Electronic Supplementary Information (ESI)

Sensitive and Specific Detection of Tumour Cells Based on Multivalent DNA Nanocreeper and Multiplexed Fluorescence Supersandwich

Lei Luo,[a] Wenshan Li,[b] Dongliu Xiang,[a] Yanyun Ma,[a] Yuan Zhou,[a] Yaqing Xu,[a] Nandi Chen,[c] Qing Wang,[a] Jin Huang,[a] Jianbo Liu,[a] Xiaohai Yang*[a] and Kemin Wang*[a]

[a]*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.*

[b]*College of Chemical Engineering, Xiangtan University, Xiangtan 411105, China.*

[c]*Department of Gastrointestinal Surgery, The Second Clinical Medicine College (Shenzhen People's Hospital) of Jinan University, Shenzhen, Guangdong 518020, China.*

* E-mail Address: yangxiaohai@hnu.edu.cn, kmwang@hnu.edu.cn.

Tel/Fax: +86-731-88821566

Contents of Electronic Supporting Information:

1. Experimental Section

ES1 Reagents and materials

All of the DNA probes used in this work were synthesized and HPLC purified by Sangon Biotech. Co., Ltd (Shanghai, China). DNA sequences are listed in Table S1. $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, NaCl, KCl, $MgCl_2 \cdot 6H_2O$, glucose, boric acid and Na_2EDTA were analytical reagents and purchased from Sinopharm Chemical Reagent Co., Ltd (China). Tris, bovine serum albumin (BSA), yeast tRNA and streptavidin were purchased from Sigma-Aldrich (USA). Agarose was obtained from Biowest (Spain). Phosphate buffered saline buffer (PBS, 10 mM, pH 7.4) was supplemented with 5 mM Mg^{2+}. Washing buffer: 4.5 g/L glucose in PBS (10 mM, pH 7.4). Binding buffer: 4.5 g/L glucose, 0.1 g/L tRNA and 1 g/L BSA in PBS (10 mM, pH 7.4). All solutions were prepared and diluted using ultrapure water (≥ 18.2 MΩ cm, 25 °C) treated by Milli-Q system (Millipore, USA). 35 mm glass bottom dishes were purchased from Cellvis (USA). Microfluidic chip was purchased from MesoBioSystem company (Wuhan, China). The human blood samples were donated by the author with informed consent.

ES2 Cell culture

Human hepatocellular carcinoma SMMC-7721 and Bel-7404 cells, normal human hepatocyte L02 cells, human colon carcinoma SW480 cells, human acute lymphatic leukemia CCRF-CEM cells, human lymphoma Ramos cells and human breast carcinoma MCF-7 cells were purchased from Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Human cervical carcinoma HeLa cells were purchased from American Type Culture Collection Cells (USA). Cells were cultured in RPMI 1640 medium supplemented with 12% fetal bovine serum (FBS, heat inactivated in 56 °C) and 100 IU/mL penicillin-streptomycin, and incubated at 37 °C with 5% CO_2 in the incubator (Thermo Fisher Scientific, USA).

ES3 Preparation of Zy1-DNC

All DNA powders were centrifuged at 400 × g and 4 °C for 1 min. Then they were dissolved with corresponding volume of PBS to certain concentration as stock solutions and stored at 4 °C. H1 and H2 were heated to 96 °C for 3 min, cooled on ice for 3 min at once, and then left at room temperature for 1 h before use, respectively. To form DNA duplex, 0.25 μM Trigger, 25 μM H1 and 25 μM H2 were mixed and incubated at 400 rpm and 25 °C for 24 h in thermostatic metal shaker bath (MB-102, Hangzhou).

And then, Zy1 and Branch were mixed (total concentration of the two DNA probes was 50 μM). Subsequently, equal volumes of DNA duplex, mixture of Zy1 and Branch, and 50 μM Streptavidin were mixed and incubated at 400 rpm and 25 ^{o}C for 1 h in thermostatic metal shaker bath to form Zy1-DNC. The resulting Zy1-DNC was diluted for further experiments and the concentration was represented by Zy1 equivalent.

ES4 Characterization of Zy1-DNC-MFS (Electrophoresis, AFM, Flow cytometry and Laser confocal imaging)

The construction process of Zy1-DNC-MFS was characterized by agarose gel electrophoresis. 2% agarose gel was prepared using 1 × TBE buffer. All of the DNA probes were diluted to 1 μM. 8 μL probes mixed with 2 μL 100 × SYBR® Gold and 2 μL 6 × loading buffer, and then electrophoresed at 80 V for 50 min in 1 × TBE buffer. The gel was subsequently photographed by Azure c150 imaging system (Azure Biosystems, USA).

The self-assembly process was also characterized by atomic force microscopy (AFM). The Zy1-DNC and Zy1-DNC-MFS with different concentration of Trigger was prepared and then dilute 250-fold with PBS. The 100 mM Ni^{2+} was pipetted on a freshly cleaved mica for 5 min absorption and then washed 3 times by ultrapure water. Thereafter, the sample was pipetted on mica for 10 min absorption and then washed 10 times by ultrapure water and gently blown dry by nitrogen gas. The prepared sample was scanned by ScanAsyst-air tips in ScanAsyst Imaging Mode on Multimode 8 Atomic Force Microscope with a NanoScope V controller (Bruker Inc.).

Flow cytometry was used to investigation of the binding ability of Zy1-DNC and the signal amplification induced by MFS. Briefly, cells were digested by trypsin and washed three times with washing buffer. After centrifuged at 300 × g for 4 min, cells were resuspended in binding buffer and counted by hemocytometer. 100 nM Zy1-FAM, 100 nM Zy1-HCR or 100 nM Zy1-DNC-MFS was incubated with 50, 000 cells in 200 μL binding buffer at 4 ^{o}C for 2 h in the dark. For Zy1-HCR, Zy1-FAM was assembled with DNA duplex in the presence of streptavidin. For Zy1-DNC-MFS, 100 nM Zy1-DNC, 1 μM SP1-FAM and 1 μM SP2-FAM were mixed and then incubated with cell suspension. For the control probes, Zy1 was replaced with random DNA sequences (Random) to construct Random-FAM, Random-HCR or Random-DNC-MFS. The concentration of all probes above were represented by Zy1 or Random. Human hepatocellular carcinoma cell line SMMC-7721 was used as the target cells, and another

human hepatocellular carcinoma cell line Bel-7404 was used as the control cells. After incubation, cells were washed three times with washing buffer and centrifuged at 300 × g for 4 min to remove unbinding probes. 10,000 cells were suspended in binding buffer prior to flow cytometry analysis (Gallios, Beckman Coulter, USA). Excite wavelength: 488 nm, collection wavelength for FAM: 525 nm band pass. All of the experiments were repeated three times.

