Electronic Supplementary Information

Proof of concept for inhibiting metastasis: circulating tumor cell – triggered localized release of anticancer agent via a structure-switching aptamer

Nandi Chen, Xiaohai Yang*, Qing Wang, Lixin Jian, Hui Shi, Shiya Qin, Kemin Wang*, Jin Huang, and Wenjing Liu

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Key Laboratory for Bio-Nanotechnology and Molecular Engineering of Hunan Province, Hunan University. Changsha 410082, China.

E-mail: kmwang@hnu.edu.cn, yangxiaohai@hnu.edu.cn; Tex/Fax:+86-731-88821566.

Experimental details

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Experimental details

S1. Materials and reagents

Avidin, Bovine serum albumin (BSA), yeast tRNA and Dulbecco's phosphate buffered saline (DPBS) were purchased from Sigma (Shanghai, China). PDMS was purchased from Dow Corning Corporation Midland, MI (Bejing, China). 1, 3-diphenylisobenzofuran (DPBF) was purchased from J&K (Guangzhou, China). Pyropheophorbide-a (Pyro) was purchased from Xianhui Pharmaceutical Co. Ltd. (Shanghai, China). RPMI 1640 cell medium was obtained from Sangon Biotechnology Company, Ltd. (Shanghai, China). Fetal bovine serum (FBS) was obtained from Tianhang Biological Technology Co., Ltd. (Zhejiang, China). Magnetic beads were purchased from Invitrogen (Dynabeads® MyOne™ Streptavidin C1, Catalog nos. 65001, Shanghai, China). Perfluoroalkoxy resin (PFA) was purchased from Iwase (EXLON-tubing, Japan, No. 5F1M18, 0.3mm×0.5mm). Household LED was purchased from Langtai Company (Jiangsu, China, LED bead: 0.5W, Color temperature: 6000-6500K. The household LED light consist of 96 LED beads and the power is 48W in total). All the oligonucleotide sequences used in this study were synthesized and purified through high performance liquid chromatography (HPLC) by Sangon Biotechnology Inc. Moreover ESI-MS was used to characterized DNA sequences in Sangon Biotechnology Inc. All the chemicals were used as received without further purification. All of the chemical reagents were of analytical grade or higher. Milli-Q-purified water (18.2 MΩ·cm) was used for all experiments.

S2. Cell culture

Ramos (CRL-1596, B-cell line, human Burkitt's lymphoma), CCRF-CEM (CCL-119, T-cell line, human acute lymphoblastic leukemia), SMMC-7721 (human hepatocellular carcinoma), Bel-7404 (human hepatocellular carcinoma) used in this study were obtained from Cell Bank of the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS, heat inactivated) and 100 U/mL penicillin-streptomycin, and cultured at 37 °C in a humidified incubator containing 5 wt %/vol CO_2.

The washing buffer was prepared by adding certain quality of glucose and $MgCl_2$ in DPBS to make the final concentration of glucose and $MgCl_2$ were 4.5 g/L and 5 mM, respectively. And the binding buffer was prepared by adding certain quality of yeast tRNA and BSA in washing buffer to make the final concentration of yeast tRNA and BSA were 0.1 mg/mL and 1 mg/mL, respectively. The cell density was determined using a hemocytometer, and this was performed prior to any experiments.

S3. Flow cytometric analyses

CEM and Ramos cells were washed and then dispersed in the binding buffer. SMMC-7721 and Bel-7404 cells were firstly washed twice with cooled D-Hank's, detached with 0.02% EDTA and 0.5% trypsin, and then dispersed in binding buffer. The density of cells was about 10^6 cells/mL. The cells were analysed on a FACScan flow cytometer by counting 10 000 events.

S3.1 The HSA probe immobilized on the magnetic beads

Magnetic beads were firstly washed with DPBS for 3 times, then incubated with the HSA probe/control probe for 1 h and washed three times before incubating with cells. 5 μL magnetic beads (approximate 5×10^7 beads) with the probes immobilized on the surface was incubated with 2×10^5 cells in 200 μL binding buffer for 30 min in dark. Then the magnetic beads were separated by an external magnetic field, and the supernatant was used for flow cytometric analyses.

S3.2 The HSA probe immobilized in micro-channel

The surface of micro-channel was modified with avidin first (see Section S6), and then the HSA probe/control probe was immobilized by the interaction between avidin and biotin. 2×10^5 cells in 200 μL binding buffer were dropped at the inlet of the channel, and aspirated at the outlet of the channel by a syringe pump (KD Scientific, Inc.). Cells collected from the outlet of the channel were used for flow cytometric analyses.

S4. Confocal imaging of cells bound with HSA probes

The cell samples used for flow cytometric analyses were also used for confocal imaging. All cellular fluorescent images were collected on an Olympus FV1000-TY1318 confocal microscope with 40× objective. 10 μL cells dispersion was dropped on a thin glass slide for confocal imaging. The 488 nm laser was the excitation source for FAM labeled HSA probes and the 633 nm laser was the excitation source for Pyro labeled HSA probes throughout the experiments.

S5. Calculate the surface coverage of HSA probes

Briefly, the surface coverage was determined by comparing molecule concentration before and after immobilization. The fluorescence of HSA probes was measured using F-7000 fluorescence spectrophotometer, and a standard curve was made by using known concentrations of HSA probes. The standard curve of the HSA probe was F =18.82 C-153.1, R=0.9996. F was the fluoresence signal of HSA probes. C was the concentration of HSA probes. The surface coverage was estimated using the following equation:

$$\text{Surface coverage} = (C_{before} - C_{after}) \times V \times N_A / S$$

C_{before} and C_{after} were measured as the molar concentration of HSA probes before and after immobilized on the magnetic beads or the micro-channel. V indicated the volume of the HSA probe solution. N_A is Avogadro constant. S was the total superficial area of the magnetic beads or the micro-channel.

S6. Design and fabrication of experimental device

As shown in Fig. 2A, this experimental device was consisting of two parts: (I) a microfluidic chip provided a micro-channel used to immobilize the HSA probe; (II) a 100 cm perfluoroalkoxy resin (PFA) capillary could be irradiated by a household LED to simulate the superficial blood vessels. A syringe pump was used to drive the cells flowing in the micro-channel and capillary.

The micro-channel was put in dark place and maintained a stable temperature by a heating board. The capillary was irradiated by household LED light when cells flowed in the capillary. The syringe pump was also placed in dark place.

The microfluidic chip was composed of two layers: a piece of slide glass and a chaotic-mixer[1] PDMS slice. The glass support was cleaned by piranha solution (H_2SO_4: 30% H_2O_2 = 3:1, v/v). The PDMS replica with a 50 cm long chaotic mixing channel (wh = 1000 × 100 μm) was produced by soft-lithography using a replicate on an SU-8 wafer. Patterned SU-8 master was fabricated by Dalian Institute of Chemical Physics, Chinese Academy of Science. For polymer molding on the patterned silica masters, a 10:1 mixture of PDMS prepolymer and the curing agent were stirred thoroughly and then degassed under vacuum. The polymer mixture was poured onto the master and cured for 2 hours at 75 °C. After curing, the PDMS replica was peeled from the master. Inlet and outlet wells were punched at the micro-channel ends in the PDMS. The PDMS replica was then reversibly attached to the glass to form a whole microfluidic chip. All solutions were introduced into the micro-channel by connecting a syringe pump to the outlet of the micro-channel.

