Supporting Information

Self-Powered 3D DNA Walker with Programmability and Signal-Amplification for Illuminating MicroRNA in Living Cells

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Experimental Section

Chemicals and Materials. Chloroauric acid (HAuCl4·4H2O), trisodium citrate were purchased from Sigma-Aldrich (Shanghai, China). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), DEPC-treated pure water (DEPC = diethyl pyrocarbonate), cell counting kit-8 (CCK-8) were from Sangon Biotechnology Co. Ltd. (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS) and Hoechst 33342 were purchased from ThermoFisher. The human cervical cancer cells (HeLa), human breast cancer cells (MCF-7) and human embryonic kidney cells (HEK293) were from KeyGen Biotech. Co. Ltd. (Nanjing, China). The human prostate cancer cells (PC-3) was obtained from BeNa Chuanglian Biology Research Institute (Beijing, China). Trizol total RNA extraction reagent was purchased from Beyotime Biotechnology Co. Ltd. (Shanghai, China). DNA sequences (details in Table S1) were synthesized and purified by Sangon Biotechnology Co. Ltd. (Shanghai, China). RNA sequences were synthesized and purified by GenScript Biotechnology Co. Ltd. (Nanjing, China). All the water used in the work was purified by a Millipore filtration system.

Table S1. DNA and RNA sequences used in the work

Name	Sequence (5’ end to 3’ end)
FAM-H1	SH-TTTTTTTTTTTCAACATCAGTCTGATAAGCTAGATGTTGAAACCTAGGTAGCTTATCAGACT-FAM
Ctrl-H1	TTTTTTTTTTTCAACATCAGTCTGATAAGCTAGATGTTGAAACCTAGGTAGCTTATCAGACT
FAM-H2	FAM-ATAAGCTACCTAGGTTTCAACATCTAGCTTATCAGACTGATGTTGAAACCTAGGCCATGTCATA-SH
Ctrl-H2	ATAAGCTACCTAGGTTTCAACATCTAGCTTATCAGACTGATGTTGAAACCTAGGCCATGTCATA
FB-H2	FAM-ATAAGCTACCTAGGTTTCAACATCTAGCTTATCAGACTGATGTTGAAACCTAGGCCATGTCATA-BHQ1
For selectivity test	
Synthetic Target (miRNA-21)	UAGCUUAUCAGACUGAUGUUGA
M1	UAGCUUAUCAGACUCAUGUUGA
M2	UAGCUUAUCAGUCUCAUGUUGA
Control sequence 3 (miRNA-221)	AGCUACAUUGUCUGCUGGGUUUC
For F-AuNP/DNA	
Random	FAM-AAACCCTAACCCTAACCCTAAAAGTTCCGTTCGAGTCCAAA-SH
C-Random	ACTCGAACGGAACTTTTAGGGTTAGGGTTAGGG
For regulation test	
Sense	UAGCUUAUCAGACUGAUGUUGAUU
Anti-sense	UCAACAUCAGUCUGAUAAGCUAUU

Apparatus. The morphology of AuNPs was characterized on JEM-2800 transmission electron microscopy (TEM, JEOL, Japan). The UV-vis absorption spectra were recorded using a UV-vis spectrophotometer (UV-3600, Shimadzu, Japan). The fluorescence emission spectra were recorded using a fluorescence spectrometer (F-7000, Hitachi, Japan). The zeta potential and dynamic light scattering analysis was obtained with a particle size analyzer (90 Plus, Brookhaven, United States). All the cell images were obtained with a Leica TCS SP8 STED 3× laser scanning confocal microscopy (Leica, Germany). The cell viability assay was performed using a Thermo Scientific Varioskan Flash (ThermoFisher, United States). TIRF images were obtained with an Olympus total internal reflection fluorescence (TIRF) microscopy (Olympus, Japan).

Construction of the DNA walker. First, the AuNPs were prepared based on the classical sodium citrate reduction method.[1] In brief, 100 mL of 0.01% $HAuCl_4$ was heated to boiling and refluxing under vigorous stirring, and then the reducing agent sodium citrate (3.5 mL, 38.8 mM) was quickly added. The mixture was refluxed with stirring for another 15 min after the color changed from pale yellow to deep wine-red. Next, the prepared AuNPs were cooled to room temperature and characterized by TEM and UV-vis absorption spectra. To prepare the DNA walking nanodevices, AuNPs were mixed with the thiolated hairpin DNAs which was reduced and annealed in advance. After stirring overnight, 5.0 M sodium chloride solution was gradually added to the above solution to achieve a final concentration of 0.5 M. Then the excess DNA sequences were removed by centrifuging at 12000 rpm and washing with PBS buffer. Finally, the AuNP/DNA nanodevices were re-dispersed in PBS and kept at 4 °C for later use.

