Electronic Supplementary Information (ESI)

Proximity-induced DNA Ag NCs enhancement with DNA-fueled molecule machine for lung cancer-associated miRNA detection

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Experimental section

Reagents and instruments

Analytical grade silver nitrate ($AgNO_3$ ≥ 99.98%), sodium borohydride ($NaBH_4$ ≥ 99.98%), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), and magnesium acetate (Mg-$(C_2H_3O_2)_2$) were purchased from Sinopharm Chemical Reagent Company Ltd. (Shanghai, China). Ammonium persulfate (APS), 6×loading buffer, and N, N, N', N'-tetramethylethylene diamine (TEMED) were ordered from Beyotime Biotech Inc. (Shanghai, China). SYBRGold was obtained from Thermo Fisher Scientific Inc. 30% acrylamide-methylene bis(acrylamide) [30% Acr-Bis (29:1)] was purchased from Biosharp Life Sciences. Human serum was collected from the North Jiangsu People's Hospital. All protocols adhered to the ethical standards of the 1964 Declaration of Helsinki and were approved by the Medical Ethics Committee of North Jiangsu People's Hospital (approval number: 2024ky313). DNA strands used in this work were synthesized and purified by Sangon Biotech. Co., Ltd. (Shanghai, China). UNlQ-10 Column Trizol Total RNA Isolation Kit from Sangon Biotech. Co., Ltd. (Shanghai, China). Water-DEPC Treated Water from Sangon Biotech. Co., Ltd. (Shanghai, China). The detailed sequences of used

DNA in this work are listed in **Table S1**.

The fluorescence spectra were recorded using an Edinburgh FS-5 fluorophotometer, the silver nanoclusters were excited at λ = 580 nm. The transmission electron microscopy (TEM) images were taken on a transmission electron microscope (Tecnai 12). BGVerMINI was applied for native polyacrylamide gel electrophoresis (PAGE). Ultraviolet−visible (UV−vis) absorption spectra were collected by a UV-2550(Shimadzu, Japan).

Preparation of DNA AgNCs

First, the Ag strand was dissolved with ultrapure water to a final concentration of 100 μM. Then, DNA-Ag NCs were synthesized according to classic methods with a minor modification[1,2]. Briefly, 50 μL of Ag strands (10 μM), 12.5 μL of fresh $AgNO_3$ (480 μM), and 12.5 μL of PB buffer (200 mM, pH=6.6) were mixed and incubated at 0°C for 1 h. Next, 12.5 μL of fresh $NaBH_4$ (240 μM) dissolved in 0°C ultrapure water was quickly dropped into the above mixture (the molar ratio of DNA/Ag^+/$NaBH_4$ remained at 1:12:6). Finally, the resulting solution was vigorously shaken for 3 min and incubated in the dark at 4°C for 4 h to form the red-emitting DNA-Ag NCs.

Polyacrylamide gel electrophoresis experiment

L/H were incubated with T, F, or (T + F) at 25°C for 3 h in 20 mM PB (pH = 7.0). Subsequently, each sample was mixed with 6×loading buffer and 100×SYBR-Gold and then transferred into the prepared 15% polyacrylamide gel. The electrophoresis was run in 1×TBE buffer at 120 V for 2 h. Finally, the experimental results were processed and imaged with a gel imaging system.

Fluorescence measurements

In order to verify its feasibility, the probes (150 nM) were incubated in a 20 mM PB buffer with or without miRNA let-7a-5p (pH=7.0) at 25°C for 3 h, and the resulting product was added to the DNA-Ag NCs (150 nM) for 90 min. For sensitivity analysis, DNA-fueled molecule machine was treated with different concentrations of miRNA let-7a-5p (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 30, 50, and 100 nM), and then, the reactants were added to the Ag NCs at 25°C for 90 min. For selectivity analysis, the same concentration of miRNA let-7 family (including let-7a-5p, let-7e, let-7g, let-7i) and miRNA-21 were added to the DNA-fueled molecule machine at 25°C for 3 h, and then the reactants were added to Ag NCs for 90 minutes at 25°C. Finally, all the fluorescence was measured by FS-5 fluorescence spectrophotometry, and the fluorescence spectra were recorded at 610-750 nm with an excitation wavelength of 580 nm.