Laser confocal imaging was used to verify the above processes. Cells were plated and cultured in glass bottom dishes for 24 h. The probes above were added respectively and incubated with cells in the same experimental conditions. Then, all samples were washed three times with washing buffer and then immerse in 200 μL binding buffer. All cellular fluorescent images were acquired on a laser confocal scanning microscopy (A1R MP, Nikon, Japan) with a 100 × oil immersion objective. Excite wavelength: 488 nm, collection wavelength for FAM: 505-525 nm.

ES5 Optimization of experiment conditions

For the optimal proportion of Zy1 to Branch, different concentrations of Zy1 and Branch (0:200, 50:150, 80:120, 100:100, 120:80, 150:50 and 200:0, with 200 nM total concentration) were used to construct Zy1-DNC. The Zy1-DNC was mixed with 0.5 μM SP1-FAM and 0.5 μM SP2-FAM. Then, these probes were instantly incubated with 50,000 SMMC-7721 in 200 μL binding buffer at 4^{o}C for 2 h. The mean fluorescence intensity of cells was determined by using flow cytometry.

For the optimal concentration of SP1-FAM and SP2-FAM, 80 nM Zy1-DNC was mixed with different concentrations of SP1-FAM and SP2-FAM (0 μM, 0.2 μM, 0.5 μM, 1.0 μM, 2.0 μM and 3.0 μM, respectively. SP1-FAM and SP2-FAM were in equal concentrations). These probes were incubated with 50,000 SMMC-7721 in 200 μL binding buffer at 4^{o}C for 2 h. The mean fluorescence intensity of cells was determined by using flow cytometry.

For the optimal incubation time, 80 nM Zy1-DNC, 1.0 μM SP1-FAM and SP2-FAM were mixed and then incubated with 50,000 SMMC-7721 in 200 μL binding buffer at 4^{o}C for different hours (0 h, 0.5 h, 1.0 h, 1.5 h, 2.0 h, 2.5 h and 3.0 h). The mean fluorescence intensity of cells was determined by using flow cytometry and the fluorescent images were captured on a laser confocal scanning microscopy.

In this section, Random-DNC-MFS and Bel-7404 were used as the control probe and the control cell, respectively. All of the experiments for the optimization were repeated

three times.

ES6 Signal-to-background ratio of Zy1-DNC-MFS binding with cells

The signal-to-background ratio was calculated according to the formula: ($F_{Zy1\text{-}DNC\text{-}MFS}$-$F_{Random\text{-}DNC\text{-}MFS}$) / $F_{SMMC\text{-}7721}$ and ($F_{Zy1\text{-}FAM}$-$F_{Random\text{-}FAM}$) / $F_{SMMC\text{-}7721}$. $F_{Zy1\text{-}DNC\text{-}MFS}$-$F_{Random\text{-}DNC\text{-}MFS}$ or $F_{Zy1\text{-}FAM}$-$F_{Random\text{-}FAM}$ was viewed as specific fluorescence signal. $F_{SMMC\text{-}7721}$ was used as background of pure cells. Different concentrations of Zy1-DNC (0, 0.5, 1, 5, 10, 50, 80, 100, 150 and 200 nM) were mixed with 1 μM SP1-FAM and 1 μM SP2-FAM. These probes were incubated with 50,000 SMMC-7721 in 200 μL binding buffer at 4 ^{o}C for 1.5 h in the dark ($F_{Zy1\text{-}DNC\text{-}MFS}$). Meanwhile, different concentrations of Zy1-FAM (0, 0.5, 1, 5, 10, 50, 80, 100, 150 and 200 nM) were incubated with SMMC-7721 ($F_{Zy1\text{-}FAM}$). Random-DNC-MFS ($F_{Random\text{-}DNC\text{-}MFS}$) or Random-FAM ($F_{Random\text{-}FAM}$) was used as the control probe for the nonspecific binding signal. The mean fluorescence intensity of cells was determined by using flow cytometry. All of the experiments were repeated three times.

Under the optimal signal-to-background condition, SMMC-7721 was incubated with Zy1-DNC-MFS or Zy1-FAM, and then the cellular fluorescent images were acquired on an inverted fluorescence microscopy (Ts2R, Nikon, Japan) with a 40 × objective. Excite wavelength: 470 nm, collection wavelength for FAM: 525 nm band pass.

ES7 Multivalent binding ability of Zy1-DNC-MFS with cells

The dissociation constant (Kd, which is inversely related to the binding affinity) was calculated according to the equation: $Y = B_{max} X / (K_d + X)$ [1], where Y was the specific fluorescence intensity ($F_{Zy1\text{-}DNC\text{-}MFS}$-$F_{Random\text{-}DNC\text{-}MFS}$ or $F_{Zy1\text{-}FAM}$-$F_{Random\text{-}FAM}$) and X was the concentration of Zy1. Fluorescein was only labeled on Zy1 or Random for fluorescence signal. Different concentration of Zy1-DNC (0, 0.5, 1, 2, 5, 10, 20 and 40 nM) was mixed with 1 μM SP1 and 1 μM SP2. These probes were incubated with 50,000 SMMC-7721 cells in 200 μL binding buffer at 4 ^{o}C for 1.5 h in the dark. Meanwhile, different concentration of Zy1-FAM (0, 0.5, 1, 2, 5, 10, 20 and 40 nM) was also incubated with SMMC-7721 cells. Random-DNC-MFS or Random-FAM was used as the control probe. The mean fluorescence intensity of cells was determined by using flow cytometry. All of the experiments for binding ability were repeated three times.