TIRF imaging and analysis. The cover slides were first cleaned in the "piranha" solution (30% H_2O_2: concentrated H_2SO_4 = 1: 3, v/v) for 30 min, followed by thoroughly washing with ddH_2O and drying with nitrogen. Then the slides were immersed in the ethanol solution containing 1% (v/v) APTES for 3 h. After that, the APTES-modified slides were washed with ethanol and dried in an oven at 120 °C for 30 min. The prepared DNA walker was deposited on the silanized cover slides for 5 min, and the redundant solution was removed. For TIRF imaging, images were obtained using a commercial TIRF microscopy with a 60× oil-immersion objective and 488 nm laser. Fluorescence intensity of single particles was analyzed by ImageJ software.

In vitro miRNA-21 detection. The AuNP/DNA nanodevices (300 μL) or control probes (300 μL) were incubated with various concentrations of target (50 μL) at 37 °C for 2 h, and then the fluorescence emission spectra were obtained. For reaction kinetics experiments, the time-dependent fluorescence curves were recorded as soon as the nanosystem were mixed with target sequences. Finally, total RNA extracts, instead of the synthetic miRNA-21 sequences, were used to assess the practicability of the walking nanodevice.

Cell culture and total RNA extraction. HeLa cells, PC-3 cells and HEK293 cells were cultured in DMEM medium supplemented with 10% fetal calf serum, penicillin (80 U/mL) and streptomycin (0.08 mg/mL) at 37 °C in an atmosphere containing 5% CO_2. MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin and streptomycin in the same incubator as the above cell lines. All kinds of cells were seed in 6-well plates (1×10^6 per well) and cultured for 24 h. Then the total RNA were extracted with Trizol according to the product manual. The concentration of extracted total RNA was determined by the UV-vis absorption spectra according to equation, $C = OD_{260} \times n \times 40$ (μg/mL), where n is the dilution factor. Finally, the extracts were diluted to the same concentration and stored at -20 °C.

Cytotoxicity assay. The cytotoxicity of the DNA walker was tested by a CCK-8 assay. HeLa cells, PC-3 cells, MCF-7 cells and HEK293 cells were seed in 96-well plates (10^4 cells/well) and cultured overnight. Then the cells were incubated with fresh medium containing the DNA walker at 37 °C for different times. Next, 100 μL of medium containing 10 μL of CCK-8 was added to each well, followed by incubation for 1 h. After that, the absorbance at 450 nm was recorded. All of the experiments were repeated three times.

Confocal fluorescence imaging of miRNA-21. For in situ imaging of intracellular miRNA-21, four kinds of cells above were seeded and cultured in confocal dishes overnight. On the second day, the cells were randomly divided into two groups, and the supernatant was replaced by fresh medium containing the DNA walker or control probe, respectively. After incubation for 5 h, the cells were stained with Hoechst 33342, a nuclear dye, and then washed three times with PBS buffer. All the cell images were obtained with a Leica TCS SP8 STED 3× laser scanning confocal microscopy under 488 nm laser excitation.

Supplementary Results and Discussion

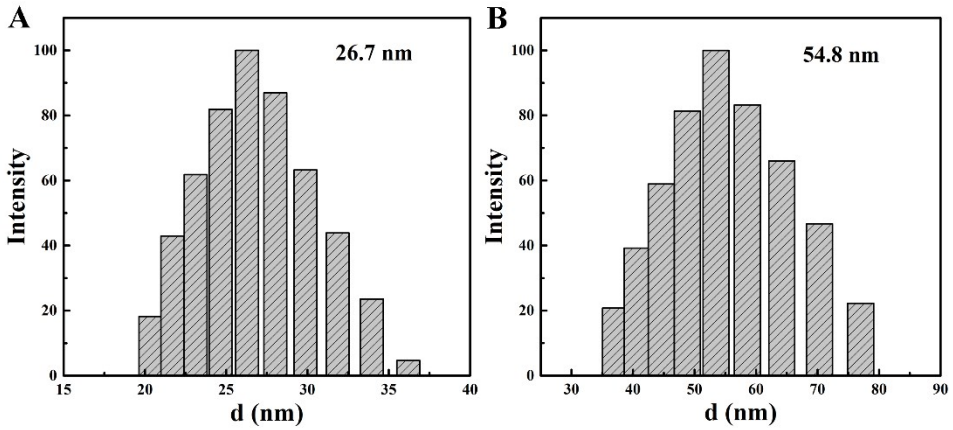

Fig. S1 DLS characterization of the AuNPs (A) and AuNP/DNA nanodevice (B).

