Electronic Supplementary Information (ESI)

An Enzyme-Initiated DNAzyme Motor for RNase H Activity Imaging in Living Cell

Namin Hu, Yafang Wang, Chang Liu, Manman He, Cunpeng Nie, Juan Zhang, Qiaoqin Yu, Chuan Zhao, Tingting Chen* and Xia Chu*

State Key Laboratory of Chemo/Bio-sensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, P. R. China

* Corresponding authors. E-mail: chenting1104@hnu.edu.cn; xiachu@hnu.edu.cn.
Tel:86-731-88821916; Fax: 86-731-88821916.

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Materials and Reagents. All oligonucleotides strands were purchased from Takara Biotechnology Co., Ltd. (Takara, China) and purified by high-performance liquid chromatography (HPLC), the sequences were listed in Table S1. Chloroauric acid ($HAuCl_4$•$4H_2O$), (3-Aminopropyl) triethoxysilane (APTES), bisbenzimide H 33342 trihydro chloride (Hoechast 33342), and tris (2-carboxyethyl) phosphine hydrochloride (TCEP•HCl) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), NaCl, $MnCl_2$, $MgCl_2$, NaH_2PO_4•$2H_2O$, Na_2HPO_4•$12H_2O$, bovine serum albumin (BSA), glycerol, dithiothreitol (DTT), tris (hydroxymethyl) aminomethane (Tris) and trisodium citrate ($C_6H_5Na_3O_7$•$2H_2O$) were supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). RNase H and RNase A were purchased from Takara Biotechnology Co., Ltd. (Takara, China). UDG, APE1were obtained from New England Biolabs (Ipswich, MA, USA). CCK-8 reagent was purchased from Dojindo Laboratories (Kumamoto, Japan). RNase H ELISA kit was purchased from Mlbio (Shanghai, China). All reagents were of analytical grade and without further purification. All cell lines were obtained from Xiangya Hospital (Changsha, China). All aqueous solutions were prepared using ultrapure water (≥18 MΩ, Milli-Q, Millipore).

Apparatus. The fluorescence spectra and the real-time fluorescence spectra were recorded by using a Hitachi FS5 fluorescence spectrometer from 500 to 600 nm under the excitation wavelength of 488 nm and the emission wavelength of 520 nm. The UV-vis absorption spectra were measured by an UV-2550 spectrometer (Shimadzu, Japan). The transmission electron microscopy (TEM) images were obtained by a JEM-2100 transmission electron microscope (JEOL, Japan). Zeta potential and dynamic light scattering (DLS) were detected by a Malvern Zetasizer Nano ZS90 (Malvern, USA). The cell viability was analyzied by a microplate reader (ELx800, BioTek). Fluorescence imaging of living cells was obtained by a Nikon confocal laser scanning fluorescence microscope (Nikon, Japan). The flow cytometry assay was evaluated by a CytoFLEX flow cytometry system (Beckman Coulter, USA). All experimental data were analyzed by origin 9.0.

Preparation of bare AuNPs. The gold nanoparticles were prepared by the reduction of trisodium citrate.[1] In brief, a chloroauric acid solution (47.67 mL, 0.3 mM) was heated to boiling and stirred constantly. The trisodium citrate (2.33 mL, 17 mM) was quickly added into the above solution, and heated for another 15 min. Then, the solution was further stirred until cool down to the room temperature and filtered by a 0.45 μm Millipore membrane filter. The prepared AuNPs (~1nm) was stored at 4 °C in the dark for future use.

Preparation of DNAzyme motor. First, thiolated anchord strand and substrate were treated with 1 mM TCEP•HCl at a molar ratio of 1:20 for 3 h at room temperature to cleave the disulfide bond. Then, 2.5 μL of 20 μM thiolated anchord strand was incubated with 2.5 μL of 20 μM locked-DNAzyme in 10 μL Tris-HCl buffer (25 mM Tris, 137 mM NaCl, pH 8.0, the final concentration of the double strands was 4.5 μM) at 37 °C for 2 h. After that, the prepared double strands and 25 μL of 20 μM substrate strand were added to 500 μL of 1 nM AuNPs solution and shaken in the dark for 16 h. The molar ratio of AuNPs, double strands and substrate strand in the solution was 1:100:1000. Then, 6.27 μL of 10% SDS solution was added to the mixture solution in order to improve the dispersion of AuNPs. The salt concentration of the above solution was gradually increased from 0.05 M to 0.3 M NaCl over an 8 h period by adding the 2 M NaCl four times. Finally, the solution was centrifuged at 13000 rpm for 30 min to remove the unconjugated oligonucleotides. Then, the above solution was washed twice times with Tris-HCl buffer (25 mM Tris, 50 mM NaCl, pH 8.0) and stored at 4 °C prior to use.

Determination of the strand loading on AuNPs. We used 20 mM DTT to determine the amount of substrate on each AuNP. Briefly, 10 μL of 2.3 nM DNAzyme motor was incubated with 50 μL of 40 mM DTT in the 100 μL Tris-HCl buffer and incubated overnight before measurement. The substrate loading amount was determined by comparing with the standard curve of FAM-labeled substrate.