ES8 Specificity of Zy1-DNC-MFS binding with cells

80 nM Zy1-DNC, 1 μM SP1-FAM and 1 μM SP2-FAM were mixed and then incubated with 50,000 different cells (SMMC-7721, Bel-7404, L02, SW480, HeLa, MCF-7, Romas and CCRF-CEM) in 200 μL binding buffer at 4 ^{o}C for 1.5 h in the dark, respectively. Random-DNC-MFS was used as the control probe. The mean fluorescence intensity of cells was determined by using flow cytometry. All of the experiments for specificity were repeated three times.

ES9 Detection sensitivity of Zy1-DNC-MFS for SMMC-7721

SMMC-7721 was counted with hemocytometer and diluted into 0, 1, 10, 50, 100, 500 and 1000 cells per sample. Each sample was incubated with Zy1-DNC-MFS (80 nM Zy1-DNC, 1 μM SP1-FAM and 1 μM SP2-FAM) in 200 μL binding buffer or 10% FBS at 4 ^{o}C for 1.5 hours in the dark. The effective cells were counted by using flow cytometry when the fluorescence was higher than the background plus three times standard deviations. All of the experiments for sensitivity were repeated three times.

ES10 Detection of spiked tumour cells in human whole blood

The tumour cells detection in human whole blood was performed in a microfluidic chip. As shown in Fig. S8, this experimental device consisted of three parts: (1) a microfluidic chip was composed of two layers and each layer has a microchannel which converged on a round membrane separation area. Each microchannel has an inlet and outlet in top layer slice. (2) A filter membrane was inserted in a round area between two layers of slice to separate and filter the fluid. (3) A syringe pump was used to drive the blood flowing in the microchannel and passing through the filter membrane.

Briefly, BSA (1 mg/mL) was introduced into the device for 1 h blocking at room temperature and washed with PBS. Then, the samples, i.e. 1 mL binding buffer spiked with SMMC-7721 cells, 1 mL whole blood without tumour cells, or 1 mL whole blood spiked with SMMC-7721 cells, were introduced into the device for filtration and then washed with PBS, respectively. Subsequently, 80 nM Zy1-DNC-MFS was introduced into the device and incubated at 4 °C for 1.5 hours in the dark and then washed with PBS. All solutions were introduced into the device at a flow rate of 100 μL/min with a syringe pump at the outlet 2 of microfluidic chip. Finally, the filter membrane was removed from the device and used to acquire images on a laser confocal scanning

microscopy.

2. Tables and Figures

Table S1. Sequences of all nucleic acid probes used [a].

DNA probe	Sequence (from 5' end to 3' end)
Trigger	TGC TGC TGC TGC TGC TGC ACG ACG
H1	Biotin-CGT CGT GCA GCA GCA GCA GCA GCA ACG GCT TGC TGC TGC TGC TGC TGC
H2	Biotin-TGC TGC TGC TGC TGC TGC ACG ACG GCA GCA GCA GCA GCA GCA AGC CGT
Zyl	ACG CGC GCG CGC ATA GCG CGC TGA GCT GAA GAT CGT ACC GTG AGC GCG TT_{10}-Biotin
Zy1-FAM	FAM-ACG CGC GCG CGC ATA GCG CGC TGA GCT GAA GAT CGT ACC GTG AGC GCG TT_{10}-Biotin
Random	$N_{49}T_{10}$-Biotin
Random-FAM	FAM- $N_{49}T_{10}$-Biotin
Branch	Biotin-T_{10}GG AGT ATT GCG GAG GAA GGT
SP1	GAG ACA GAA GAC TGA AAA TAA CCT TCC TCC GCA ATA CTC C
SP1-FAM	GAG ACA GAA GAC TGA AAA TAA CCT TCC TCC GCA ATA CTC C-FAM
SP2	TAT TTT CAG TCT TCT GTC TCG GAG TAT TGC GGA GGA AGG T
SP2-FAM	FAM-TAT TTT CAG TCT TCT GTC TCG GAG TAT TGC GGA GGA AGG T

[a] N in Random or Random-FAM represents any one of the four deoxynucleotides (A, T, C and G).

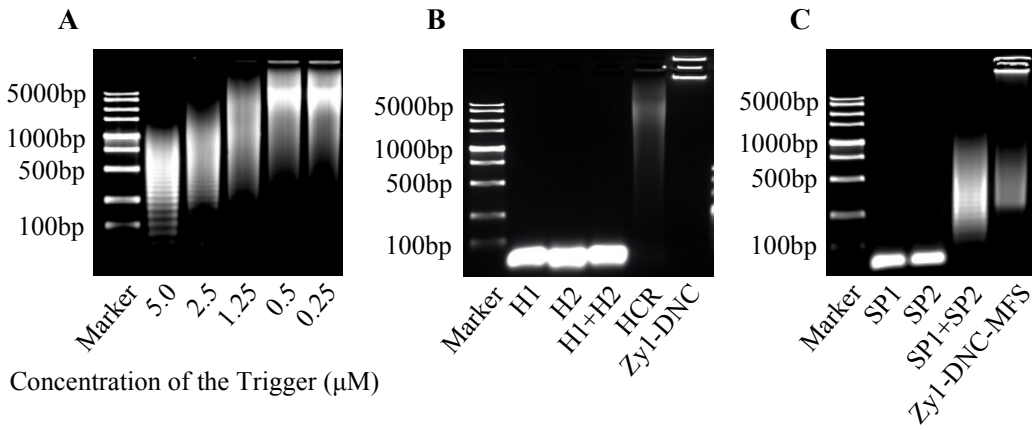


Fig. S1 Electrophoresis characterization of self-assembly process of Zy1-DNC-MFS. (A) Investigation of the length of HCR product. 25 μM H1 and 25 μM H2 were assembled with different concentration of Trigger. (B) Self-assembly of Zy1-DNC. From left to right: Marker, 50 μM H1, 50 μM H2, 25 μM H1 + 25 μM H2, 0.25 μM Trigger + 25 μM H1 + 25 μM H2 (HCR), 0.25 μM Trigger +25 μM H1 + 25 μM H2 + 50 μM Streptavidin + 25 μM Zy1 + 25 μM Branch (Zy1-DNC). (C) Self-assembly of Zy1-DNC-MFS. From left to right: Marker, 100 μM SP1, 100 μM SP2, 50 μM SP1 + 50 μM SP2, 0.25 μM Trig-ger +25 μM H1 + 25 μM H2 + 50 μM Streptavidin + 25 μM Zy1 + 25 μM Branch + 50 μM SP1 + 50 μM SP2 (Zy1-DNC-MFS).

