Supporting Information:

Molecular Recognition-activatable DNA Nanofirecracker Enables Signal-enhanced Imaging in Living Cells

Xiaojing Xing,[*b,c,‡] Jin Li,[c,‡] Liping Qiu,[*a,c] and Weihong Tan[c]

[a] NHC Key Laboratory of Birth Defect for Research and Prevention, Hunan Provincial Maternal and Child Health Care Hospital, Changsha 410000, China

[b] Engineering Technology Research Center of Henan Province for Solar Catalysis, College of Chemistry and Pharma-ceutical Engineering, Nanyang Normal University, Nanyang, Henan 473061, China

[c] Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China

‡ These authors contributed equally to this work.

* E-mail: xjxing@hnu.edu.cn; qiuliping@hnu.edu.cn

Tables of Contents

1. Experimental section.....S-3

2. DNA sequences designed in this work…….....S-6

3. Detailed working principle of the DNA nanofirecracker.....S-7

4. Gel electrophoresis analysis of the nanofircracker.....S-8

5. Working principle for measuring the payload of the nanofirecracker S-9

6. Quantifying the payload of the nanofirecracker…….....S-10

7. Impact of ATP on the disassembly of the nanofirecracker.....S-11

8. Optimizing the sequence design of H1 and H2.....S-12

9. Detection capability of Apt-SE-RP in buffer solution.....S-13

10. Detection selectivity of Apt-SE-RP.....S-14

11. Target-responsive fluorescence enhancement of Apt-SE-RP, DSP and MB.....S-15

12. Capability of Apt-SE-RP for distinguishing DNA mismatch.....S-16

13. Cellular internalization of Cy5-labelled AS1411.....S-17

14. Cell-type specificity of Apt-SE-RP S-18

15. Cytotoxicity of Apt-SE-RP.....S-19

16. CLSM images of MCF-7 cells with different treatments.....S-20

17. Relative signal intensity of MCF-7 cells with different treatments…….....S-21

18. CLSM images of fixed MCF-7 cells with different treatments.....S-22

19. Ratiometric detection of mRNA in cordycepin-pretreated MCF-7 cells.....S-23

Experimental section

Chemicals and Materials. Lipopolysaccharide (LPS) and cordycepin were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Agarose and ethidium bromide (EB) were purchased from Beijing Dingguo Changsheng Biotechnology Co. Adenosine 5'-triphosphate, disodium salt (ATP) solution, DNA marker, and DNase I were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All oligonucleotides (Table S1, Supporting Information) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). RPMI 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin (100×) were purchased from Life Technologies (Gibco, Carlsbad, CA). Unless otherwise specified, all other used reagents were of analytical grade and used without further treatment. All solutions used in the experiments were prepared using ultrapure water (resistance > 18 MΩ cm).

Preparation of the nanofirecracker probe. H1 and H2 were individually heated at 95 °C for 5 min, incubated on ice for 3 min, and kept at room temperature (RT) for 2 h. Subsequently, H1 (10 μL, 10 μM) and H2 (10 μL, 10 μM) were mixed in Tris-HCl buffer (20 mM, pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$) with a final volume 99 μL, then the mixture was incubated at RT overnight. After that, the target recognition probe (0.5 μL, 100 μM) and the cell targeting ligand (aptamer AS1411, 0.5 μL, 100 μM) were added and incubated for 30 min. Finally, the mixture with a final volume of 100 μL was purified using an Amicon Ultra Centrifugal Filter (100 kD, Millipore, USA) to remove excess single-stranded DNA and assemblies of small sizes.

Characterization of the nanofirecracker probe. For atomic Force Microscopy (AFM) assay, the H1/H2 duplex or free H1 were deposited on mica for 3 min, rinsed three times with ultrapure water, and dried using nitrogen gas. The resultant products were imaged with a Multimode 8 (Bruker, USA) using ScanAsyst mode in ambient air. The corresponding concentration of H1 and H2 was fixed at 1 μM. For agarose gel electrophoresis, 5 μL of the sample were mixed with 1 μL 6×loading buffer and loaded onto a 3% agarose gel stained with EB. The electrophoresis was performed in TBE buffer (1%) at 100 V for 60 min, and the gel was imaged with an AlphaImager IS-2200 (Alpha Innotech, CA, USA). The corresponding concentration of H1 and H2 was fixed at 2 μM.

Fluorescence measurements. Typically, the nanofirecracker probe (5 μL, 50 nM) was mixed with the analyte of different concentration and ATP of the final concentration of 4 mM in 10 μL Tris-HCl buffer in a 0.5-mL centrifuge tube. After incubation at 37 °C for 2 h, 90 μL Tris-HCl buffer was added and the fluorescence spectrum was recorded with a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ). The excitation wavelength for the TAMRA fluorophore was fixed at 545 nm with an emission wavelength ranging from 560 nm to 700 nm. The excitation wavelength for the Cy5 fluorophore was fixed at 625 nm with an emission wavelength ranging from 640 nm to 750 nm. For comparison of the signal amplification effect, two control probes, a molecular beacon (MB) and a double-stranded DNA probe (DSP), were synthesized and tested under identical conditions as that of the nanofirecracker probe.

Cell lines and cell culture. Both MCF-7 cells and LO2 cells were purchased from ATCC. They were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 atmosphere.