Avidin (1 mg/mL) was immobilized on the surface of the micro-channel at flow rate of 200 μL/min and then washed the micro-channel at a flow rate of 300

μL/min for three times with 0.2 mL DPBS each time. Similarly, then the HSA probe/control probe was immobilized at a flow rate of 200 μL/min by the interaction between avidin and biotin, and the channel was washed at a flow rate of 300 μL/min for three times with 0.2 mL DPBS each time.

S7. Cytotoxicity investigation

The cytotoxicity study was performed by MTS assay using a CellTiter 96 cell proliferation assay (Promega, Madison, WI) for SMCC-7721 and Bel-7404 cell lines in a 96-well cell culture plate at 20 000/well, 250 μL. Four groups of cell samples were set up as follows: Group 1, cells collected from the micro-channel without Pyro labeled probe and treated without LED irradition when they flowed in the capillary (Control LED-); Group 2, cells collected from the micro-channel without Pyro labeled probe and treated with LED irradiation when they flowed in the capillary (Control LED+); Group 3, cells collected from the micro-channel with Pyro labeled probe and treated without LED irradition when they flowed in the capillary (HSA LED-); Group 4, cells collected from the micro-channel with Pyro labeled probe and treated with LED irradition when they flowed in the capillary (HSA LED+). The household LED light irradiated the cells when they flowed in the capillary for 15 min. The illuminance was 6.0×10^4 lux. The flow rate was 100 μL/min. Afterwards, all cells were incubated in a CO_2 incubator at 37 ^oC for 24 h. Finally, CellTiter reagent solution (30 μL/well) was added to each well and incubated at 37 ^oC for 3 h. The absorbance value at 570 nm was determined by using a multi-function plate reader Infinite® M1000 (Tecan, Switzerland).

Table S1. Sequences of DNA used in this study.

Name	Sequence
Sgc8-FAM	5'-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GA- FAM-3'
Sgc8-Control	5'-T_{31}-TA CGG TTA GA- FAM-3'
Sgc8-cDNA with BHQ1	5'-BHQ1-TCT AAC CGT ATT TTT TTT TT -biotin-3'
Sgc8-cDNA	5'-TCT AAC CGT ATT TTT TTT TT -biotin-3'
ZY1-FAM	5'-FAM-AAA GCG CGC GCG CGC ATA GCG CGC TGA GCT GAA GAT CGT ACC GTG AGC GCG CT-3'
ZY1-Pyro	5'-Pyro-AAA GCG CGC GCG CGC ATA GCG CGC TGA GCT GAA GAT CGT ACC GTG AGC GCG CT-3'
ZY1-Control-FAM	5'-FAM-AAA GCG CGC G-T_{43}-3'
ZY1-Control-Pyro	5'-Pyro-AAA GCG CGC G-T_{43}-3'
ZY1-cDNA	5'-CGC GCG CTT TTT TTT TTT -biotin-3'

The underlined sequence in Sgc8-FAM and Sgc8-Control could hybridize to the underlined sequence in Sgc8-cDNA with BHQ1 and Sgc8-cDNA.

The underlined sequence in ZY1-FAM, ZY1-Pyro, ZY1-Control-FAM and ZY1-Control-Pyro could hybridize to the underlined sequence in ZY1-cDNA.

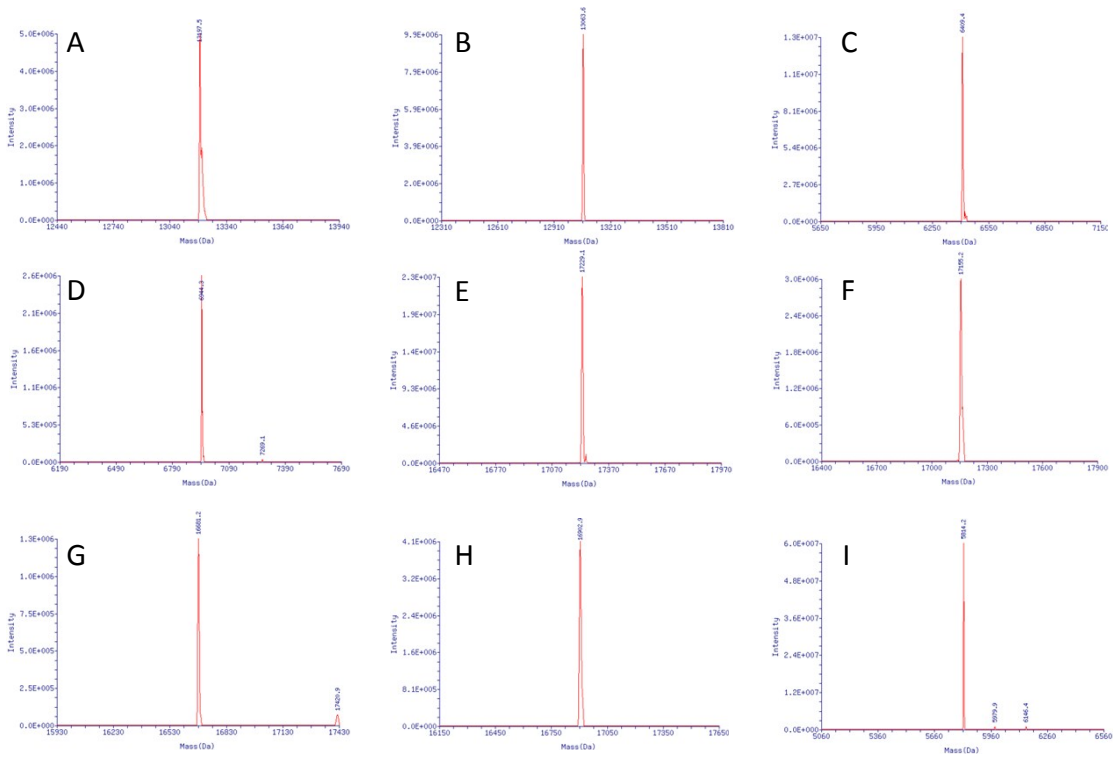

Fig. S1 DNA sequences characterized by ESI-MS

All DNA sequences used in this research were synthesized and purified through high performance liquid chromatography (HPLC) by Sangon Biotechnology Inc. Moreover ESI-MS was used to characterize DNA sequences in Sangon Biotechnology Inc.

