Supporting Information

A DNAzyme-based normalized fluorescence strategy for direct quantification of endogenous zinc in living cells

*Xuanhao Zhang,[a] Zhi-Ling Song, *[a] Qiqi Chao,[a] Qian Li,[a] Rongmei Kong,[b] Gao-Chao Fan,[a] and Xiliang Luo*[a]*

[a]Key Laboratory of Optic-electric Sensing and Analytical Chemistry for Life Science, MOE, Shandong Key Laboratory of Biochemical Analysis, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, China

[b]School of Chemistry and Chemical Engineering, Qufu Normal University, Qufu, Shandong 273165, People's Republic of China

Corresponding Author
**Email: zhilingsong@qust.edu.cn (Z. L. S.)*
**Email: xiliangluo@qust.edu.cn (X. L.)*

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1. Supplemental Materials and Methods

Reagents. Oligonucleotide sequences were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and purified by high-performance liquid chromatography (HPLC). Lipofectamine 3000 were bought from Thermo Fisher Scientific (China) Co., Ltd. N,N,N',N'-Tetramethylethylenediamine (TEMED) was bought from Shanghai Aladdin Biochemical Technology Co., Ltd. Ammonium persulphate (APS) was bought from Yantai Far East Fine Chemical Co. Ltd. 20 bp DNA Ladder and 6× Loading-Buffer were bought from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Penicillin streptomycin, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). Doubly distilled water (resistance > 18 M Ω•cm) purified from a Milli-Q Integral System was used throughout all experiments

Apparatus. FL-4600 fluorescence spectrophotometer (Hitachi, Japan) was used to detect all fluorescence spectra. Fluorescence confocal images were recorded by Olympus FV1000. Polyacrylamide gel electrophoresis (PAGE) was performed on a DYCZ-24K (LIUYI, China) electrophoresis apparatus. All UV-vis absorption spectra were recorded by UV-2600 spectrophotometer (Shimadzu). WD-9413B (LIUYI, China) gel imaging system was used to image polyacrylamide gel.

Prepration of DNAzyme system. The labeled enzyme (E) DNA strands and substrate (S) DNA strands (ES) were dispersed in Tris-HCl buffer (50 mM Tris, 45.7 mM HCl, 150 mM NaCl, pH 7.4) and stored at -20 °C for the further experiment. For the preperation of DNAzyme probe, the E and S strands were first denatured at 95 °C for 5 min and then slowly cooled down to room temperature. The obtained DNAzyme probe were stored at 4℃ for the in vitro and intracellular assay.

Polyacrylamide gel electrophoresis. 6 % polyacrylamide gel was employed for the verification of the formation of the DNAzyme probe and Zn^{2+} triggered cleavage of

substrate strand. 10 μL DNAzyme product (1μM) was loaded on the 1.5 mm thin gel. Then electrophoresis was carried out at 135 V for 1 min and 80 V for 60 min at room temperature in 1×TAE buffer. After separation, the gel was stained by 0.2 μg/mL ethidium bromide for 30 min and imaged with the WD-9413B gel imaging system.

In vitro detection. 20 μL DNAzyme probes were were first denatured at 95 °C for 5 min and then slowly cooled down to room temperature. Then the obtained DNAzyme probes were mixed with 80 μL Tris-HCl solution containing different concentrations of Zn^{2+}. After incubation at 37 ℃ for 3 h, the fluorescence spectra were detected by an F-4600 fluorescence spectrophotometer, in which the measured fluorescence intensities of FAM fluorophore at 518 nm and TMR at 580 nm were named as F_{FAM} and $F_{TMR,}$ respectively. For the detection of $F_{FAM\text{-}max}$, 80 μL Tris-HCl solution containing excess Zn^{2+} (150 μM) was added into 20 μL DNAzyme probes and incubated for 10 min at 37 ℃, then the maximum fluorescence of FAM fluorophore at 518 nm was measured, called as $F_{FAM\text{-}max}$. The adopted excitation/emission wavelengths of FAM fluorophore and TMR were 488/518 nm and 543/580 nm respectively. After spectra measurement, the transformation of absolute fluorescence response F_{FAM} into normalized fluorescence $F_{FAM}/F_{FAM\text{-}max}$ was performed.

Cell culture. HeLa, MCF-7 and L02 cells were cultured in DMEM containing 10% premium fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

DNAzyme transfection. DNAzyme probes transfection was performed using Lipofectamine 3000 reagent (Invitrogen) with a little modification according to the protocol provided by the manufacturer. Briefly, Lipofectamine 3000 (5 μL) and DNAzyme probes (2 nmol) were incubated separately in Opti-MEM media (Invitrogen) for 3 minutes at room temperature. Then these two solutions were mixed and allowed to incubate for an additional 25 minutes to form DNAzyme-

Lipofectamine complex. Next, the prepared DNAzyme-Lipofectamine complex was mixed with DMEM without FBS or antibiotics and added into cells for 3 hours.