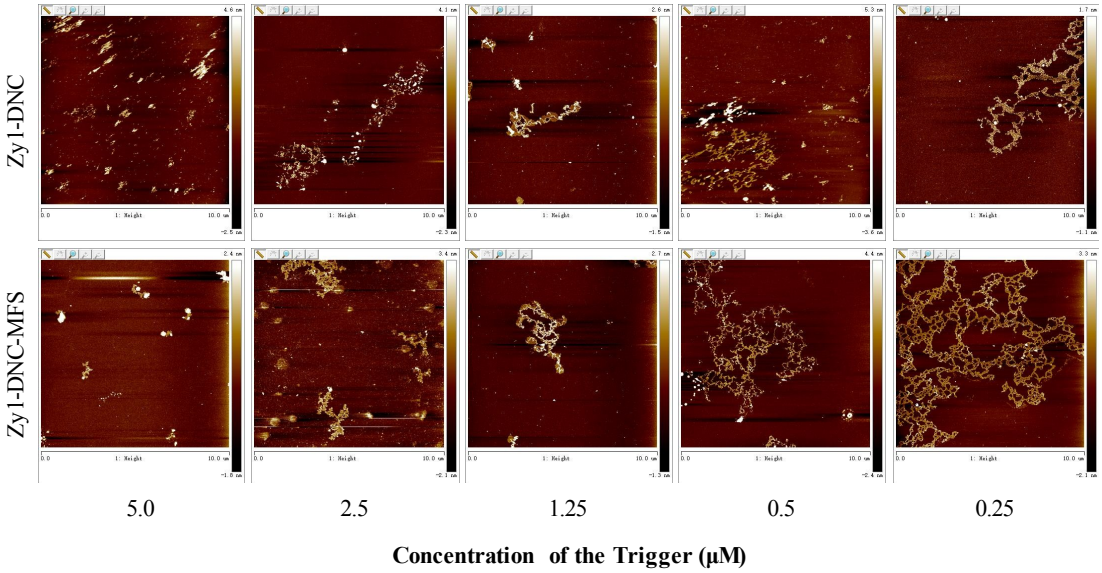


Fig. S2 Atomic force microscopy (AFM) images of Zy1-DNC and Zy1-DNC-MFS. AFM images of the DNA nanocreeper with different concentration of Trigger after addition of Zy1 and Branch (Zy1-DNC, first row); after addition of SP1 and SP2 (Zy1-DNC-MFS, last row).

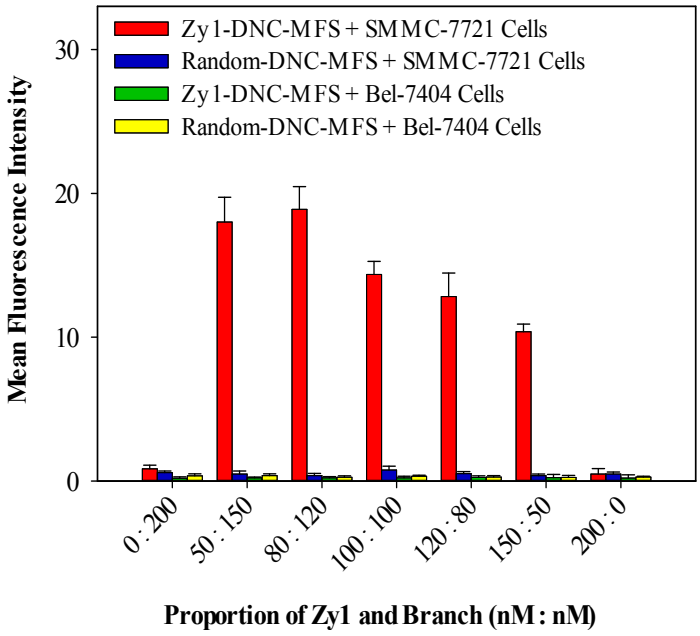


Fig. S3 Effects of concentration proportion of Zy1 to Branch on cell fluorescence intensity. Zy1-DNC with different concentration ratio of Zy1 to Branch (total concentration remained 200 nM), 0.5 μM SP1-FAM and 0.5 μM SP2-FAM were mixed and then incubated with SMMC-7721 at 4 °C for 2 hours in the dark. Random-DNC-MFS and Bel-7404 were used as controls. The mean fluorescence intensity of cells was determined by using flow cytometry. The error bars were the standard deviations of three parallel experiments.

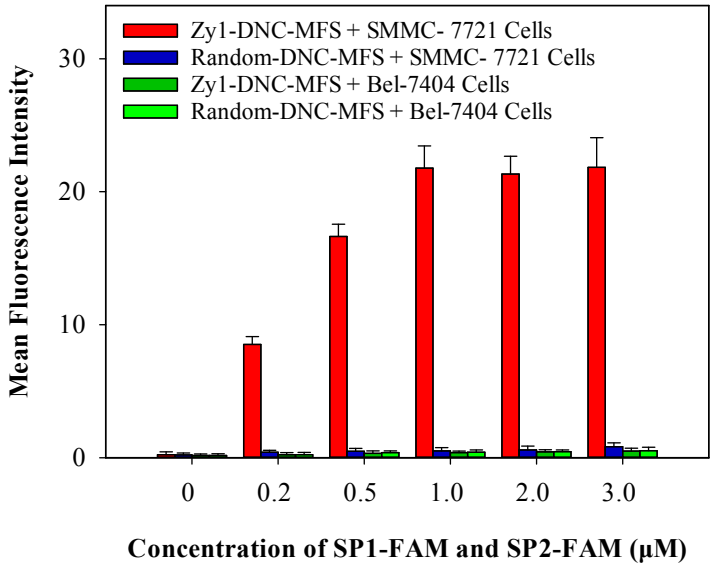


Fig. S4 Effects of concentration of SP1-FAM and SP2-FAM on cell fluorescence intensity. 80 nM Zy1-DNC and different concentrations of SP1-FAM and SP2-FAM were mixed and then incubated with SMMC-7721 at 4 °C for 2 hours in the dark. Random-DNC-MFS and Bel-7404 were used as controls. The mean fluorescence intensity of cells was determined by using flow cytometry. The error bars were the standard deviations of three parallel experiments.