Detection of miRNA let-7a-5p in Human Serum Samples

0.1% human serum was diluted with PB (pH = 7.0, 20 mM), and then different concentrations (0, 0.01, 0.1, 0.5, 1 nM) of miRNA let-7a-5p were added to 0.1% human serum. The obtained samples were analyzed using the proposed sensing platform. Subsequently, miRNA let-7a-5p levels is measured in the serum samples from lung cancer patients and healthy volunteers. Human serum RNA was extracted from six lung cancer patients and six healthy volunteers with RNA isolation kit. The extracted RNA was added to DNA-fueled molecule machine at 25°C for 3 h, and then added to Ag NCs for 90 min. The method was according to a previous report with a slight modification[3].

Quantitative real-time polymerase chain reaction (RT-qPCR)

The concentration of the extracted RNA was then measured using agarose gel electrophoresis. cDNA was synthesized using the reverse transcription reaction program of the miRNA cDNA synthesis kit (Thermo Scientific™ EP0733) according to the manufacturer's instructions. The resulting cDNA sample was diluted 10-fold and quantified using qPCR. The polymerase chain reaction (PCR) instrument was ABI Stepone plus. qPCR detection was performed using a SG Fast qPCR Master Mix (Sangon Biotech. Co., Ltd.). The reaction conditions were as follows: 95°C for 30 seconds; 95°C for 10 seconds interval; 60°C for 30 seconds interval, 45 cycles. The specific PCR primers of miRNA-let-7a-5p and U-6 were purchased by Sangon Biotechnology Co., Ltd. (Shanghai, China). All reactions were performed in triplicate, and U6 was used as the control. The data were normalized with U6 and the relative expression of miRNA-let-7a-5p was determined by the $2^{-\Delta\Delta Ct}$ method. The GraphPad Prism 8 software was applied to analyze the RT-qPCR results. The paired t-test was applied to compare the miRNA-let-7a-5p expression level between case and control.

Table S1. All oligonucleotide sequences used in experiment section are listed as following (from 5' to 3').

L	**AACTAT**ACAACCTACTACCTCA**CTGACAT**GACTCCTG
H	ATGTCAGTGAGGTAGTAGGTTGTCACTGACAT
F	GCTGTGCATCTATGTCAGTGAGGTAGTAGGTTGT
T (let-7a-5p)	TGAGGTAGTAGGTTGTATAGTT
Ag	GACTCCTGCCCCCCCAGGAGTCAGATGCACAGCTA
Let-7e	TGAGGTAGGAGGTTGTATAGTT
Let-7g	TGAGGTAGTAGTTTGTACAGTT
Let-7i	TGAGGTAGTAGTTTGTGCTGTT
miRNA-21	TAGCTTATCAGACTGATGTTGA

Note: The bold font of the underline represents the toehold to recognize with a target (T) and fuel DNA (F). The red font denotes mismatched-base.

Supporting Figures：

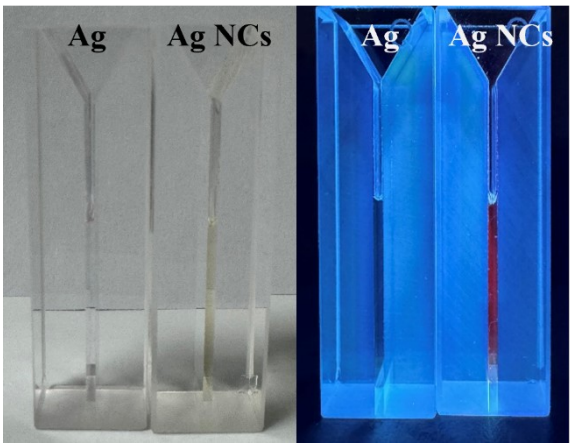

Fig. S1 The photograph of DNA-Ag strand and DNA-templated Ag NCs irradiated sunlight and UV lamp. Hairpin-templated DNA-Ag NCs show a light yellow under natural light, whereas a strong red emission can be seen under ultraviolet irradiation. The concentration of the DNA-Ag NCs and DNA-Ag are 5 μM.

