Supplementary Information (SI) for Journal of Materials Chemistry B.

DNA nanowire based-DNAzyme walker for amplified imaging of microRNA in tumor cells

Haoqi Yang, Ziyong Wu, Shujuan Sun*, Shusheng Zhang and Pengfei Shi*

Shandong Provincial Key Laboratory of Detection Technology for Tumor Markers, College of Chemistry and Chemical Engineering, Linyi University, Linyi 276000, PR China

Email: sjswmu@sina.com; shipengfei913@163.com

Table of Contents

4. Section D: References

1. Section A: Experimental Procedures

SA.1 Assembly of DNA nanowire based-DNAzyme Walker (D-Walker)

Preparation of D-MB

Locked-DNAzyme was prepared by adding 2 μL of Lock-d (10 μM) and 2 μL of DNAzyme (10 μM) into 6 μL of TAMg buffer (40 mM Tris, 7.6 mM $MgCl_2 \cdot 6H_2O$, pH 7.40). The solution was annealed at 90 °C for 5 min and then cooled down to room temperature. Molecular beacon (MB) was obtained by mixing F-S (2 μL ,10 μM), D-S (2 μL ,10 μM) and 6 μL of TAMg buffer following the same procedure as above. Then, the two solutions (locked-DNAzyme and MB) were mixed for 2 h at room temperature, resulting in DNAzyme based-molecular beacon (D-MB) solution (20 μL, 1 μM).

Preparation of NWs

For the self-assembly of NWs, equal volumes of S1 and S2 (1 μL, 10 μM each) were mixed with 6 μL of TAMg buffer. The mixture was annealed at 90°C for 5 min and then slowly cooled to room temperature to obtain Unit-1 (U-1). Unit-2 (U-2) was assembled from S3 and S4 following the same procedure. Then, U-1 and U-2 solutions were mixed and incubated at room temperature for 1 h to obtain the NWs solution (16 μL).

Preparation of D-Walker

For Figure 1 and Figure S4, D-Walker was assembled by mixing the previously prepared D-MB solution (20 μL, 1 μM) with NWs solution (16 μL), then, 2 μL each of Apt and Linker (10 μM) were added to the mixture. After thoroughly mixing, it was incubated at room temperature for 2 h to obtain the D-Walker solution (40 μL, 0.5 μM). The concentration of D-Walker was defined as the concentration of F-S.

For other experiments, the pre-prepared D-MB and MB were mixed at a molar concentration of 1:4 and then assembled with NW to obtain D-Walker. Specifically, D-MB solution (4 μL, 1 μM) was mixed with MB solution (8 μL, 2 μM), then NWs solution (16 μL), Apt (2 μL, 10 μM), Linker (2 μL, 10 μM) and 8 μL of TAMg were added to the mixture, after thoroughly mixing, it was incubated at room temperature for 2 h to obtain the D-Walker solution (40 μL, 0.5 μM).

SA.2 Electrophoretic mobility analysis

15% native polyacrylamide gel electrophoresis (nPAGE) and 10% denatured polyacrylamide gel electrophoresis (dPAGE) were performed at a constant voltage of 80 V, and 0.5×TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) was used as working buffer. For nPAGE analysis, 8 μL of reaction solution was mixed with 2 μL of 6× loading buffer, and 2 μL of SYBR Green I was used for staining DNA. For dPAGE analysis, 5 μL of the above reaction mixture was mixed with 5 μL of 2× loading buffer, and heaten at 95℃ for 7 min, followed by cooling on ice for 2 min. Finally, the resulting samples were performed 10% dPAGE analysis. The gel images were obtained by chemiDox XRS Imaging system (Bio-RAD, U.S.A.).

The sample preparation method for Figure 1C is as follows:

For Lane 1-5 and Lane 11: 1 μL of the corresponding DNA sequence was added to TAMg buffer to total volume of 20 μL each. After thoroughly mixing, the solution was annealed at 90°C for 5 min and then gradually cooled to room temperature.

For Lane 6: Equal amount (1 μL) of F-S and D-S strand were mixed together. The resulting solution was supplemented to 19 μL with TAMg buffer and annealed at 90 ℃ for 5 min. After cooling gradually down to room temperature, 1 μL of Linker was mixed and incubated at room temperature for 2 h.

For Lane 7 and Lane 9: The same sample was prepared according to the procedure adopted in Lane 6, but the volume of TAMg was supplemented to 18 μL. Then, 1 μL of S2/S4 was added and mixed thoroughly. The resulting solution was incubated at room temperature for 2 h.

For Lane 8 and Lane 10: Equal amount (1 μL) of F-S and D-S strand were mixed together. The resulting solution was supplemented to 9 μL with TAMg buffer and annealed at 90 ℃ for 5 min. Lock-d and DNAzyme following the same procedure. Mixed the obtained two parts of the solution, the D-MB solution was obtained. Then 1 μL of S2/S4 and 1 μL of Linker were added. After thoroughly mixing, the resulting solution was incubated at room temperature for 2 h.

For Lane 12: The same sample was prepared according to the procedure adopted in Lane 8, but the volume of TAMg was supplemented to 8 μL and the volume of Linker

was 2 μL. Then, 1 μL of S4 was added and mixed thoroughly. The resulting solution was incubated at room temperature for 2 h.

For Lane 13: The same sample was prepared according to the procedure adopted in Lane 12, but the volume of TAMg was supplemented to 7 μL. Then, 1 μL of S1 and S3 and 2 μL of Apt were added and mixed thoroughly. The resulting solution was incubated at room temperature for 2 h.

The preparation method of samples for Figure 1D was follows:

D-Walker was prepared according to the section of "**SA.1 Assembly of DNA nanowire based-DNAzyme Walker (D-Walker)**".

For Lane 1: 33.5 μL of pre-prepared D-Walker (0.5 μM) was mixed with 6.5 μL of ddH_2O to obtain the sample.

For Lane 2: 33.5 μL of pre-prepared D-Walker (0.5 μM) and 2.5 μL of D-miR-21 were mixed thoroughly, then 4 μL of ddH_2O was added, the resulting solution was incubated at room temperature for 10 min.

For Lane 3: 33.5 μL of pre-prepared D-Walker (0.5 μM) and 4 μL of NaCl (5 M) were mixed thoroughly, then 2.5 μL of ddH_2O was added, the resulting solution was incubated at room temperature for 10 min.