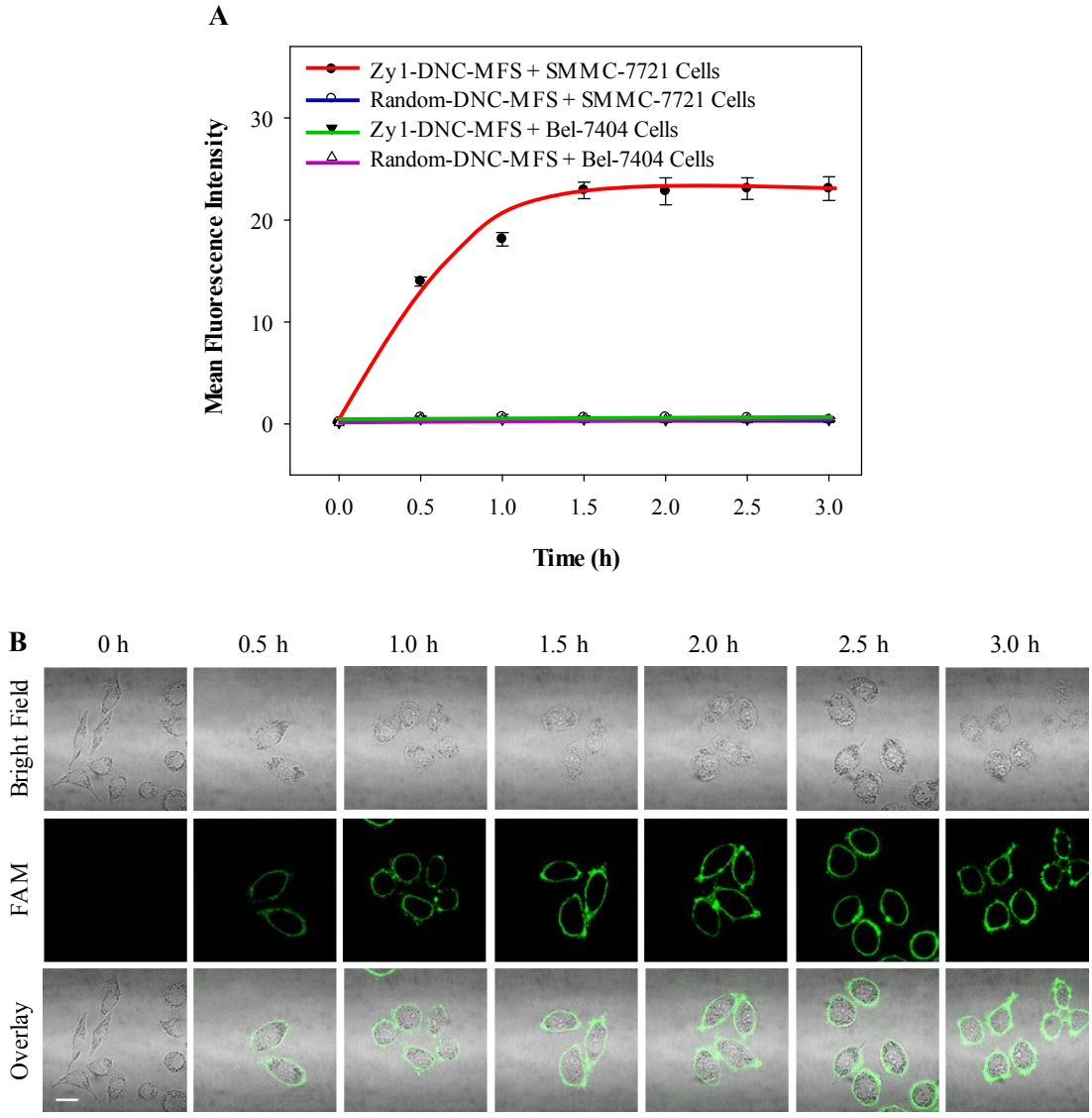


Fig. S5 Effects of incubation time of Zy1-DNC-MFS with cells on cell fluorescence intensity. 80 nM Zy1-DNC, 1 μM SP1-FAM and 1 μM SP2-FAM were mixed and then incubated with SMMC-7721 at 4 °C for different hours in the dark. Random-DNC-MFS and Bel-7404 were used as controls. The mean fluorescence intensity of cells was determined by using flow cytometry (A) and fluorescent images were captured with laser confocal scanning microscopy (B). The error bars were the standard deviations of three parallel experiments. Scale bar: 20 μm.

