Supplementary information

Two photon excited fluorescent probes for calcium based on internal charge transfer

Xiaohu Dong[a], Yiyao Yang[a], Jian Sun[b], Zhihong Liu[*a,c] and Bi-Feng Liu[*b]

[a] College of Chemistry & Molecular Sciences, Wuhan University, Wuhan 430072 (China). Fax: 86-27-68754067; Tel: 86-27-87218754; E-mail: zhhliu.whu@163.com

[b] The Key Laboratory of Biomedical Photonics of MOE - Hubei Bioinformatics & Molecular Imaging Key Laboratory – Division of Biomedical Photonics at Wuhan National Laboratory for Optoelectronics, Department of Systems Biology, College of Life Science & Technology, Huazhong Univ Sci & Technol, Wuhan 430074 (China). Fax: 86-27-87792170; Tel: 86-27-87792203; E-mail: bfliu@mail.hust.edu.cn

[c] Key Laboratory of Analytical Chemistry for Biology and Medicine (Wuhan University), Ministry of Education

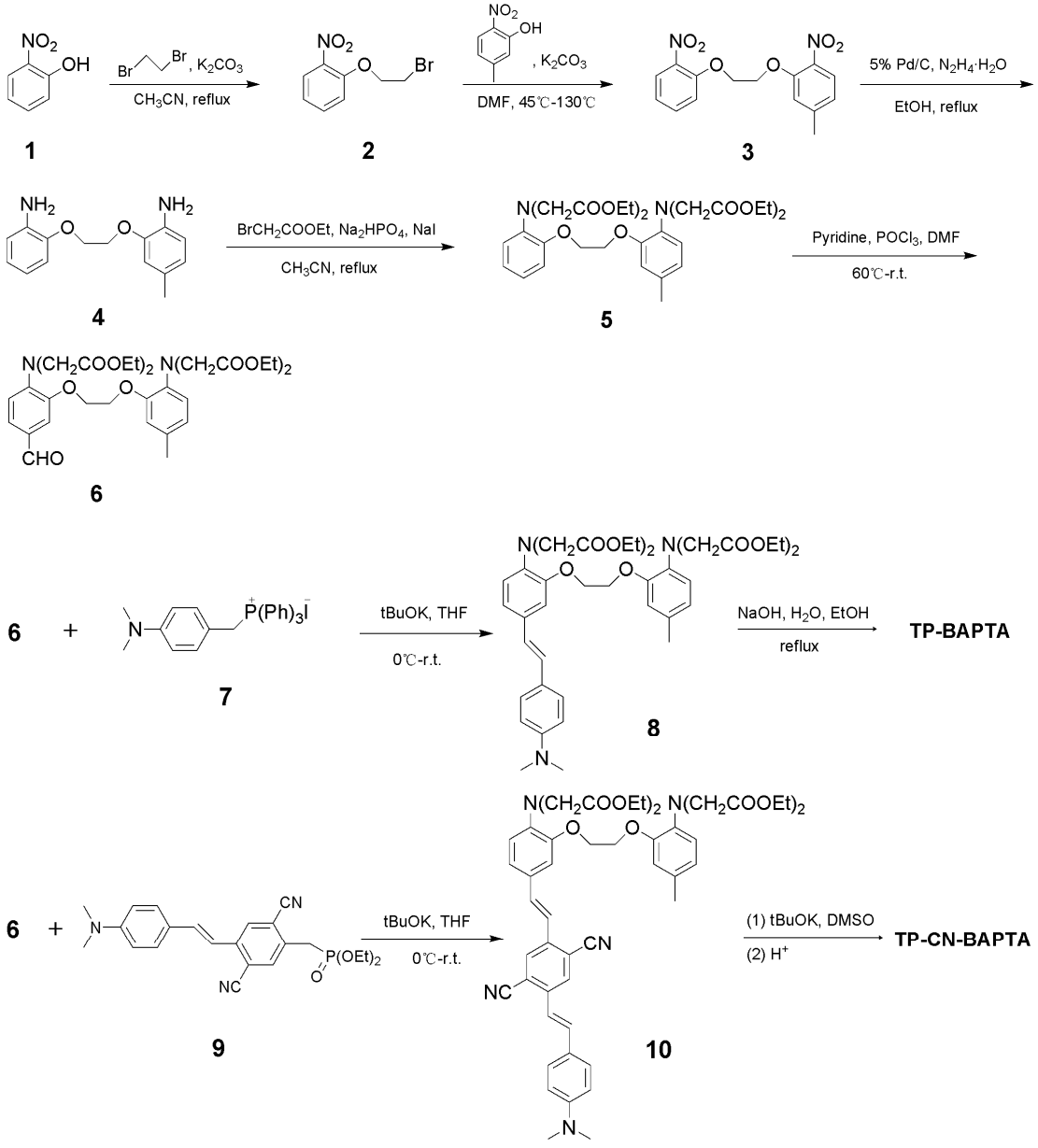
Synthesis of TP-BAPTA and TP-CN-BAPTA

Materials

All solvents and reagents were used as received without further purification unless for special needs. 2-nitro-5-methylphenoxide and potassium tert-butoxide were purchased from Alfa Aesor (Ward Hill, MA). Other regents were bought from Sinopharm Chemical Reagent Co. (Shanghai, China).

Synthetic Procedures

The synthesis route for TP-BAPTA and TP-CN-BAPTA is described in Scheme S1. 5-aldehyde-5'-methly-BAPTA-tetraethyl ester was prepared according to the method in literatures.[1] Compounds 7 and 9 were synthesized in our previous work.[2,3]



Scheme S1: The synthesis route for TP-BAPTA and TP-CN-BAPTA.