Cell viability test. Cytotoxicity of Apt-SA-RP was assessed by Cell Counting Kit-8 (CCK-8) assay. MCF-7 cells (1×10^4 cells/well) were seeded in a 96-well plate and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h to reach 80 % confluency. The cells were treated with Apt-SE-RP of different concentrations for 2 h, 4 h, or 6 h. After removal of free probe, the cells were added with 10 % (v/v) CCK-8 reagent and incubated for 30 min. Finally, the cell viability was tested by measuring the absorbance at 450 nm with a multimode microplate reader.

CLSM imaging. MCF-7 cells (2×10^5) were seeded in a 25-mm glass-bottom confocal dish and cultured overnight. Generally, for regulating the expression level of MnSOD mRNA, MCF-7 cells were pretreated with cordycepin (100 μg/mL, downregulation) and LPS of specific concentrations (upregulation) for 2 h. Then, the cells were thoroughly washed with PBS and incubated with the nanofirecracker or control probes at 37 °C in a 5% CO_2 atmosphere for 2 h. Unless otherwise stating, the corresponding probe concentration was fixed at 50 nM. After being washed three times, the cells were imaged with a LSM880 confocal laser scanning microscope (Carl Zeiss, Germany) using a 60× oil immersion objective. The data were analyzed with an ImageJ software.

For FISH assay. MCF-7 cells were washed three times with Diethyl pyrocarbonate (DEPC)-containing PBS, the cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 20 min and washed three times. Subsequently, the cells were permeabilized with 0.2% Triton for 25 min, washed three times and incubated with 50 nM DNA probes in DEPC-containing PBS plus 4 mM ATP at 37 °C for 2 h. The resultant cells were analyzed with CLSM imaging.

Flow cytometry assay. MCF-7 cells (1.5×10^5) were seeded in 12-well plates and cultured overnight. The cells were incubated with detection probes at 37 °C in a 5% CO_2 atmosphere for 2 h. After removal of free probes, the cells were detached with 0.25 % (w/v) trypsin containing EDTA, resuspended in 200 μL PBS and detected with a BD FACSVerse™ flow cytometer. Data were analyzed with a FlowJo software.

Table S1. Sequences of DNA oligonucleotides used in this work

Name	Sequence (5′-3′)
H1 (14, 15)	TAMRA-TTC CTT ***CC TGG GGG AGT ATT GCG GAG GAA GG*** AAT GTA AT
H2 (14, 15)	CCC CCA GG AAG GAA AT(BHQ2) T AC ATT CC TTC CT
H1 (15, 15)	TAMRA-A TTC CTT ***CC TGG GGG AGT ATT GCG GAG GAA GG*** AAT GTA AT
H2 (15, 15)	CCC CCA GG AAG GAA T(BHQ2) ATT AC ATT CC TTC CT
H1 (17, 15)	TAMRA-AGC TTC CTT ***CC TGG GGG AGT ATT GCG GAG GAA GG*** AAT GTA AT
H2 (17, 15)	CCC CCA GG AAG GAA GCT(BHQ2) ATT AC ATT CC TTC CT
Target model	G GAA TGT AAT CAA CTG GGA GAA TGT AAC TGA AAG ATA CAT
RP	TA GTA TCT TTC AGT TAC ATT CTC CCA GTT GAT TAC ATT CC TTC CT CCG
Control RP	NN NNN NNN NNN NNN NNN NNN NNN NNN N AT TAC ATT CC TTC CT CCG
Cy5-Apt	Cy5-TTTT ***GGT GGT GGT GGT TGT GGT GGT GGT GG*** TTT AG GAA GG AAT GTA AT
Control Cy5-Apt	Cy5-TTTT NNN NNN NNN NNN NNN NNN NNN NNN NN TTT AG GAA GG AAT GTA AT
MB	TAMRA-CCG AGC AGT TAC ATT CTC CCA GTT GAT TGC TCG G-BHQ2
Control MB	TAMRA-CCG AGC ANN NNN NNN NNN NNN NNN TGC TCG G-BHQ2
Forward primer	GGCCTACGTGAACAACCTGAA
Reverse primer	CTGTAACATCTCCCTTGGCCA
M1	GGAATGTAATCAACTGGGATAATGTAACTGAAAGATACAT
M2	GGAATGTAATCATCTGGGAGAATGTAATTGAAAGATACAT
M3	GGAATGTTATCAACTGGTAGAATGTAACTGATAGATACAT
F-DSP	CGGAGGAAGGAATGTAAT-TAMRA
Q-RP	TA GTA TCT TTC AGT TAC ATT CTC CCA GTT(BHQ) GAT TAC ATT CC TTC CT CCG
Control Q-RP	NNN NNN NNN NNN NN NNN NNN NNN NNN NNT (BHQ) GAT TAC ATT CC TTC CT CCG

Note: Italic bold letters in H1 and Cy5-Apt represent the sequence of the ATP aptamer and the AS1411 aptamer, respectively. The underlined fragments of H1 is complementary to the underlined fragments of H2. RP is the recognition probe for the target mRNA. The red letters in M1, M2, and M3 represent the corresponding mutation points of the target sequence.

