Electronic Supplementary Information

FRET-based Nanoprobes for Simultaneous Monitoring of Multiple mRNAs in Living Cells using a Single Wavelength Excitation

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Experimental details:

Materials. Tamoxifen, β-Estradiol Bovine insulin, 3-(4,5-dimethyl-thiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), Dynasore and Chlorpromazine were purchased from Sigma Chemical Company; Ethylisopropylamiloride (EIPA) was purchased from J&K Scientific Ltd., Filipin was purchased from Cayman Chemical Company; deoxyribonuclease I (DNase I) was purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China); Hydrogen tetrachloroaurate(III) ($HAuCl_4 \cdot 4H_2O$, 99.99%), Trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), $MgCl_2$ and KCl were purchased from China National Pharmaceutical Group Corporation (Shanghai, China). RNAStore was purchased from Tiangen biotech Co., Ltd. (Beijing,China); Cell culture products, unless mentioned otherwise, were purchased from GIBCO. All the chemicals were of analytical grade and used without further purification. Sartorius ultrapure water (18.2 MΩ cm) was used throughout the experiments. DNA oligonucleotides were

synthesized and purified by TAKARA Biotechnology (Dalian, China) and Sangon Biotechnology Co., Ltd (Shanghai, China). The human breast cancer cell line MCF-7 was purchased from KeyGEN biotechnology Company (Nanjing, China), the normal immortalized human mammary epithelial cell line MCF-10A was purchased from Shanghai Bioleaf Biotechnology Company (Shanghai, China)； Human hepatocellular liver carcinoma cell line HepG2 and human hepatocyte cell line HL-7702 were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

Instruments. High resolution transmission electron microscopy (HRTEM) was carried out on a JEM-2100 electron microscope. Absorption spectra were measured on a pharmaspec UV-1700 UV-visible spectrophotometer (Shimadzu, Japan). Fluorescence spectra were obtained with FLS-920 Edinburgh Fluorescence Spectrometer with a Xenon lamp and 1.0 cm quartz cells at the slits of 3.0/3.0 nm. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. Absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) in the MTT assay. Flow cytometry was performed using a Beckman Coulter Epics XL (Beckman Coulter, Inc., Brea, CA). Confocal fluorescence imaging were performed with a TCS SP5 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with an objective lens (×20). RT-PCR was carried out with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA).

Preparation of gold nanoparticles. The gold nanoparticles (AuNPs) of 13 nm were

prepared using the sodium citrate reduction method reported before. All glassware was cleaned in aqua regia (HCl/HNO_3, 3:1), rinsed with H_2O, and oven-dried before the experiments. 100 mL $HAuCl_4$ (0.01 %) was heated to boiling with vigorous stirring, then 2.0 mL trisodium citrate (1%) was added under stirring. The color of the solution turned from pale yellow to colorless and finally to burgundy. Boiling was continued for an additional 10 min. The colloid was stirred until the solution reached room temperature. Then it was filtered through a 0.45 μm Millipore membrane filter. The prepared AuNPs were stored at 4 °C.

MB structure. The potential secondary structure of MB was predicted by using UNAfold on www.idtdna.com. It indicated that the "stem and loop" conformation has formed for all the four MBs.

Preparation of nanoprobe. Equimolar MBs (labeled by Alexa Fluor 488 or Cy3) were mixed and then added to a solution of Au NPs (1 nM) with a final concentration of 50 nM each and shaken for 6h. Then SDS solution (10%) was added to the mixture with final concentration of 0.1 %. After that the solution was shaken overnight, then phosphate buffer (0.1 M; pH = 7.4) was added to the mixture to achieve 0.01 M phosphate concentration and the NaCl concentration of the mixture was slowly increased to 0.1 M over an eight-hour period. Afterwards the nanoprobe solution was centrifuged (13500 g, 30 min) and resuspended in phosphate buffered saline (PBS) for three times. Then the nanoprobe was sterilized using a 0.22 μm acetate syringe filter and resuspended in PBS with a concentration of 3 nM as stock solution stored at 4 °C. The nanoprobe was diluted to certain concentration for use in all subsequent

experiments. The concentration of Au NPs was determined by measuring their extinction at 524 nm ($\varepsilon = 2.7\times10^8$ L mol^{-1} cm^{-1}).

Quantitation of each MB loaded on the nanoprobe. The two MBs loaded on Au NPs were quantitated according to the published protocol. 20 mM mercaptoethanol (ME) was added to the probe solution (1 nM). After incubated overnight with shaking at room temperature, the MBs were released. Then the released MBs were separated through centrifugation and the fluorescence was measured with a fluorescence spectrometer. The fluorescence of Alexa Fluor 488 labeled MB was excited at 488 nm and measured at 515 nm and the fluorescence of Cy3 labeled MB was excited at 550 nm and measured at 560 nm. The fluorescence was converted to molar concentrations of MB by interpolation from a standard linear calibration curve prepared with known concentrations of MB with identical buffer pH, ionic strength and ME concentrations. By dividing molar concentrations of each MB by the original nanoprobe concentration, the amount of MBs per nanoprobe was calculated. The MBs loaded on the three-color and four-color FRET nanoprobes were quantitated according to the above method.

Kinetics. Cell lysis was used in this experiment. The nanoprobe (1 nM) was mixed with cell lysis solution (3×10^6 cells/mL), then the FRET fluorescence intensity was measured with increasing time (0, 5, 10, 15, 20, 30, 40, 60, 80, 100, 120, 140 minutes). The FRET fluorescence was excited at 488 nm and measured at 560 nm.

FRET active experiment. The nanoprobe (1 nM) was incubated with cell lysis of increasing concentrations (8, 16, 24, 32, 40, 48, 56, 64×10^4 cells/mL). After

incubation for 1h at 37 °C, the fluorescence was monitored at appropriate excitation wavelengths. The FRET fluorescence was excited at 488 nm and measured at 560 nm.