***1-(2-bromoethoxy)-2-nitrobenzene (2)*:** A mixture of **1** (5.56g, 40mmol), 1,2-dibromoethane (34.6mL, 400mmol) and K_2CO_3 (11.4g, 80mmol) in 120mL acetonitrile was stirred under reflux for 3 hours. The solution was filtered and the

precipitate was washed with CH_2Cl_2 until the filtrate became colorless. After evaporation, the residue was dissolved in hot methanol and was recrystallized under ice bath. After filtration, the combined filtrate was evaporated and the residue was recrystallized from ether-hexane to yield 7.86g, 80.3%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 7.83 (d, 1H, J=9.0), 7.53 (t, 1H, J=7.5), 7.09 (m, 2H), 4.41 (t, 2H, J=6.6), 3.67 (t, 2H, J=6.3).

***4-methyl-1-nitro-2-(2-(2-nitrophenoxy)ethoxy)benzene (3)*:** A mixture of 2-nitro-5-methylphenoxide (2.69g, 17.6mmol) and K_2CO_3 (4.86g, 35.2mmol) in 15mL DMF was heated to 45℃ for 30min. Then **2** (3.94g, 16mmol) was added and heated to 130℃ for 3h. The mixture was diluted to 100mL with water and filtered. The precipitate was washed with 10% (w/w) $NaHCO_3$ and water. Recrystallization from ethanol (contain 0.4mL acetic acid per 100mL) yielded 4.31g, 84.6%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 7.83 (d, 1H, J=8.1), 7.78 (d, 1H, J=9.0), 7.57 (t, 1H, J=7.5), 7.24 (s, 1H), 7.07 (m, 2H), 6.87 (d, 1H, J=9.0), 4.54 (s, 4H), 2.45 (s, 3H).

***2-(2-(2-aminophenoxy)ethoxy)-4-methylaniline (4)*: 3** (5.05g, 15.8mmol) and 5% Pd/C (0.82g) were heated in ethanol under N_2 atmosphere. 35mL $N_2H_4 \cdot H_2O$ was added dropwise. The mixture was refluxed for 2h. It was filtered while hot and washed with hot ethanol. The combined filtrate was evaporated and recrystallized from ethanol to give 3.07g white crystals. Yield: 75%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 6.82 (m, 3H), 6.71 (m, 3H), 6.62 (d, 1H, J=6.6), 4.35 (t, 4H, J=3.0), 3.2-3.8 (br s, 4H), 2.25 (s, 3H).