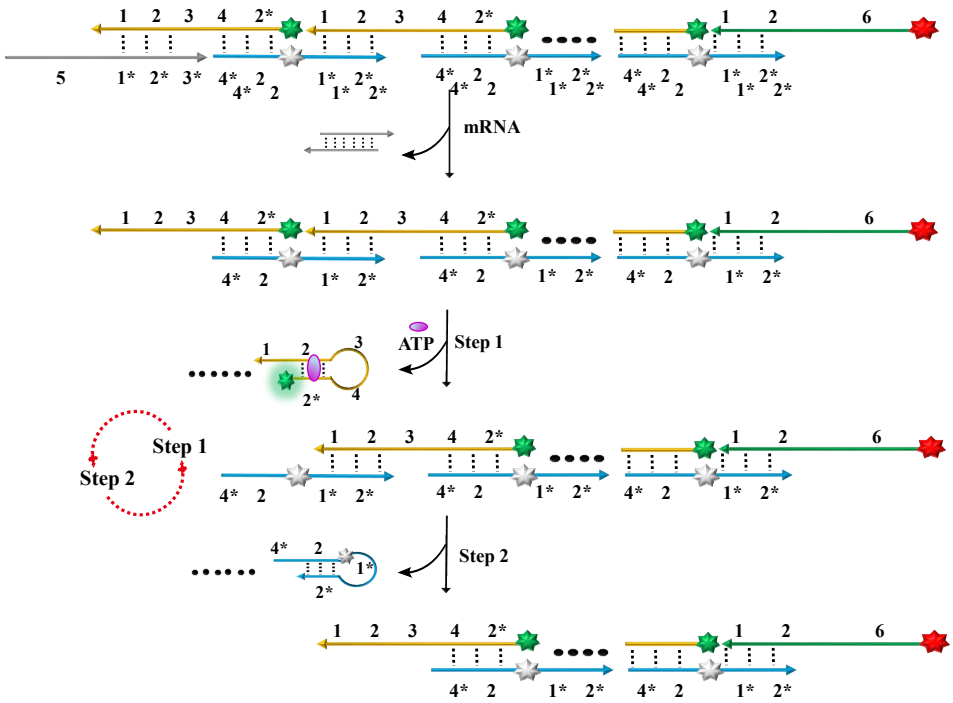

Fig. S1 Working principle of the DNA nanofirecracker for target detection. The DNA nanofirecracker (Apt-SE-RP) is constructed through DNA self-assembly, and composed of three modules: the cell targeting unit (Apt, green line), the recognition probe (RP, gray line), and the signal enhancing (SE) section in between. Domain x is design to complementary to domain x*. The section of SA was formed by tandem hybridization of two DNA monomers, H1 (1-2-3-4-2*, orange line) and H2 (4*-2-1-2*, blue line). The domain 2-3-4 in H1 represents the sequence of the ATP aptamer. The domains 5-1* of RP is complementary to the target. On hybridization with the target, RP would be released from Apt-SE-RP through forming the RP/target complex with higher stability (the hybridization stability of the target/$RP_{5\text{-}1*}$ complex is higher than that of the $RP_{1*\text{-}2*\text{-}3*}$/$H1_{1\text{-}2\text{-}3}$ complex), thus exposing the toehold region (domain 1-2-3) of H1. As such, H1 is activated to bind with ATP through formation of a specific tertiary conformation, resulting in liberation of H1 from Apt-SE-RP (step 1). Subsequently, H2 is released from Apt-SE-RP by self-hybridization to form a hairpin structure (step 2), thereby releasing another recognition site of H1 for ATP. In this way, the target-activated Apt-SE-RP would be dissociated step-by-step, thus generating multiple signal outputs for indicating the presence of the target.

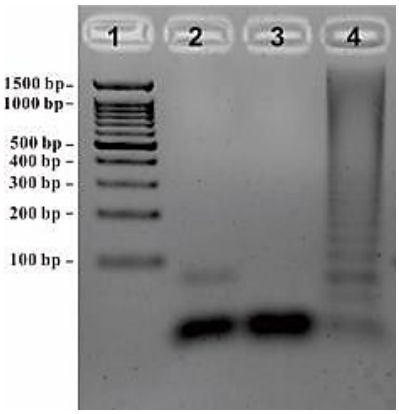

Fig. S2 Gel electrophoresis assay. From Lane 1 to Lane 4: DNA marker, H1, H2, nanofirecracker.

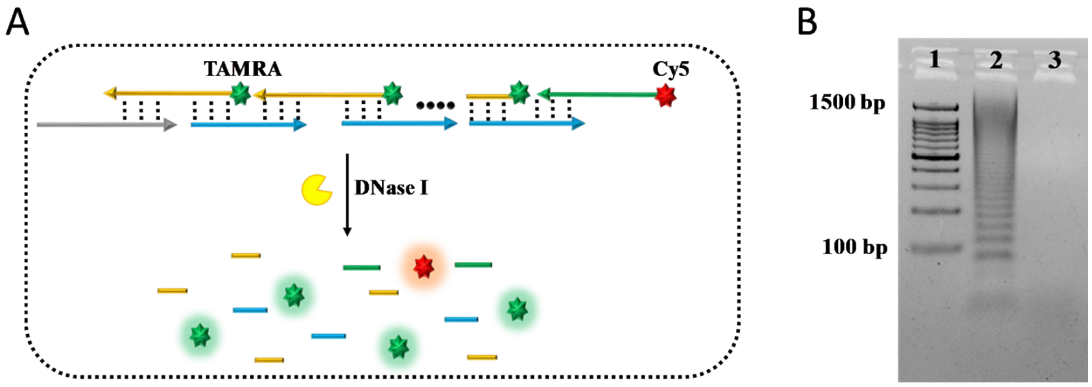

Fig. S3 (A) Schematic illustration of DNase I-based fluorescence assay for measuring the payload of the nanofirecracker. In order to minimize the interference of the BHQ2 quencher on the fluorescence emission of both the TAMRA fluorophore and the Cy5 fluorophore, the purified nanofirecracker probe was pretreated with DNase I (0.2 μL, 1.0 U/μL) at 37 ℃ for 30 min. Then, the TAMRA fluorescence (at 585 nm) and the Cy5 fluorescence (at 665 nm) was measured with a Fluoromax-4 spectrofluorometer at the excitation wavelength of 545 nm and 625 nm, respectively. The average payload of the nanofirecracker was quantified according to the fluorescence calibration curve of both the TAMRA-labelled H1 monomer and the Cy5-labelled Apt, based on the assumption that the nanofirecracker had one and only one Apt. (B) Agarose electrophoresis assay of the nanofirecracker before (2) and after (3) treatment with 0.2 U DNase I. Lane 1 represents the DNA marker.