A) Sgc8-FAM: 13197.5 Da B) Sgc8-Control: 13063.6 Da C) Sgc8-cDNA with BHQ1: 6409.4 Da D) Sgc8-cDNA:6944.3 Da E) ZY1-FAM: 172291.1 Da F) ZY1-Pyro: 17155.2 Da G) ZY1-Control-FAM: 16681.2 Da H) ZY1-Control-Pyro:16902.9 Da I) ZY1-cDNA: 5814.2 Da

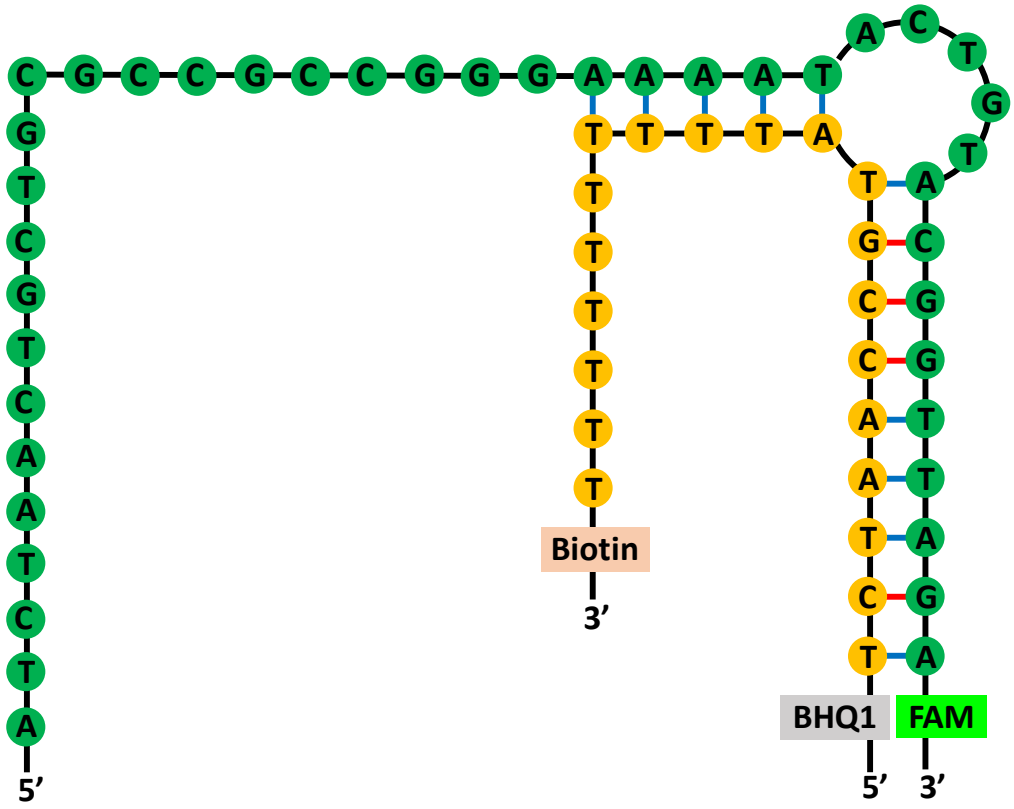

Fig. S2 The secondary structure of probes for CCRF-CEM.

The FAM was attached at the 3’ end of Sgc8-FAM and BHQ1 was attached at the 5’ end of Sgc8-cDNA with BHQ1. Therefore, the BHQ1 could quench the FAM well. The Tm temperature between Sgc8-FAM and Sgc8-cDNA with BHQ1 calculated by the Mfold website server was 40.4 ^{o}C.[2]

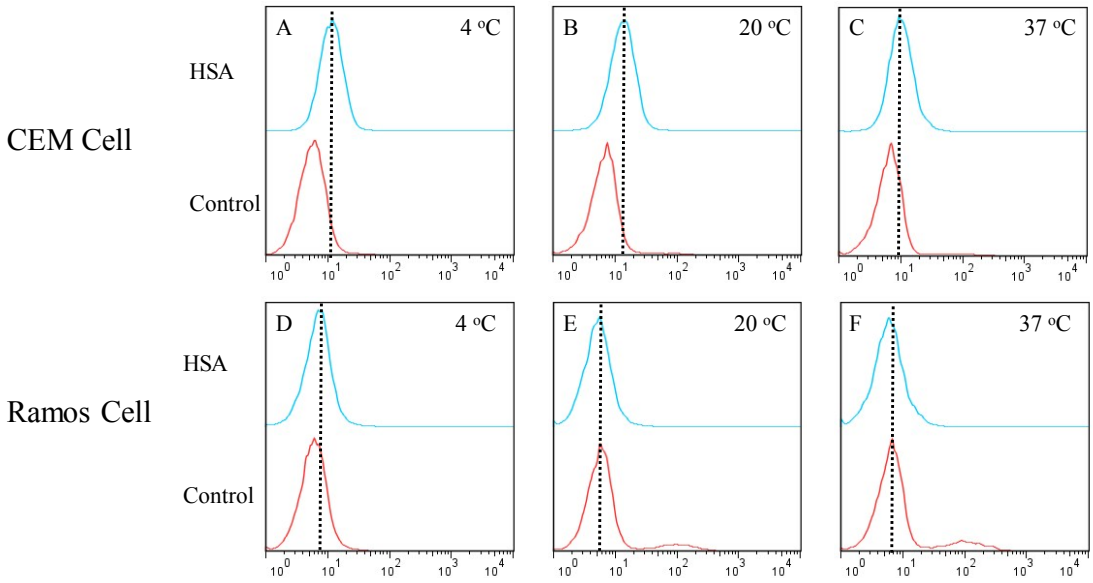

Fig. S3 The binding ability of probes immobilized on magnetic beads in binding buffer at different temperatures (CEM and Ramos).

Flow cytometry results of probes immobilized on magnetic beads. The results showed the selective binding of HSA probes (Sgc8-FAM + Sgc8-cDNA with BHQ1) to the target CEM cells, but not negative control Ramos cells. Control probe: Sgc8-Control + Sgc8-cDNA with BHQ1. Concentration of probes: 250 nM.

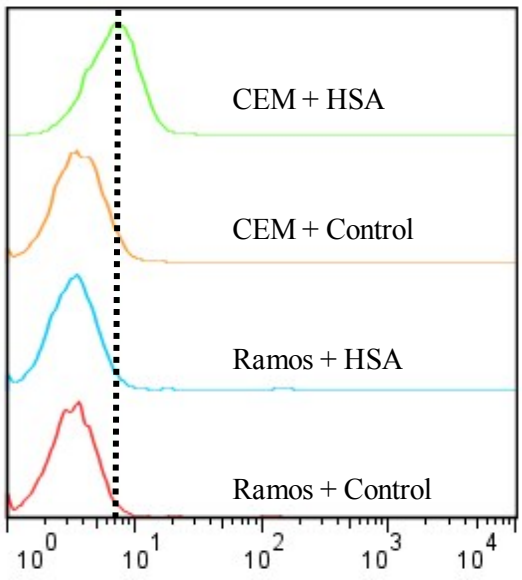

Fig. S4 The binding ability of probes immobilized on magnetic beads without quencher in binding buffer.