5-methyl-BAPTA-tetraethyl ester (5): A mixture of **4** (1.63g, 6.32mmol), ethyl

bromoacetate (4mL, 36.4mmol), Na_2HPO_4 (4.49g, 31.6mmol) and NaI (0.38g, 2.53mmol) in 25mL anhydrous acetonitrile was refluxed under nitrogen for 18h. Toluene was added after distilling the solvent. The solution was washed with water and saturated brine. Organic layer was dried over $MgSO_4$ and evaporated. Recrystallization from ethanol gave 2.68g product as white powder. Yield: 70.4%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 6.91-6.67 (m, 7H), 4.27 (s, 4H), 4.16 (s, 4H), 4.12 (s, 4H), 4.06 (m, 8H), 2.26 (s, 3H), 1.15 (m, 12H).

5-aldehyde-5'-methly-BAPTA-tetraethyl ester (6): **5** (0.903g, 1.5mmol) was dissolved in 3mL DMF. 150μL pyridine was added. 1.2mL $POCl_3$ was dropped slowly at 0℃. The reaction mixture was stirred at room temperature for 30min and heated to 60℃ for 1h and then back to room temperature over night . The mixture was poured into aqueous NaOH mixed with ice. The aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with water and saturated brine. After drying and evaporation, the residue was chromatographed on silica in petroleum ether-EtOAc 4:1 (v/v) to yield 459mg **6**, 48.5%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 9.78 (s, 1H), 6.76-6.65 (m, 7H), 4.30 (d, 4H, J=5.1), 4.22 (s, 4H), 4.14 (s, 4H), 4.04 (q, 8H), 2.25 (s, 3H), 1.13 (m, 12H).

TP-BAPTA tetraethyl ester (8): To the solution of **7** (105mg, 0.24mmol) in 10mL THF, potassium tert-butoxide (40mg, 0.36mmol) was added at 0□ and nitrogen atmosphere. The solution turned into dark red immediately. After 20min, **6** (126mg, 0.2mmol) dissolved in dry THF was slowly added. The mixture was stirred at room temperature for 2h, then water was added. The solution was extracted with ethyl

acetate. Organic layer was washed with saturated brine. After drying and evaporation, the residue was chromatographed on silica in petroleum ether-EtOAc 5:1 (v/v) to yield 110mg **8**, 73.6%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 7.39 (d, 1H, J=8.7), 7.18 (d, 1H, J=7.8), 6.98 (d, 1H, J=9), 6.82 (d, 1H, J=8.7), 6.77 (m, 3H), 6.64 (m, 3H), 6.40 (d, 1H, J=8.7), 4.33 (d, 4H, J=8.7), 4.16 (s, 4H), 4.14 (s, 4H), 4.02 (m, 8H), 2.91 (s, 6H), 2.05 (s, 3H), 1.12 (m, 12H). **HRMS**: calc. for $C_{41}H_{53}N_3O_{10}$ $(M+Na)^+$: 770.3629, found: 770.3624.

TP-CN-BAPTA tetraethyl ester (10): The process was similar to the synthesis of **8**. 235mg (0.37mmol) **6** and 144mg (0.34mmol) **9** gave 209mg **10**, 68.5%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 7.97 (d, 2H, J=4.5), 7.48 (d, 2H, J=8.7), 7.21-7.04 (m, 6H), 6.79-6.68 (m, 6H), 4.33 (s, 2H,), 4.29 (s, 2H), 4.19 (s, 4H), 4.13 (s, 4H), 4.05 (m, 8H), 3.03 (s, 6H), 2.26 (s, 3H), 1.15 (m, 12H). **HRMS**: calc. for $C_{51}H_{57}N_5O_{10}$ $(M+Na)^+$: 922.4003, found: 922.3998.

TP-BAPTA: **8** (110mg, 0.15mmol) was hydrolyzed in aqueous NaOH / EtOH. EtOH was removed after reaction and diluted HCl was added. The formed precipitate was washed with water to yield 72mg TP-BAPTA, 76%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 7.35 (d, 2H, J=9), 7.12-6.78 (m, 6H), 6.69-6.57 (m, 4H), 4.26 (s, 4H), 4.04 (s, 4H), 3.99 (s, 4H), 2.89 (s, 6H), 2.19 (s, 3H). **HRMS**: calc. for $C_{33}H_{37}N_3O_{10}$ $(M+Na)^+$: 658.2377, found: 658.2375.