FRET ratio. The nanoprobe (1nM) loaded with Alex fluor 488-MB_1 was incubated with cell lysis solution (3×10^6 cells/mL) for 1h at 37 °C. Then the fluorescence of Alex fluor 488 was measured with 488 nm excitation and the fluorescence intensity at 515 was set as F_1. The nanoprobe (1nM) loaded with Alex fluor 488-MB_1 and Cy3-MB_2 or 488-MB_1, Cy3-MB_2 and Rox-MB_3 or 488-MB_1, Cy3-MB_2, Rox-MB_3 and Cy5-MB_4 was incubated with cell lysis solution (3×10^6 cells/mL) for 1h at 37 °C. Then the fluorescence of was measured with 488 nm excitation and the fluorescence intensity at 515 was set as F_2. The FRET ratio was calculated as follow: FRET ratio = $(F_1-F_2)/F_1$

Specificity experiment. The complementary or single base mismatched DNA targets for each MB and other DNA targets were combined differently（sur-t, tk1-t, sur-t+tk1-t, sur-t+tk1-mt, sur-mt+tk1-t, myc-t, gt-t） and added to 1 mL hybridization buffer containing 1 nM nanoprobe, while the DNA targets were 100 nM for each kind. All experiments were repeated at least three times.

Nuclease assay. Two groups of nanoprobe (1 nM in buffer) were placed in a 96-well fluorescence microplate at 37 ºC. After allowing the samples to equilibrate (10 minutes), 1.3 μL of DNase I in assay buffer (2 U/L) was added to one group. The fluorescence of these samples was monitored for 60 min. After that 100 nM DNA targets for each MB were added into the two samples with incubation for 1 h at 37 ºC, then the FRET fluorescence was measured at appropriate excitation wavelengths after

the solution was cooled to room temperature.

Cell culture. All the cells were cultured in Dulbecco's modified Eagles medium (DMEM) and were supplemented with 10% fetal bovine serum and 100 U/ml 1% antibiotics penicillin/streptomycin and maintained at 37 °C in a 100% humidified atmosphere containing 5% CO_2 at 37 °C.

MTT assay. MTT assay was performed to study the cytotoxicity of the nanoprobe. MCF-7 cells (1×10^6 cells/well) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL $well^{-1}$. Plates were maintained at 37 °C in a 5% CO_2 / 95% air incubator for 24 h. After the original medium has been removed, the MCF-7 cells were incubated with naked-Au NPs (1 nM), nanoprobe (1nM) for 3 h, 6 h, 12 h, 24 h. The cells incubated with the culture medium only were served as control. Then the cells were washed with PBS for three times and 100 μL MTT solutions (0.5 mg mL^{-1} in PBS) were added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a microplate reader (Synergy 2, Biotek, USA).

Confocal fluorescence imaging. MCF-10A, MCF-7, HepG2 and HL-7702 cells were used in these experiments and tamoxifen and β-Estradiol were used to down regulate and up regulate the expression of TK1 mRNA. In comparative experiment of cancer cells and normal cells, all cells were plated on chamber slides for 24 h. Then the nanoprobe (1 nM) was respectively delivered into different cells in culture medium at 37 °C in 5 % CO_2 for 4 h. The cells were examined by confocal laser scanning

microscopy (CLSM) with different laser transmitters. In the experiments in which TK1 mRNA was regulated, MCF-7 or HepG2 cells were pretreated with β-Estradiol (10^{-8} mol/L) or tamoxifen (10^{-6} mol/L) for 48 h. Then the other steps performed as described above using the nanoprobe(1 nM). After that the cells were monitored by CLSM with 543 nm exciation for Cy3 fluorescence and 488 nm exciation for FRET fluorescence.

Mechanisms of nanoprobe uptake. Chlorpromazine was used to inhibit clathrin-mediated endocytosis (CME), Filipin was used to inhibit cavolin-mediated endocytosis and dynasore was used to inhibit both pathways above. Ethylisopropylamiloride (EIPA) was used to inhibit macropinocytosis. MCF-7 cells were divided into five groups, one group was set as control group and the other groups were pre-incubated with endocytosis inhibitors: Chlorpromazine 10μM, Filipin 5μM, Dynasore 100μM and EIPA 50μM. Then nanoprobe (1nM) was added into each group. After 4 h incubation Confocal fluorescence imaging studies were carried out and FRET signal was monitored by 488nm exciation.

Flow cytometry. MCF-10A, MCF-7, HepG2, HL-7702, tamoxifen treated HepG2 and tamoxifen treated MCF-7 cells（HepG2 and MCF-7 cells were pretreated by tamoxifen for 48h）were used in this experiment. The nanoprobe was added to all of the cells and incubated with the cells for 4 h, respectively. After treatment, cells were detached from culture flasks using trypsin. Flow cytometry was performed using a Beckman Coulter Epics XL, with excitation at 488 nm to monitored FRET fluorescence.

RT-PCR. MCF-10A, MCF-7, HepG2, HL-7702, tamoxifen treated HepG2 and tamoxifen treated MCF-7 cells （HepG2 and MCF-7 cells were pretreated by tamoxifen for 48h）were used in this experiment. Total RNA from sorted cells was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA synthesis was performed using an iScript kit (Bio-Rad). RT-PCR was carried out with SYBR Green I (Qiagen) on an ABI PRISM 7000 sequence detection system. Relative level of tumor mRNA was calculated from the quantity of tumor mRNA PCR products and the quantity of GAPDH PCR products. The primers used in this experiment were TK1 forward, 5'-TATGCCAAAGACACTCGCTAC-3'; TK1 reverse, 5'-GCAGAACTCCACGATGTCAG-3'; GAPDH forward, 5'-GGGAAACTGTGGCGTGAT-3'; GAPDH reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'.

Supplementary Table:

Table S1. DNA sequences employed in this work.