For Lane 4: 33.5 μL of pre-prepared D-Walker (0.5 μM), 2.5 μL of D-miR-21 and 4 μL of NaCl (5 M) were mixed thoroughly. The resulting solution was incubated at room temperature for 10 min.

SA.3 Anti-degradation assay

D-MB and D-Walker (35 μL, 0.5 μM) were prepared according to the section of "**SA.1 Assembly of DNA nanowire based-DNAzyme Walker (D-Walker)**". Respectively, with the difference being the use of short chains instead of D-S. 4 μL of the above samples were incubated with DNase I (4 μL, 2 U/mL) respectively at 37°C for different time (0, 30, 60, 90 and 120 min). Subsequently, 0.9 μL of EDTA (25 mM) was added, and the resulting mixtures were incubated at 65°C for 10 min for stopping the enzymatic degradation.

SA.4 Fluorescence detection

To validate the feasibility of D-Walker to detect target, pre-prepared D-Walker (40 μL, 0.5 μM) was mixed with 10 μL of NaCl (5 M) and 149 μL of TAMg, after adding the target D-miR-21 (1 μL, 10 μM), real-time fluorescence detection was immediately conducted at room temperature for 12 min. For the blank group, 1 μL of ddH_2O was used instead of D-miR-21, and other steps were same. For the group of D-MB and its blank, the experimental methods were the same as above.

Fluorescence detection was performed on the F-4600 fluorescence spectrophotometer (Hitachi, Japan), with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Both excitation and emission slits were set to 5 nm, and the detector voltage was set to 800 V.

To investigate the quantitative analysis capability of D-Walker for the target, a series of target D-miR-21 solutions at different concentrations (0.01 μM, 0.02 μM, 0.04 μM, 0.1 μM, 0.2 μM, 1 μM, 2 μM, 3 μM, 4 μM) were prepared. Each concentration of the target D-miR-21 (1 μL) was sequentially mixed with the previously prepared D-Walker (40 μL, 0.5 μM), NaCl (10 μL, 5 M), and 149 μL of TAMg. After incubating at 37°C for 4 h, real-time fluorescence detection was performed. For the blank group, 1 μL of ddH_2O was used instead of D-miR-21, and other steps were same.

SA.5 Confocal fluorescence imaging

In order to evaluate the imaging effect of D-Walker on miRNA-21 in target cells, HeLa cells were used in confocal fluorescence imaging experiments. HeLa cells were cultured in complete DMEM medium (DMEM supplemented with 10% FBS and 1% PS solution) for 24 h. When the cells reached approximately 80% confluence in confocal dishes (35 mm), they were washed three times with 500 μL PBS. The pre-prepared D-Walker (50 μL, 0.5 μM) was mixed with 450 μL of DMEM, then the resulting solution was used for incubating with HeLa cells in a humidified incubator at 37°C at 5% CO_2 for 4 h. After removing the residual D-Walker, the cells were washed three times with 500 μL PBS and fixed with 4% paraformaldehyde for 15 min. The cells were washed again three times with 500 μL PBS and then stained with Hoechst 33342 (500 μL, 10 μg/mL) at 37°C for 15 min to stain the nuclei. Finally, before

confocal fluorescence imaging, the cells were washed with 500 μL PBS to remove the staining solution. For D-MB group, D-MB (50 μL, 0.5 μM) was used instead of D-Walker, and other procedures were same as above. For R-Walker group, R-Walker was prepared as the methods in "Preparation of D-Walker" except that Random (2 μL, 10 μM) was instead of Apt. Other steps were same as the D-Walker group.

To evaluate the performance of D-Walker in identifying different expression levels of miRNA-21 in cells, MCF-7 cells were used in confocal fluorescence imaging experiments. MCF-7 cells were cultured in 500 μL of complete DMEM medium (DMEM supplemented with 10% FBS and 1% PS solution) with the addition of E_2 solution (0.5 μL, 10 μM) for 24 h. When the cells reached approximately 80% confluence in confocal dishes (35 mm), they were washed three times with 500 μL of PBS. D-Walker (50 μL, 0.5 μM) was prepared using Cy5-modified S1 (S1-Cy5) instead of S1. The above solution was mixed with 450 μL of DMEM and then incubated with HeLa cells in a humidified incubator at 37°C with 5% CO_2 for 4 h. Before performing confocal fluorescence imaging, the incubation mixtures were removed, and the cells were fixed and stained following the same procedure as described above. The difference in the control group was that the MCF-7 cells were not treated with the E_2 solution.

Confocal fluorescence imaging was recorded on A1+ CellManipulator Plus confocal microscope (Nikon, Japan). The Hoechst, FAM and Cy5 were excited using lasers with wavelengths of 405 nm, 488 nm, and 638 nm, respectively. All confocal fluorescence images were analyzed using NIS-Elements Viewer 5.21.

SA.6 Flow cytometry analysis

The required D-Walker, R-Walker, and D-MB were synthesized using the previously established assembly method. HeLa cells were inoculated into a 6-well plate and cultured for 24 h. Following this, they were incubated with the 1 mL of DMEM medium containing pre-prepared D-Walker (100 μL, 0.5 μM) or other counterparts in a humidified incubator at 37°C at 5% CO_2 for 4 h. Subsequently, the cells were digested using trypsin and washed with PBS (500 μL) until the suspension became colorless,

finally, the cells were dissolved in 500 μL of PBS and analyzed using flow cytometry (Cutoflex, Beckman Coulter).

SA.7 Quantitative reverse transcription-PCR (qRT-PCR) analysis

Total RNA was extracted from both E_2-treated MCF-7 cells and untreated MCF-7 cells using RNA isolater Total RNA Extraction Reagent Kit. Subsequently, the cDNAs were synthesized using the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) method. The resulting cDNA samples were then subjected to qPCR analysis using the miRNA Universal SYBR qPCR Master Mix kit on the Quant Studio 1 plus qPCR instrument (Thermo Fisher, USA). The PCR reaction program was as follows: an initial denaturation stage at 95°C for 5 min followed there were 40 cycles, each comprising a denaturation step at 95°C for 10 s, followed by an annealing/extension step at 60°C for 60 s, then 95°C for 15 s finally. The relative expression level of miRNA-21 was calculated by using the $2^{-\triangle\triangle Ct}$ method. The reagent kits used for qRT-PCR were all purchased from Vazyme Biotech Co., Ltd (Nanjing, China).