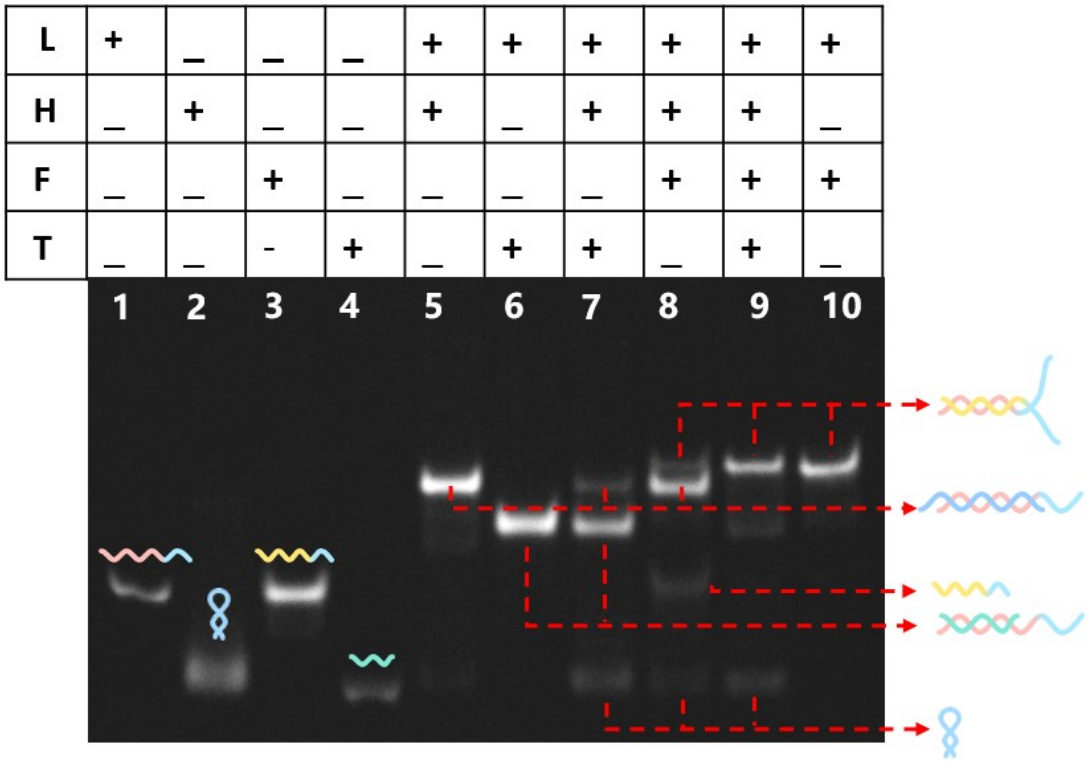

Fig. S2. PAGE analysis of the mechanism of strand displacement reaction at each step. Firstly, the complex of L/H (1:1.5), L/T (1:1) and L/F (1:1) was heated to 95 ◦C for 5 min with PB buffer (20 mM, pH = 7), followed by cooling to room temperature to ensure the formation of stable complexes. The final concentration of the DNA in lanes 1-4 is 4 μM and the mixture concentration in lanes 5-10 is 1 μM, respectively. The bands from lane 1 to lane 4 denoted L, H, F and T, respectively. L/H complex existed in lane 5. Lane 6 and lane 10 represented L/T complex and L/F duplex, respectively, which acted as control bands. It was found the same band appears in lane 7 as lane 6, which indicates that T could replace H to generate L/T complex. When F was applied to the L/H reaction system, there were a one bright and three weak bands, and these bands from top to bottom were represent L/F duplex, L/H duplex, F, H, respectively, indicating that F could not replace H. At the same time, when T and F were simultaneously added to the L/H reaction system, a reactant L/H band almost completely disappeared and a new band corresponding to the L/F duplex (lane 10) was produced in lane 9. Notably, only when T and F were added simultaneously to the L/H complex, a bright L/F band would appear. It was evident that DNA-fueled molecule machine could gradually work as expected. L/H were incubated with T, F, or (T + F) at 25°C for 3 h in 20 mM PB (pH = 7.0).