Cytotoxicity test. Good biocompatibility is a vital attribute for an intracellular nanoprobe. We investigated the cytotoxicity of the DNAzyme probe in different concentrations for the MCF-7 cells by MTS assay. The cell viability measurement was evaluated based on the reduction of MTS to formazan crystals by the mitochondrial dehydrogenases. 1×10^3 MCF-7 cells in 50 μL washing buffer (Dulbecco`s phosphate buffered saline, DPBS, Gibco) were pre-seeded to each test well in 96-well plate and then incubated with PRMI-1640 culture medium for 24 h.Next, the culture medium was taken out and fresh culture medium with different concentrations of probes (0-300 nM) was added. The cells were incubated for 12 h and then 100 μL fresh PRMI-1640 medium and 20 μL MTS solution were added into each well and incubated for another 0.5 h. Finally, the absorbance intesity at 490 nm was recorded by a Bio-Tek Multi-Mode Microplate Reader (Winooski, VT) to assess the cell viability. All the experiments were conducted at least 3 times.

Fluorescence imaging in living cells. After incubation with DNAzyme-Lipofectamine complex for 3 hours, cells were washed several times with PBS buffer and covered with DMEM without FBS or antibiotics. Then cell imaging was analyzed at scanning confocal microscope under the 488 nm or 543 nm laser, and emissions were recorded in the green or red channel.

Calculation of intracellular zinc concentration. For the calculation of intracellular Zinc concentration in the single MCF-7 cell (Fig. S6), an important step in achieving this goal was to obtain the fluorescence intensity of F_{FAM}, $F_{FAM\text{-}max}$ in living cells. The procedure was similar to the in vitro testing. After the DNAzyme probes transfection into MCF-7 cells, the fluorescence intensity of F_{FAM} was observed through the cell imaging. Then, in order to obtain the $F_{FAM\text{-}max}$, excess Zn^{2+} (150 μM) was added into

the cell medium, and the real-time imaging was analyzed at scanning confocal microscope under the 488 nm laser. The observed maximum fluorescence intensity was appointed as the $F_{FAM\text{-}max}$. Therefore, based on the F_{FAM} and $F_{FAM\text{-}max}$ in confocal imaging, the normalized intensity $F_{FAM}/F_{FAM\text{-}max}$ could be calculated in the single model MCF-7 cell. Moreover, plugging the data $F_{FAM}/F_{FAM\text{-}max}$ into the vitro linear equation, the endogenous zinc concentration in the MCF-7 cell could be calculated.

For the comparation of the intracellular Zinc concentration in different cells, the procedure was a little change from the above one. Typically, excess Zn^{2+} (150 μM) was mixed with the DNAzyme probes and incubated for 10 min to obtain the fully cleaved DNAzyme probes. After the transfection of fully cleaved DNAzyme probes into cells, cell imaging was analyzed at scanning confocal microscope under the 488 nm or 543 nm laser, which showed the corresponding $F_{FAM\text{-}max}$ and F_{TMR}. Then the corresponding ratio k ($k = F_{FAM\text{-}max}/F_{TMR}$, equation S1) could be calculated. Next, the untreated DNAzyme probes were performed to image the endogenous zinc ions in cells, which showed the intensity of F_{FAM} and F_{TMR}. According to the known k value, the $F_{FAM\text{-}max}$ could be calculated from the equation $F_{FAM\text{-}max} = kF_{TMR}$ (equation S2), followed by the calculation of $F_{FAM}/F_{FAM\text{-}max}$ (equation S3). Finally, the quantification of the endogenous zinc ions in living cells was achieved by the in-vitro linear equation.

$$k = F_{FAM\text{-}max}/F_{TMR} \text{ (equation S1)}$$

$$F_{FAM\text{-}max} = kF_{TMR} \text{ (equation S2)}$$

$$F_{FAM}/F_{FAM\text{-}max} = F_{FAM}/kF_{TMR} \text{ (equation S3)}$$

ICP-MS measurement of intracellular zinc concentration. Intracellular zinc quantification with ICP-MS was performed according to the previous report[S1] with a little modification. First of all, the cells were detached by trypsin followed by resuspension in water. The cell density was evaluated by using a hemocytometer. Then, the cell suspension was treated with sonication for about 1 h. Then, the mixed solution obtained above was filtered through 0.22 μm pore-diameter membranes, and

then filtrate was collected for later ICP-MS experiment. Finally, the total content of zinc was quantified by ICP-MS, and the amount of zinc in a single cell was calculated.

Supplementary Figures

Table S1. DNA sequences used in this study.