Flow cytometry results of probes immobilized on magnetic beads. The results showed the selective binding of HSA probes (Sgc8-FAM + Sgc8-cDNA) to the target CEM cells, but not negative control Ramos cells. Control probe: Sgc8-Control + Sgc8-cDNA. Concentration of probes: 250 nM. Temperature: 20 ^{o}C.

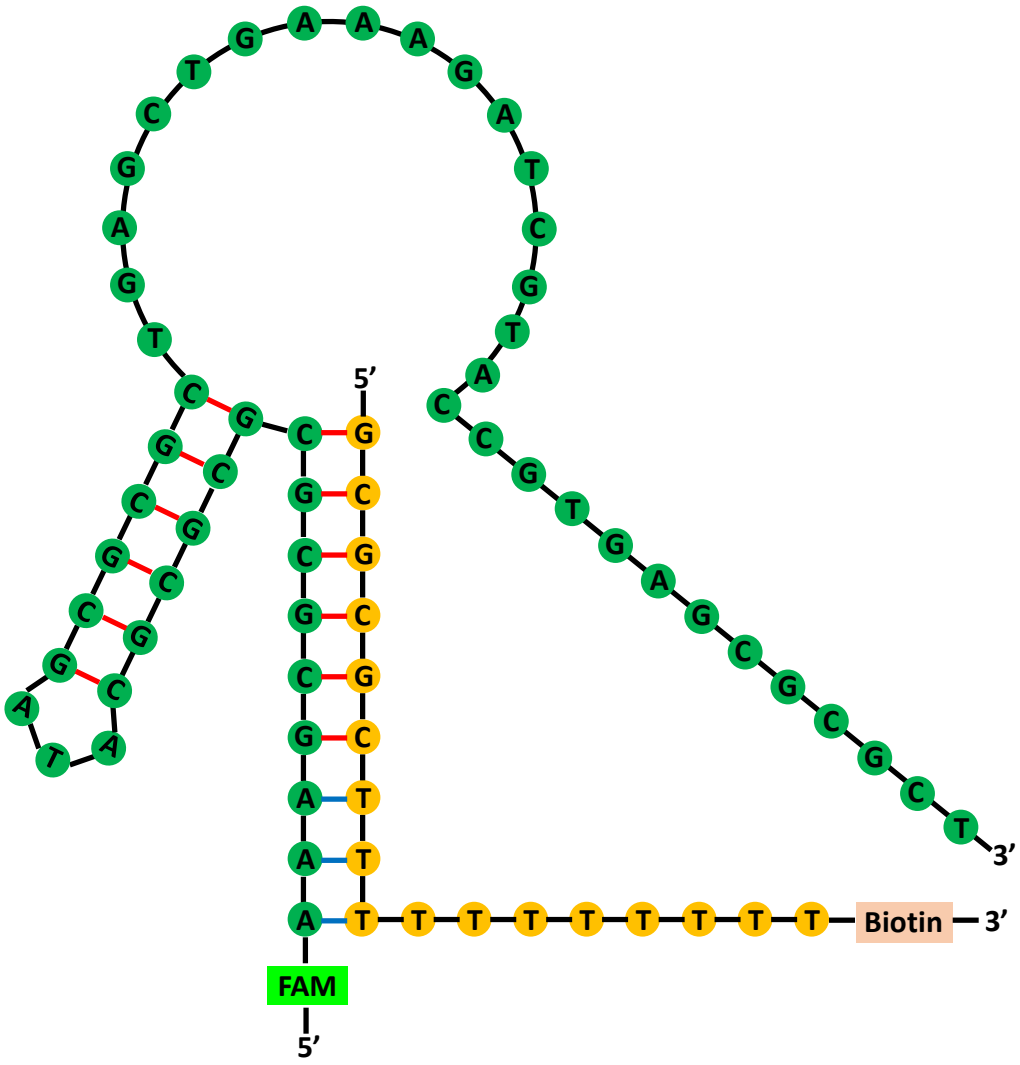

Fig. S5 The secondary structure of probes for SMMC-7721.

The melting temperature between ZY1-FAM and ZY1-cDNA calculated by the Mfold website server was 62.1 ^{o}C. FAM or Pyro was labeled at 5’ end of the ZY1-FAM/ZY1-Pyro. In our strategy the anticancer agent were not released and activated in the same place of the body, the qunencher was not added in the HSA probe for SMMC-7721 cell. Moreover, it is much easier to label the photosensitizer at the 5’ end than the 3’ end.

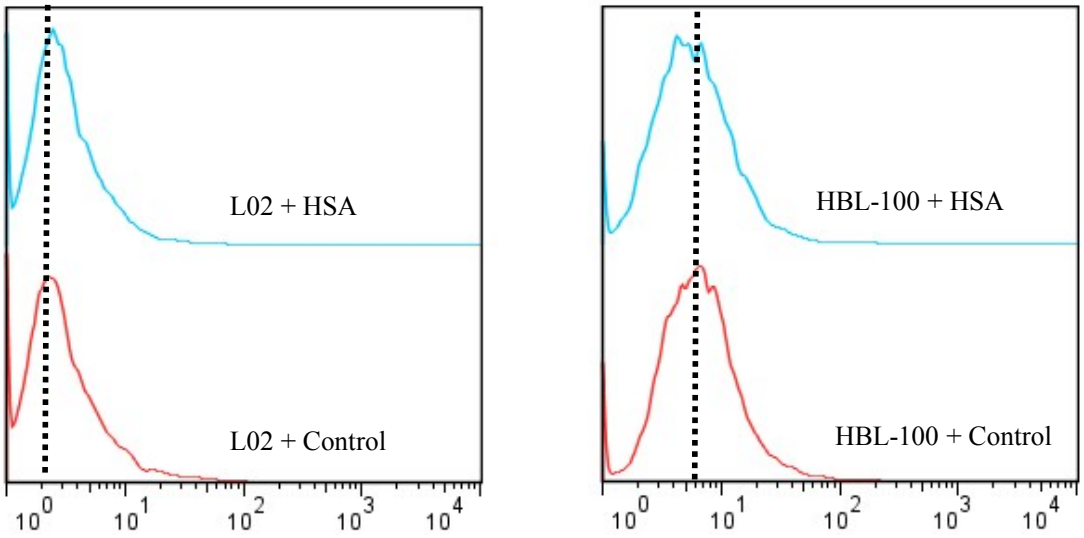

Fig. S6 The selectivity of HSA probe for normal cell.

Flow cytometry results showed HSA probes (ZY1-FAM + ZY1-cDNA) do not bind both L02 and HBL-100. L02 is human normal hepatic cell line and HBL-100 is human normal breast cell line. Both L02 and HBL-100 are non-malignant cells unlike target cell (SMCC-7721) and control cell (Bel-7404). Therefore, this HSA probe shows well selectivity to its target cell. Temperature: 4 ^{o}C. Concentration of probes: 250 nM. Control probe: ZY1-Control-FAM + ZY1-cDNA.