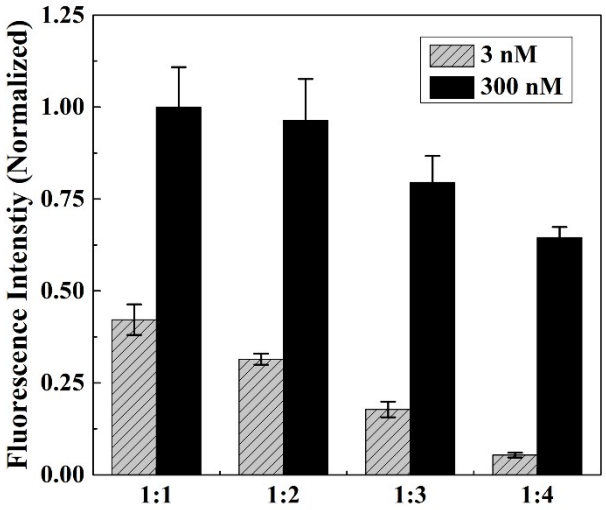

Fig. S2 Normalized fluorescence intensity at 517 nm of the AuNP/DNA nanodevice with various H1/H2 ratios after incubation with 3 or 300 nM target sequence. Error bars indicate standard deviation of triplicate tests.

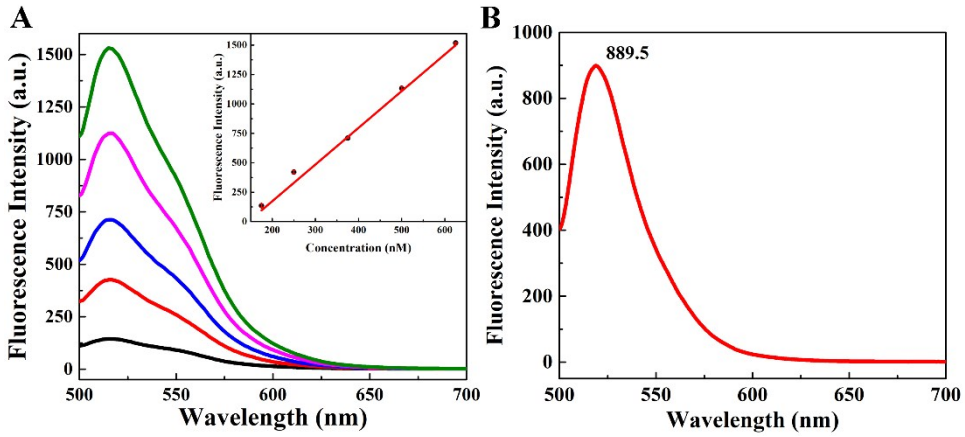

Fig. S3 Quantification of H1 and H2 in the reaction system. (A) Fluorescence spectra of different concentrations of FAM labeled H1 sequences. Inset: the linear relationship between fluorescence intensity and concentration. Error bars indicate standard deviation of triplicate tests. (B) Fluorescence spectrum of the supernatant. λ_{ex} = 488 nm, λ_{em} = 517 nm.

According to our previous work, the ability of two structurally similar thiolated DNA sequences to attach to the surface of AuNP showed a slight difference.[1] To quantify the concentration of H1 and H2 in the prepared probe solution, fluorescence spectra (Fig. S3A) of series concentrations of FAM labeled H1 sequences were first measured to obtain the calibration curve. As the concentration increased, the fluorescence intensity at 517 nm was enhanced in a linear way, I_F = -450.4 + 3.119 × C (10^{-9} M), where the correlation coefficient R^2 was 0.995. Then 50 μL of mercaptoethanol (ME) was added to the prepared nanodevice solution (300 μL) to replace the H1 strands on the surface of AuNPs. After shaking at room temperature for 2 h, the released oligonucleotides were separated by centrifugation. The fluorescence spectrum (Fig. S3B) of the supernatant was recorded and the concentration of the released H1 sequences was calculated to be 429.6 nM. So the concentration of H1 (or H2) in the original AuNP/DNA nanodevice was determined to be 250.6 nM (429.6 × 350/300 × 0.5 = 250.6).