Oligonucleotide	**Sequences (from 5' to 3')**
Enzyme strand (E)	CACGTCCATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT
TMR-E	CACGTCCATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-TMR
Substrate strand (S)	ACTCACTATrAGGAAGAGATGGACGTG
FAM-S-TMR	FAM-ACTCACTATrAGGAAGAGATGGACGTG-TMR
S1	ACTCACTAT
FAM-S1	FAM-ACTCACTAT
S2	GGAAGAGATGGACGTG
TMR-S2	GGAAGAGATGGACGTG

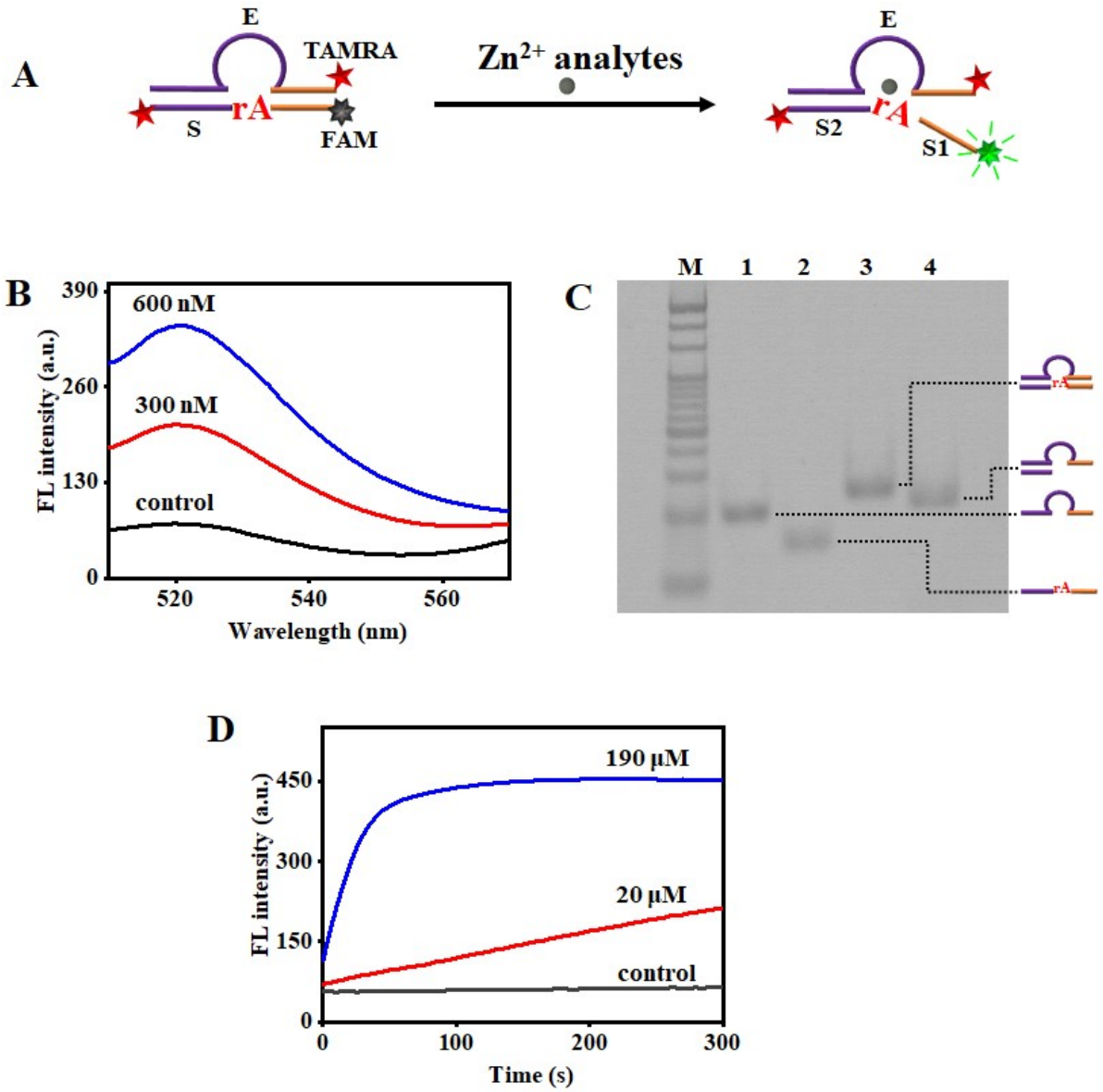

Fig. S1. (A) The schematic diagram of traditional DNAzyme method for the detection of Zn^{2+}. (B) The fluorescence spectra of DNAzyme probe (50 nM) mixed with different concentrations of Zn^{2+}: 0, 300 nM and 600 nM. (C) Native PAGE of DNAzyme probe in the presence of Zn^{2+}. M: DNA ladder; Lane 1: enzyme strand; Lane 2: substrate strand; Lane 3: enzyme strand+substrate strand; Lane 4: lane 3+ Zn^{2+}. The dotted lines (from bottom to top) represent substrate strand, enzyme strand, cleaved DNAzyme, and DNAzyme. (D) Kinetic curve responses of the DNAzyme probe for different concentrations of Zn^{2+} (0, 20 and 190 μM).