Gel Electrophoresis. For gel electrophoresis of oligonucleotide strands, 10 μM locked-DNAzyme was incubated with different concentrations substrate strand (the molar ratio of locked-DNAzyme to substrate strand was 1:1 and 1:3) followed by

adding 200 U/mL RNase H and 0.75 mM $MnCl_2$ in 10 μL Tris-HCl buffer solution (40 mM Tris, 4 mM $MgCl_2$, 4% glycerol, 0.003% BSA, pH 7.7) at 37 °C for 2 h. Then, 2 μL of 6 × loading buffer was added to the above solution. The mixture was performed on 20% PAGE electrophoresis with 1 × TBE buffer at 150 V for 100 min.

For gel electrophoresis of functionalized AuNPs, 4.6 nM different functionalized AuNPs were incubated with 100 U/mL RNase H and 0.75 mM Mn^{2+} in 10 μL Tris-HCl buffer (40 mM Tris, 4 mM $MgCl_2$, 4% glycerol, 0.003% BSA, pH 7.7) at 37 °C for 2 h. Then, 2 μL of 60% glycerol was introduced into above mixture. The mixture was performed on 1% agarose electrophoresis with 1 × TBE buffer at 110 V for 60 min.

Fluorescence Experiments. In a typical experiment, 0.23 nM DNAzyme motor was incubated with different concentrations of RNase H and 0.75 mM Mn^{2+} in 100 μL Tris-HCl buffer (40 mM Tris, 4 mM $MgCl_2$, 4% glycerol, 0.003% BSA, pH 7.7) for 2 h at 37 °C before measurement. The real-time fluorescence detection was measured every 10 min for 2.5 h.

Total Internal Reflection Fluorescence (TIRF) Imaging and Analysis. The cleaned confocal dish was incubated with a solution of APTES (5%, v/v) for aminosilanization to promoted the deposition of AuNPs on the dish surface. Then, amino-coated confocal dish was washed with ultrapure water for three times. Then, 2.3 nM DNAzyme motor (or control DNAzyme motor) was incubated with 50 U/mL RNase H and 0.75 mM Mn^{2+} in 10 μL Tris-HCl buffer at 37 °C for 2 h and deposited on the confocal dish for 10 min. All images were obtained using an 100 × oil dipping objective with 488-nm laser by a TIRF microscopy (Nikon) .

Cell culture. All cells were cultured in DMEM medium with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 U/ml) and maintained in humidified atmosphere including 5% CO_2 at 37 °C.

Confocal Fluorescence Imaging. The cells were seeded on a 35 mm confocal dish with a 10 mm well and maintained in 2 mL DMEM medium at 37 °C for 24 h. Then, the cells were incubated with 0.3 nM DNAzyme motor and 0.5 mM Mn^{2+} in 1 mL of culture medium at 37 °C for 8 h, and washed three times with cold PBS followed by

treating with 1 mL of 10 μg/mL Hoechast 33342 before measurement. All fluorescence images were obtained using an 100 × oil dipping objective on a Nikon confocal laser scanning fluorescence microscope.

CCK-8 Assay. Hela cells (1×10^4) were cultured in 200 μL of DMEM medium in a 96-well plate at 37 °C for 24 h. Then, the cells were incubated with different concentrations of DNAzyme motor (0.3 nM, 0.5 nM, 1 nM) in 100 μL of culture medium at 37 °C for different times. After washed three times by cold PBS, the cells were treated with 10 μL CCK-8 reagent in 100 μL of culture medium for another 3 h. The absorbance was measured by an ELx800™ microplate reader at 450 nm.

Flow Cytometric Assay. The cells (1×10^5) were treated with 0.3 nM DNAzyme motor and 0.5 mM Mn^{2+} in 500 μL DMEM medium under the given conditions. After 8 h incubation, the cells were treated with 50 μL of 0.25% trypsin for 3 min and centrifuged at 2000 rpm for 3 min followed by washing twice times with cold PBS. Finally, the cells were resuspended in 500 μL cold PBS for flow cytometry assay.

Inductively coupled plasma mass spectrometry (ICP-MS) detection. The cells (1×10^5) were seeded on a 35 mm culture plates in 2 mL DMEM medium at 37 °C for 24 h. Then, the cells were incubated with 0.3 nM DNAzyme motor in 1 mL culture medium at 37 °C for 8 h, and washed five times by 700 μL cold PBS. The all washing solution (4.5 mL in total) were collected and diluted with ultrapure water for 2000 times for ICP-MS detection.

Quantification of RNase H in Cell-Free Extracts. The cells (5×10^6) were digested by 0.25% trypsin for 3 min and centrifuged at 2000 rpm for 3 min. Then, the cells were washed three times with 10 mL of cold PBS followed by resuspended in 500 μL ice-cold cell lysis buffer on ice for 5 min. After that, the cells were pulse-sonicated on ice 5 times for 5 s each. The extracts were centrifuged at 15,000g for 20 min at 4 °C and the supernatants were collected. The quantification of RNase H in cell-free extracts was performed by a Human RNase H ELISA kit according to the manufacturer's instructions.