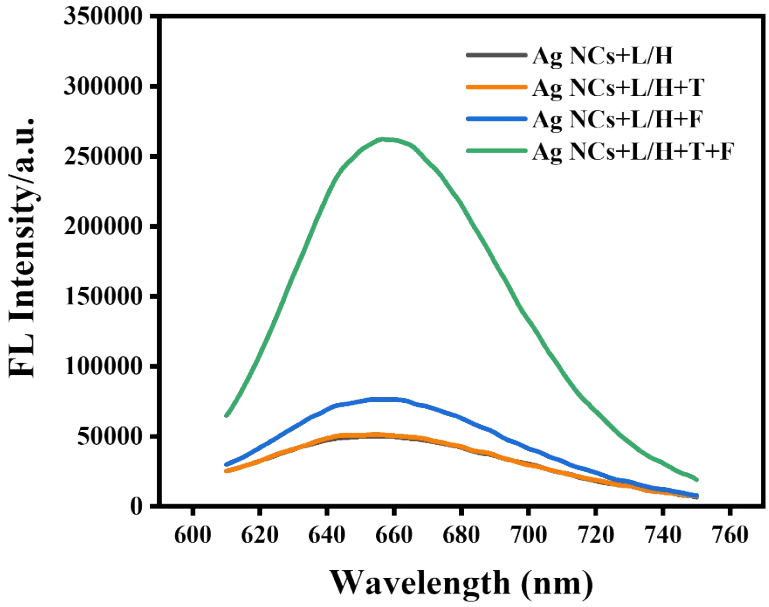

Fig. S3. Fluorescence feasibility analysis of proximity-induced Ag NCs enhancement system in response to let-7a-5p. 200 nM L/H was incubated with 50 nM T and 300 nM F in 20 mM PB at 25°C for 3 h under different experimental conditions, and then treated with Ag NCs for 90 min. The fluorescence spectra were recorded at 610 nm-750 nm with the excitation wavelength at 580 nm.

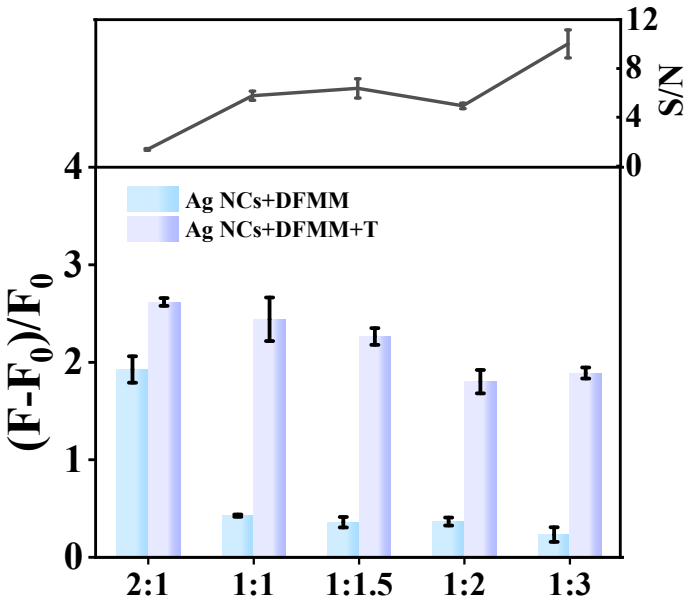

Fig. S4. The fluorescence intensity changes of DFMM with the different ratio (2:1, 1:1, 1:1.5, 1:2, 1:3) of L to H strands in the absence of T and in the presence of T. The DFMM was incubated with 50 nM T at 25°C for 3 h in PB (pH=7.0), and the products were treated with Ag NCs at 25°C for 90 min in PB (pH=7.0). F_0 denote the fluorescence of 150 nM Ag NCs treated with 200 nM L/H, and F denote the fluorescence of 150 nM Ag NCs treated with the DFMM in the absence of T and in the presence of T. F_0 denote the fluorescence of 150 nM Ag NCs treated with 200 nM L/H, and F denote the fluorescence of 150 nM Ag NCs treated with the DFMM in the absence of T and in the presence of T. The error bars denoted the standard deviation of three parallel measurements. Error bars represented three independent repeated experiments.