Oligonucleotide	Sequence
MB1	5'-Alexa Fluor 488- GACATGTAGAGATGCGGTGGTC-CATGTCAAAAAA-$(CH_2)_3$-SH-3'
MB2	5'-Cy3-ACGACGCCAGGGAGAACAGAAACCGTCGTAAAAAA-$(CH_2)_3$-SH-3'
MB3	5'-Rox-CAGTTGGTGAAGCTAACGTTGAGCAACTGAAAAAA-$(CH_2)_3$-SH-3'
MB4	5'-Cy5-CAGTGTCTTATGCGGATAGTGAAACACTGAAAAAA-$(CH_2)_3$-SH-3'
survivin perfectly matched target	5'-GACCACCGCATCTCTA-3'
survivin single-base mismatched target	5'-GACCACCTCATCTCTA-3'
TK1 perfectly matched target	5'-GTTTCTGTTCTCCCTGG-3'
TK1 single-base mismatched target	5'-GTTTCTGTGCTCCCTGG-3'
c-myc perfectly matched target	5'-CTCAACGTTAGCTTCAC-3'
c-myc single-base mismatched target	5'-CTCAACGTGAGCTTCAC-3'
GalNAc-T target	5'-TTCACTATCCGCATAAG-3'
GalNAc-T single-base mismatched target	5'-TTCACTATGCGCATAAG-3'

[a]Underlined letters represent the stem sequence; [b]Letters in red represent the mismatched site.

Supplementary Figures:

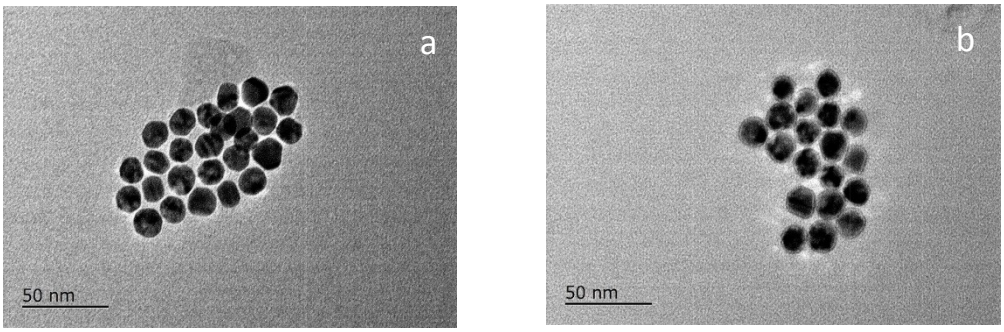


Fig. S1 HRTEM images of AuNPs (a) and the FRET nanoprobe (b).

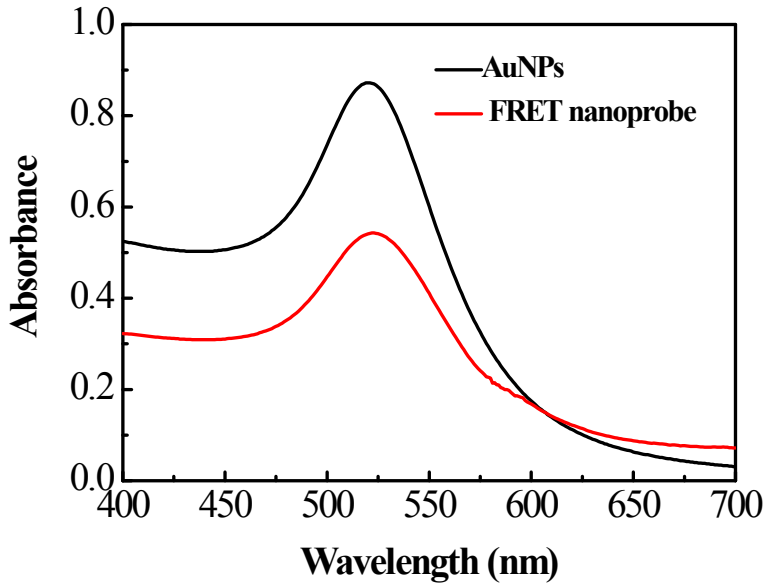


Fig. S2 UV-vis spectra for AuNPs and the FRET nanoprobe.