2. Section B: Supporting Tables

Table S1. DNA sequences designed in the work[a]

	Names	Sequences (from 5′ to 3′)
DNA nanowire	Strand-1 (S1)	GAAAGAAACAACCCTTGCGCATTCGAGTCTCCCTCATTTAGT TCTCATCCTTTCCATTCGAGTCTCC
	Strand-2 (S2)	TATTGTTTCTTGGTAGTCTTTAGCTGCAGTGTGAAAGGATGA GAACTAAATGAGTTAGCTGCAGTGT
	Strand-3 (S3)	GAAAGAAACAACCCTTGCGGGAGACTCGAATGTTACTATCCC ACATTCTCTTTCGGAGACTCGAATG
	Strand-4 (S4)	TATTGTTTCTTGGTAGTCTACACTGCAGCTAAGAAAGAGAAT GTGGGATAGTAAACACTGCAGCTAA
DNAzyme based-molecular beacon (D-MB)	DNAzyme	GCGGCGGTACCAGGTCAAAGGTGGGTGAGGGGACGCCAAGAG TCCCCGCGGTTAGATAGAGATTAGCTTATCAGACT
	Lock-d	TCAACATCAGTCTGATAAGCTAATCTCTAT
	FAM-Substrate (F-S)	**FAM**-CTGAGCAGTTTTTTTTCTACTCTATCTAT**/rA/**GGAAG TACCGCCGCTGCTTTTTTCTGCTCAGTACTCTCTTAACTCTC
	Dabcyl-Short strand (D-S)	GACATCTGTCTTTTTTTTGAGAGTTAAGAGAGTA-**Dabcyl**
Other related-DNA strands	Linker	AAAAAAAGACAGATGTCTTTAGACTACCAAGAAACAATA
	Aptamer-sgc8 (Apt)	CGCAAGGGTTGTTTCTTTCTTTATCTAACTGCTGCGCCGCCG GGAAAATACTGTACGGTTAGA
	Random	CGCAAGGGTTGTTTCTTTCTTTGCGAGTATGTTGTCAACTGC CTTCCACTCTGATATAAACCA
	Strand 1-Cy5 (S1-Cy5)	**Cy5**-GAAAGAAACAACCCTTGCGCATTCGAGTCTCCCTCATTTAGT TCTCATCCTTTCCATTCGAGTCTCC
	Substrate	CTGAGCAGTTTTTTTTCTACTCTATCTAT**/rA/**GGAAGTACC GCGCTGCTTTTTTCTGCTCAGTACTCTCTTAACTCTC
	Short strand	GACATCTGTCTTTTTTTTGAGAGTTAAGAGAGTA
Lock strands of other lengths	Lock-a	TCAACATCAGTCTGATAAGCTAATCTCTATCTA
	Lock-b	TCAACATCAGTCTGATAAGCTAATCTCTATCT
	Lock-c	TCAACATCAGTCTGATAAGCTAATCTCTATC
	Lock-e	TCAACATCAGTCTGATAAGCTAATCTCT
	Lock-f	TCAACATCAGTCTGATAAGCTAATCTC
Mismatched non-targets	mismatch-1 (mis-1)	TATCTTATCAGACTGATGTTGA
	mismatch-2 (mis-2)	TATATTATCAGACTGATGTTGA
	mismatch-3 (mis-3)	TATATTATAAGACTGATGTTGA
	mismatch-4	TATATTATAATACTGATGTTGA

	(mis-4)	
	mismatch-5 (mis-5)	TATATTATTATAATGATGTTGA
Target or non-targets	D-miR-21	TAGCTTATCAGACTGATGTTGA
	D-miR-141	TAACACTGTCTGGTAAAGATGG
	D-let-7d	AGAGGTAGTAGGTTGCATAGTT
	D-miR-200b	TAATACTGCCTGGTAATGATGA
	D-miR-429	TAATACTGTCTGGTAAAACCGT
Primers for Quantitative Real-time PCR	21-Stem-loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACTCAACA
	F-primer-miR-21	GCGCGTAGCTTATCAGACTGA
	R-primer-miR-21	AGTGCAGGGTCCGAGGTATT
	6-Stem-loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACAACGCT
	F-primer-U6	GCGCGCTCGCTTCGGCAGCACA
	R-primer-U6	AGTGCAGGGTCCGAGGTATT

[a]The base sequences in the underlined parts of S1 and S2 are complementary; the base sequences in the underlined parts of S3 and S4 are complementary; the base sequences in the red font of S1 and S3 complement each other; the base sequences in the green font of S2 and S4 are complementary; the base sequences with gray backgrounds in S1 and S3 sequences can complement the base sequences with gray backgrounds in Aptamer sgc8 and Random. The base sequences of yellow background in DNAzyme and Lock strands (Lock-a, Lock-b, Lock-c, Lock-d, Lock-e, Lock-f) were complementary; the base sequences of the green font in DNAzyme and F-S complement each other; the two red font base sequences of F-S complement each other; the blue font base sequences in F-S and D-S are complementary. The purple font base sequences of Linker and D-S complement each other; the blue background base sequences in S2, S4 and Linker are complementary; the underlined base sequences in Lock strands and D-miR-21 are complementary; the red background parts in Mismatched non-targets represent mismatched bases.

Table S2. The average Ct values of miR-21 in E_2-treated or untreated MCF-7 cells determined by qRT-PCR[b]

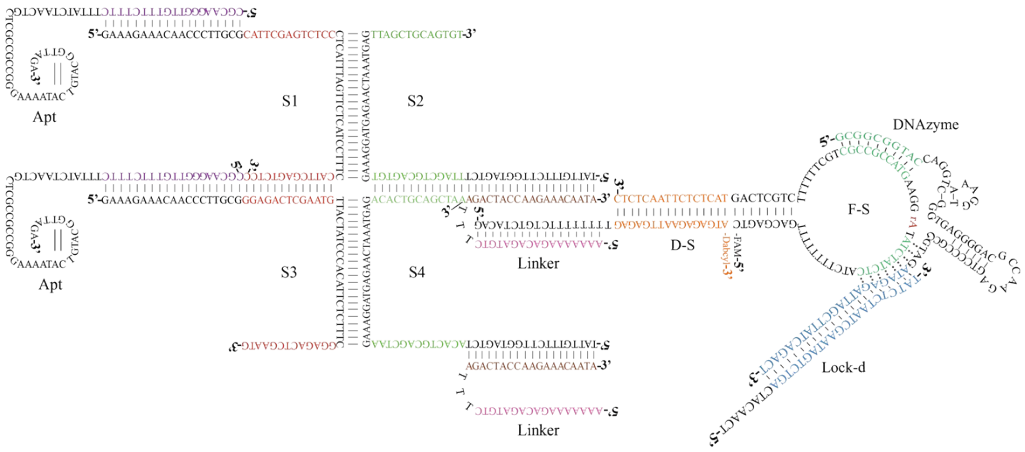
Cell line	miR-21	U6	ΔCt	ΔΔCt	$2^{-(\Delta\Delta Ct)}$
MCF-7	20.561	21.547	-0.986	0.000	1.000
MCF-7-E_2	20.097	21.905	-1.808	0.822	0.566

[b]The relative expression level of miRNA-21 under the influence of E_2 was calculated by using the $2^{-\triangle\triangle Ct}$ method.