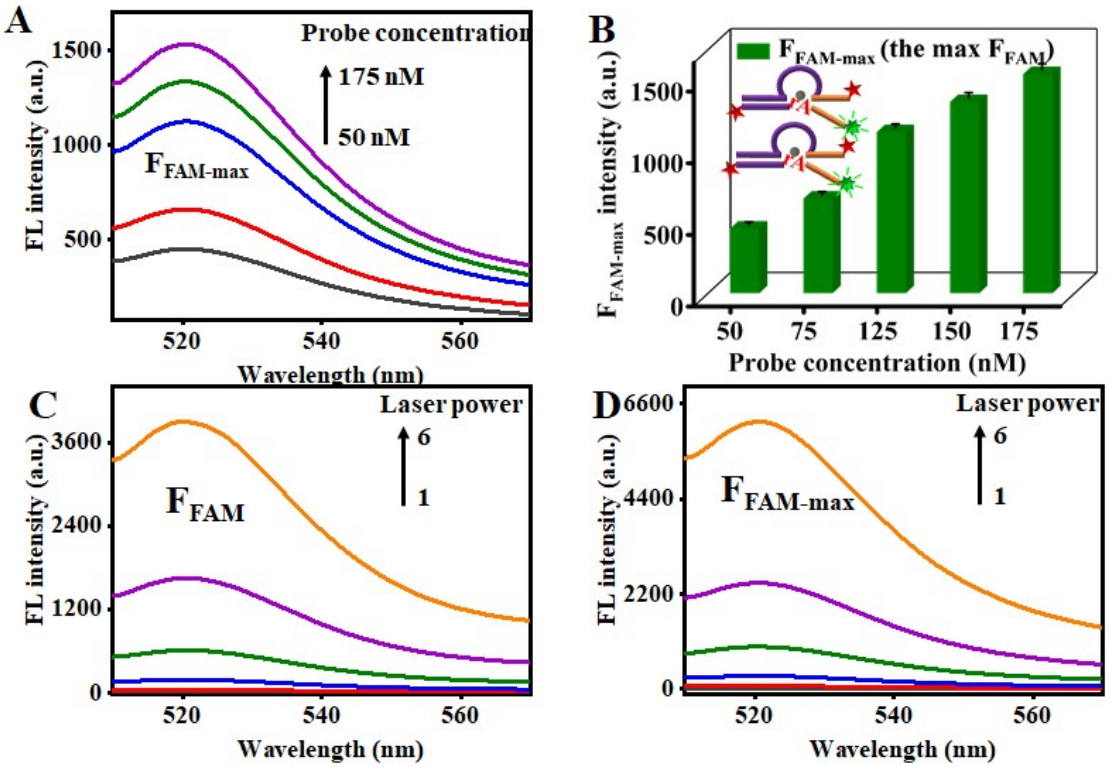

Fig. S2. Self-calibrating capability of the normalized DNAzyme strategy under different probe concentrations and laser powers. (A) The fluorescence responses of different concentrations of DNAzyme probe (50, 75, 125, 150 and 175 nM) to excess Zn^{2+} (190 μM). (B) The corresponding histogram of part A. (C) The fluorescence responses of DNAzyme probe to the same concentration of target Zn^{2+} (450 nM) under different laser powers. (D) The fluorescence responses of DNAzyme probe to excess Zn^{2+} (190 μM) under different laser powers.