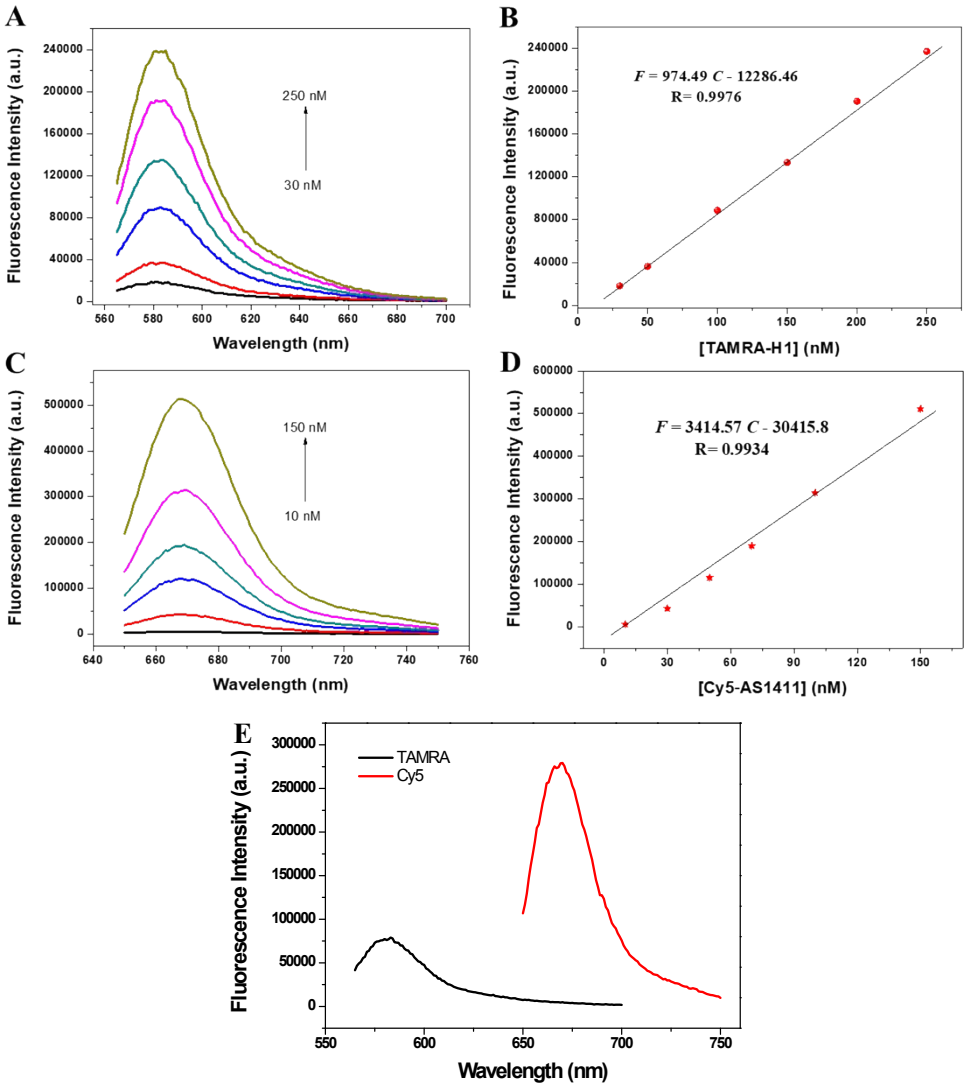

Fig. S4 Measuring the payload of the nanofirecracker. (A) Fluorescence spectra of TAMRA-labelled H1 at different concentrations. (B) Fluorescence calibration curve of the TAMRA fluorescence intensity (585 nm) versus the H1 concentration. (C) Fluorescence spectra of Cy5-labelled Apt at different concentrations. (D) Fluorescence calibration curve of the Cy5 fluorescence intensity (665 nm) versus the Apt concentration. (E) Fluorescence spectra of the nanofirecracker after DNase I treatment. Of note, the nanofirecracker was pre-purified through ultrafiltration with Amicon Ultra centrifugal filter units (100 K MWCO) to remove free DNA strands and small-sized assemblies before this assay.

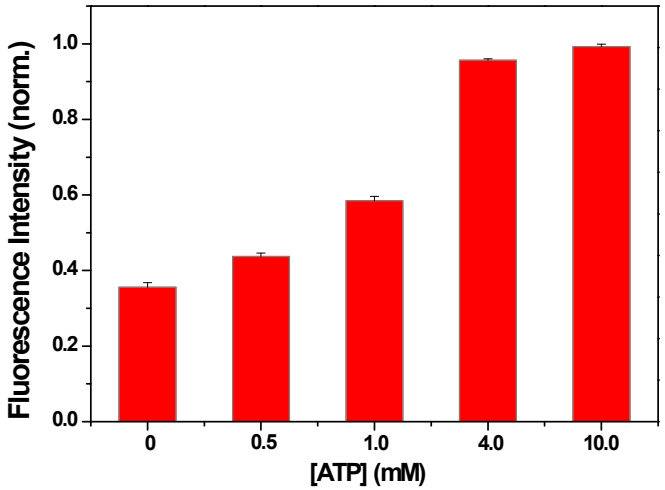

Fig. S5 Relative TAMRA fluorescence intensity (585 nm) of the H1/H2 complex in the presence of ATP at different concentrations. The H1/H2 complex (5 μL 50 nM) was mixed with 5 μL ATP of different concentrations in a 0.5-mL centrifuge tube. After incubation at 37 °C for 2 h, 90 μL Tris-HCl buffer was added and the fluorescence spectrum was recorded.