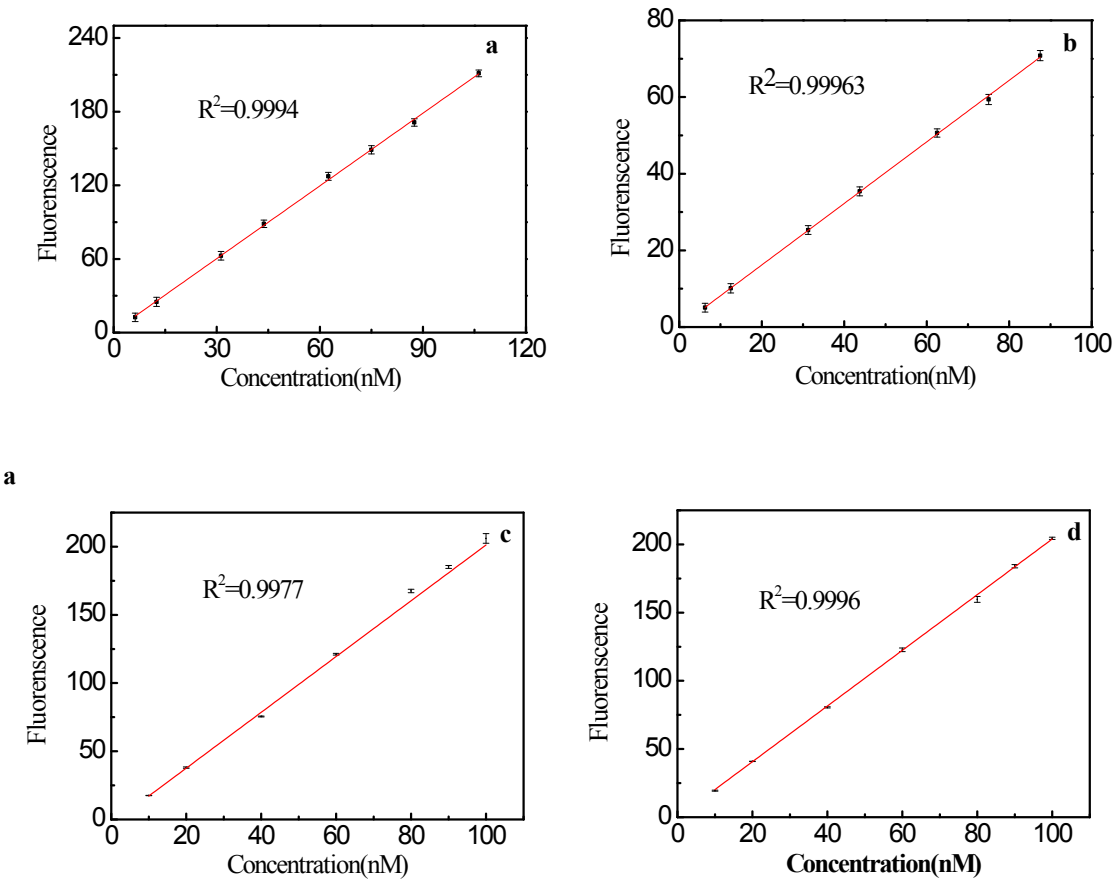


Fig. S3 Standard linear calibration curves of fluorescent dyes. a) Alexa Fluor 488, b) Cy3, c) Rox, d) Cy5.

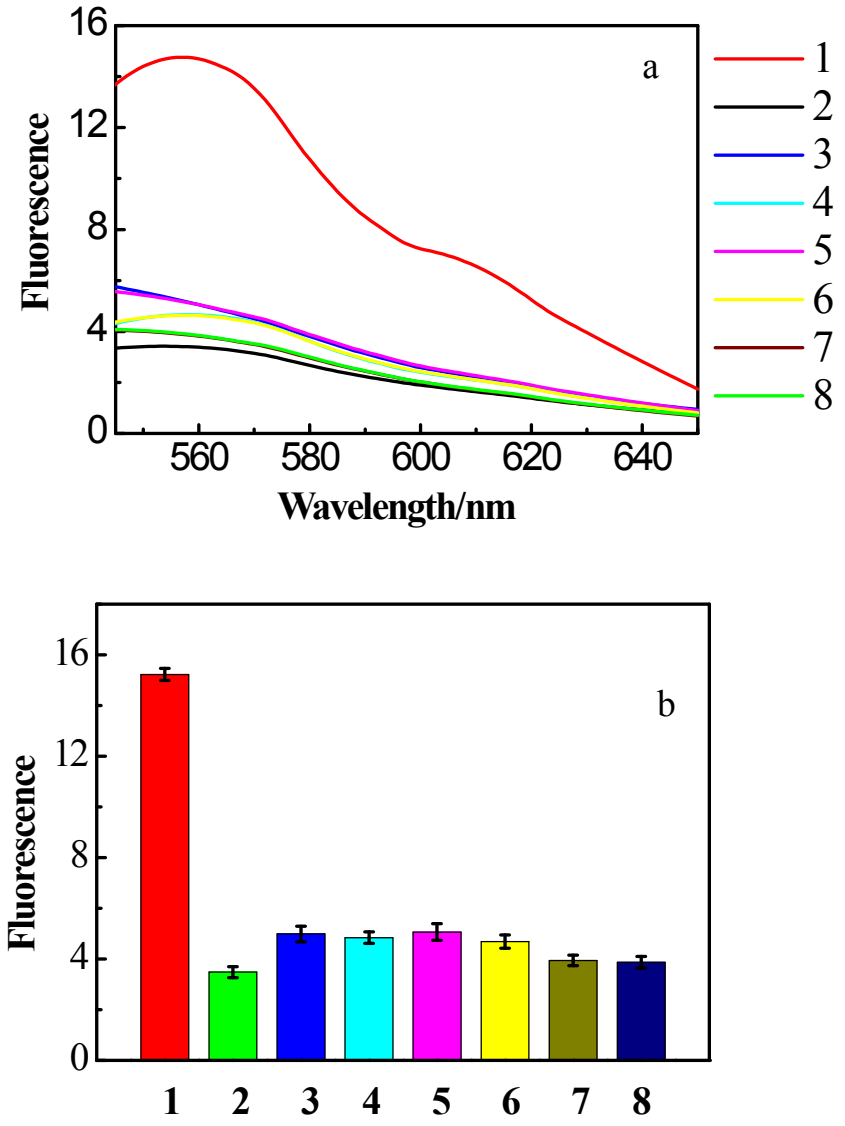


Fig. S4 Specificity of the FRET nanoprobe over several DNA targets. The fluorescence intensity was recorded when the nanoprobe was mixed with the perfectly matched target, single-base mismatched target and other mRNA targets. 1. perfectly matched survivin target and perfectly matched TK1 target, 2. Control, 3.perfectly matched surviving target, 4.perfectly matched TK1 target, 5.perfectly matched survivin target and single-base mismatched TK1 target, 6.perfectly matched TK1 target and single-base mismatched survivin target, 7.perfectly matched c-myc target, 8.perfectly matched GalNAc-T target. The excitation wavelength was 488 nm, and the recorded

emission wavelength was 565nm.

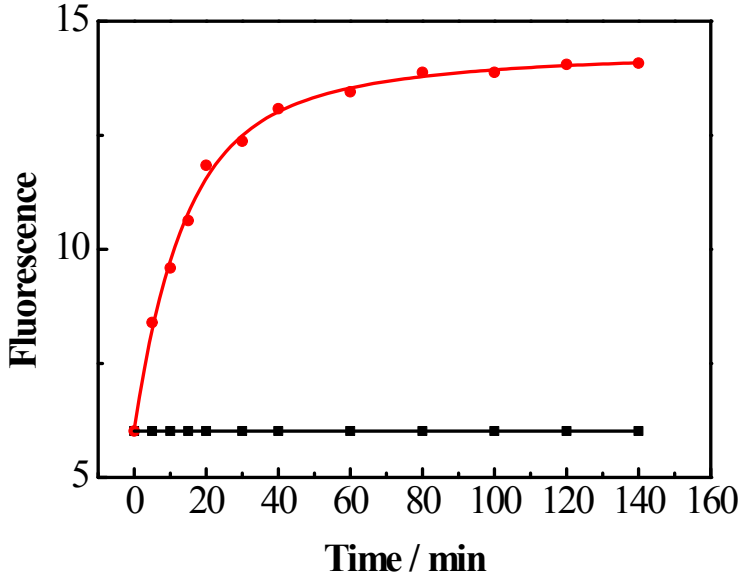


Fig. S5 Kinetics of the bi-color FRET nanoprobe in MCF-7 cell extracts. The excitation wavelength was 488 nm, and the recorded emission wavelength was 565nm.