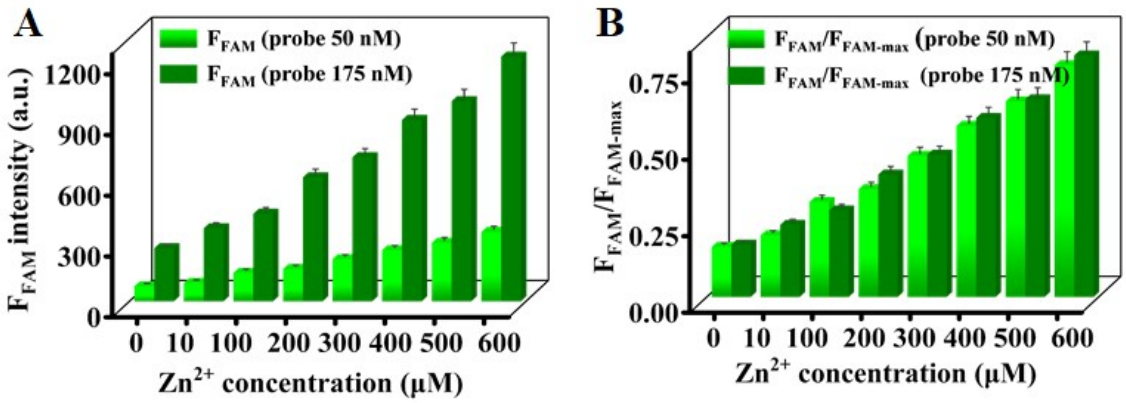

Fig. S3. (A) Comparation of the fluorescence responses of DNAzyme probe at 50 nM and175 nM to different concentrations of Zn^{2+} (0-600 nM). (B) The comparation of the normalized fluorescence responses of DNAzyme probe at 50 nM and 175 nM to Zn^{2+} (0-600 nM).

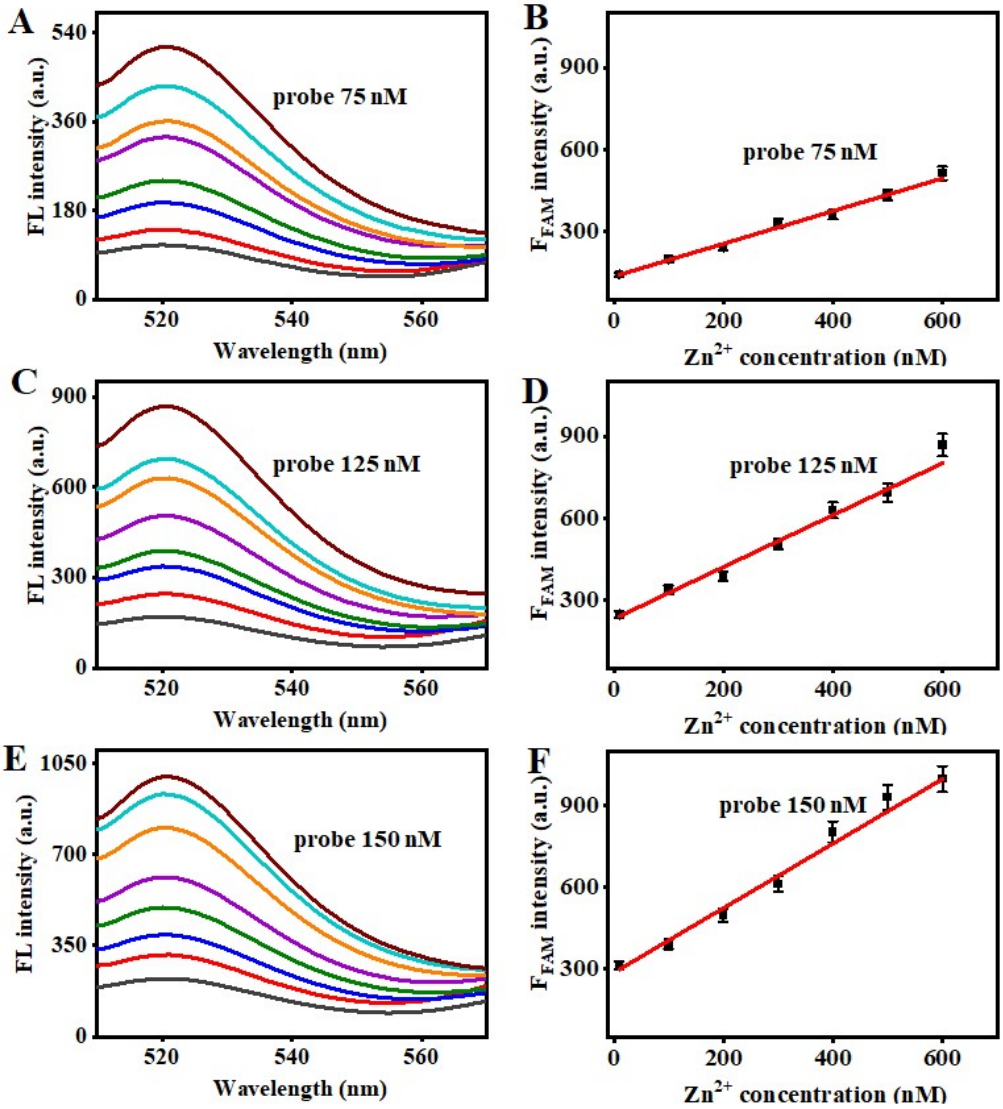

Fig. S4. Fluorescence responses of various concentrations of DNAzyme probes (A, 75 nM; C, 125 nM; E, 150 nM) to different concentrations of Zn^{2+} (0, 10, 100, 200, 300, 400, 500, and 600 nM). The corresponding relationship of fluorescence intensity *versus* the concentration of Zn^{2+} when the probe was at 75 nM (B), 125 nM (D), and 150 nM (F).