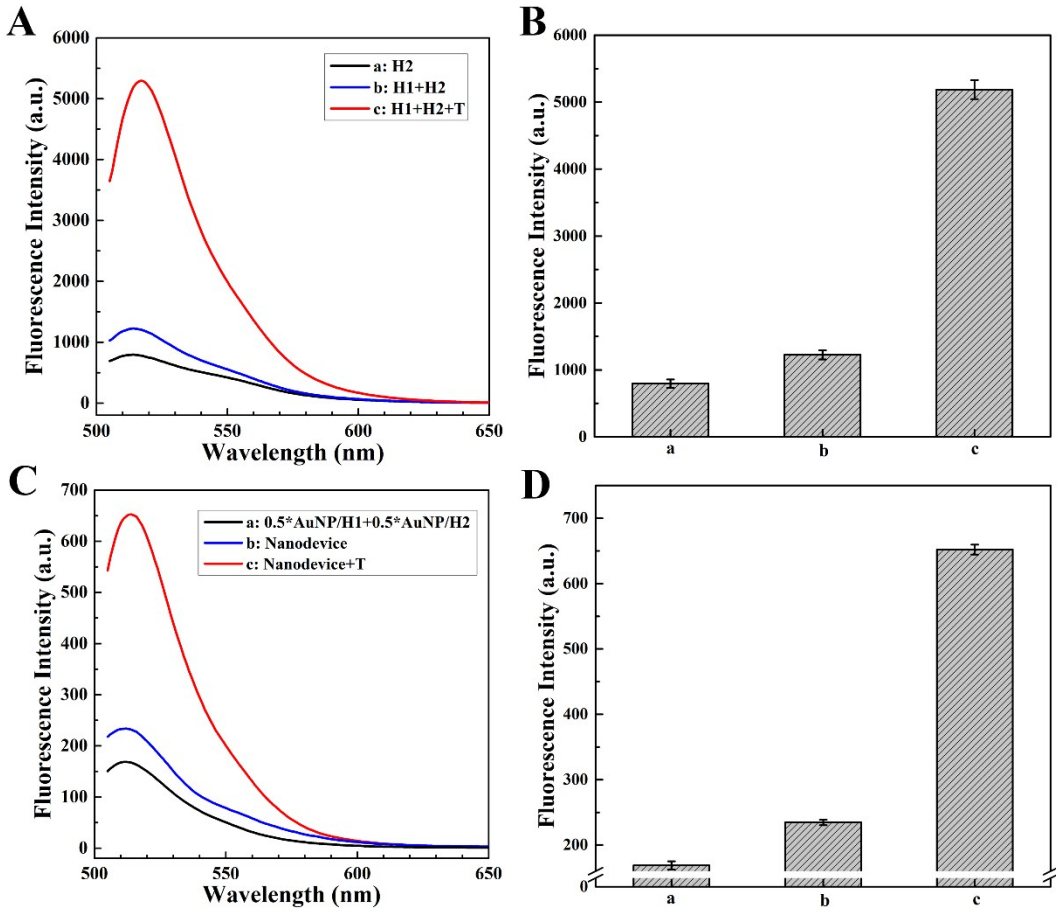

Fig. S4 (A) The fluorescence spectra and (B) fluorescence intensity of different reaction systems (H1: Ctrl-H1 sequence; H2: FB-H2 sequence; $C_{H1} = C_{H2} = 2$ μM). (C) The fluorescence spectra and (D) fluorescence intensity of the AuNP/DNA nanodevice with or without target sequence. $\lambda_{ex} = 488$ nm, $\lambda_{em} = 517$ nm. Error bars indicate standard deviation of triplicate tests.

According to Fig. S4A and S4B, 9.79% ($(F_b - F_a) / (F_c - F_a) = 0.979$) of H1 hybridized with H2 in the absence of target sequence after the mixture was left at 37 °C for 7 days. On the other hand, to investigate the nonspecific background of the proposed nanodevice, AuNPs modified with the hairpin DNA H1 or H2 (termed as AuNP/H1 and AuNP/H2) were mixed and used as the control group. After one week at 37 °C, 13.6% ($(F_b - F_a) / (F_c - F_a) = 0.136$) background was generated by the nonspecific hybridization of H1 and H2 assembled on AuNP (Fig. S4C and S4D).

Table S2. Concentration of miRNA-21 (C_T) used in Fig. 3. The concentration of H1 in the reaction system was calculated to be 214.8 nM (250.6 × 300 ÷ 350 = 214.8).

T/H1	C_T (nM)
5.0×10^{-4}	1.074×10^{-1}
1.0×10^{-3}	2.148×10^{-1}
1.5×10^{-3}	3.222×10^{-1}
2.0×10^{-3}	4.296×10^{-1}
7.5×10^{-3}	1.611
1.5×10^{-2}	3.222
7.5×10^{-2}	16.11
1.5×10^{-1}	32.22
7.5×10^{-1}	161.1
1.5	322.2

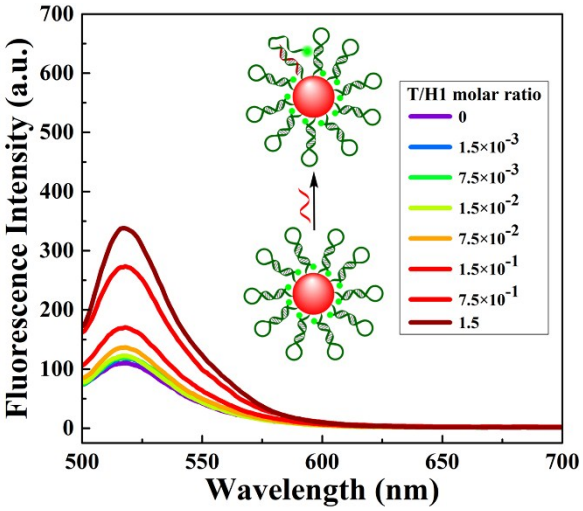

Fig. S5 Fluorescence emission spectra of control probe after incubation with miRNA-21 of different concentrations. λ_{ex} = 488 nm.