Table S3. Comparison of imaging performance between D-Walker and other methods.

Number	Method	LOD	The basis of cell penetration	Biostability	Ref
1	MnO2 nanotube-based probes	0.6 nM	MnO_2 nanotube	/	[1]
2	Genetically encoded fluorescent RNA sensor	0.3 pM	Lipofectamine 3000	/	[2]
3	Ratiometric fluorescent biosensor	0.2 nM	Protonated Phenyl-doped carbon nitride nanosheets	/	[3]
4	DNA "Nano wheel" based localized DNA cascade reaction	85.3 pM	DNA nanowire	/	[4]
5	Hairpin-fuelled catalytic beacons	67 pM	Gold nanoparticles	At least 60 min in DNase I.	[5]
6	DNAzyme mediated signal amplification	23 pM	Lipofect-amine 3000	At least 50 min in DNase I (50 U/mL).	[6]
7	Toehold-initiated rolling circle amplification	/	20% formamide	/	[7]
8	DNAzyme-mediated signal amplification	61 pM	DNA nanowires decorated with aptamers	At least 120 min in DNase I (2 U/mL) and 240 min in cell lysate.	This study

Section C: Supporting Figures



Scheme S1. The Watson-Crick base pairing between oligonucleotide sequences for the assembly of D-Walker.

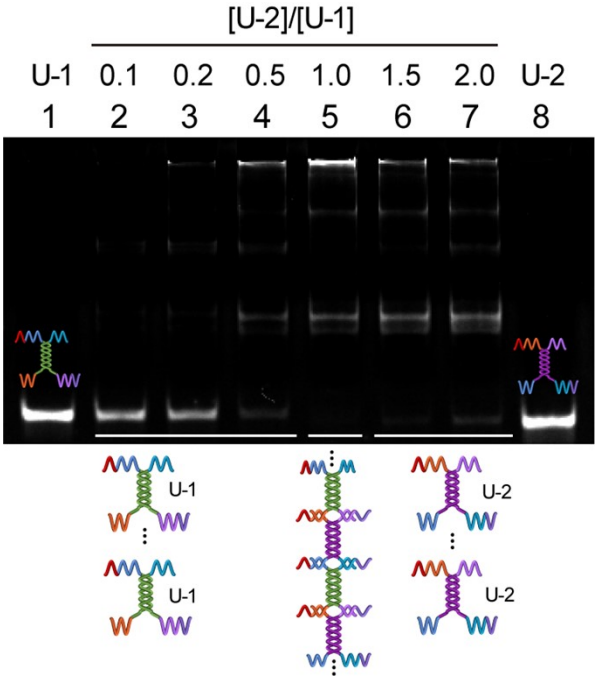


Figure S1. Native PAGE analysis to optimize nanowire assembly at various U-2 to U-1 ratios. U-1 concentration was constant (250 nM).

Experimental procedure:

The preparation process for U-1 and U-2 was consistent with the "**SA.1 Assembly of DNA nanowire based-DNAzyme Walker (D-Walker)**" section. The analytical samples were prepared as follows. Pre-prepared U-1 (Lanes 1-7 each contain 2.5 μL, while Lane 8 contains no addition) and U-2 (0 μL, 0.25 μL, 0.5 μL, 1.25 μL, 2.5 μL,

3.75 μL, 5 μL, 2.5 μL sequentially) were mixed with TAMg buffer to each total volume of 10 μL, after thoroughly mixing, they were incubated at room temperature for 1 h to obtain the samples of the various concentration ratio of U-2 to U-1.

Discussion:

From Lane 1 and Lane 8, distinct bands of U-1 and U-2 can be clearly observed. By increasing or decreasing the ratio of U-2/U-1, the integration of the two components could be finely tuned. When the ratio of U-2 to U-1 was 1, theoretically achieving optimal assembly with the main DNA strands concentrated at the top in the Lane 5. Moreover, compared to U-2 or U-1 alone, Lanes 2-7 exhibit reduced intensity and the emergence of new bands. This aligns with the assembly mechanism of DNA nanowires, where once one unit is depleted, even with additional units available, the assembly process terminates.

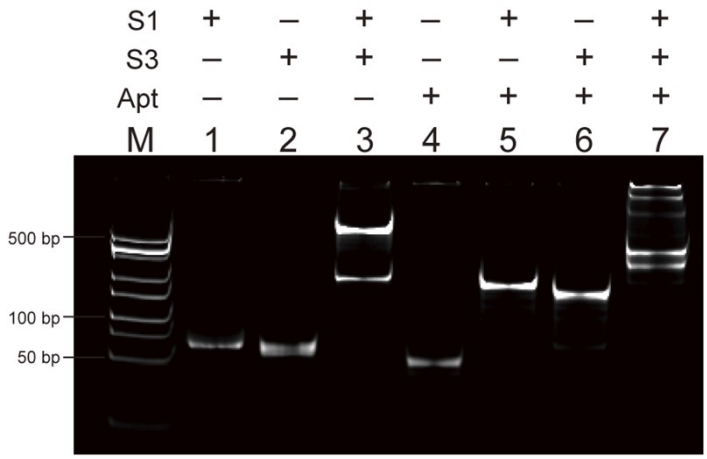


Figure S2. Native PAGE analysis to verify the binding of S1, S3 and Apt respectively. Lane 1, S1; Lane 2, S3; Lane 3, S1 + S3; Lane 4, Apt; Lane 5, S1 + Apt; Lane 6, S3 + Apt; line7, S1 + S3 + Apt.