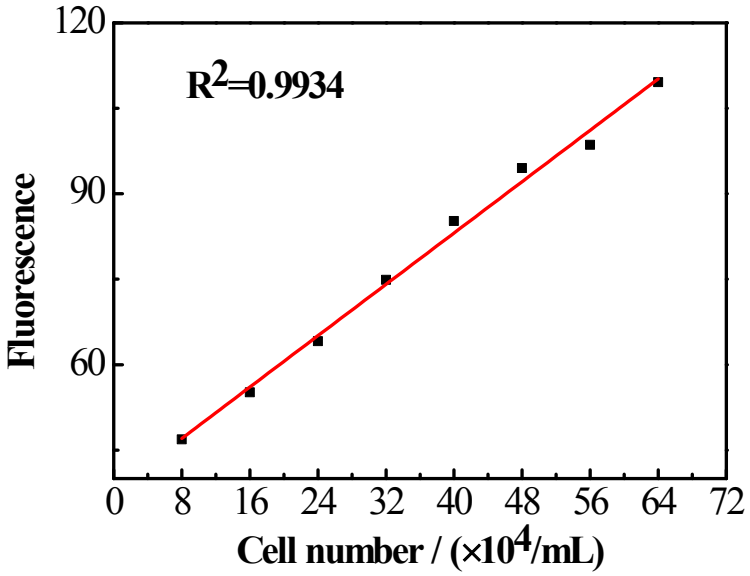


Fig. S6 Fluorescence response of the FRET nanoprobe in MCF-7 cell extracts with the increase of cell number. The excitation wavelength was 488 nm, and the recorded emission wavelength was 565nm.

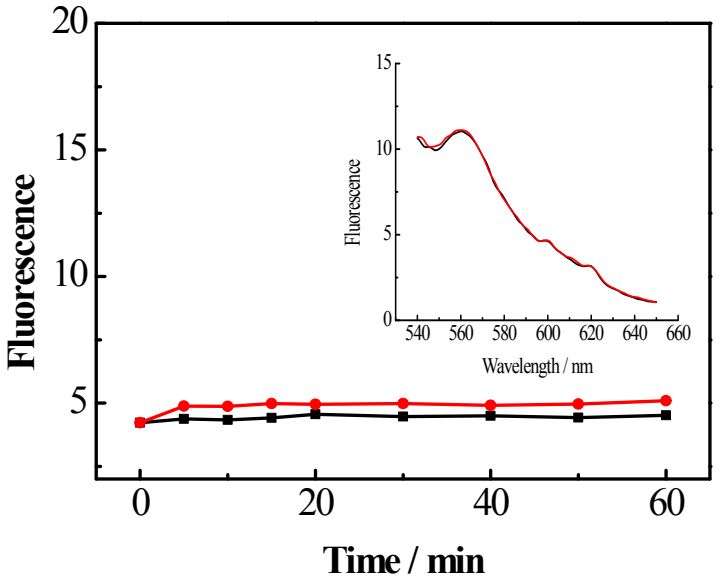


Fig. S7 Nuclease stability of the bi-color FRET nanoprobe in the presence or absence of DNase I. Fluorescence curve of the nanoprobe (1 nM) in buffer without DNase I (black trace) or in the presence of DNase I (red trace) as a function of time. Insets: fluorescence spectra after hybridization of the nanoprobe with the two perfectly matched DNA targets in the presence of DNase I (red curve) and absence of DNase I (black curve). The excitation wavelength was 488 nm.

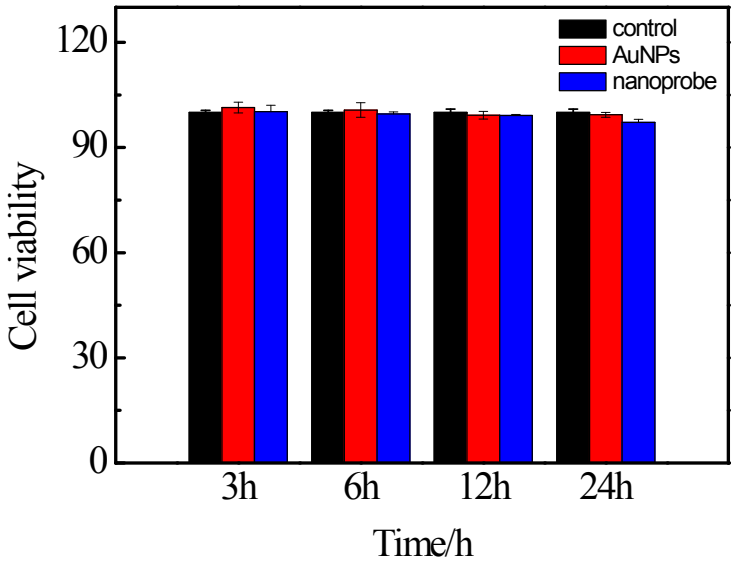


Fig. S8 Growth inhibition assay (MTT). MCF-7 cells were incubated with unmodified AuNPs (1 nM), the bi-color FRET nanoprobe (1 nM) for 3 h, 6 h, 12 h, 24 h. Black bars stand for the control, red bars stand for the unmodified AuNPs; blue bars stand for the nanoprobe (1 nM).