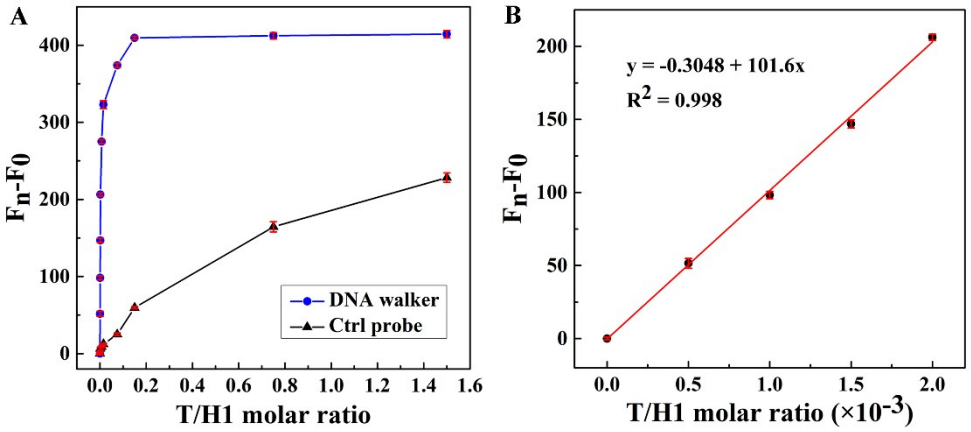

Fig. S6 (A) The relationships of fluorescence intensity of the AuNP/DNA nanodevice or control probe *vs* the T/H1 molar ratio. (B) The linear relationship between the fluorescence intensity of the nanodevice and the T/H1 molar ratio. λ_{ex} = 488 nm, λ_{em} = 517 nm.

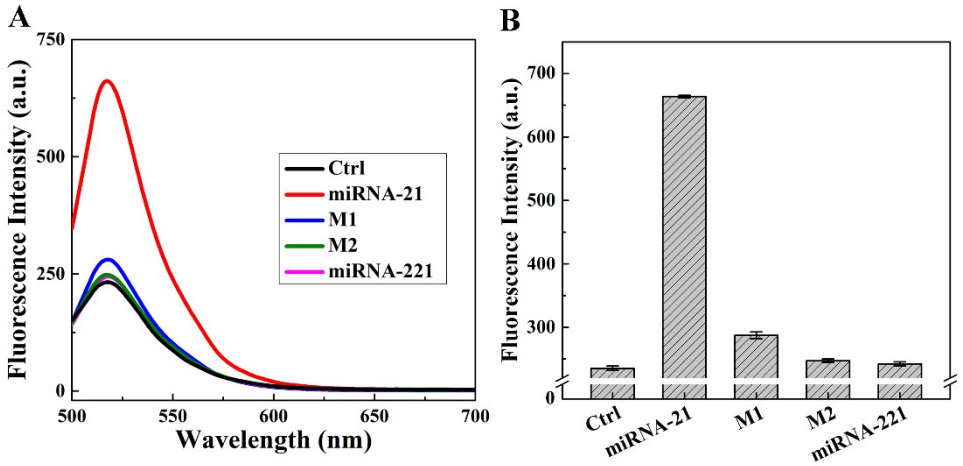

Fig. S7 Fluorescence emission spectra (A) and the fluorescence intensity at 517 nm (B) of the AuNP/DNA nanodevice incubated with different miRNA sequences. λ_{ex} = 488 nm. Error bars indicate standard deviation of triplicate tests.

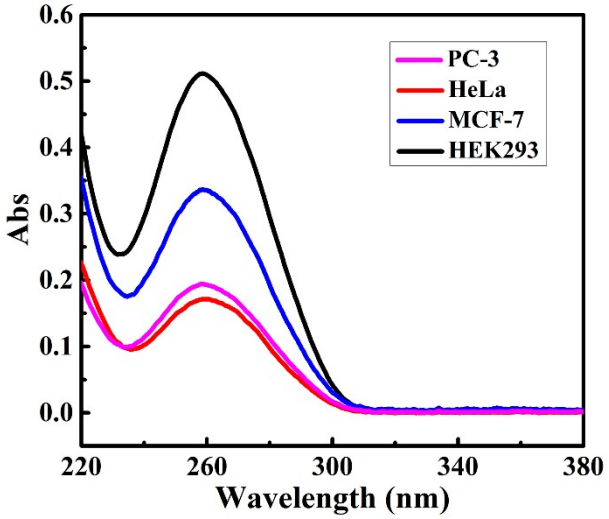

Fig. S8 UV-vis absorption spectra of the diluted total RNA extracted from different cells.