TP-CN-BAPTA: This ester **10** was hydrolyzed according to the reported method.[4] Potassium tert-butoxide (50mg, 0.45mmol) was added to the solution of **10** (60mg, 0.066mmol) in 10mL DMSO. The reaction was stirred at room temperature for 2h.

The product was precipitated on addition of acetonitrile. The precipitate was dissolved in water. To the solution was added diluted HCl. The formed precipitate was washed and dried to yield 35mg as red powder, 67%. **^{1}H NMR** ($CDCl_3$, 300MHz), δ: 8.46 (s, 1H), 8.41 (s, 1H), 7.65-7.46 (m, 4H), 7.22-7.01 (m, 4H), 6.76-6.65 (m, 6H), 4.25 (s, 4H), 4.08 (s, 4H), 4.00 (s, 4H), 2.95 (s, 6H), 2.20 (s, 3H). **HRMS**: calc. for $C_{43}H_{41}N_5O_{10}$ $(M+Na)^+$: 810.2751, found 810.2747.

Apparatus for Absorption and Fluorescence Measurements

UV-vis absorption spectra of probes were measured on a TU-1900 UV-vis spectrophotometer (Beijing Purkinje General Instrument). One-photon excited fluorescence was recorded on a Perkin-Elmer LS 55 fluoremeter equipped with 1-cm cell. A PMT was used as the detector. Two-photon excited fluorescence was measured using a mode-locked Ti:sapphire pulsed laser (Mira900, Coherent Inc., Santa Clara, CA). The anti-Stokes photoluminescence was recorded on a liquid nitrogen-cooled CCD detector (SPEC-10, Princeton Instruments, Trenton, NJ) through a monochromator (Spectrapro 2500i, Acton Research Corp., Acton, MA).

Determination of the Fluorescence Quantum Yield

The fluorescence quantum yield was measured by the relative method as literature reported.[5] Quinine sulfate in 0.1N sulfuric acid (η = 0.79) and Rhodamine B in methol (η = 0.71) were respectively used as reference for TP-BAPTA and TP-CN-BAPTA. The fluorescence quantum yield was calculated according to the following equation:[6]

$$\eta_s = \frac{A_r I_s n_s^2}{A_s I_r n_r^2} \eta_r \, (A \le 0.05)$$

The subscripts *s* and *r* represent the sample and the reference molecule respectively. η

is the fluorescence quantum yield. *A* is the absorbance of molecules that is controlled below 0.05 for both molecules in the same wavelength. *I* means the integrated emission area and *n* is the refractive index of the solvent.

Determination of Two-Photon Absorption Cross-Section

The two-photon absorption (TPA) cross-section was measured using the two-photon induced fluorescence method under the excitation of 800 nm. TP-BAPTA (1.7×10^{-4}M) and TP-CN-BAPTA (2.3×10^{-4}M) was dissolved in DMF respectively. Rhodamine B in methanol (1.0×10^{-4}M) was used as the reference with the reported value of 150 GM (1GM = 1×10^{-50} cm^4 s $photon^{-1}$) around 800 nm.[7] The TPA cross-section was determined by the following equation:

$$\delta_s = \frac{S_s \eta_r \phi_r C_r}{S_r \eta_s \phi_s C_s} \delta_r$$

Similar to the calculation of fluorescence quantum yield, subscripts *s* and *r* denote the sample and the reference molecules, respectively. *S* represents the measured two-photon excited fluorescence intensity, η is the fluorescence quantum yield, *C* is the concentration of the solution, and ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The refractive indexes (*n*) of the solvents are used here instead of ϕ since all the experiments of both samples and reference were done under the same instrumental conditions.

Determination of Apparent Dissociation Constant

A series of calibration solutions were prepared by adding $CaCl_2$ solutions with different concentrations. Each solution contained 1μM TP-BAPTA, 10mM K_2EGTA, 100mM KCl and 30mM MOPS in pH7.2. Appropriate TP-BAPTA was dissolved in