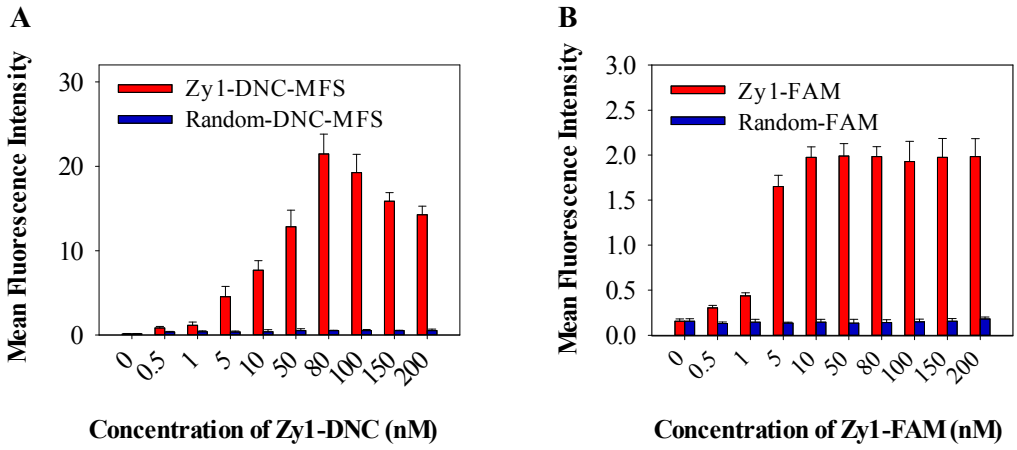


Fig. S6 Comparison of different concentrations of Zy1-DNC-MFS and Zy1-FAM on cell fluorescence intensity. (A) Different concentrations of Zy1-DNC with fixed ratio of Zy1 and Branch (80 : 120), 1 μM SP1-FAM and 1 μM SP2-FAM were mixed and then incubated with SMMC-7721 at 4 °C for 1.5 h in the dark. Random-DNC-MFS was treated as control. (B) Different concentrations of Zy1-FAM were incubated with SMMC-7721 at 4 °C for 1.5 h in the dark. Random-FAM was treated as control. The mean fluorescence intensity of cells was determined by using flow cytometry. The error bars were the standard deviations of three parallel experiments.

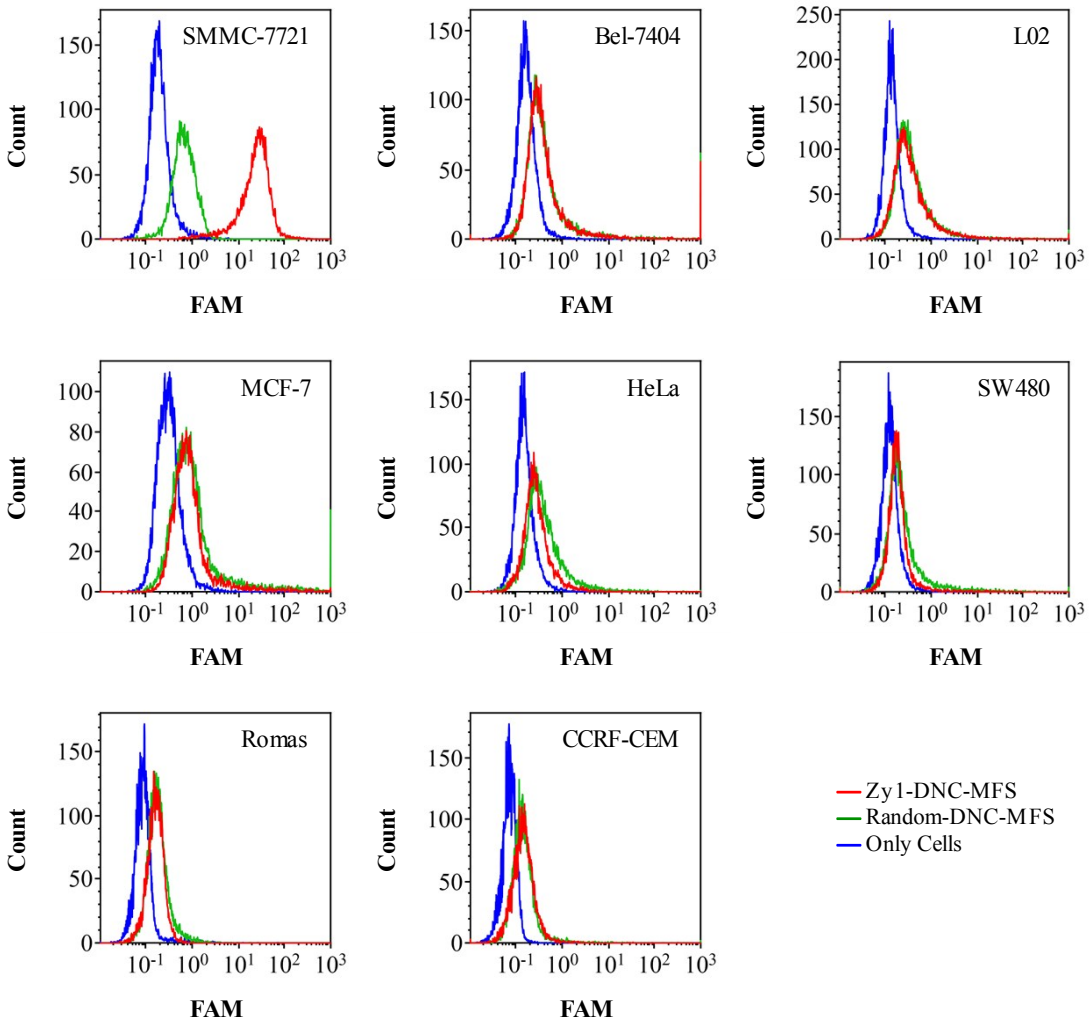
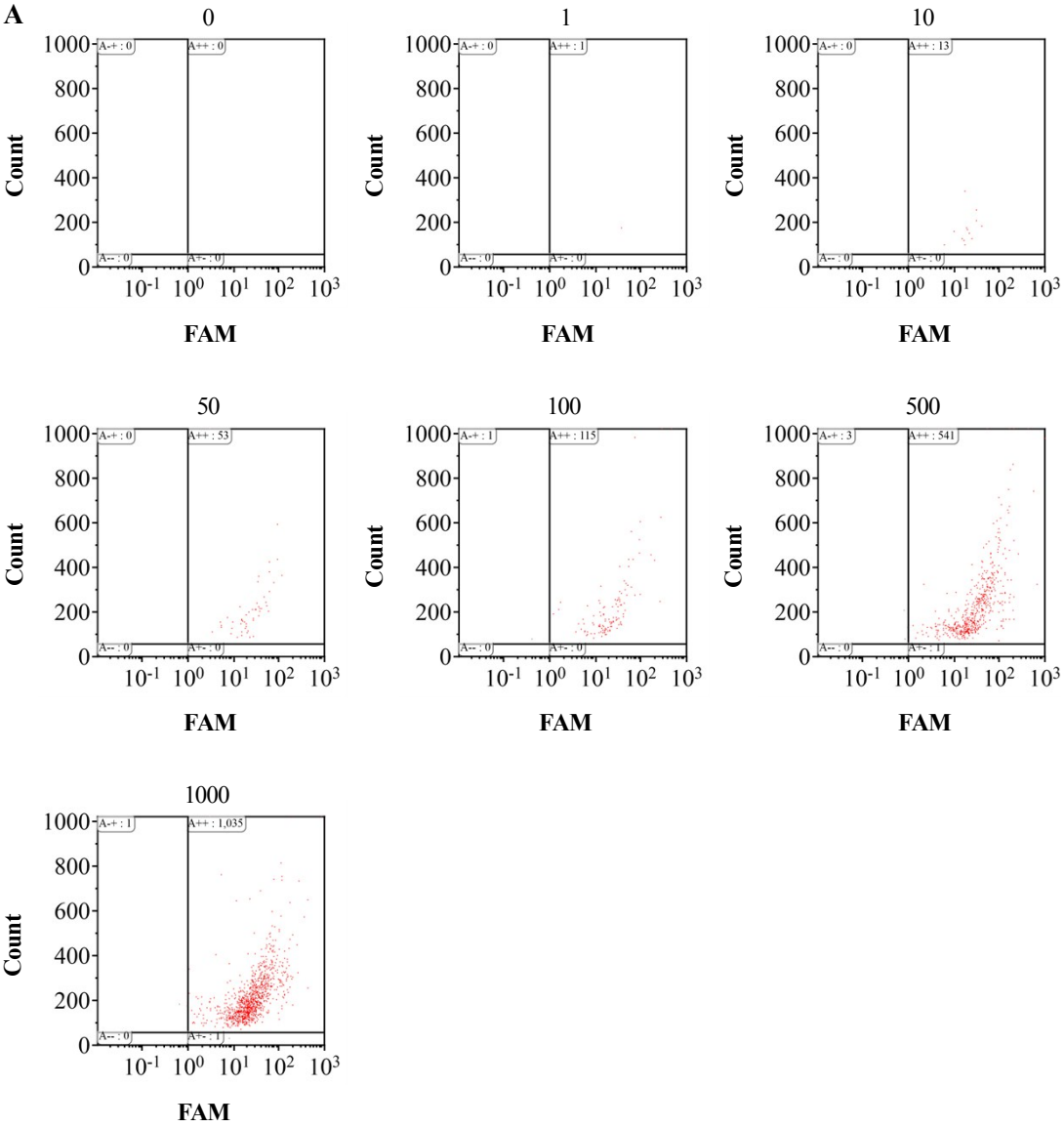