Table S1. The sequence of all DNA or DNA-RNA chimeric strands in this work.

Name	Sequence (5′-3′)
substrate strand	HS-$(T)_{14}$CACTATrAGGAAGAGAT-6-FAM
H1	ACCAGCTCAGTGTGATTTTTTTTTTTTTTTTTCTCTTCTC CGAGCCGGTCGAAATAGTTTTTT(ACUAUUUCGACC)r
anchored strands (44T)	TCACACTGAGCTGGTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTT-HS
anchored strands (40T)	TCACACTGAGCTGGTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTT-HS
active DNAzyme	ACCAGCTCAGTGTGATTTTTTTTTTTTTTTTTCTCTTCTC CGAGCCGGTCGAAATAGTTTTTT
locked-DNAzyme	ACCAGCTCAGTGTGATTTTTTTTTTTTTTTTTCTCTTCTC CGAGCCGGTCGAAATAGTTTTTT(ACUAUUUCGACCG G)r
control locked-DNAzyme	ACCAGCTCAGTGTGATTTTTTTTTTTTTTTTTCTCTTCTC CGAGCCGGTCGAAATAGTTTTTTACTATTTCGACCGG

Table S2. Comparison of our analytical method with other reported methods for the detection of RNase H.

Strategy	Detection Modes	Detection limit	Reference
DNA tetrahedron	Fluorescence	3.41 U/mL	S2
Tb^{3+}-induced G-quadruplex conjugates	Fluorescence	2 U/mL	S3
Gold nanoparticle	Colorimetric	27 U/mL	S4
Light-Switching Excimer Beacon	Fluorescence	5 U/mL	S5
Gold nanoparticle	Fluorescence	0.023 U/mL	This work

References

S1 M. A. Vetten, N. Tlotleng, D. T. Rascher, A. Skepu, F. K. Keter, K. Boodhia, L.-A. Koekemoer, C. Andraos, R. Tshikhudo and M. Gulumian, Part. Fibre Toxicol., 2013, **10**, 50.

S2 K. Zhang, W. Huang, Y. Huang, H. Li, K. Wang, X. Zhu and M. Xie, Anal. Chem., 2019, **91**, 7086−7096.

S3 K. Wu, C. Ma, H. Liu, H. He, W. Zeng and K. Wang, Anal. Methods., 2017, **9**, 3055−3060.

S4 X. Xie, W. Xu, T. Li and X. Liu, Small, 2011, **7**, 1393−1396.

S5 Y. Chen, C. J. Yang, Y. Wu, P. Conlon, Y. Kim, H. Lin and W. Tan, ChemBioChem., 2008, **9**, 355−359.

Fig. S1 Characterization of bare AuNPs and the DNAzyme motor. TEM images of (A) bare AuNPs and (B) the DNAzyme motor. (C) UV-vis absorption spectra of bare AuNPs (red) and the DNAzyme motor (black). (D) Zeta potential. Dynamic light scattering (DLS) measurements of (E) bare AuNPs and (F) the DNAzyme motor.