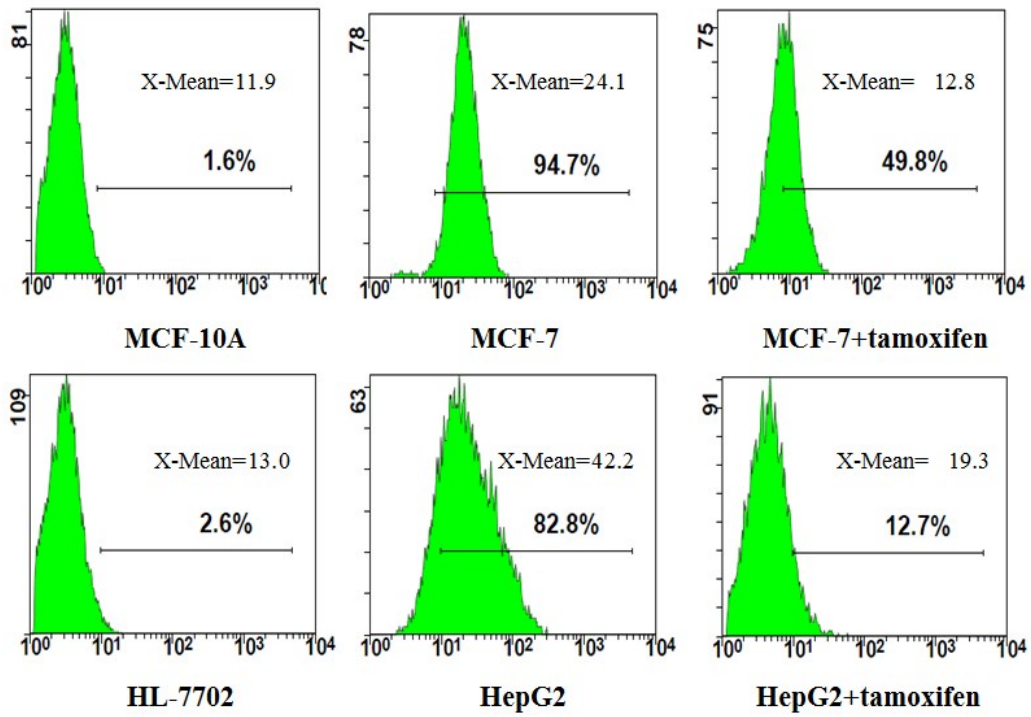


Fig. S9 Flow cytometry data of the bi-color FRET nanoprobe incubated with MCF-7 cells, MCF-10A cells, HepG2 cells, HL-7702 cells, treated MCF-7 cells and treated HepG2 cells.

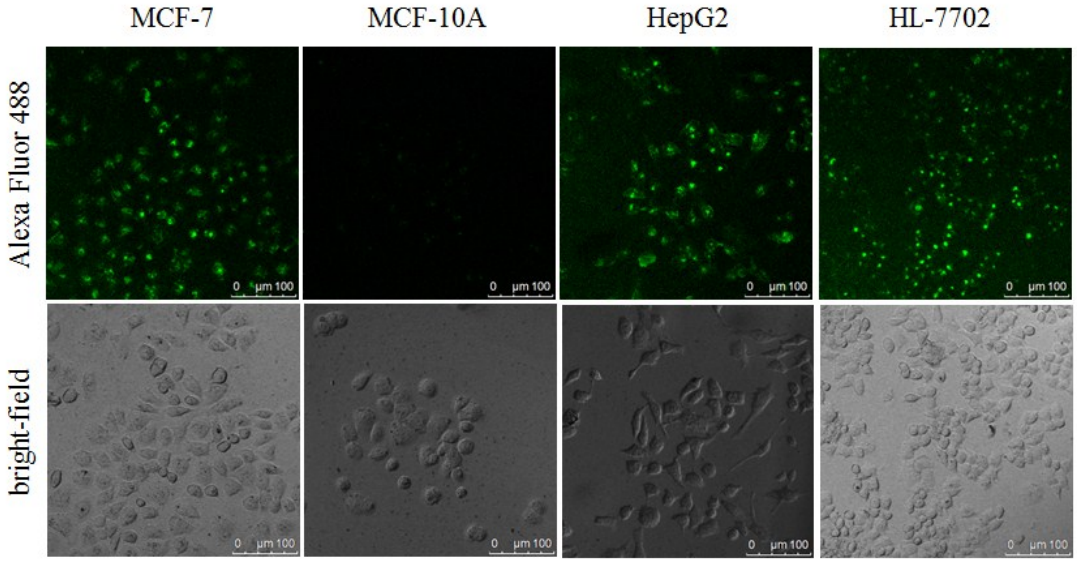


Fig. S10 Intracellular imaging of the survivin mRNAs under CLSM. MCF-7, HepG2, MCF-10A, and HL-7702 cells were incubated with the single-color nanoprobe (1 nM) for 4 hours at 37°C. The excitation wavelength was 488 nm. Scale bars are 100μm.