To determine the concentration of the total RNA extracts, their UV-vis absorption spectra were recorded. Then the concentration of total RNA from different cells was calculated according to the equation, $C = OD_{260} \times n \times 40$ (μg/mL), where the dilution factor n was 1000 (listed in Table S2). All the extracts were diluted to be 2 μg/μL for later use.

Table S3. Quantification of concentration of total RNA extracted from HeLa, MCF-7, PC-3 and HEK293 cells.

Cell type	OD_{260}	Concentration (μg/μL)
HEK293	0.5094	20.3760
HeLa	0.1711	6.8440
MCF-7	0.3357	13.4280
PC-3	0.1929	7.7160

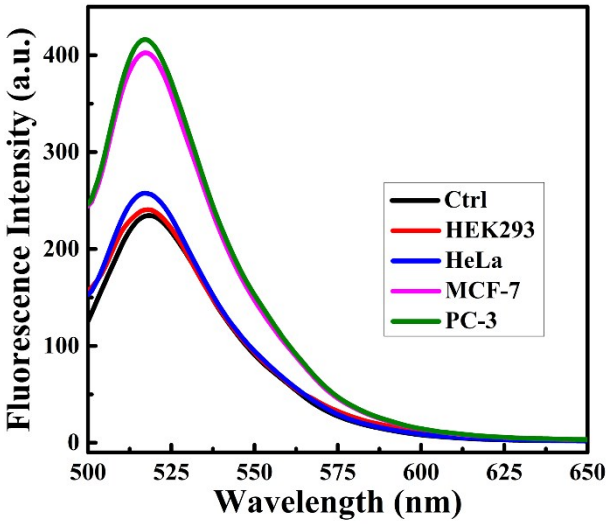

Fig. S9 Fluorescence emission spectra of the AuNP/DNA nanodevice incubated with total RNA extracted from HEK293, HeLa, MCF-7 and PC-3 cells. λ_{ex} = 488 nm, λ_{em} = 517 nm.

Table S4. Determination of the copy numbers of miRNA-21 in each pg total RNA extracted from HeLa, MCF-7, PC-3 and HEK293 cells.

Cell type	F_n-F_0	T/H1 ($\times10^{-5}$)	C_{miRNA} (pM)	miRNA copies ($\times10^{11}$)	miRNA copies per pg total RNA
HEK293	5.95±2.52	6.26±2.48	13.24±5.34	2.79±1.12	28±11
HeLa	22.34±3.19	22.29±3.14	47.92±6.75	12.10±1.42	101±14
MCF-7	167.46±2.85	165.13±2.81	355.02±6.03	74.83±1.27	748±13
PC-3	181.84±2.07	179.27±2.04	385.44±4.38	81.24±0.92	812±9

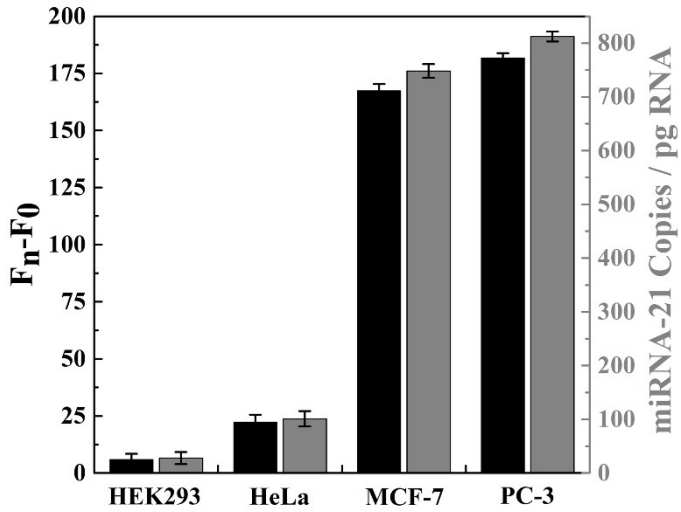

Fig. S10 Fluorescence intensity of the nanodevice incubated with total RNA extracted from HEK293, HeLa, MCF-7 and PC-3 cells, and copy numbers of miRNA-21 in each pg total RNA from these cells. Error bars indicate standard deviation of triplicate tests.