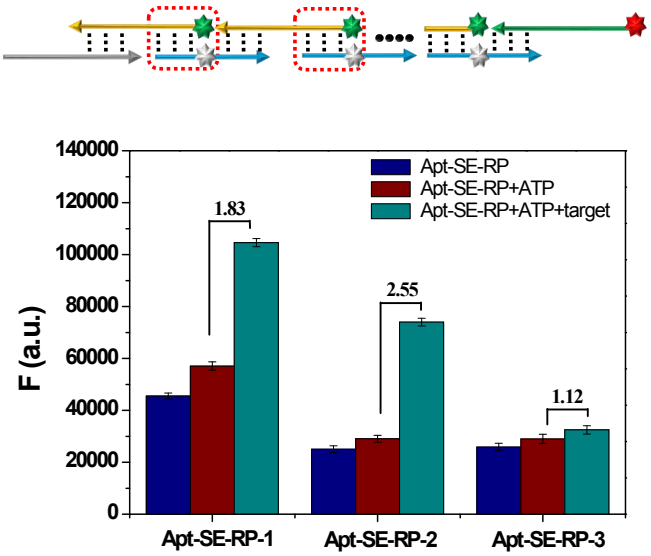

Fig. S6 Optimizing the sequence design of H1 and H2. Apt-SE-RP-1 was composed of H1 (14, 15) and H2 (14, 15). Apt-SE-RP-2 was composed of H1 (15, 15) and H2 (15, 15). Apt-SE-RP-3 was composed of H1 (17, 15) and H2 (17, 15). Generally, the nanofirecracker probe (5 μL 50 nM) was mixed with the target (5 μL, 100 nM) in Tris-HCl buffer containing 4 mM ATP. After incubation at 37 °C for 2 h, 90 μL Tris-HCl buffer was added and the fluorescence spectrum was recorded. The signal-to-noise ratio of Apt-SE-RP-1, Apt-SE-RP-2, and Apt-SE-RP-3, is 1.83, 2.55, and 1.12, respectively.

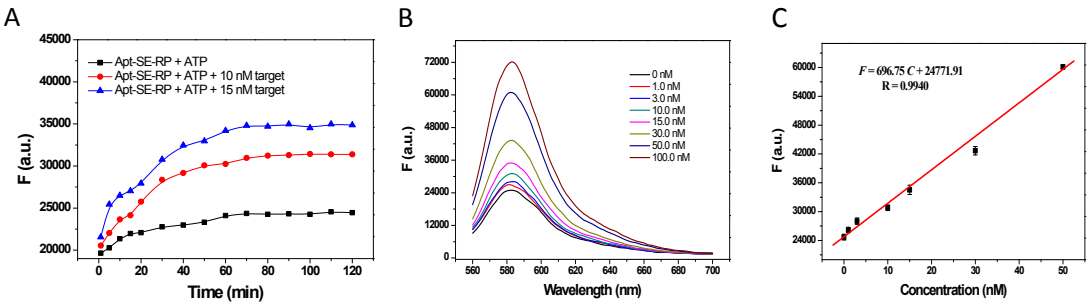

Fig. S7 (A) Fluorescence kinetics of Apt-SE-RP in the absence or presence of the target. The TAMRA fluorescence intensity (585 nm) of each sample was plotted with the reaction time. **(B)** Fluorescence spectra of Apt-SE-RP in response to the target of different concentrations. **(C)** The linear relationship between the TAMRA fluorescence intensity and the target concentration. In these experiments, Apt-SE-RP (5 μL 50 nM) was mixed with the target of different concentrations (5 μL) in Tris-HCl buffer containing 4 mM ATP. After incubation at 37 °C for 2 h, 90 μL Tris-HCl buffer was added and the fluorescence spectrum was recorded.

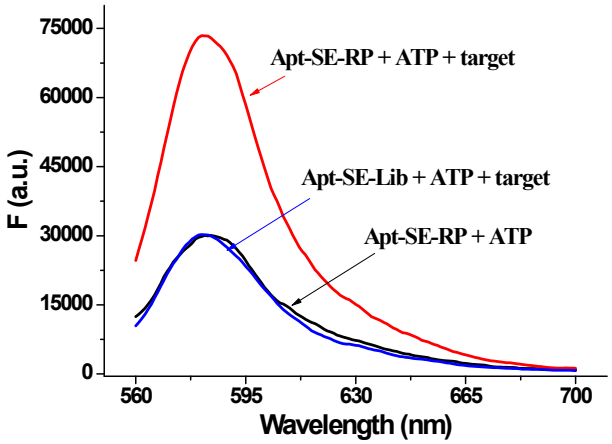

Fig. S8 Fluorescence spectra of Apt-SE-RP or Apt-SE-Lib in different conditions. Concentration of Apt-SE-RP, Apt-SE-Lib, target and ATP in these measurements were 50 nM, 50 nM, 50 nM and 4 mM, respectively.