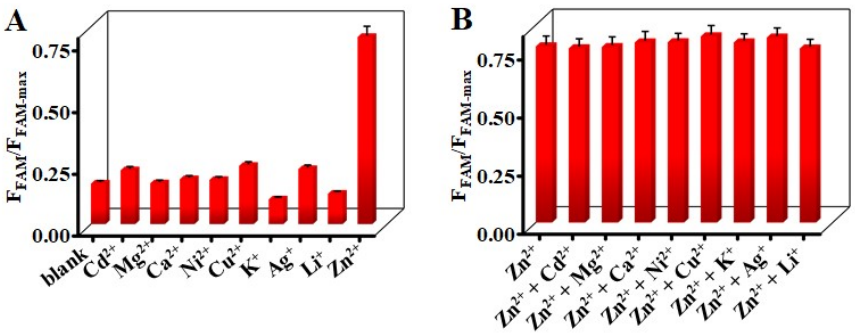

Fig. S5. Specificity of the DNAzyme probe (50 nM) for Zn^{2+}. Zn^{2+} was at 600 nM, K^{+} was at 1 mM and all the other metal ions were at 1 μM.

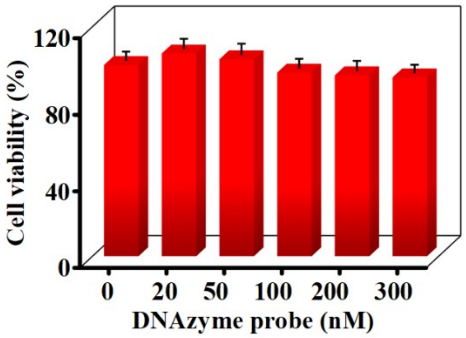

Fig. S6. (A) Cell viability of MCF-7 cells treated with different concentrations (0-300 nM) of DNAzyme probe for 12 h.

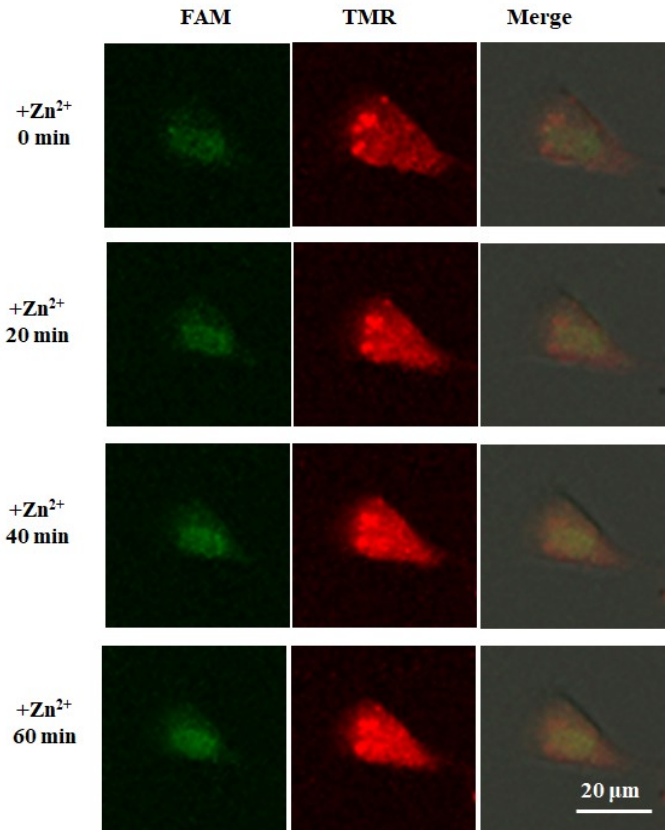

Fig. S7. Confocal images of probe-pretreated MCF-7 cells after the addition of extra Zn^{2+} for different time. The green and red channels were excited under the 488 nm laser and the 543 nm laser, respectively.

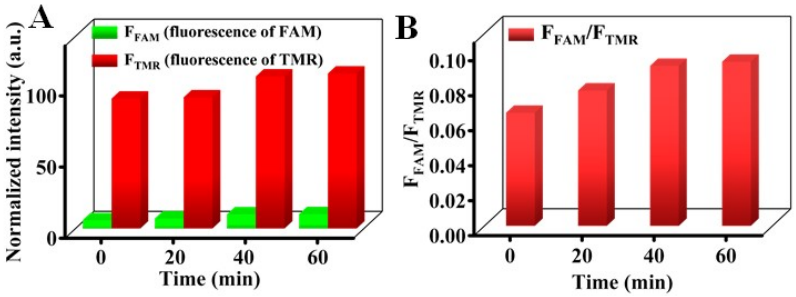

Fig. S8. (A) The corresponding alterations of green fluorescence of FAM and red fluorescence of TMR in the cytoplasm of treated MCF-7 cells. (B) The changes in the ratio of green fluorescence to red fluorescence in the cytoplasm of probe-pretreated MCF-7 cells after the addition of extra Zn^{2+} for different time.