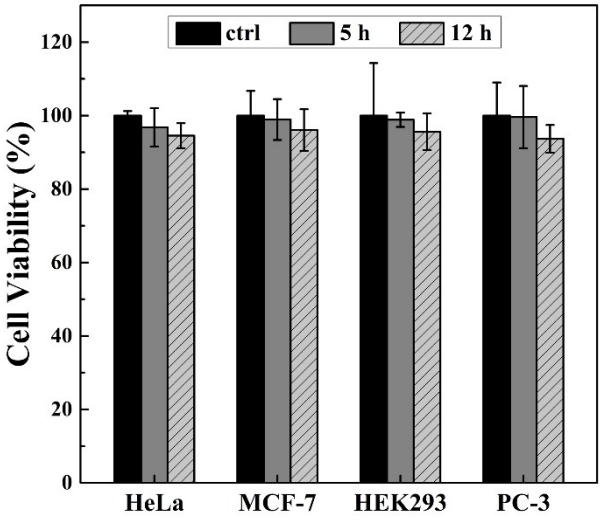

Fig. S11 Viability of HeLa, MCF-7, HEK293 and PC-3 cells (100 μL, 5×10^5 /mL) after incubated with the AuNP/DNA nanodevice for different times. Error bars indicate standard deviation of triplicate tests.

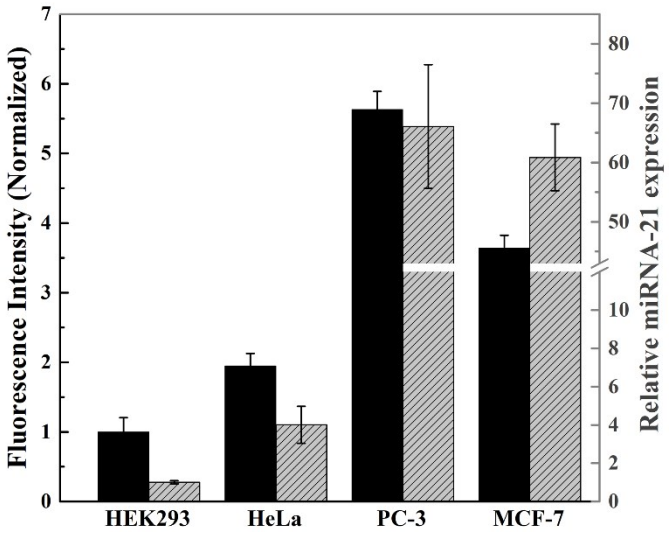

Fig. S12 (Left) The normalized fluorescence intensity of different cells after incubated with the DNA walker for 5 h. The data were processed by ImageJ software based on Fig. 4. (Right) The miRNA-21 expression levels of different cells determined by qPCR. Error bars indicate standard deviation of triplicate tests.