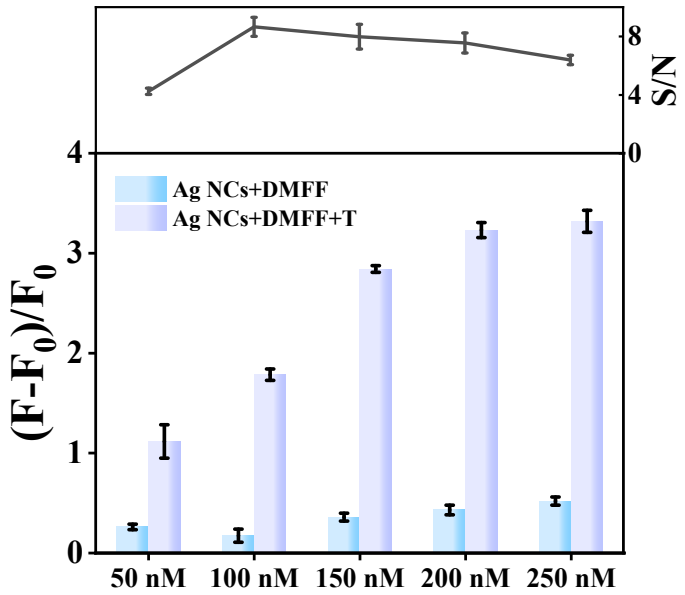

Fig. S5. The fluorescence intensity changes of DFMM with different concentration (50, 100, 150, 200, 250 nM) of L/H in the presence of T and the absence of T at 25°C in 20 mM PB (pH=7.0) for 3 h, and the products were treated with 150 nM Ag NCs at 25°C for 90 min in PB (pH=7.0). F_0 denote the fluorescence of 150 nM Ag NCs treated with L/H, and F denote the fluorescence of 150 nM Ag NCs treated with the DFMM in the absence of T and in the presence of T. The error bars represented three independent repeated experiments.

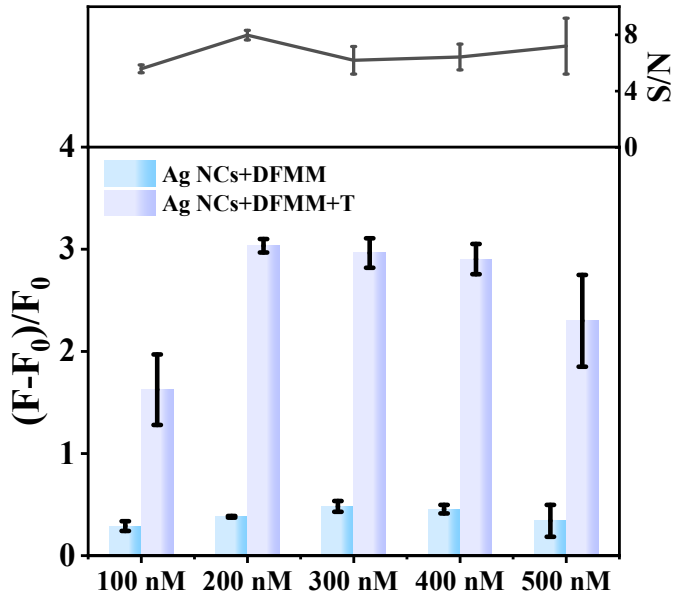

Fig. S6 The fluorescence intensity changes of DFMM with different concentration (100, 200, 300, 400, 500 nM) of F in the presence of T and the absence of T at 25°C in 20 mM PB (pH=7.0) for 3 h, and the products were treated with 150 nM Ag NCs at 25°C for 90 min in PB (pH=7.0). F_0 denote the fluorescence of 150 nM Ag NCs treated with 200 nM L/H, and F denote the fluorescence of 150 nM Ag NCs treated with the DFMM in the absence of T and in the presence of T. The error bars represented three independent repeated experiments.

