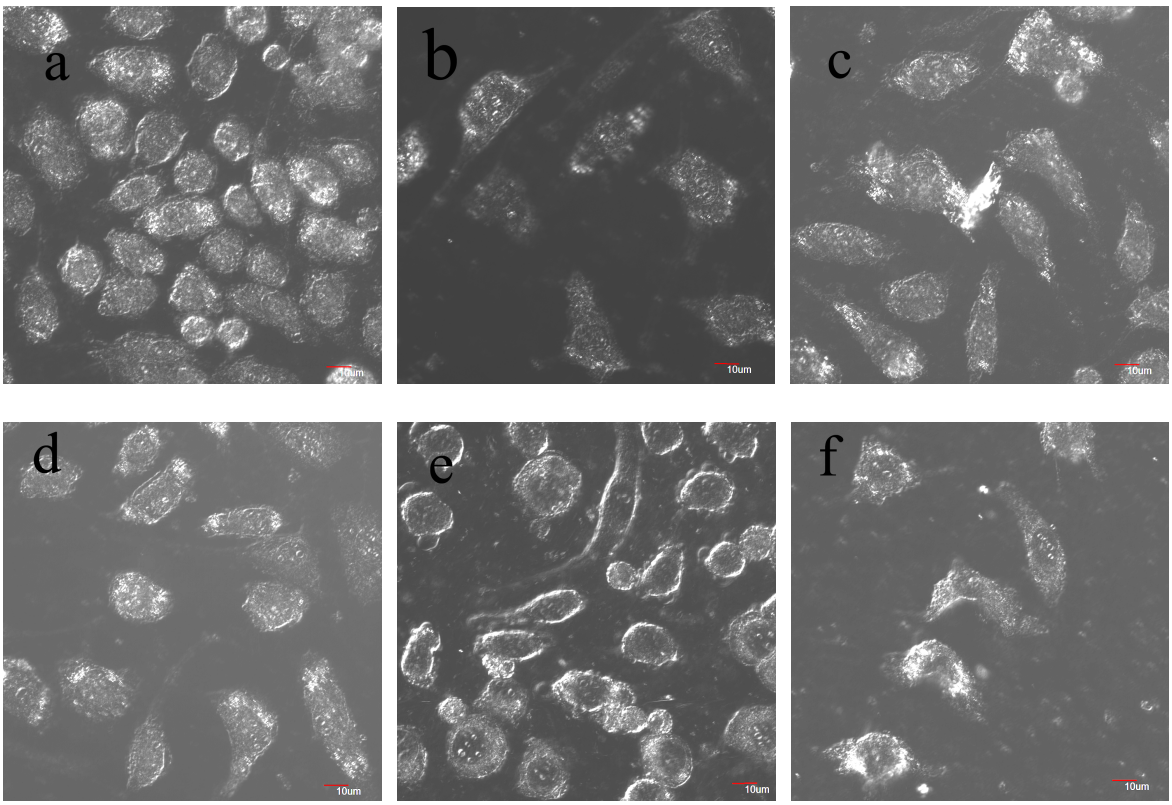

Fig. S4 Bright-field images of Fig. 3

9. References

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