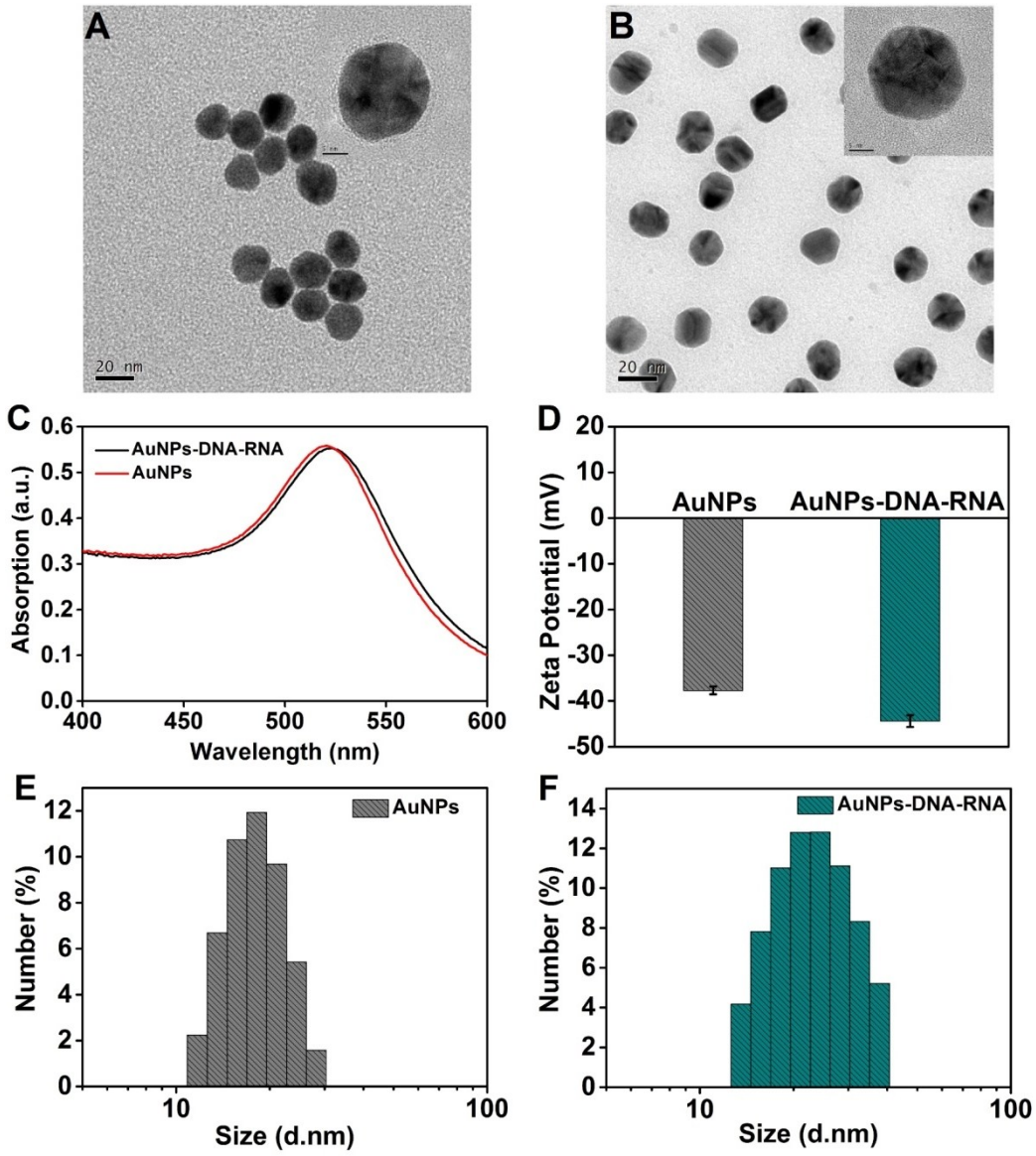


Fig. S2 Polyacrylamide gel electrophoresis images of the locked-DNAzyme and its substrate after different treatment.

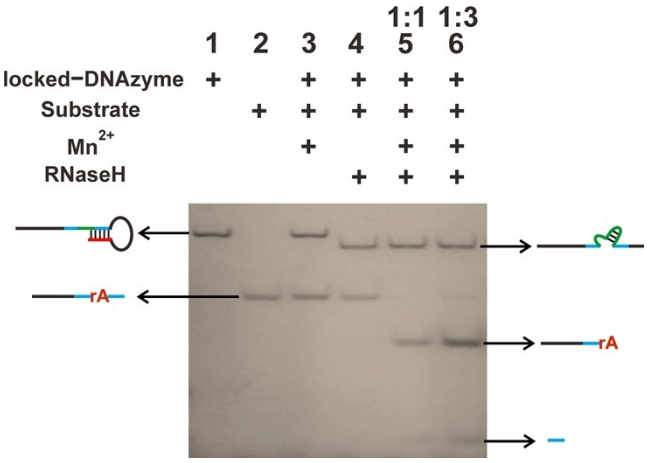
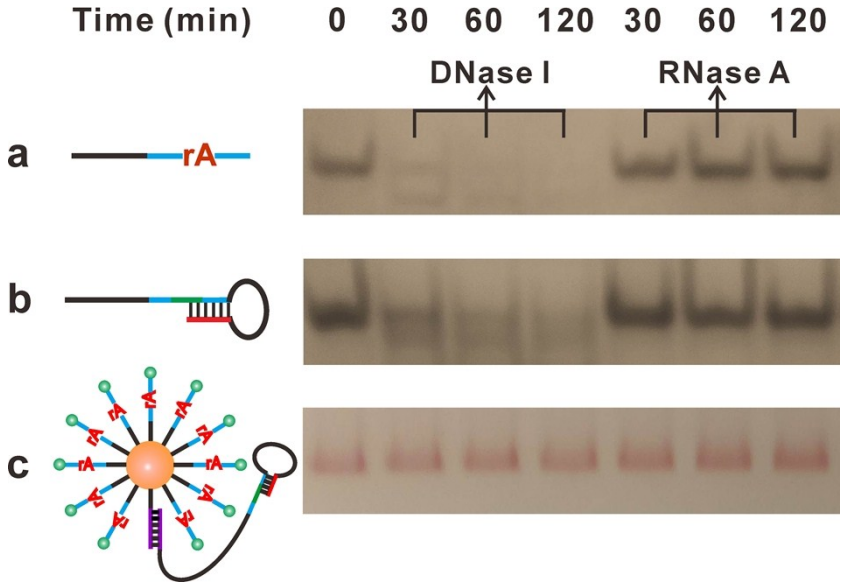


Fig. S3 The stability experiment of different probes against DNase I and RNase A. (a) the substrate, (b) locked-DNAzyme and (c) DNAzyme motor.