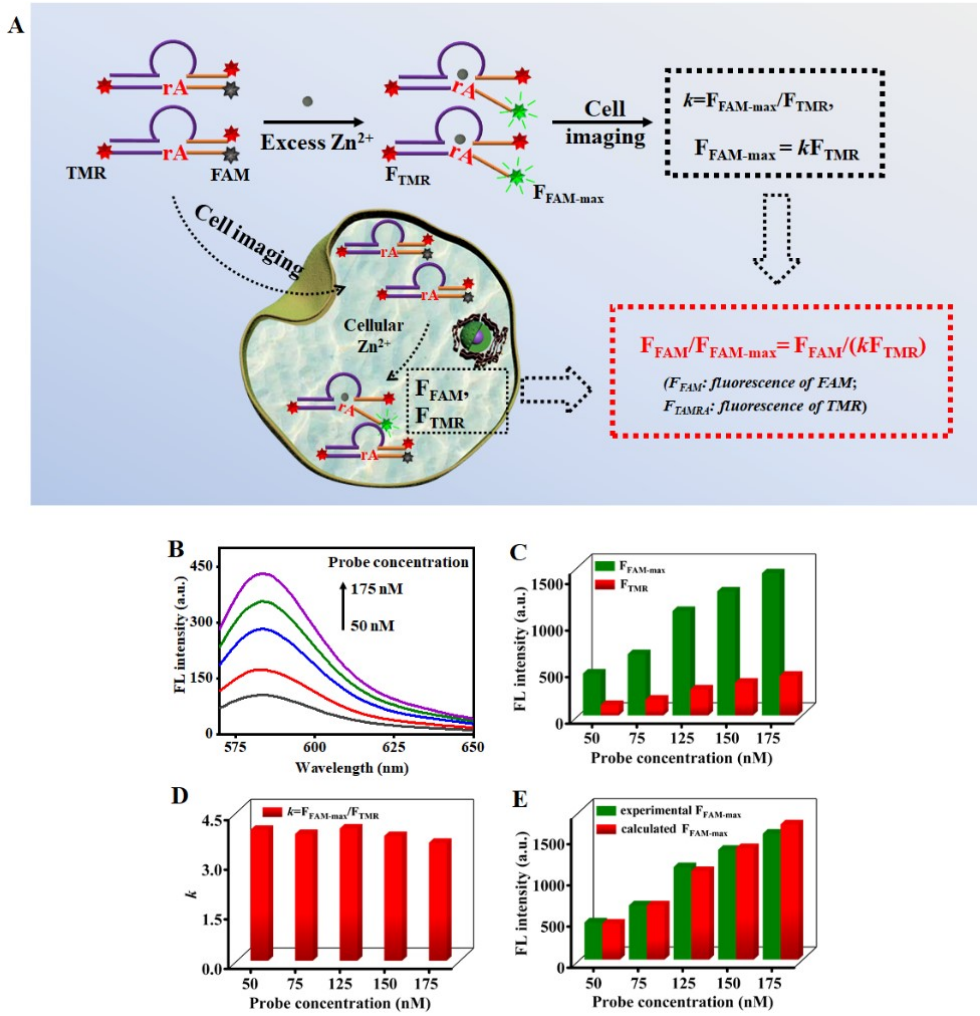

Fig. S9. (A) Schematic illustration of DNAzyme-based normalized strategy for the zinc imaging and quantification in living cell. (B) The fluorescence spectra of TMR in different concentrations of DNAzyme probes (50, 75, 125, 150 and 175 nM). (C) The changes of the maximum green FAM fluorescence ($F_{FAM\text{-}max}$) and red TMR fluorescence (F_{TMR}) in different concentrations of completely cleaved DNAzyme probes. (D) The calculated ratios (k) of $F_{FAM\text{-}max}$ to F_{TMR} corresponding to part C. (E) Comparation of the experimental $F_{FAM\text{-}max}$ with that calculated from k (k was 0.20061 when probe was at 75 nM).