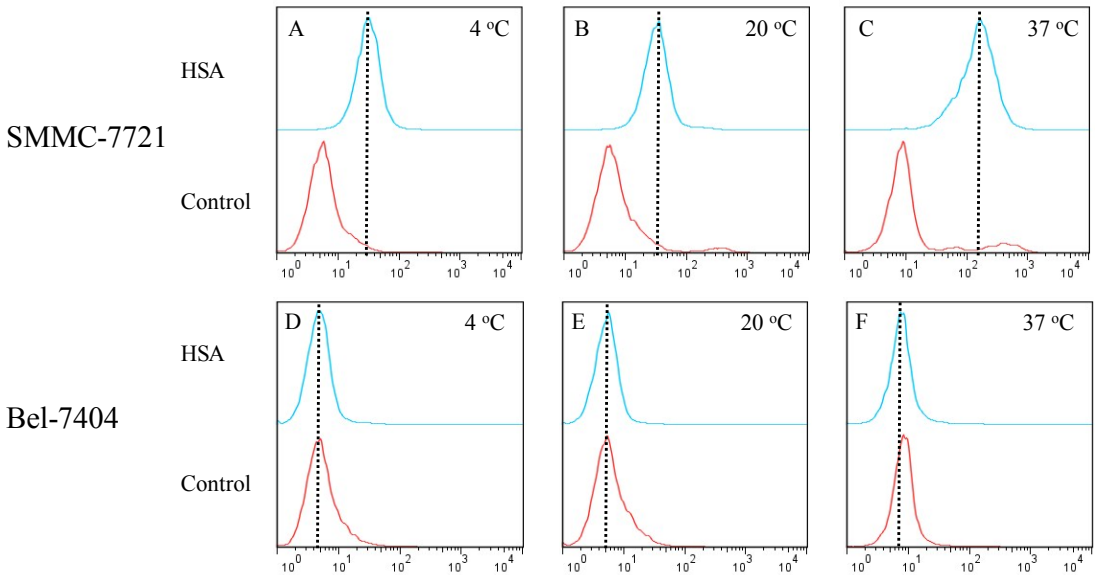

Fig. S7 The binding ability of probes immobilized on magnetic beads in binding buffer at different temperatures (SMMC-7721 and Bel-7404).

Flow cytometry results showed the selective binding of HSA probes (ZY1-FAM + ZY1-cDNA) to the target SMMC-7721 cells, but not negative control Bel-7404 cells. Due to the high melting temperature between aptamer of SMMC-7721 and its cDNA complex, 37 °C was the optimized temperature. Concentration of probes: 250 nM. Control probe: ZY1-Control-FAM + ZY1-cDNA.

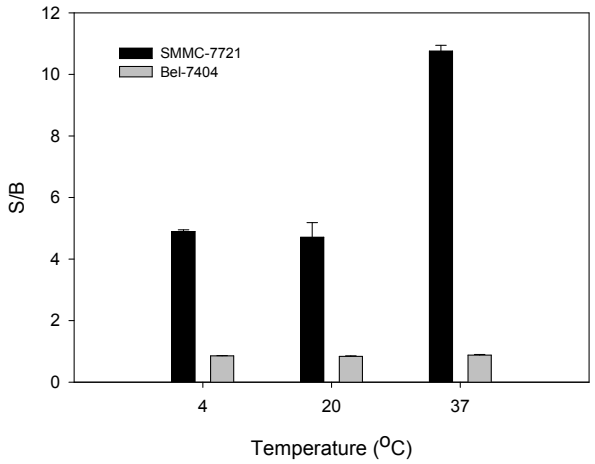

Fig. S8 Effect of temperature on the recognition of target SMMC-7721 cell.

In order to keep the affinity of the HSA probe in high temperature, 37 °C, we designed the HSA probe for SMMC-7721 more strictly. The melting temperature between ZY1-FAM and ZY1-cDNA calculated by the Mfold website server was 62.1 °C. Therefore, even the aptamer ZY1 can recognize the SMMC-7721 target cell at low temperature. It is hard for the SMMC-7721 target cell to disturb the DNA hybridization between ZY1-FAM and ZY1-cDNA in low temperature. Thus, 37 °C was the optimized temperature. HSA probes: ZY1-FAM + ZY1-cDNA. Concentration of probes: 250 nM. Control probe: ZY1-Control-FAM + ZY1-cDNA.

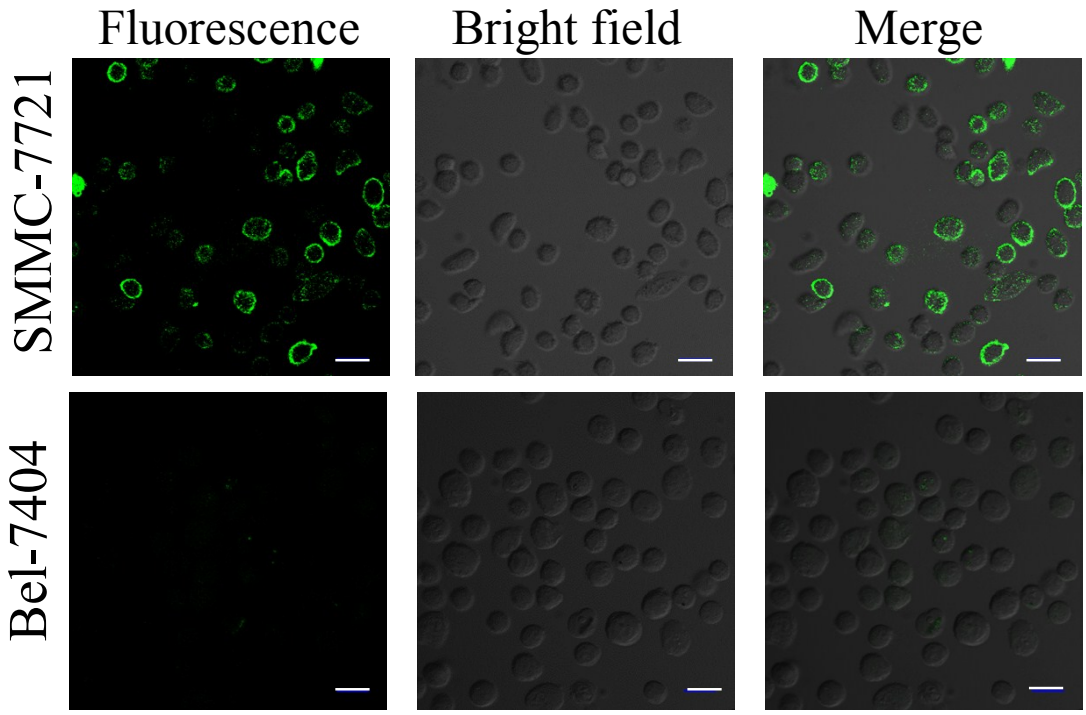

Fig. S9 Confocal imaging of SMMC-7721 and Bel-7404 after recognizing by the HSA probe.