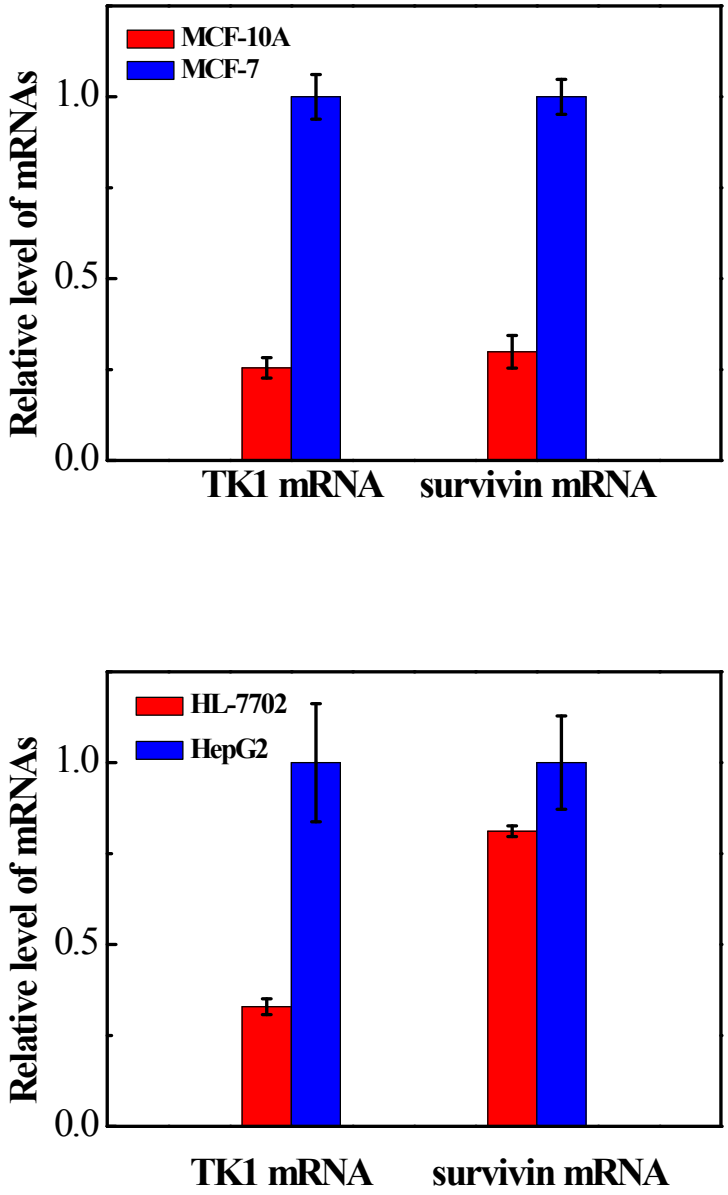
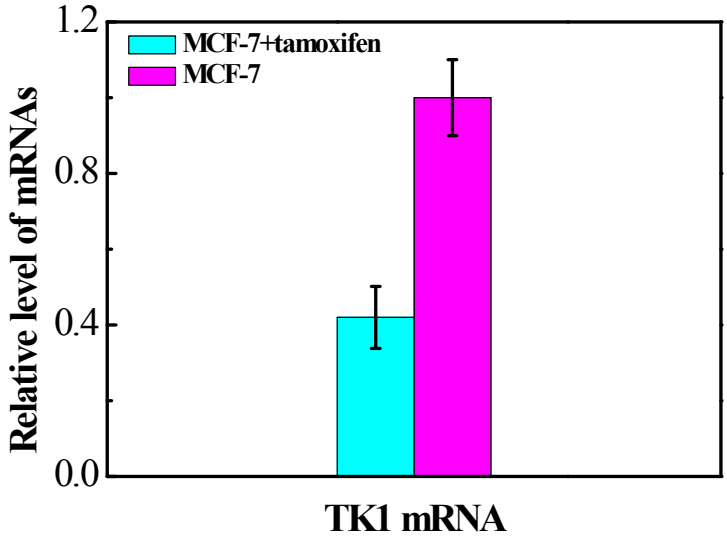


Fig. S11 Detection of the levels of the two mRNAs by RT-PCR in a: MCF-10A cells and MCF-7 cells, b:HL-7702 cells and HepG2 cells. The relative level of tumor mRNA was calculated from the quantity of tumor mRNA PCR products and the quantity of GAPDH PCR products and normalized to the expression level in cancer cells.



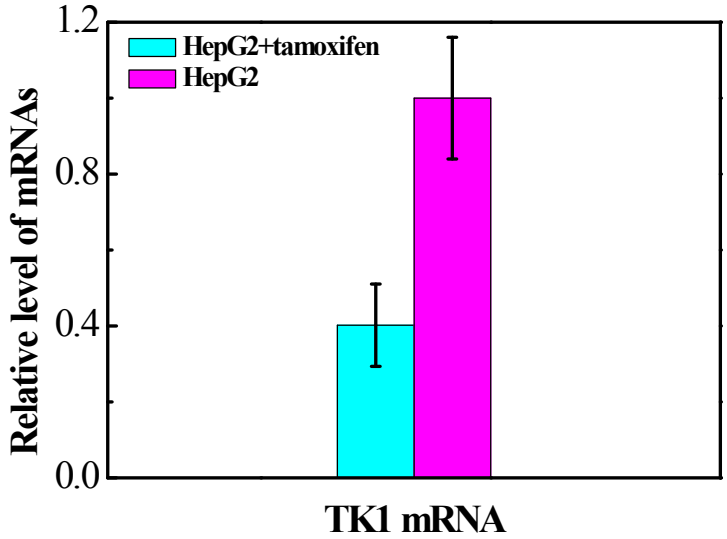


Fig. S12 Detection of the levels of TK1 mRNAs by RT-PCR in a: MCF-7 cells and treated MCF-7 cells, b: HepG2 cells and treated HepG2 cells. The relative level of tumor mRNA was calculated from the quantity of tumor mRNA PCR products and the quantity of GAPDH PCR products and normalized to the expression level in untreated cells.