Experimental procedure:

The experimental procedure was consistent with the "**SA.2 Electrophoretic mobility analysis**" section. The specific samples preparation method was as follows: 1 μL of the corresponding DNA sequence was added to the TAMg buffer to the total volume of 10 μL. Then, they were heated at 90°C for 5 min and gradually cooled to room temperature.

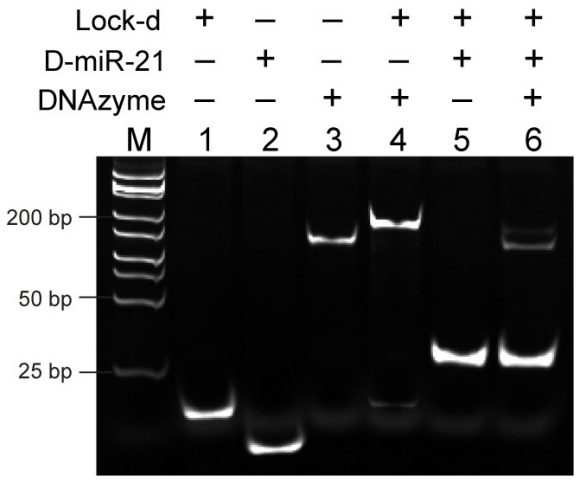


Figure S3. Native PAGE for validation of Locked-DNAzyme responds to target D-miR-21. Lane 1, lock-d; Lane 2, D-miR-21; Lane 3, DNAzyme; Lane 4, lock-d + DNAzyme; Lane 5, lock-d + D-miR-21; Lane 6, lock-d + DNAzyme + D-miR-21.

Experimental procedure:

For Lanes 1-3: 1 μL of the corresponding DNA sequence was added to 9 μL TAMg buffer and heated at 90°C for 5 min, gradually cooled to room temperature.

For Lane 4 and Lane 5: Two corresponding DNA strands (1 μL, each) were diluted in TAMg to total volume of 10 μL each. After thoroughly mixing, the solution was annealed at 90°C for 5 min and then gradually cooled to room temperature.

For Lane 6: DNAzyme (1 μL), lock-d (1.2 μL) and TAMg (6.3 μL) were mixed, and the solution was annealed at 90°C for 5 min then gradually cooled to room temperature. Subsequently, 1.5 μL of D-miR-21 was added and the mixture was incubated at room temperature for 1 h.

Discussion:

Lanes 1-3 respectively represent the lock-d, D-miR-21 and DNAzyme band. In Lane 4, neither the DNAzyme strand band nor the lock-d band was observed; instead, a new band appeared. Similarly, in Lane 5, both the target D-miR-21 band and the lock-d strand band disappeared, being replaced by a new band. This indicated the formation of the locked-DNAzyme strand hybrid and the target D-miR-21/lock-d strand hybrid, respectively. In Lane 6, a band with similar brightness and position to Lane 5 could be observed. This indicated that the lock-d strand readily hybridized with the target D-

miR-21, thereby activating the previously inactive DNAzyme.

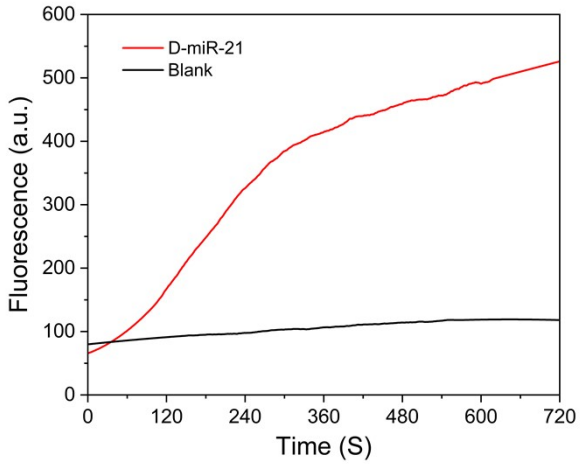


Figure S4. Real-time monitoring of fluorescence intensity of D-MB in the presence of D-miR-21.

Discussion:

Figure S4 displayed the fluorescence changes of D-MB induced by the target D-miR-21 as detected in real-time. When D-miR-21 was not exists, the fluorescence intensity remained at a baseline level (black curve), whereas in the presence of D-miR-21, the fluorescence intensity of D-MB exhibited a rapid initial increase followed by a plateau (red curve), indicating that D-MB could respond quickly to D-miR-21.

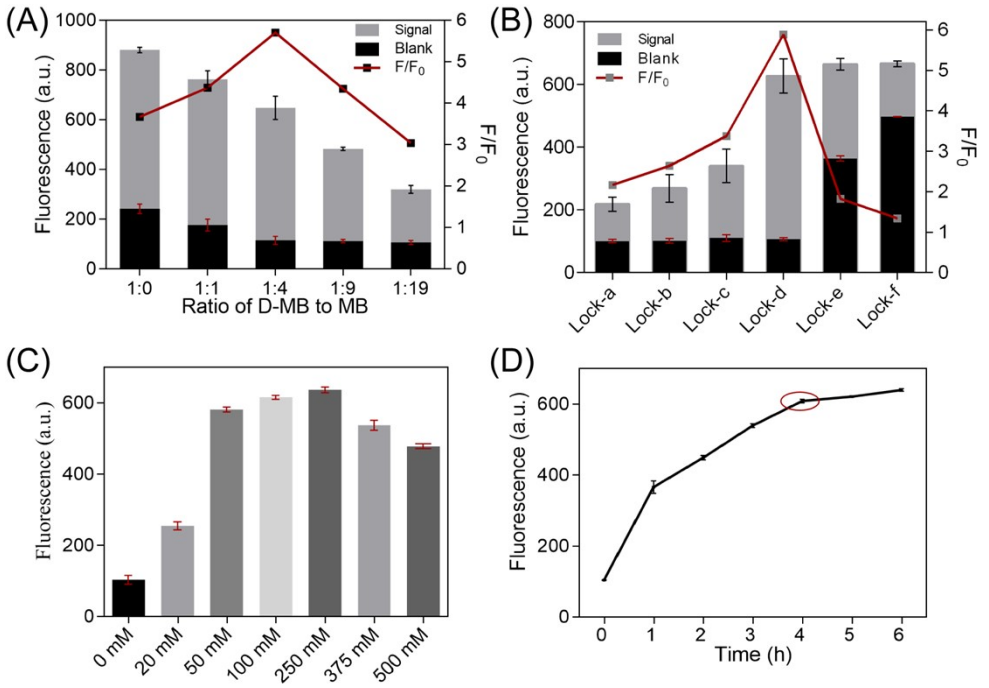


Figure S5. Optimization of construction and sensing conditions for D-Walker. (A) Fluorescence intensity of D-Walkers assembled by different ratios of D-MB to MB incubated with D-miR-21. (B) Fluorescence intensity of D-Walkers assembled by lock strands with different base lengths incubated with D-miR-21. For (A) and (B), relative fluorescence intensity was calculated by F/F_0, where F and F_0 represent the fluorescence intensity induced by target D-miR-21 and blank, respectively. (C) Fluorescence intensity of D-Walker induced by D-miR-21 in different concentrations of Na^+. (D) Fluorescence intensity of D-Walker incubated with D-miR-21 at different times. Error bars represent standard deviations from three parallel assays.