Confocal laser scanning microscopy images of the cells, indicating the specific binding between HSA probe (ZY1-FAM + ZY1-cDNA) and SMMC-7721 rather than Bel-7404. Temperature: 37 ^{o}C. Concentration of probes: 250 nM. Scale bar: 20 μm.

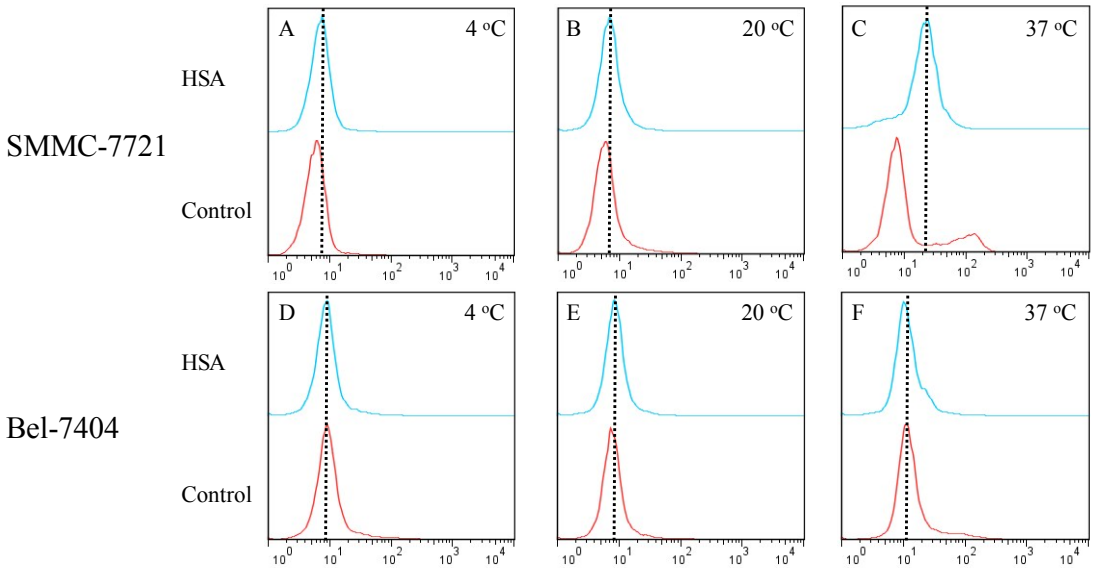

Fig. S10 The binding ability of probes immobilized on magnetic beads in culture medium at different temperatures (SMMC-7721 and Bel-7404).

The binding affinity of the HSA probe decreased in 4 °C, 20 °C and 37 °C because of the complex culture medium with 10% FBS. There was nonspecific adsorption between proteins in FBS and HSA probes. In binding buffer, it showed the highest S/B at 37 °C. Therefore, 37 °C was the optimized temperature in culture medium. Concentration of HSA probes: 250 nM. HSA probe: ZY1-Control-FAM + ZY1-cDNA. Control probe: ZY1-Control-FAM + ZY1-cDNA.

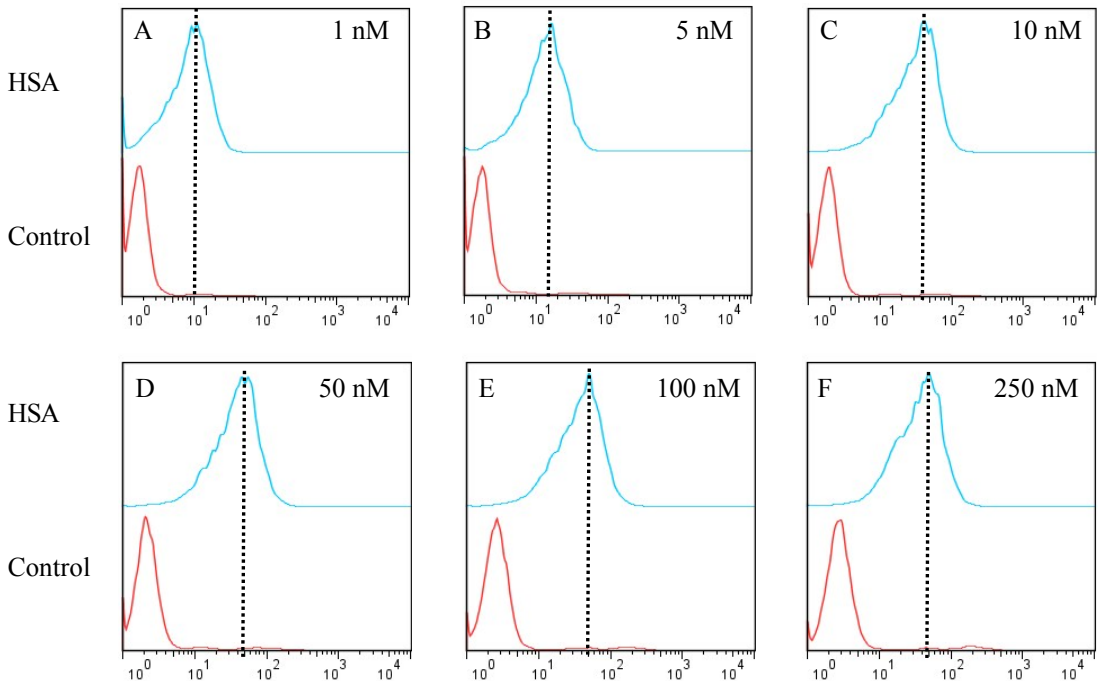

Fig. S11 The binding ability of different concentration of probe for immobilization onto magnetic beads (SMMC-7721 and Bel-7404).

For the target SMMC-7721 cell, the fluorescence signal increased and reached a plateau when the HSA probe (ZY1-FAM + ZY1-cDNA) concentration was more than 100 nM. However, the control probe (ZY1-Control-FAM + ZY1-cDNA) did not increase obviously. Temperature: 37 ^{o}C.