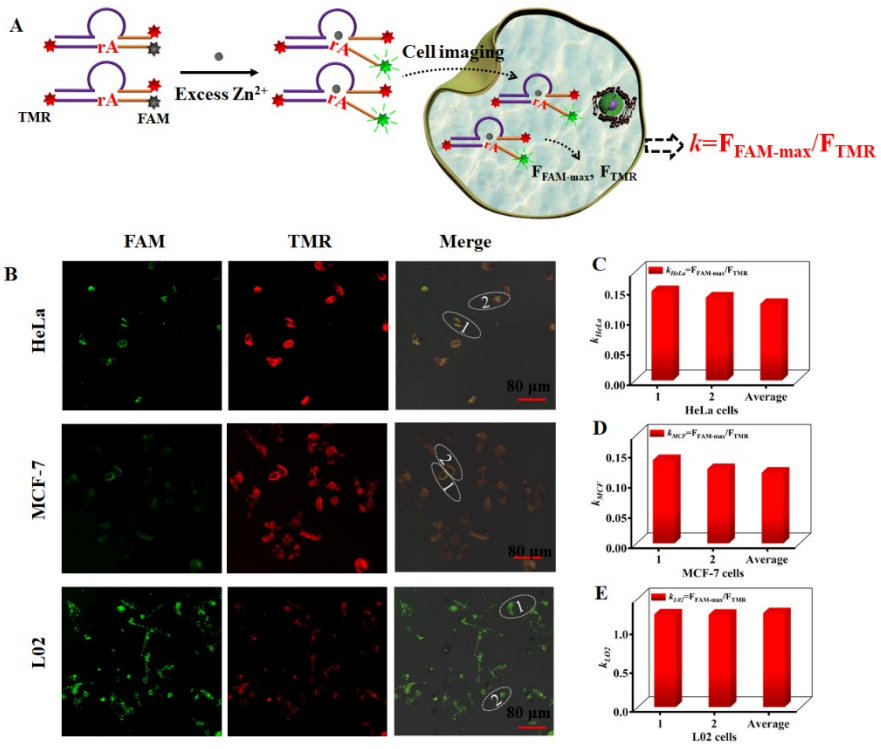

Fig. S10. (A) Schematic illustration of DNAzyme-based normalized strategy for the *k* measurement in different cell lines before the intracellular zinc quantification. (B) Confocal images of HeLa, MCF-7 and L02 cells after the addition of completely cleaved DNAzyme probes. (C-E) The corresponding alterations of the *k* ratios calculated from the green FAM fluorescence ($F_{FAM-max}$) and the red TMR fluorescence (F_{TMR}) in treated HeLa, MCF-7 and L02 cells.

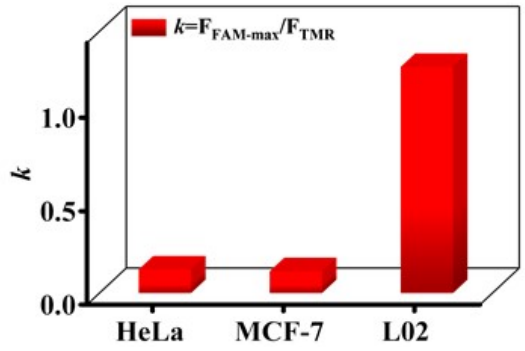

Fig. S11. The averaged *k* values calculated by the green FAM fluorescence and red TMR fluorescence in the treated HeLa, MCF-7 and L02 cells.

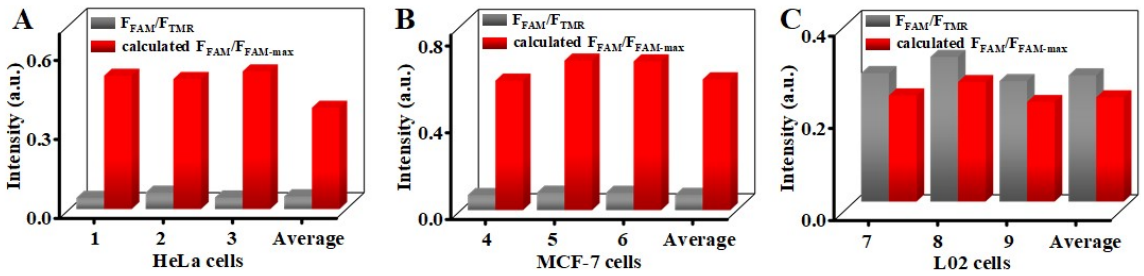

Fig. S12. The alterations of F_{FAM}/F_{TMR} and $F_{FAM}/F_{FAM-max}$ values in the corresponding HeLa, MCF-7 and L02 cells.

Table S2. Intracellular Zinc content in MCF-7, HeLa and L02 cells obtained by ICP-MS detection.

Cell line	ICP-MS result (fg cell^{-1})
MCF-7	10.8
HeLa	4.2
L02	0.8

References:

1. Y. Shu, N. Zheng, A.-Q. Zheng, T.-T. Guo, Y.-L. Yu and J.-H. Wang, *Anal. Chem.* 2019, 91, 4157-4163.