In the experiment, nanoprobe (4.6 nM, the concentration of substrate strand on the AuNP is 809.6 nM), substrate strand (1 μM) and the locked-DNAzyme strand (1 μM) were incubated with 2.5 U/mL DNase I or RNase A for different time periods, respectively. The results showed that the bands of the nanoprobe were still clear after incubation with DNase I for different time periods, while the bands of the substrate strand and the locked-DNAzyme strand were blurred and even disappeared when incubated with the same concentration of the DNase I. This was because that DNase I could hydrolyze single or double stranded DNA while the nanoparticle could protect nucleic acid probes against the degradation of the nuclease. These data suggested that the stability of nanoprobe was better than that of the free DNA strand. The results that treated with RNase A showed that the bands of the nanoprobe, substrate strand and the locked-DNAzyme strand were still clear after incubation with RNase A for different time periods. This was because that the RNase A could only hydrolyze single RNA strand. These data implied that our system had good selectivity and reliability.

Fig. S4 Real-time fluorescence monitoring the operation of the motor under the different conditions.

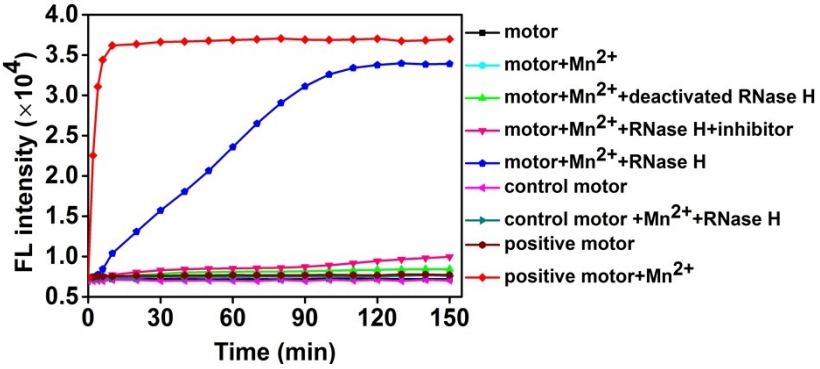


Fig. S5 TIRF images of different motor. (A) (a) DNAzyme motor; (b) DNAzyme motor incubated with Mn^{2+} and RNase H; (c) control DNAzyme motor; (d) control DNAzyme motor incubated with Mn^{2+} and RNase H. Scale bar: 5 μm. (B) Real-time images of the DNAzyme motor incubated with Mn^{2+} and RNase H.

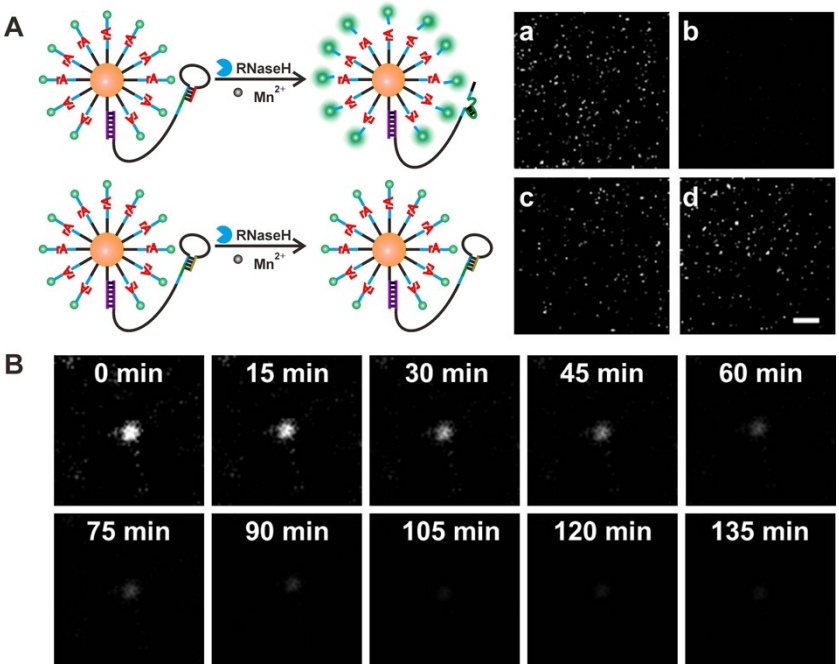


Fig. S6 Polyacrylamide gel electrophoresis images of different locked-DNAzyme and its substrate after different treatment.

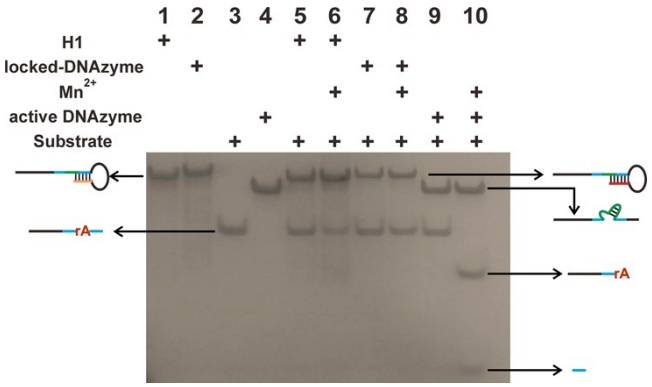


Fig. S7 The optimization of the length of thymines part in anchored strands. Error bars were estimated from three replicate measurements.