Fig. S7 Characterization of Zy1-DNC-MFS binding specificity with different cells. 80 nM Zy1-DNC-MFS (80 nM Zy1-DNC, 1 μM SP1-FAM and 1 μM SP2-FAM) incubated with SMMC-7721 at 4 °C for 1.5 h in the dark. Fluorescence histogram plot was determined by using flow cytometry. Random-DNC-MFS was treated as control.

A


0
A-+ : 0
A++ : 0
A-- : 0
A+- : 0
1
A-+ : 0
A++ : 1
A-- : 0
A+- : 0
10
A-+ : 0
A++ : 13
A-- : 0
A+- : 0
50
A-+ : 0
A++ : 53
A-- : 0
A+- : 0
100
A-+ : 1
A++ : 115
A-- : 0
A+- : 0
500
A-+ : 3
A++ : 541
A-- : 0
A+- : 1
1000
A-+ : 1
A++ : 1,035
A-- : 0
A+- : 1
Count
FAM
0
200
400
600
800
1000
10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}

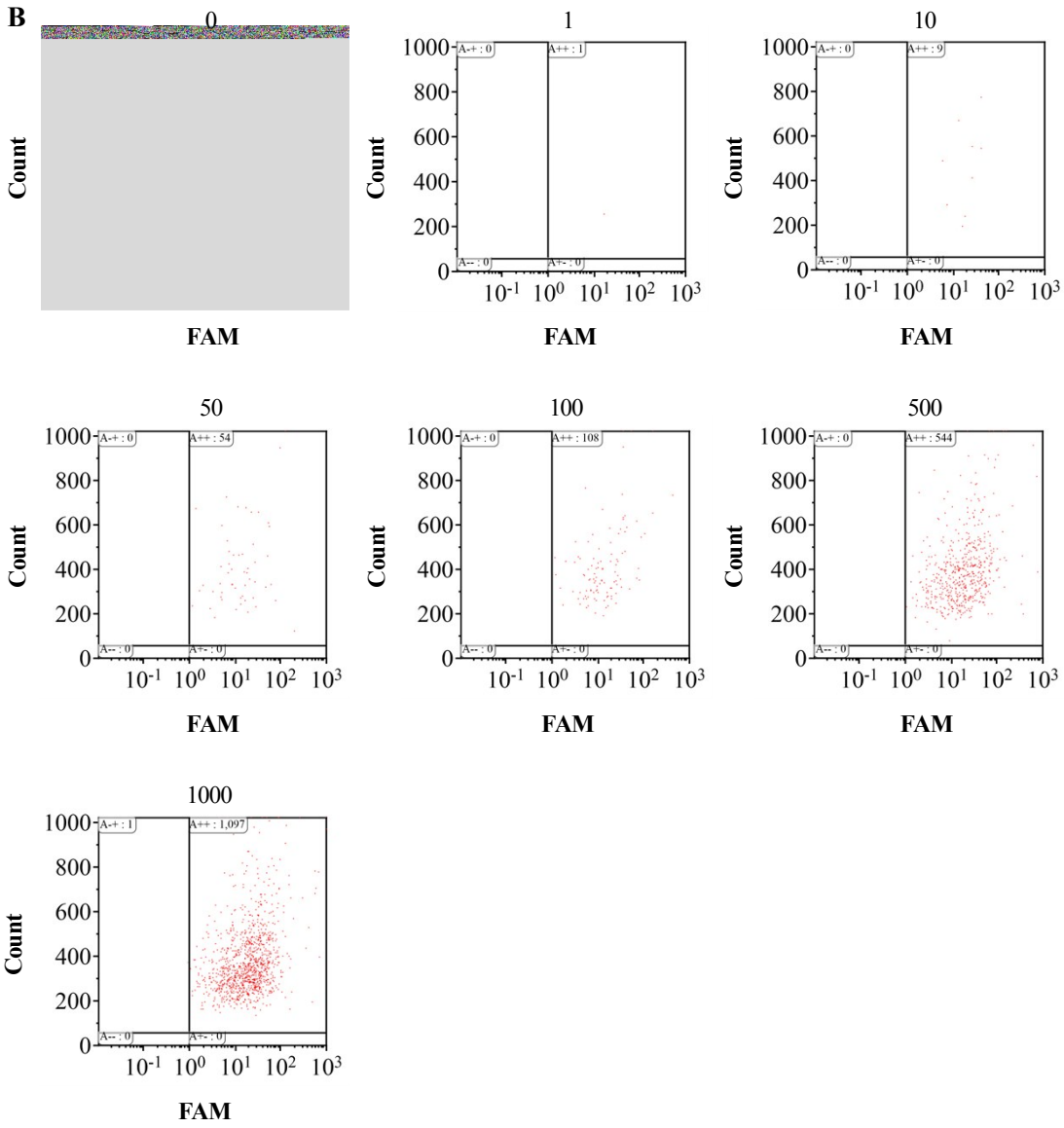


Fig. S8 Cell detection in binding buffer (A) and 10% FBS (B). 80 nM Zy1-DNC-MFS (80 nM Zy1-DNC, 1 μM SP1-FAM and 1 μM SP2-FAM) incubated with 0, 1, 10, 50, 100, 500 and 1000 cells at 4 °C for 1.5 h in the dark. Cells were detected by using flow cytometry.