DMSO to prepare a 1.0×10^{-3}M stock solution and then diluted to 1.0×10^{-5}M with water. Buffer solution was prepared including 100mM K_2EGTA, 1000mM KCl and 300mM MOPS in pH7.2. After adding 100μL solution of TP-BAPTA (1.0×10^{-5}M) and 100μL buffer solution, various $CaCl_2$ solutions were added, and then all solution were diluted to 1mL to make sure that the final concentrations of total Ca^{2+} were 0, 1, 2, 3, 4, 5, 6, 7, 8, 8.5, 8.7, 9, 9.5mM. Free [Ca^{2+}] was controlled by EGTA to be 0.017, 0.038, 0.064, 0.1, 0.15, 0.23, 0.35, 0.6, 0.85, 1, 1.35 and 2.84μM respectively. It could be calculated from the K_d of EGTA for Ca^{2+} using following equation:[8]

$$[Ca^{2+}]_{free} = K_d^{EGTA} \times \frac{[CaEGTA]}{[K_2EGTA]}$$

K_d was calculated from the Hill plot in which x axes was $\log[Ca^{2+}]_{free}$, y axes was $\log((F_{max}-F)/(F-F_{min}))$. F_{max} was the maximum fluorescence intensity, F_{min} was the minimum fluorescence intensity and F was the observed fluorescence intensity.

Cell Culture

TP-CN-BAPTA-ESTER working solution was diluted from 1 mM stock solution with Ca^{2+}-free Tyrode's buffer to a final concentration of 5 μM. 20 μM ATP was prepared in Ca^{2+}-free Tyrode's buffer (pH 7.2).

HeLa cells were grown in culture flasks containing Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 U/ml penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. Confluent cells were harvested by treatment with trypsin (0.25%) for 2 minutes at room temperature and then cultured in 35 mm petri dishes for overnight. Following rinsing with Ca^{2+}-free Tyrode's buffer for three times, adherent cells were incubated with TP-CN-BAPTA-ESTER work solution for 1

hour at 37°C. After incubation, cells were rinsed with Ca^{2+}-free Tyrode's buffer for three more times and ready for experiments.

Calcium Imaging by Two-Photon Microscopy

The Olympus FV1000 confocal microscope was used for calcium imaging with two-photon excitation fluorescence (800 nm). Fluorescent images were recorded at a rate of 3 frames per second. ATP solutions were manually applied to the adherent cells using micropipette.

Cytotoxicity assay

HeLa cells (3×10^5 cells/mL) were seeded onto a 96-well cell culture plate containing Dulbecco's modified Eagle's medium supplemented with varying concentrations (0, 1.5, 3, 6, 12, 24 μM) of TP-CN-BAPTA-ester or TP-BAPTA-ester. Each concentration of chelator was repeated 5 times. After 12-hour incubation at 37℃, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well to a final concentration of 0.5 mg/mL, followed by an additional incubation for 4 hours. Subsequently 100 μL DMSO (Sigma) was added to each sample and the spectrophotometrical absorbance (490 nm) was measured using an ELISA reader. The reference wavelength was 620 nm.

Figures

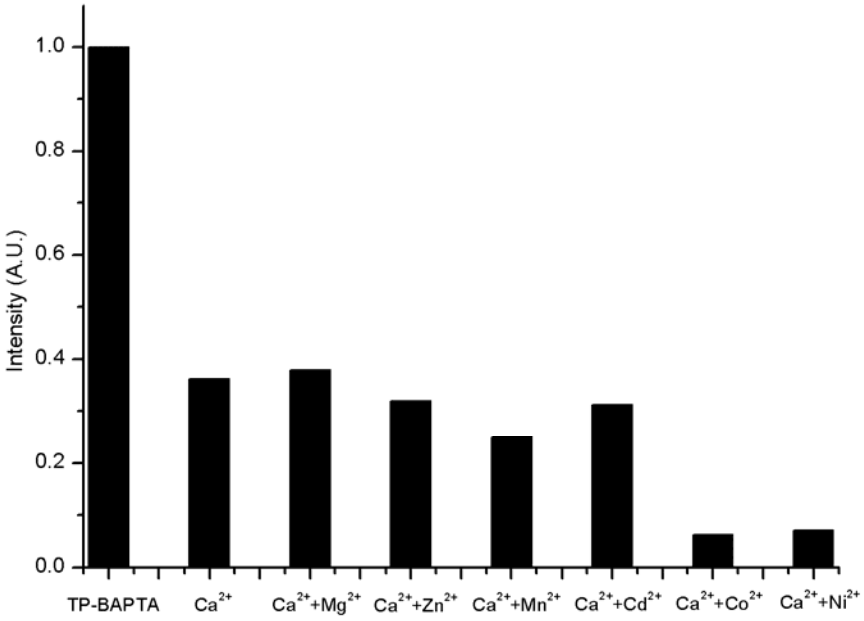


Fig.S1 Effect of co-existing metal ions on the fluorescence quenching of 1μM TP-BAPTA in 30mM MOPS solutoin, 10mM EGTA, 100mM KCl and pH7.2. These data were measured in the presence of 9mM Ca^{2+} together with 9mM tested ions. The excitation wavelength was 352nm.