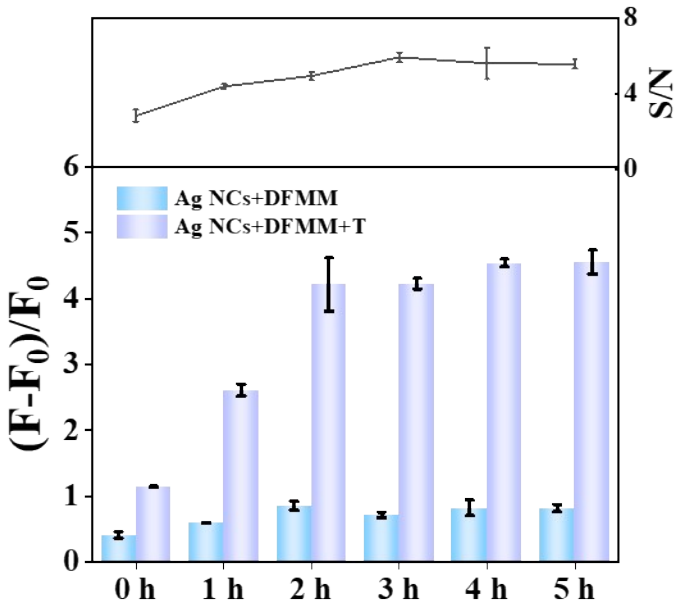

Fig. S7 Optimizing of DFMM reaction time. DFMM was treated with 50 nM T and without T at 25oC in 20 m M PB (pH=7.0) at different times, and the products were treated with 150 nM Ag NCs at 25°C for 90 min in PB (pH=7.0). F_0 denote the fluorescence of 150 nM Ag NCs treated with 200 nM L/H, and F denote the fluorescence of 150 nM Ag NCs treated with the DFMM in the absence of T and in the presence of T. The error bars represented three independent repeated experiments.

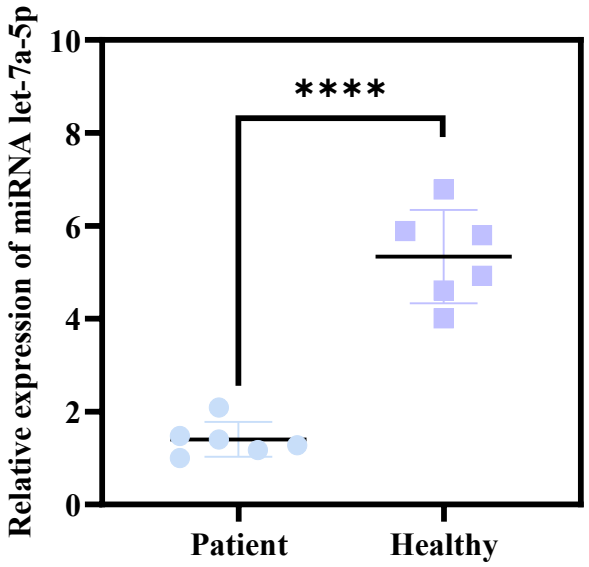

Fig. S8 The relative expression level of miRNA let-7a-5p in serum samples from healthy controls (n=6) and lung cancer patients (n=6) using RT-qPCR (****P＜0.0001). The data is analyzed by $2^{-\Delta\Delta Ct}$ method.

Table S2. Comparison of our proposed DFMM with other DNA-Ag NCs-based biosensors for miRNA detection.

Strategy	Limit of Detection	Linear Range	References
CHA, NCB	38 pM	0.75-15 nM	4
FRET	6.9 nM	12-300 nM	5
PET	0.06 nM	0.1 nM-8 μM	6
HCR	0.1 nM	0.1-1.6 nM	7
G-quadruplex	67 pM	0.25-1000 nM	8
EDC	0.4 pM	1-1000 pM	This work

Table S3. Detection of miRNA let-7a-5p in human serum samples (n=3).

Samples	Added (pM)	Detected (pM)	Recovery(%)	RSD(%)
1	10	10.82	108.20	1.53
2	100	105.94	105.94	3.11
3	500	484.05	96.81	2.26
4	1000	985.78	98.58	1.71

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