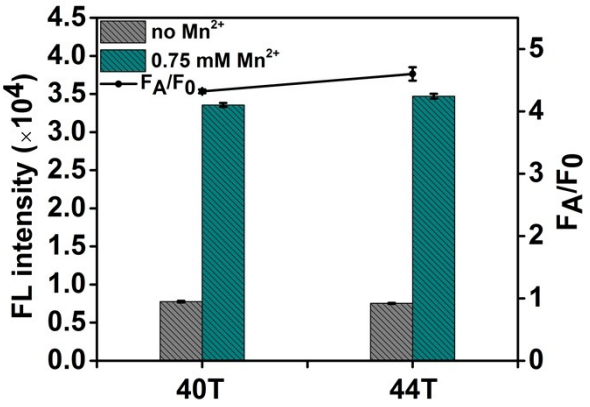


Fig. S8 Optimization of experimental conditions. (A) Fluorescence emission spectra of the DNAzyme motor against different Mn^{2+} concentrations. (B) The optimization of different molar ratio of locked-DNAzyme to substrate strands. Error bars were estimated from three replicate measurements.

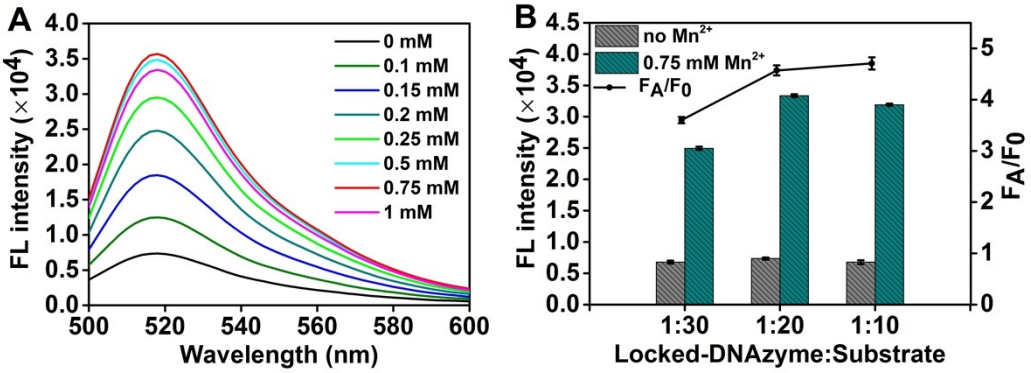


Fig. S9 Real-time fluorescence monitoring the operation of the DNAzyme motor responding to different concentrations of RNase H. Error bars were estimated from three replicate measurements.

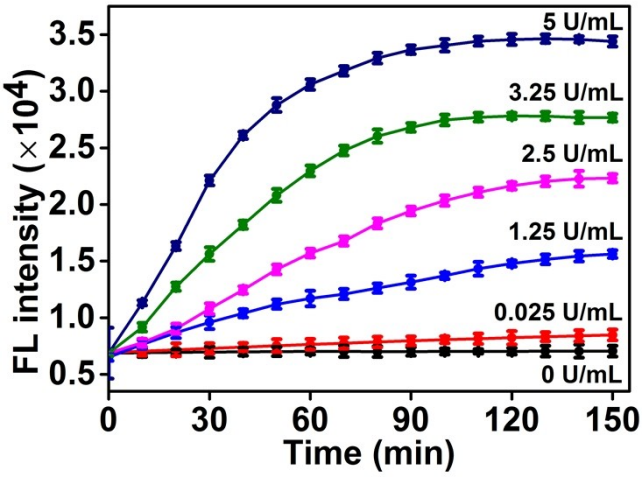


Fig. S10 The specificity of the DNAzyme motor in response to RNase H. Error bars were estimated from three replicate measurements.

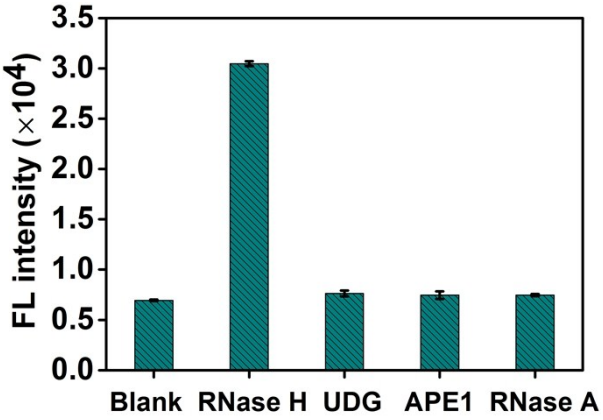


Fig. S11 CCK-8 assay. Hela cells incubated with different concentrations of DNAzyme motor for 6 h, 8 h, 12 h and 24 h, respectively. Error bars were estimated from three replicate measurements.