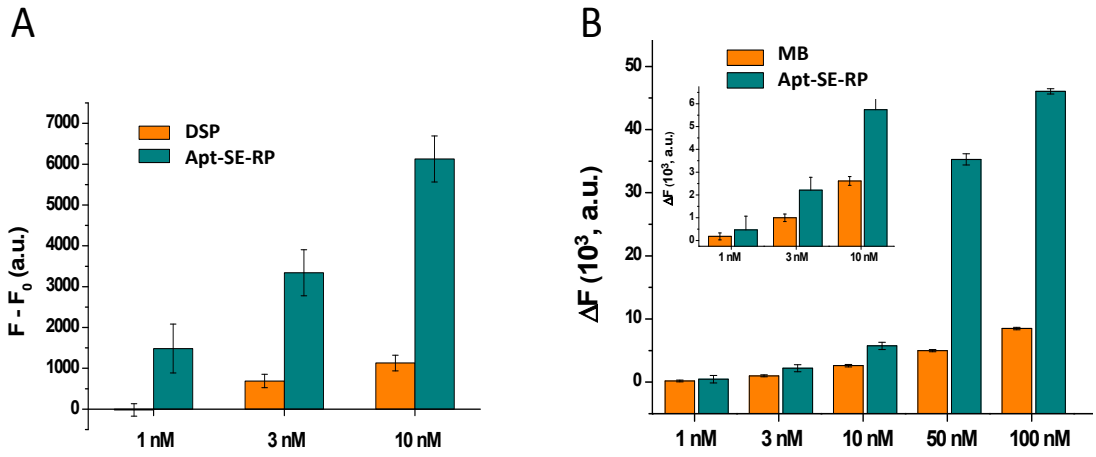

Fig. S9 (A) Target-responsive fluorescence enhancement of Apt-SE-RP and DSP. **(B)** Target-responsive fluorescence enhancement of Apt-SE-RP and MB. In these experiments, 5 μL 50 nM detection probes (Apt-SE-RP, DSP or MB) was mixed with 5 μL the target of specific concentration in Tris-HCl buffer containing 4 mM ATP in a 0.5-mL centrifuge tube. After incubation at 37 °C for 2 h, 90 μL Tris-HCl buffer was added and the fluorescence spectrum was recorded.

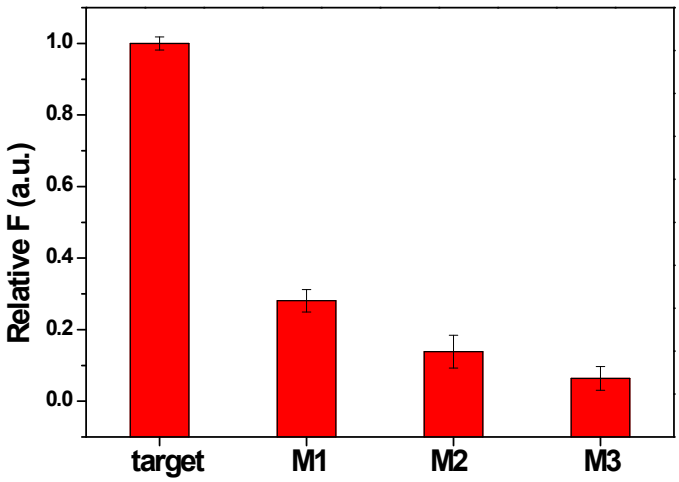

Fig. S10 Relative fluorescence enhancement of Apt-SE-RP induced by 100 nM target (fully complementary), 1-mismatch (M1), 2-mismatch (M2) or 3-mismatch (M3). The signal of the target sample was set as 1, and that from other samples was normalized accordingly.

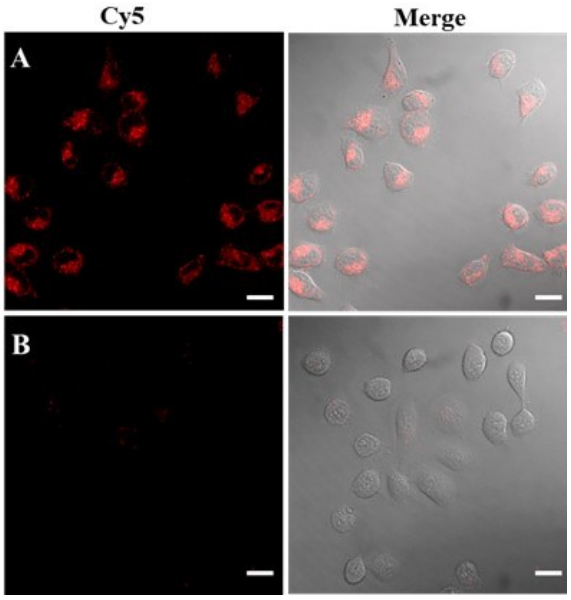

Fig. S11 Cellular internalization of Cy5-labelled AS1411. CLSM images of MCF-7 cells after incubation with 50 nM Cy5-labelled AS1411 (A) or 50 nM Cy5-labelled library sequence (B) at 37 ^{o}C for 2 h. Scale bars represent 20 μm.