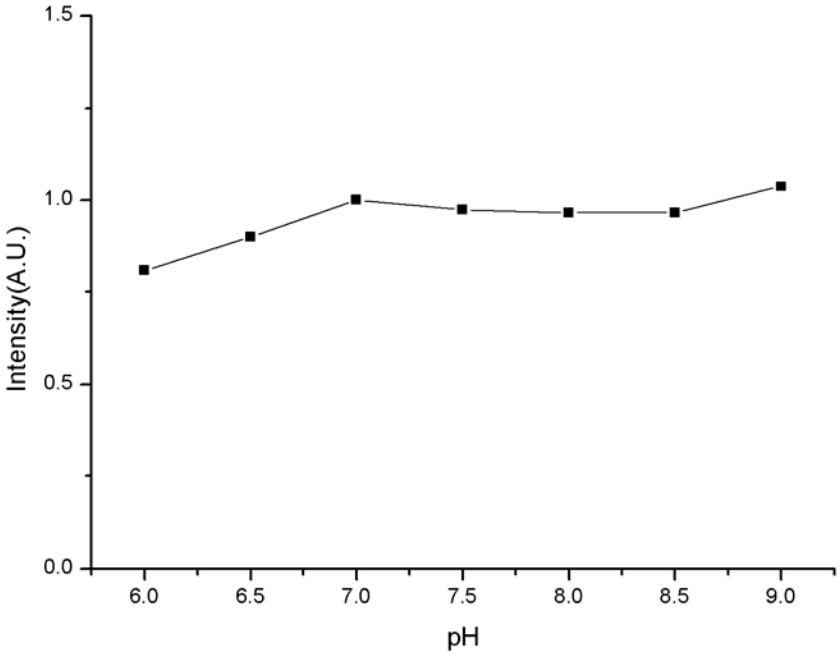


Fig.S2 Effect of pH on the fluorescence intensity of 1μM TP-BAPTA in 30mM MOPS, 10mM EGTA, 100mM KCl and pH6.0-9.0. The excitation wavelength was 352nm.