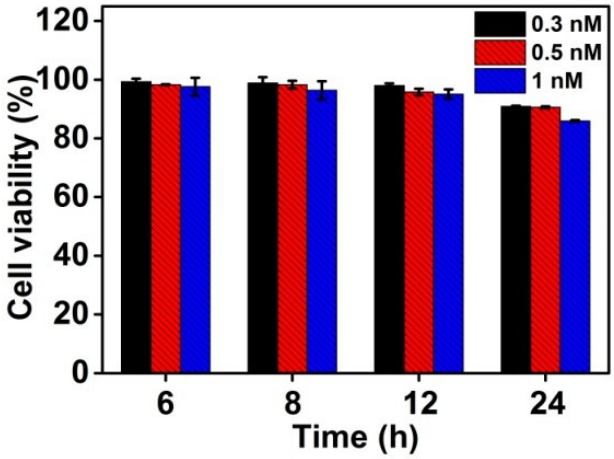


Fig. S12 Confocal imaging of the Hela cells after incubated with different motor and Mn^{2+}.

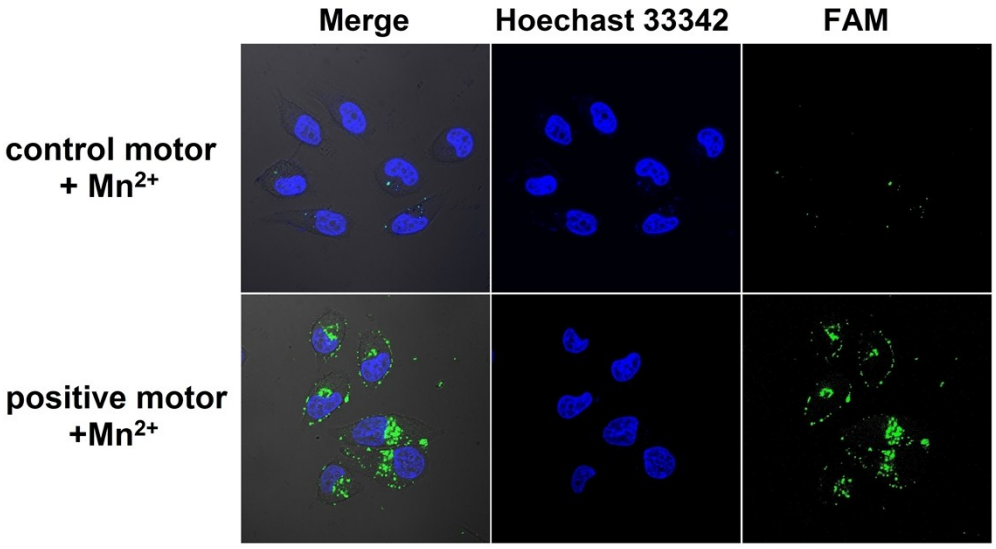


Fig. S13 Mean fluorescence intensity of figure 2 and the corresponding flow cytometric assays of Hela cells after different treatment. 1: motor, 2: motor and Mn^{2+}, 3: motor, Mn^{2+} and inhibitor, 4: control motor and Mn^{2+}, 5: positive motor and Mn^{2+}. Error bars were estimated from three replicate measurements.

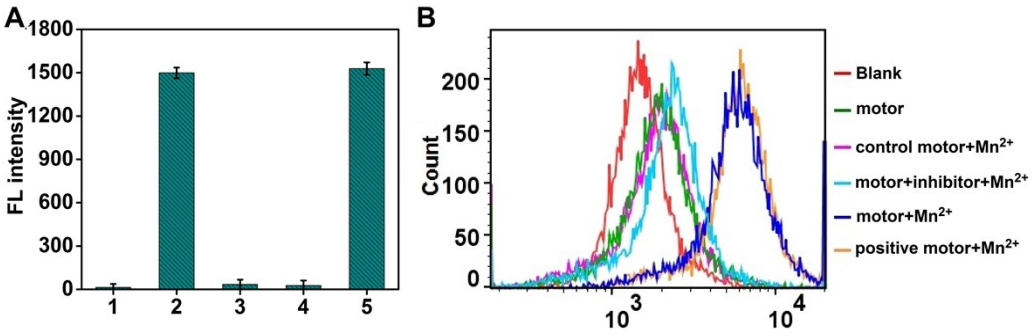


Fig. S14 Mean fluorescence intensity of figure 3 and the corresponding flow cytometric assays of different cells after incubated with DNAzyme motor and Mn^{2+}. Error bars were estimated from three replicate measurements.