Experimental procedure:

Pre-prepared D-MB and MB were mixed at various molar concentration ratio of 1:0, 1:1, 1:4, 1:9 and 1:19 respectively, and then assembled with NW to obtain D-Walker. Specifically, D-MB solution (20 μL, 10 μL, 4 μL, 2 μL, 1 μL sequentially, 1 μM each), MB solution (0 μL, 5 μL, 8 μL, 9 μL, 9.5 μL sequentially, 2 μM each) were mixed with TAMg (0 μL, 5 μL, 8 μL, 9 μL, 9.5 μL sequentially), then NWs solution (16 μL), Apt (2 μL, 10 μM), Linker (2 μL, 10 μM) and 8 μL of TAMg were added to the mixture, after thoroughly mixing, they were incubated at room temperature for 1 h to obtain the D-Walker solution (40 μL, 0.5 μM).

Each ratio of D-Walker was sequentially mixed with the target D-miR-21 (1 μL, 10 μM), NaCl (10 μL, 5 M), and added TAMg to make the total volume to 200 μL. After incubating at 37°C for 4 h, real-time fluorescence detection was performed. For

the blank group, 1 μL of ddH_2O was used instead of D-miR-21, and other steps were same.

By varying the length of the locking strand, the concentration of the cofactor Na^+, and the incubation time individually, while keeping other experimental procedures consistent with the "**SA.4 Fluorescence detection**" protocol.

Discussion:

As we designed the detector based on the DNA walker, considering the issue of probe walking, it was not necessarily the case that more locked-DNAzyme led to better detection performance. Therefore, it was necessary to explore the ratio of D-MB to MB. As shown in Figure S5A, D-Walker exhibited the bigest F/F_0 and optimal performance when the ratio of D-MB to MB was 1:4. Increasing or decreasing the amount of Locked-DNAzyme both decreased F/F_0, affecting the detection performance. Therefore, the final study employed this ratio to prepare D-Walker.

The key to suppressing fluorescence background was that the DNAzyme remained dormant until the D-miR-21 was added. This was also the purpose of designing the lock strands. To optimize the length of the lock strand, we evaluated the response signals of detectors synthesized using six different lock strands, as shown in Figure S5B. It was evident that detector assembled by lock-d yielded the highest signal-to-blank ratio, indicating the most effective silencing of DNAzyme. Although lock-e and lock-f showed very high response signals to the D-miR-21, the background signal was too strong, resulting in a lower signal-to-blank ratio. Therefore, lock-d was chosen as the best locking strand.

Given that the D-Walker we designed relied on Na^+ to undergo conformational changes and achieve activation, cleavage, and walking, the concentration of Na^+ also impacted the performance of the D-Walker. Consequently, we investigated the correlation between Na^+ concentration and fluorescence signal, as depicted in Figure S5C. In the absence of Na^+, there was nearly no fluorescence signal, highlighting the essential role of Na^+ co-factor for D-Walker functionality. Notably, at a Na^+ concentration of 250 mM, the fluorescence signal reached its highest peak, consistent

with prior findings (Oriented Tetrahedron-Mediated Protection of Catalytic DNA Molecular-Scale Detector against in Vivo Degradation for Intracellular miRNA Detection).

It was imperative to explore the reaction time to determine the optimal performance of the detector. The relationship between reaction time and fluorescence intensity was illustrated in Figure S4D. After 4 h, although there was a slight increase in fluorescence intensity over time, the magnitude of enhancement was minimal. Therefore, 4 h was chosen as the optimal reaction time.

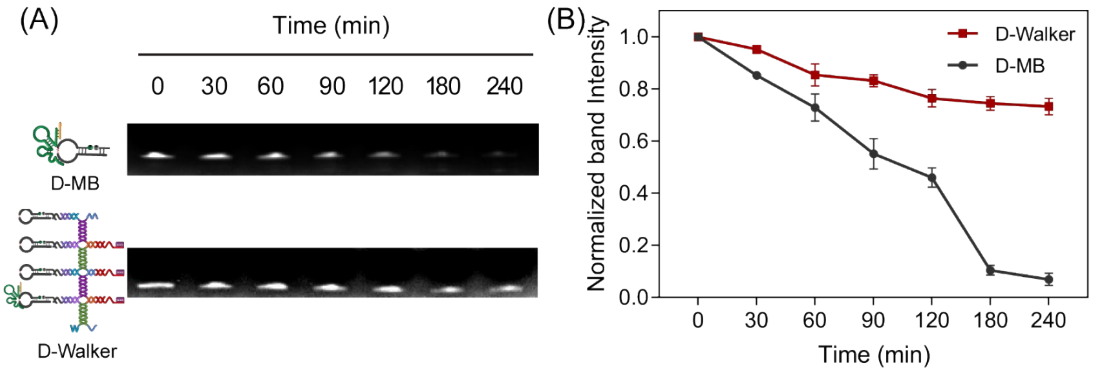


Figure S6. The stability analysis of D-Walker in cell lysate. (A)The dPAGE analysis of the stability of D-Walker in HeLa cell lysate. (B) Quantitative evaluation of the fluorescence intensity of the bands shown in (A), error bars represent standard deviations from three independent experiments.