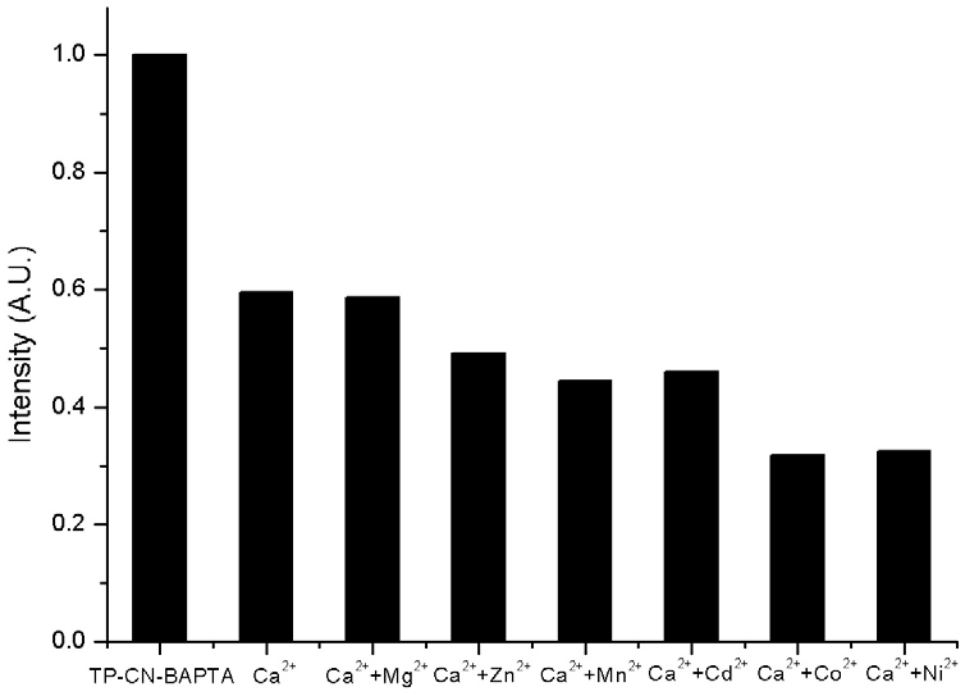


Fig.S3 Effect of co-existing metal ions on the fluorescence quenching of $3\times10^{-5}\mu M$ TP-CN-BAPTA in 30mM MOPS solution, 10mM EGTA, 100mM KCl and pH7.2. These data were measured in the presence of 9.5mM Ca^{2+} together with 9.5mM tested ions. The excitation wavelength was 437nm.

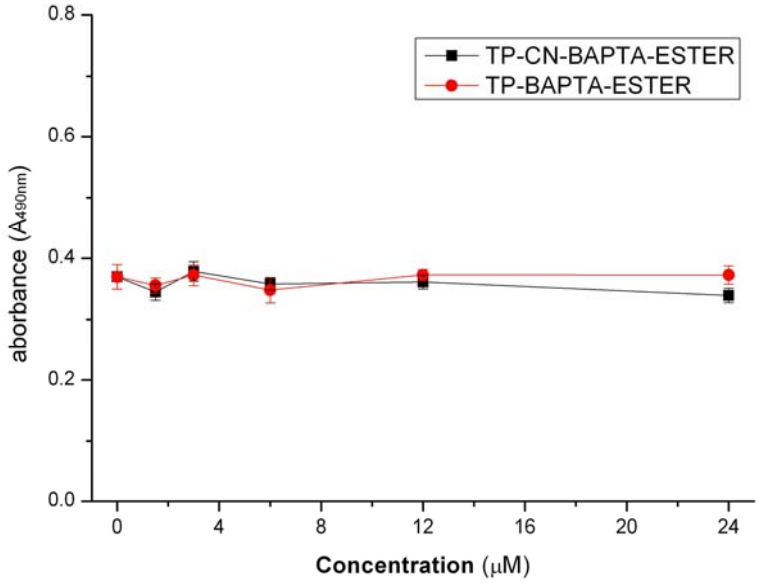


Fig.S4 Spectrophotometrical absorbance of HeLa cells cultured with varying concentrations of TP-CN-BAPTA-ester or TP-BAPTA-ester.

References

1 G. Grynkiewicz, M. Poenie, and R. Y. Tsien, *J. Biol. Chem.,* 1985, **260**, 3440; S. Veravong, V. Ruangpornvisuti, B. Pipoosananakaton, M. Sukwattanasinitt and T. Tuntulani, *Science Asia,* 2000, **26**, 163; S. Chandra and R. Kumar, *Transition Met. Chem.,* 2004, **29**, 269.

2 L. Z. Liu, G. H. Wei, Z. H. Liu, Z. K. He, S. Xiao, Q. Q. Wang, *Bioconjugate Chem.*, 2008, **19**, 574.

3 L. Z. Liu, M. Shao, X. H. Dong, X. F. Yu, Z. H. Liu, Z. K. He, Q. Q. Wang, *Anal. Chem.*, 2008, **80**, 7735.

4 E. Roussakis, F. Liepouri, Artemissia-Phoebe Nifli, E. Castanas, T. G.

Deligeorgiev, H. E. Katerinopoulos, *Cell Calcium,* 2006, **39**, 3.

5 J. N. Demas, G. A. Crosby, *J. Phys. Chem.,* 1971, **75**, 991.

6 H. S. Joshi, R. Jamshidi, Y. Tor, *Angew. Chem., Int. Ed.,*1999, **38**, 2721.

7 C. Xu, W. W. Webb., *J. Opt. Soc. Am. B,* 1996, **13**, 481.

8 R. Y. Tsien, T. Pozzan, T. J. Rink, *J. Cell Biol.* 1982, **94**, 325; S. Bagh and M. F. Paige, *J. Phys. Chem. A,* 2006, **110**, 7057.