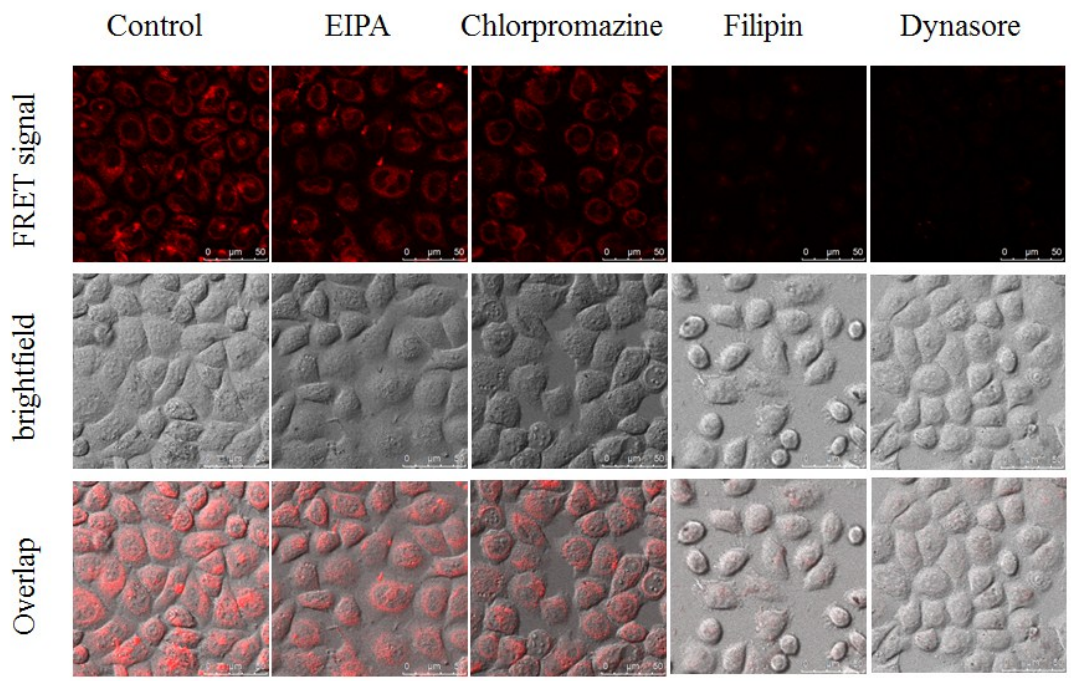


Fig. S13 Intracellular imaging of MCF-7 cells treated with chlorpromazine, filipin, EIPA and dynasore. The FRET signal was obtained using the excitation wavelength of 488 nm. Scale bars are 100 μm.

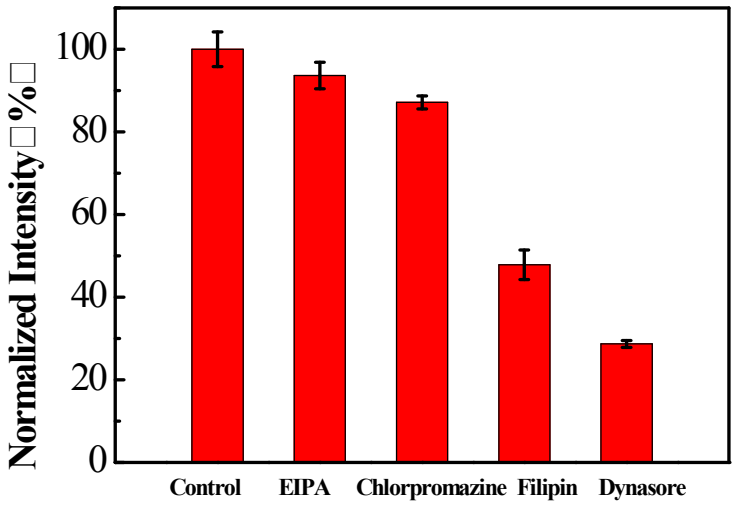


Fig. S14 Normalized fluorescence intensity of MCF-7 cells treated with chlorpromazine, filipin, EIPA and dynasore.

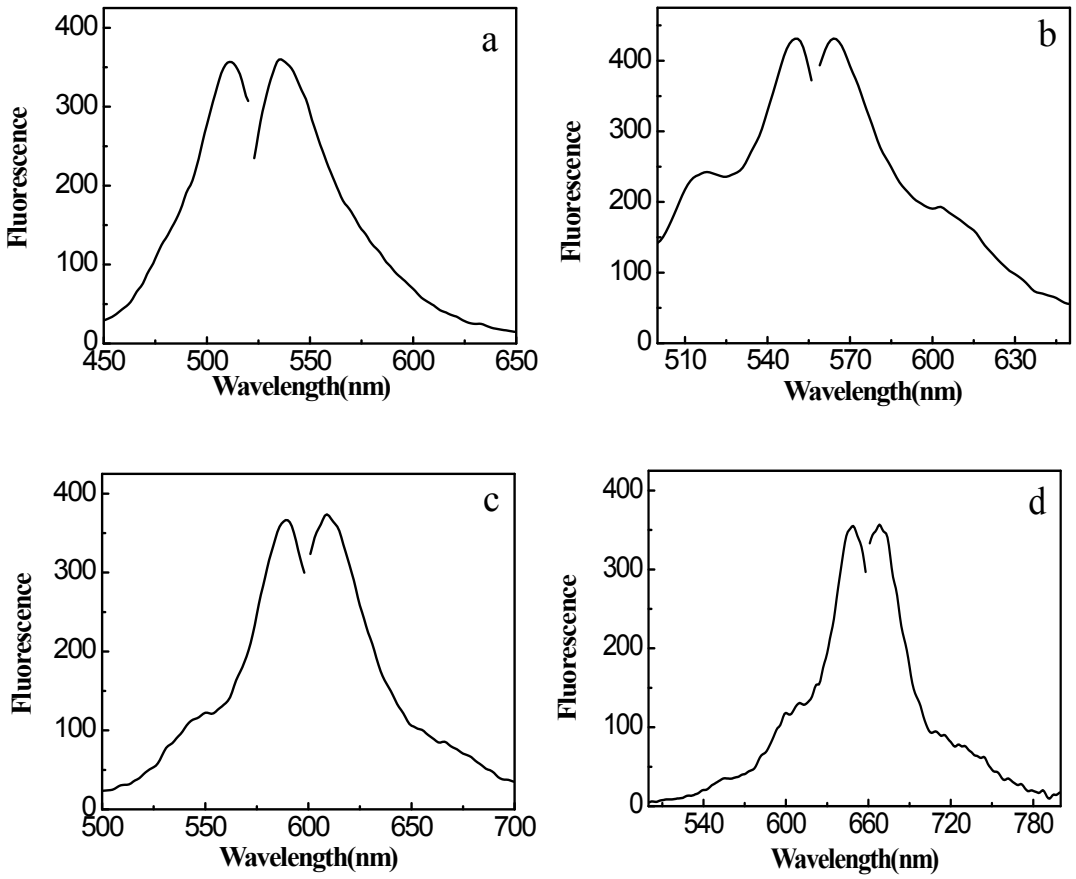


Fig. S15 The excitation spectra and emission spectra of the four fluorophores. a: Alexa Fluor 488, b: Cy3, c: Rox, d: Cy5.