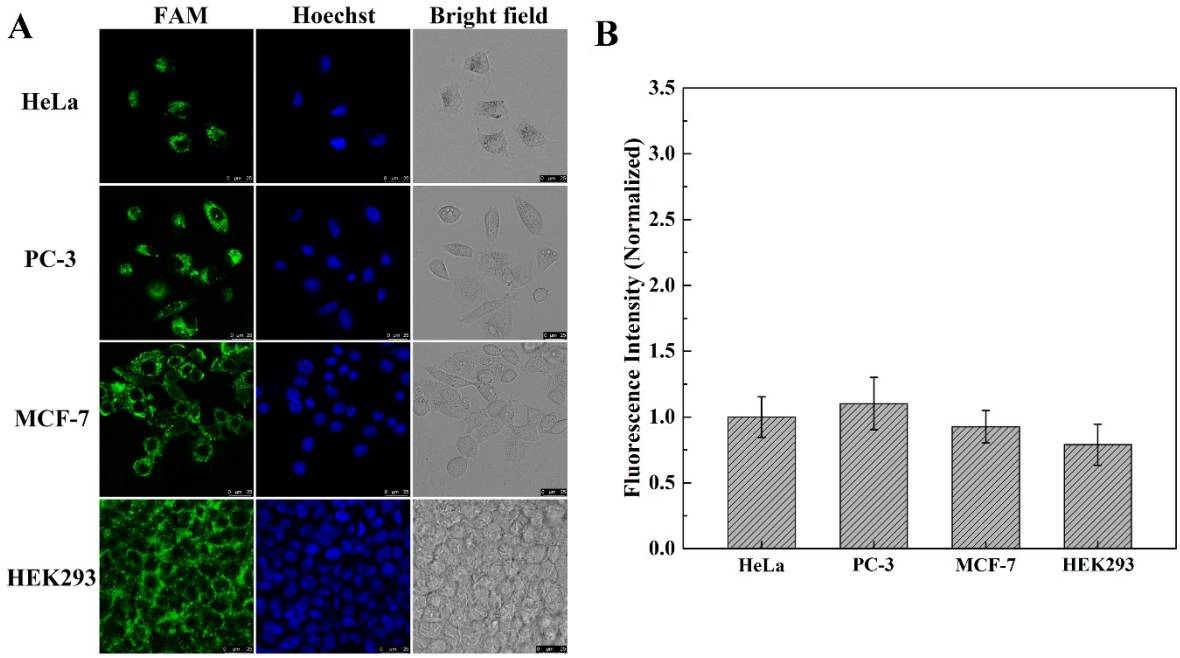

Fig. S13 (A) Confocal images of different cells after incubation with the F-AuNP/DNA probe that was always fluorescent for 5 h. (B) Normalized fluorescence intensity of different cell lines. The data were processed by ImageJ software.

To evaluate the effect of nanodevice accumulation in different cell lines on fluorescence signal, the control probe (named as F-AuNP/DNA) which could be always fluorescent was prepared by

functionalizing AuNP with duplexes of Random sequence and C-Random sequence (Table S1). Various cell lines (HeLa, PC-3, MCF-7 and HEK293 cells) were incubated with the probe at 37 °C for 5 h. The confocal images (Fig. S13) showed that there was no significant difference in fluorescence intensity of all the tumor cell lines, while HEK293 cells exhibited slightly weaker fluorescense signal. Furthermore, even the fluorescence signal of HEK293 cells were much stronger than any of the signals shown in Fig. 4, indicating that the cell uptake of the probe was excessive. Therefore, the intracellular accumulation would not affect the light-up signal of the DNA walker in different cell lines.

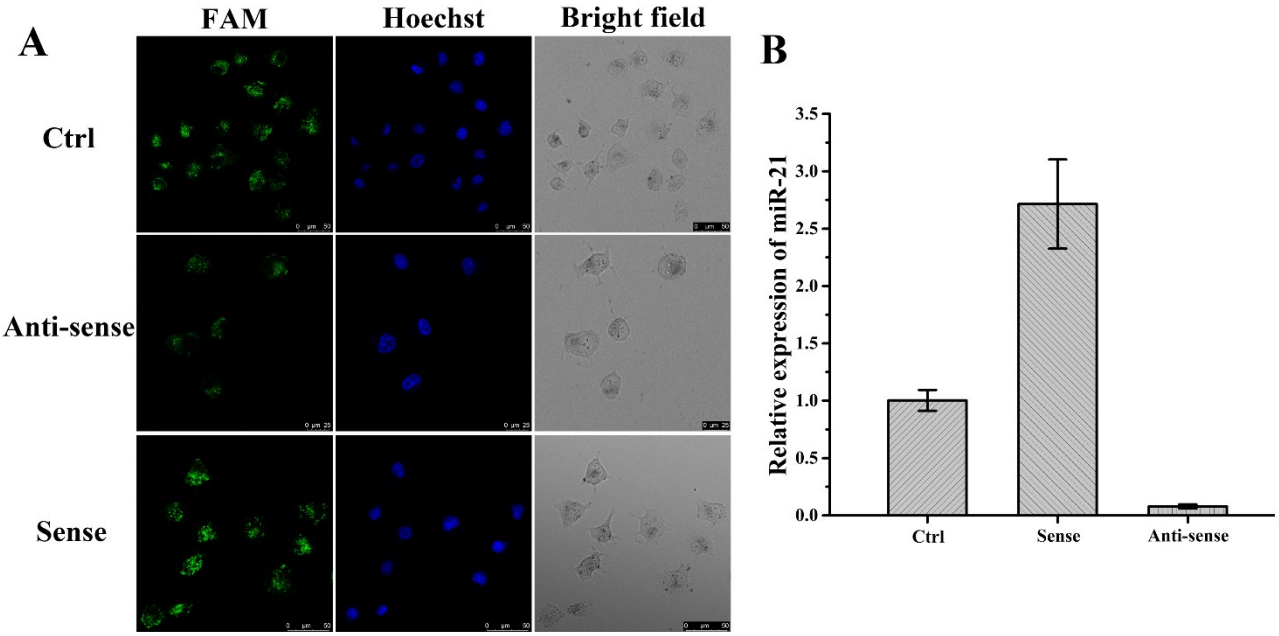

Fig. S14 (A) Live-cell imaging of miRNA-21 with the AuNP/DNA nanodevice in HeLa cells after pre-transfected with different sequences. (B) The miRNA-21 expression levels of HeLa cells determined by qPCR. Error bars indicate standard deviation of triplicate tests.

References

1 X. J. Yang, K. Zhang, J. J. Xu and H. Y. Chen, *Anal. Chem.*, 2018, **90**, 6199-6205.