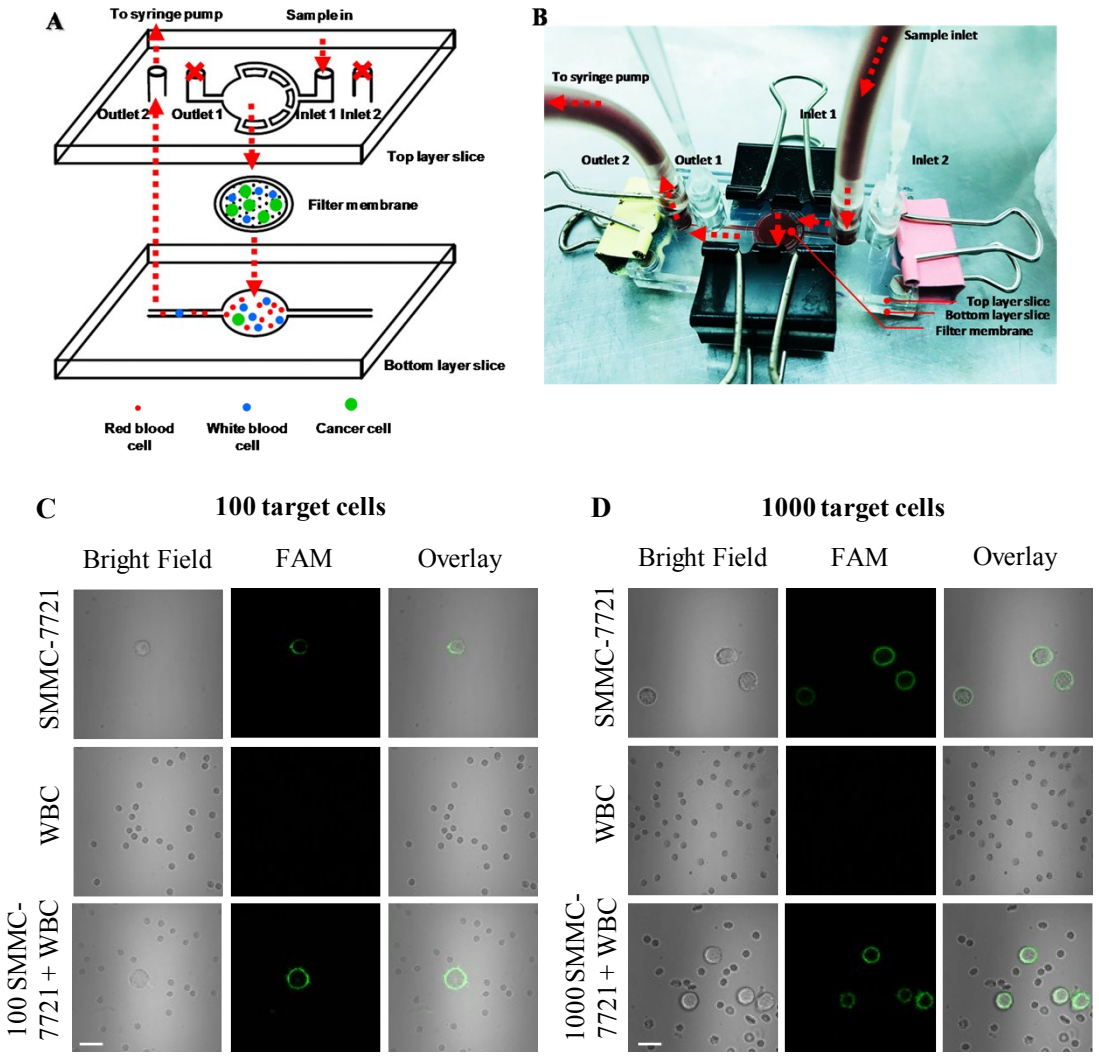


Fig. S9 Microfluidic chip for the spiked tumour cells detection in human whole blood. The illustration (A) and photo (B) of microfluidic chip for detection of tumour cells spiked in human whole blood. The fluid was introduced from inlet 1 and flow into microchannel of top layer slice. Then, the fluid passed through the filter membrane on which tumour cells and a few of white blood cells were intercepted. The rest of blood including all red blood cells and most of white blood cells was drained from the microchannel of bottom layer slice into outlet 2 which connected to a syringe pump. The outlet 1 and inlet 2 was blocked. Target cells intercepted on filter membrane were counted by using confocal microscopy: whole blood sample containing 100 (C) and 1000 (D) SMMC-7721 cells. Scale bar: 20 μm.

References

1 C. T. Nguyen, K. Tanaka, Y. Cao, S. H. Cho, D. Xu and G. Stacey, *PLoS One*, 2016, **11**, e0161894.