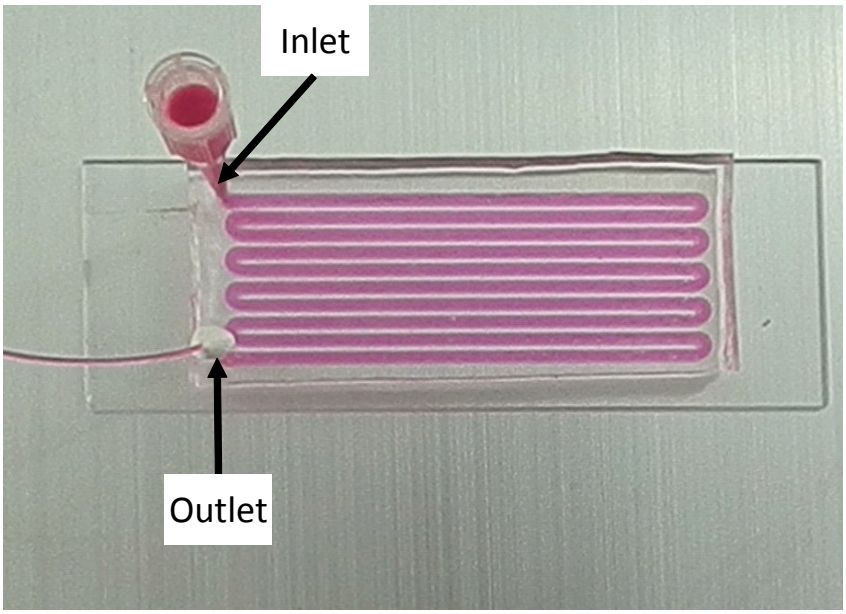

Fig. S12 The photo of microfluidic device.

A microfluidic chip provided a micro-channel used to immobilize the HSA probe. It was placed on a heating board, which was made by our lab to maintain a stable temperature of the micro-channel. A tip was inserted into the inlet well, which could contain 200 μL solution. Solutions were introduced into the micro-channel by connecting a syringe pump to the outlet of the micro-channel.

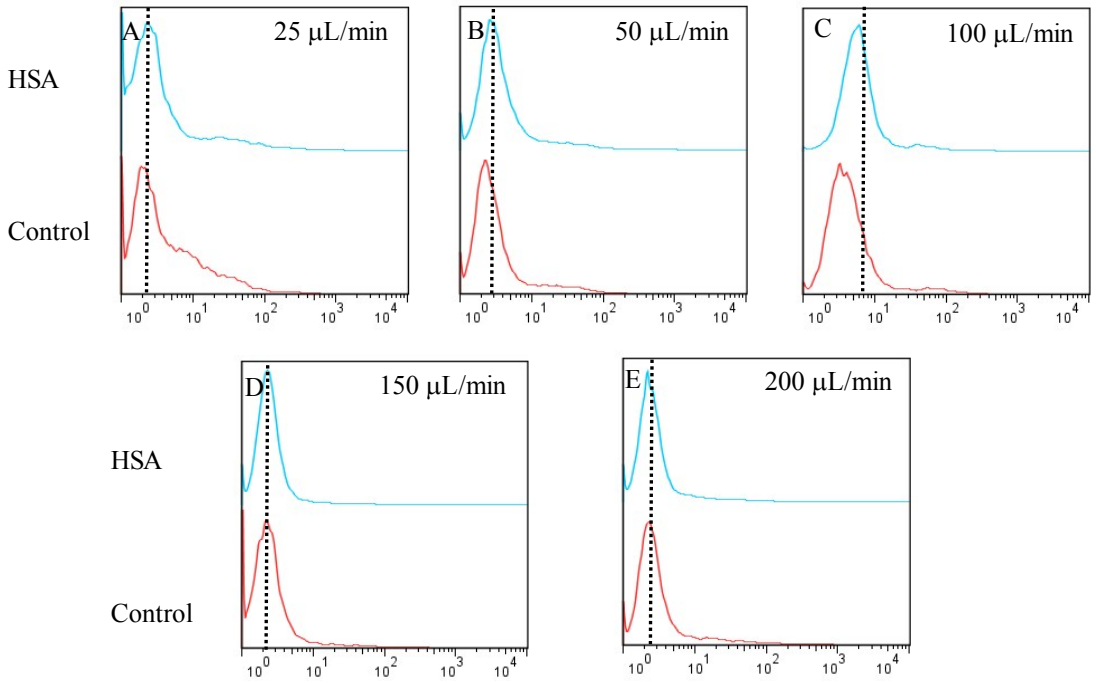

Fig. S13 Optimize the flow rate of cell dispersion in micro-channel.

For the target SMMC-7721 cell, the higher flow rate decreased the fluorescence of target obviously, since there was not sufficient time for the HSA probe (ZY1- FAM + ZY1-cDNA) to recognize the target cell. Whereas, the low flow rate may trap cells in the channel and not easy to recover. Hence, a flow rate of 100 μL/min was chosen. Temperature: 37 ^{o}C. Concentration: 20 μM. Control probe: ZY1-Control-FAM + ZY1-cDNA.

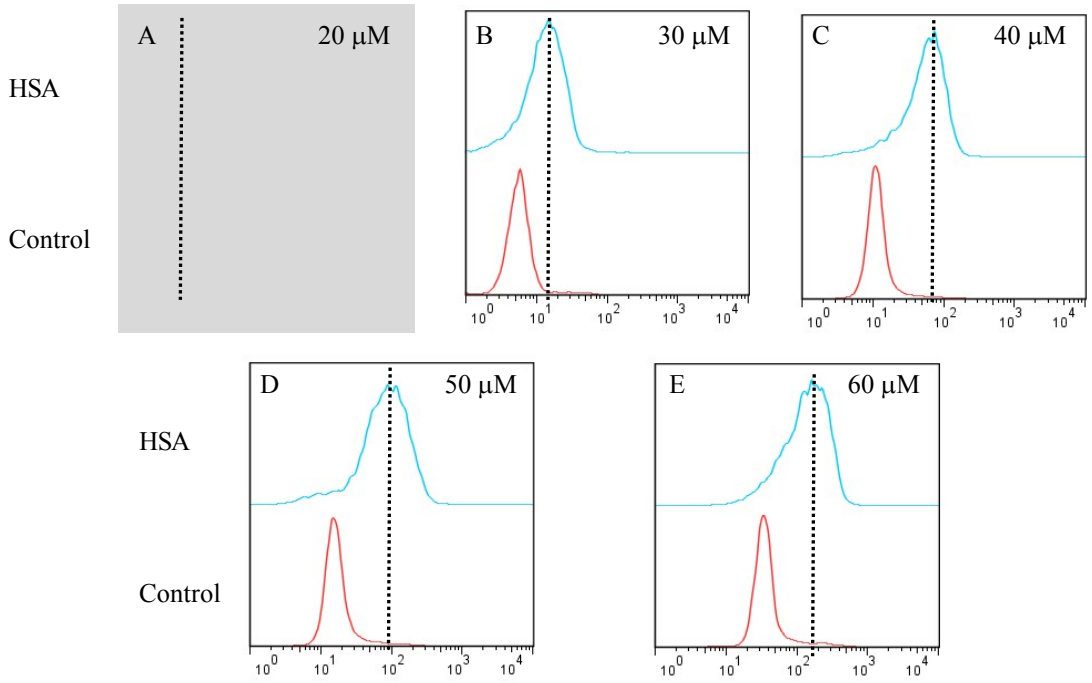

Fig. S14 Optimize the concentration of the HSA probe for immobilization onto the surface of micro-channel.