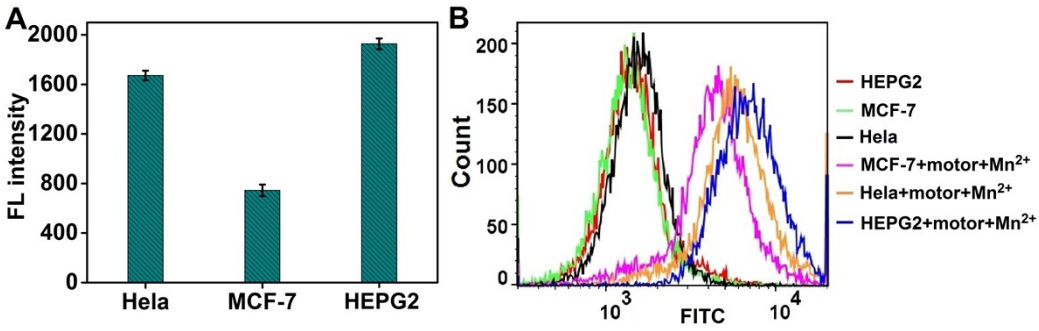


Fig. S15 The detection of RNase H levels in different cell lines by using ELISA kit. A) The standard curve of the RNase H; B) The RNase H levels in different cells. Error bars were estimated from three replicate measurements.

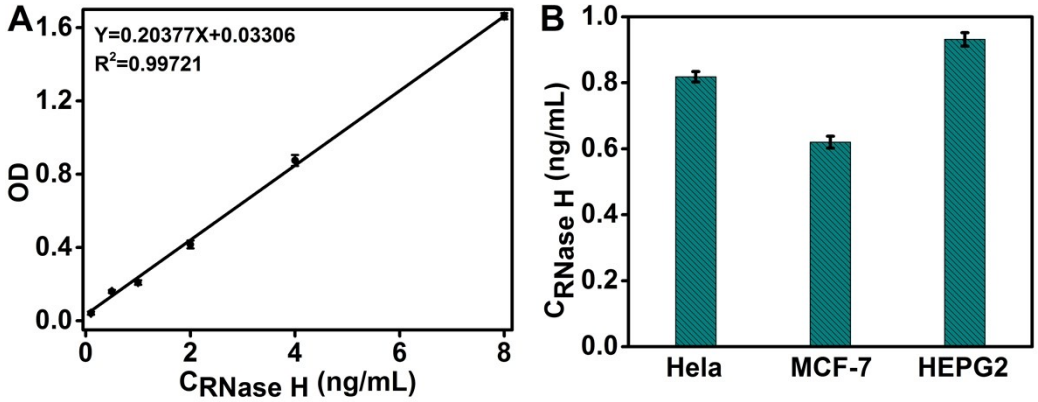


Fig. S16 Dark-field microscopy imaging (A) and ICP-MS detection (B) for different cells after incubated with DNAzyme motor. (a) Hela cells, (b) MCF-7 cells, (c) HEPG2 cells. Error bars were estimated from three replicate measurements.

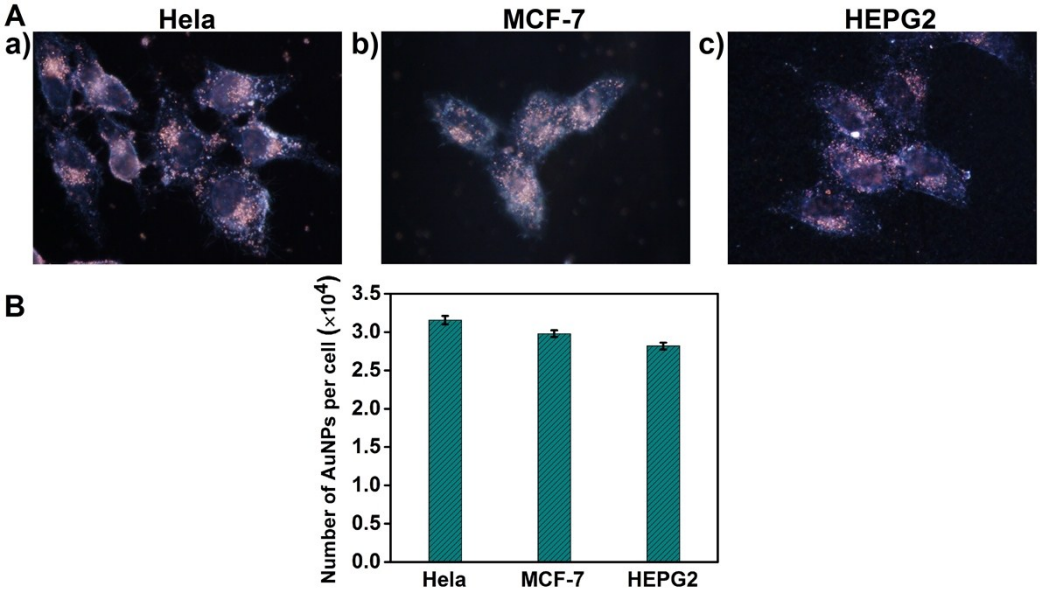


Fig. S17 The RNase H inhibitor screening in the living cells. (A) Confocal imaging of the Hela cells after incubated with motor, Mn^{2+} and other treatments. (B) The effect of different concentrations of antibiotics on RNase H activity.