Experimental procedure:

Preparation of Cell Lysis. HeLa cells were cultured in a flask with a growth area of 25 cm^2 until they reached 80% confluence, then washed with PBS and treated with 500 μL of trypsin. The cells were centrifuged at 900 rpm for 3 min, the supernatant was discarded and then the cells were resuspended in PBS. After removing the PBS, 500 μL of lysis buffer (including 50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, and protease inhibitor at a 1:100 concentration) was added, and the mixture was incubated on ice with shaking for 20 min. The lysate was then centrifuged at 12000 rpm for 30 min at 4°C. The supernatant was collected and stored at -20°C for further use. D-MB and D-Walker (0.5 μM) were prepared according to the section of "**SA.1 Assembly of DNA nanowire based-DNAzyme Walker (D-Walker)**". 4 μL of the

above samples were incubated with 4 μL of the cell lysate respectively at 37°C for different time (0, 30, 60, 90, 120, 180 and 240 min).

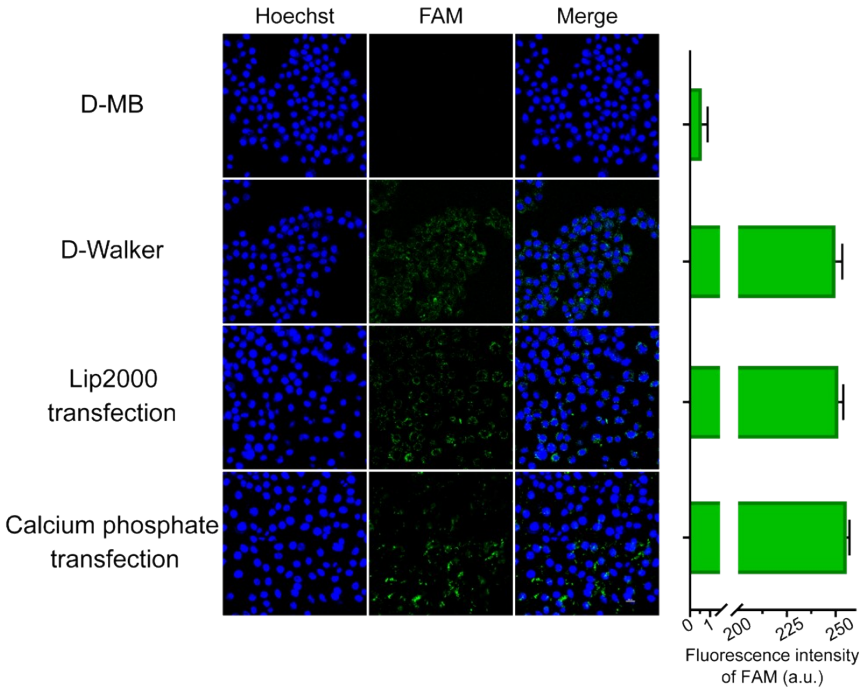


Figure S7. Confocal microscopy images of cellular uptake efficiency of D-Walker. The quantitative analysis of FAM fluorescence intensity shown in the right panel using ImageJ. Scale bar: 20 μm.

Experimental procedure:

For the D-MB and D-Walker groups:

The experimental method was the same as the corresponding group in "SA.5 confocal fluorescence Imaging", except that the Dabcyl-short strand (D-S) was replaced by a Short strand without Dabcyl group. For each group, the equivalent amount of MB was 25 pmol.

For the Lipo2000 transfection group:

To prepare the DNA dilution, 50 μL of Opti-MEM I culture medium was mixed with the prepared D-MB solution (50 μL, 0.5 μM, the equivalent amount of MB was 25 pmol). Next, 2.0 μL of Lip2000 was diluted in 50 μL of Opti-MEM I to create the Lip2000 dilution. After allowing the Lip2000 dilution to sit at room temperature for 5 min, it was combined with the DNA dilution and incubated at room temperature for 20

min to form the DNA-Lip2000 complex. Subsequently, 0.5-2 × 10^5 HeLa cells were seeded in a confocal dish using 500 μL of Opti-MEM I culture medium and incubated for 24 h until the cells reached 80% confluency. The cells were then washed three times with PBS, and the DNA-Lip2000 complex was added.

For the CCK-8 toxicity experiment, the same protocol was followed, with the only difference being that the cells were seeded in a 96-well plate, necessitating a corresponding reduction in sample volumes. Specifically, the volumes for D-MB, Lip2000, and Opti-MEM I culture medium were adjusted from 50 μL, 2.0 μL, and 50 μL to 10 μL, 0.5 μL, and 25 μL, respectively. The equivalent amount of MB was 5 pmol.

For the Calcium phosphate transfection group:

Considering the limited DNA volume recommended in the instructions of the Calcium Phosphate Cell Transfection Kit, the assembly method of D-MB was slightly adjusted. To prepare D-MB, Locked-DNAzyme was prepared by adding 2.5 μL of Lock-d (10 μM) and 2.5 μL of DNAzyme (10 μM) into 5 μL of TAMg buffer. The solution was annealed at 90 °C for 5 min and then cooled down to room temperature. MB was obtained by mixing F-S (2.5 μL, 10 μM), Short strand (2.5 μL, 10 μM) and 5 μL of TAMg buffer following the same procedure as above. Then, the two solutions (locked-DNAzyme and MB) were mixed for 2 h at room temperature, resulting in the D-MB solution (20 μL, 1.25 μM, the equivalent amount of MB was 25 pmol).

HeLa cells were seeded in a confocal dish and incubated for 24 h until they reached 80% confluency. Subsequently, 500 μL of Opti-MEM I was added, and the cells were incubated in a 37°C, 5% CO_2 incubator for 4 h before adding the DNA-$CaCl_2$-BBS mixture. The preparation of the DNA-$CaCl_2$-BBS solution was as follows: 100 μL of $CaCl_2$ solution was added to the pre-prepared D-MB (20 μL, 1.25 μM), and mixed thoroughly to obtain the DNA-$CaCl_2$ solution. Then, 100 μL of BBS solution was added, and the mixture was allowed to stand at room temperature for 30 min to form the DNA-$CaCl_2$-BBS solution.

For the CCK-8 toxicity experiment, the cells were seeded in a 96-well plate, and the volumes for D-MB, $CaCl_2$, and BBS were adjusted from 20 μL, 100 μL, and 100

μL to 4 μL, 25 μL, and 25 μL, respectively. The equivalent amount of MB was 5 pmol.