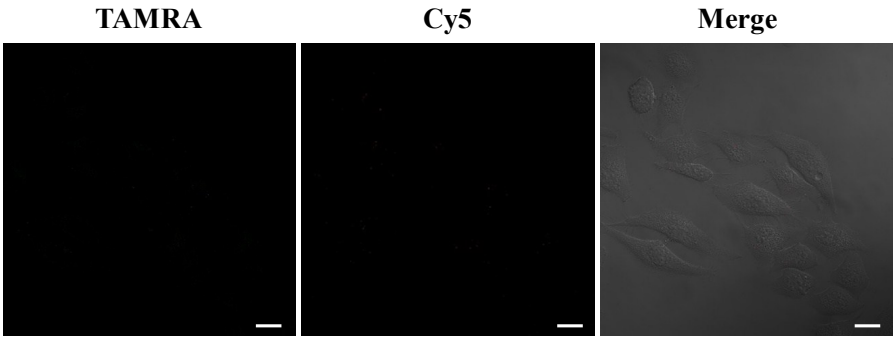

Fig. S12 CLSM images of LO2 cells incubated with 50 nM Apt-SE-RP at 37 °C for 2 h. Scale bars represent 20 μm. The Apt-SE-RP probe here was consisted of TAMRA-labelled H1 monomer, unmodified H2 monomer, RP and Cy5-labelled Apt.

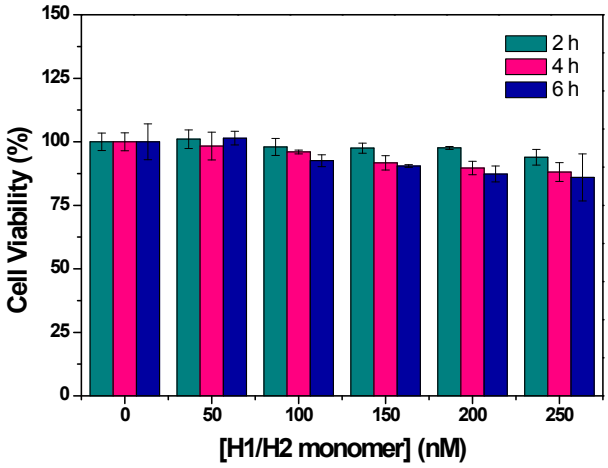

Fig. S13 Cell viability of MCF-7 cells incubated with Apt-SE-RP of different concentrations for 2 h, 4 h, and 6 h. The cytotoxicity of Apt-SE-RP was assessed by Cell Counting Kit-8 (CCK-8) assay.

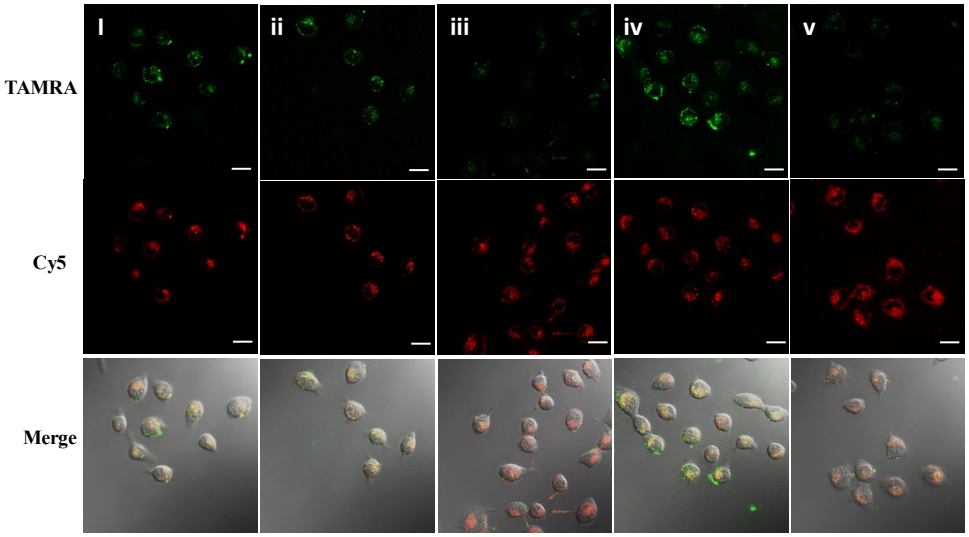

Fig. S14 CLSM images of MCF-7 cells with different treatments: Apt-SE (i), Apt-SE-RP (ii), Apt-SE-Lib (iii), Apt-SE-RP + pre-treatment with 1 μg/mL LPS (iv), Apt-SE-RP + pretreatment with 100 μg/mL cordycepin (v). Scale bars represent 20 μm.

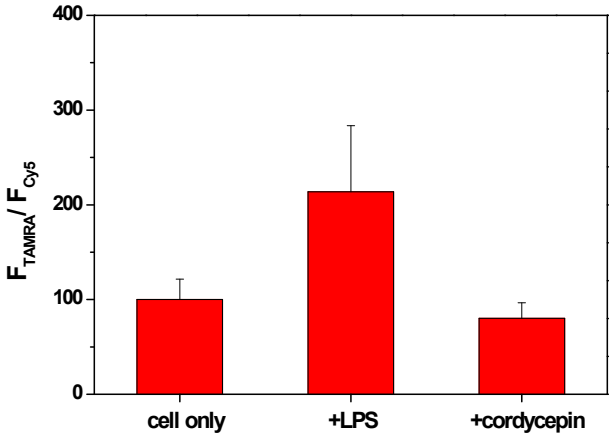

Fig. S15 Signal ratio between TAMRA and Cy5 ($F_{\mathrm{TAMRA}}/F_{\mathrm{Cy5}}$) of MCF-7 cells with different treatments. More than thirty cells were measured with an ImageJ software in each experiment, and three independent experiments were performed.