For the target SMMC-7721 cell, the fluorescence signal enhanced obviously from 20 μM to 50 μM of the HSA probe (ZY1-FAM + ZY1-cDNA). Meanwhile, the fluorescence signal of control probe (ZY1-Control-FAM + ZY1-cDNA)was also enhanced. With comprehensive consideration, 40 μM was chosen as the optimized concentration. Temperature: 37 ^{o}C. Flow rate: 100 μL/min.

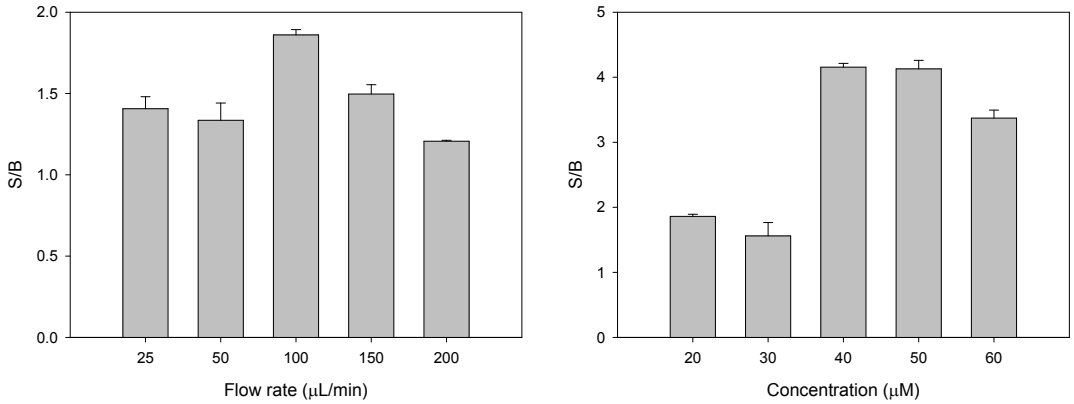

Fig. S15 The statistical result of optimized condition in micro-channel.

Left: Effect of the flow rate on the recognition of target SMMC-7721 cell in micro-channel. The best performance was achieved at a flow rate of 100 μL/min based on the ratio of signal and background(S/B). Temperature: 37 ^{o}C. Concentration: 20 μM. HSA probe: ZY1-FAM + ZY1-cDNA. Control probe: ZY1-Control-FAM + ZY1-cDNA.

Right: Effect of the HSA probe concentration for immobilization onto the surface of micro-channel. The best performance was achieved at 40 μM HSA probe used to immobilize onto the surface of micro-channel based on the ratio of signal and background(S/B). Temperature: 37 ^{o}C. Flow rate: 100 μL/min. HSA probe: ZY1-FAM + ZY1-cDNA. Control probe: ZY1-Control-FAM + ZY1-cDNA.

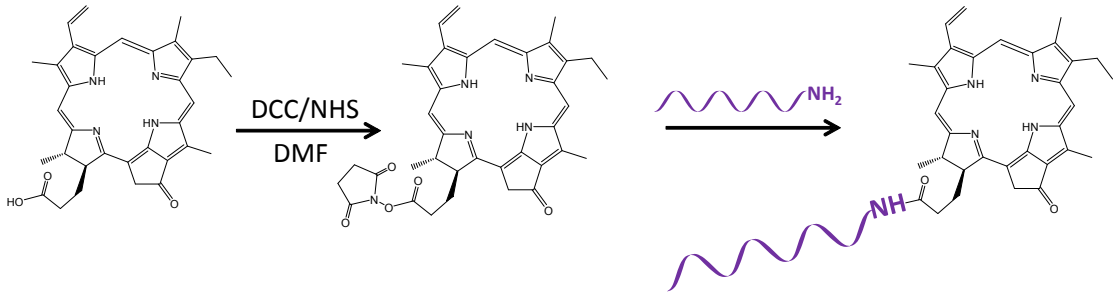

Fig. S16 Synthesis of pyropheophorbide-a labeled DNA

Pyropheophorbide-a (Pyro) as covalently linked to the 5' end of the aptamer through the reaction between -COOH and $-NH_2$ by N,N'-Dicyclohexylcarbodiimide (DCC)/ N-Hydroxysuccinimide (NHS) in N,N-Dimethylformamide (DMF).

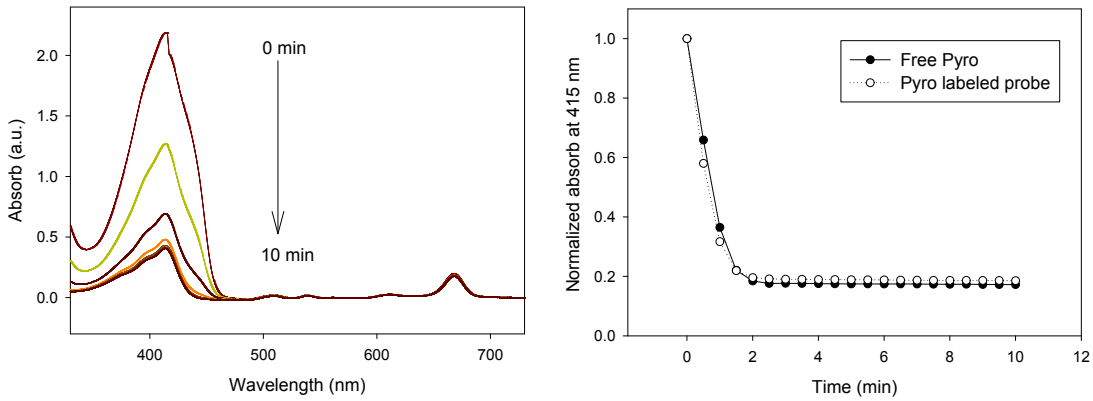

Fig. S17 The efficiency of $^1O_2$ generated by pyropheophorbide-a.

The concentration of DPBF and Pyro/Pyro labeled probe were 100 mM and 4 μM respectively. The photobleaching during labeling process may reduce the $^1O_2$ generated by the Pyro labeled on the probe.

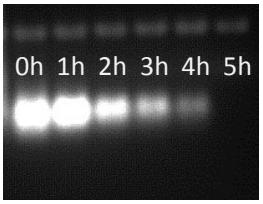

Fig. S18 The stability of the HSA probe in 50% FBS.

The first band is the protein and DNA in FBS. Therefore, this band appeared in every line and did not change with time extending. The band of HSA become lighter with time extending since the HSA probe was partly degraded by the nuclease in FBS.

Reference:

1 S. Wang, K. Liu, J. Liu, Z. T. F. Yu, X. Xu, L. Zhao, L. Tom, E. K. Lee, J. Reiss, Y. K. Lee, L. W. K. Chung, J. Huang, M. Retting, D. Seligson, K. N. Duraiswamy, C. K.-F. Shen and H. R. Tseng, *Angew. Chem. Int. Ed.*, 2011, **50**, 3084-3088.

2 N. R. Markham, M. Zuker, *Nucleic Acids Res.*, 2005, **33**, W577-W581.