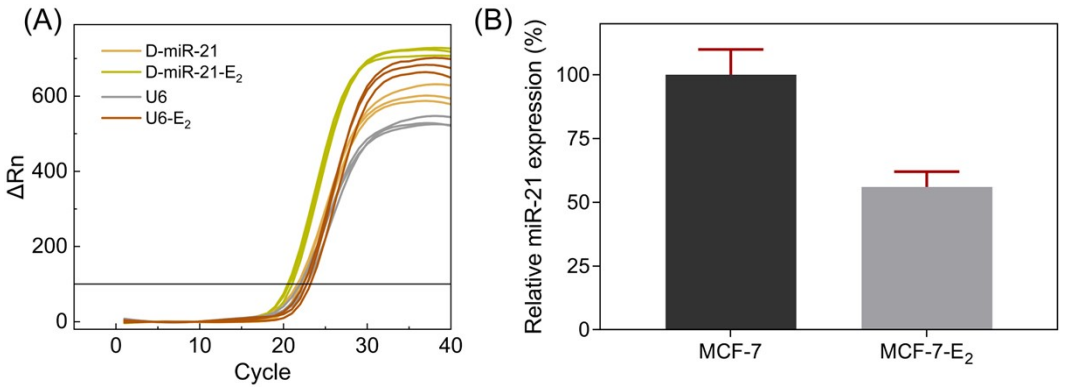


Figure S8. The qRT-PCR analysis of miR-21 extracted within MCF-7 cells treated with E_2. (A) qRT-PCR curves for miR-21 and U6. U6 small RNA as an internal control. The threshold line is represented by the black horizontal line, and the threshold value is 100. (B) Quantification of miR-21 expression level. Relative expression levels of miRNA-21 calculated by the $2^{-(\Delta\Delta Ct)}$ method, while the miR-21 expression in MCF-7 cells defined as 100%. Error bars represent standard deviations from three parallel assays.

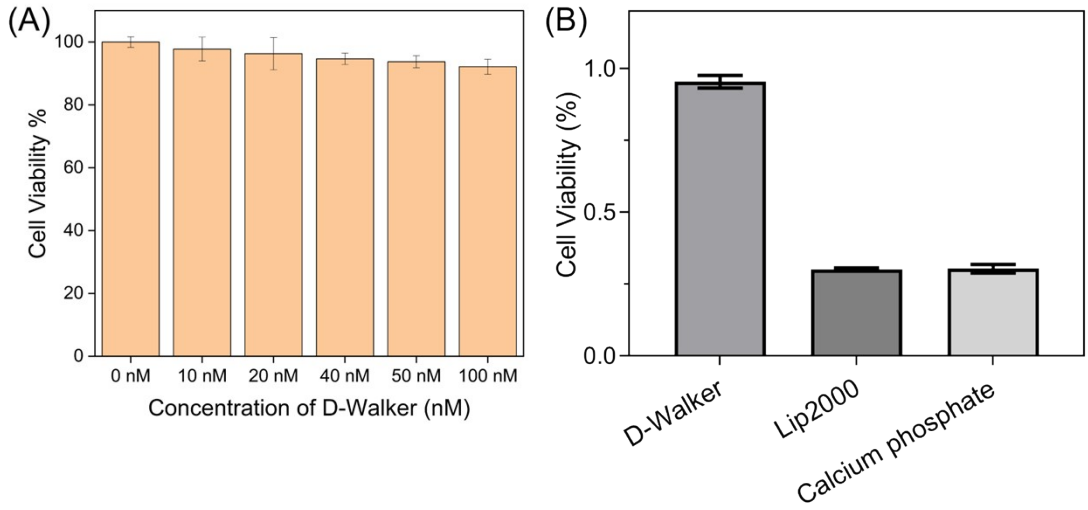


Figure S9. Cell cytotoxic analysis of D-Walker toward HeLa cells. (A) Cell viability under incubation with different concentrations of D-Walker. (B) Cell viability of D-Walker, Lip2000, and Calcium phosphate transfection treatment groups. The same equivalent amount (5 pmol) of MB was used for each group. Data are shown as means ± SD (n = 3).

Experimental procedure:

HeLa cells were seeded in a 96-well plate and incubated for 24 h before washing with PBS. Subsequently, the appropriate samples were added, and the cells were incubated with Opti-MEM I for 4 h. After another wash with PBS, the medium was

replaced with DMEM. Following a 24-hour incubation, each well received 100 μL of DMEM and 10 μL of CCK-8. The absorbance at 450 nm was measured after 20 min using a microplate reader (Synergy HTX, USA).

4. Section D: References

1. Lu, Q.; Ericson, D.; Song, Y.; Zhu, C.; Ye, R.; Liu, S.; Spernyak, J. A.; Du, D.; Li, H.; Wu, Y., MnO_2 nanotube-based nanoSearchlight for imaging of multiple microRNAs in live cells. *ACS Appl. Mater. Interfaces* **2017,** *9* (28), 23325-23332.
2. Ying, Z.-M.; Wu, Z.; Tu, B.; Tan, W.; Jiang, J.-H., Genetically encoded fluorescent RNA sensor for ratiometric imaging of microRNA in living tumor cells. *J. Am. Chem. Soc.* **2017,** *139* (29), 9779-9782.
3. Zhou, D.; Liu, X.; Liu, X.; Xu, Y.; Chen, R.; Lin, C.; Guo, L.; Fu, F., Ratiometric fluorescent biosensor for microRNAs imaging in living cells. *Sens. Actuators B Chem.* **2020,** *322*, 128632.
4. Yang, F.; Cheng, Y.; Cao, Y.; Zhang, Y.; Dong, H.; Lu, H.; Zhang, X., MicroRNA triggered DNA "nano wheel" for visualizing intracellular microRNA via localized DNA cascade reaction. *Anal. Chem.* **2019,** *91* (15), 9828-9835.
5. Wang, J.; Huang, J.; Quan, K.; Li, J.; Wu, Y.; Wei, Q.; Yang, X.; Wang, K., Hairpin-fuelled catalytic nanobeacons for amplified microRNA imaging in live cells. *Chem. Commun.* **2018,** *54* (73), 10336-10339.
6. Yang, Y.; He, Y.; Deng, Z.; Li, J.; Li, X.; Huang, J.; Zhong, S., An autonomous self-cleavage DNAzyme walker for live cell microRNA imaging. *ACS Appl. Bio Mater.* **2020,** *3* (9), 6310-6318.
7. Deng, R.; Tang, L.; Tian, Q.; Wang, Y.; Lin, L.; Li, J., Toehold-initiated rolling circle amplification for visualizing individual microRNAs in situ in single cells. *Angew. Chem. Int. Ed. Engl.* **2014,** *53* (9), 2389-93.