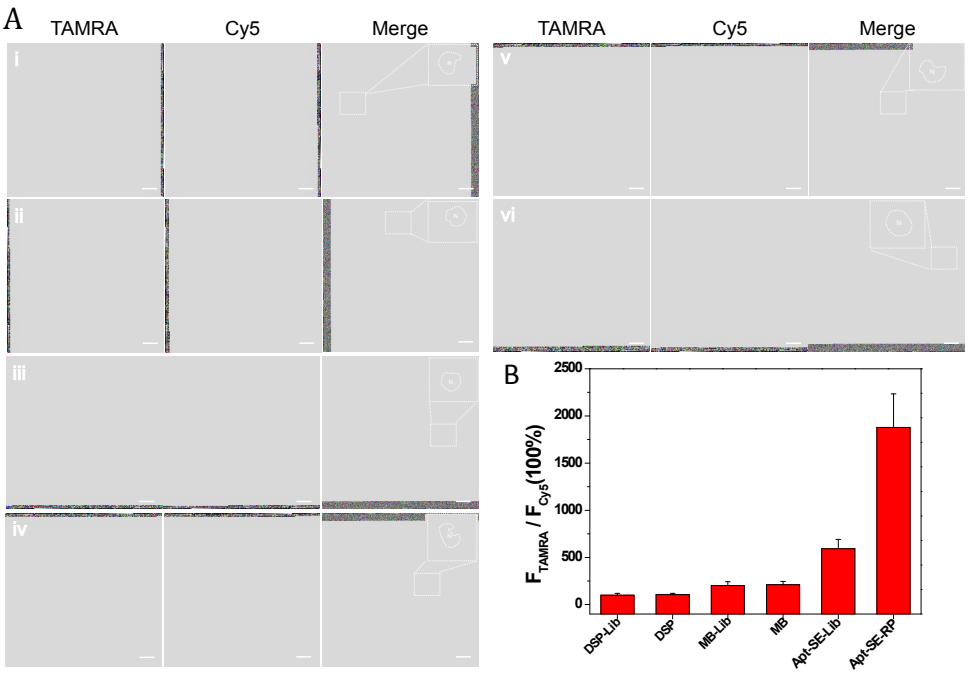

Fig. S16 (A) CLSM images of fixed MCF-7 cells after incubation with DSP-Lib (i), DSP (ii), MB-Lib (iii), MB (iv), Apt-SE-Lib (v) or Apt-SE-RP (vi). Scale bars represent 20 μm. The region of nucleus in the enlarged view was labelled with the character of "N". (B) Signal ratio between TAMRA and Cy5 (F_{TAMRA}/F_{Cy5}) of different samples in the cytoplasm. The F_{TAMRA}/F_{Cy5} value of the cells treated with DSP-Lib was set as 100%, and other samples were calculated accordingly. The cytoplasmic fluorescence signal of more than thirty cells were measured using an ImageJ software in each experiment, and three independent experiments were performed.

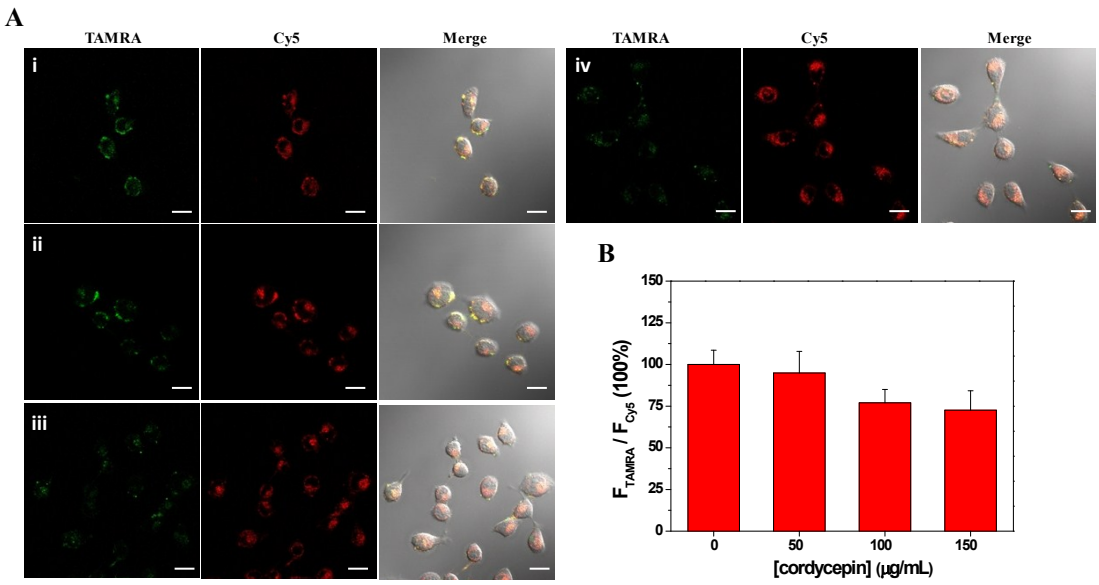

Fig. S17 (A) CLSM images of MCF-7 cells pretreated with cordycepin of 0 μg/mL (i), 50 μg/mL (ii), 100 μg/mL (iii) or 150 μg/mL (iv), and then incubated with Apt-SE-RP (50 nM). Scale bars represent 20 μm. (B) Corresponding normalized signal ratio of F_{TAMRA} to F_{Cy5}. The $F_{\mathrm{TAMRA}}/F_{\mathrm{Cy5}}$ value of the cells without cordycepin treatment was set as 100%. More than thirty cells were measured using an ImageJ software in each experiment, and three independent experiments were performed.