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Self-delivery DNA Nanoprobe for Reliable MicroRNA Imaging in Live Cell by Aggregation Induced Red-shift-Emission

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1. Chemicals and materials.

Reagents for DNA synthesis were purchased from Honeywell Burdick & Jackson (Muskegon, USA). Universal controlled pore glass (CPG) and standard phosphoramidites were purchased from Hai Phoenix Technology, LLC (Arizona, USA). 5-DMT-1,1-dihydro-2-O-Hexynl-3-CE phosphoramidite was purchased from Chemgenes Corporation (Wilmington, USA). All the RNA sequences were purified by HPLC and ordered from Genewiz Corporation (Nanjing, China). The detailed sequence information is listed in Table S1. N, N, N', N'-tetramethylethylenediamine (TEMED), acrylamide, bis-acrylamide and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Ammonium persulfate (APS) and 5 × tris/boric acid/EDTA (TBE) buffer were purchased from Bioson Corporation (Beijing, China). 1-(Azidomethyl) pyrene (Py-N_3), Copper (I) bromide, tris [(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (TBTA) ethyl alcohol, acetonitrile, Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF) and dichloromethane (DCM) were obtained from Aladdin Co., Ltd (Shanghai, China). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin were obtained from Gibco BRL Co., Ltd (Grand Island, USA). HeLa MCF-7, and A549 cells were kindly provided by Professor Ying Sun at the Department of Biology of Southern University of Science and Technology (SUSTech).

2. DNA Synthesis and Purification

All the DNA sequences used in the work were synthesized on a H-8 DNA synthesizer (K&A, Germany) by using standard phosphoramidite chemistry on a universal CPG support. After cut from the CPG beads, the raw products of the synthesized ssDNA, pyrene-modified ssDNA and FAM- modified ssDNA were purified via reverse-phase high-performance liquid chromatography (HPLC (Agilent Co., USA)) through a C-18

reverse column using a binary gradient (buffer A: 100 mM triethylammonium acetate (TEAA), buffer B: acetonitrile; and the flow rate was set at 1 mL/min). After collected the crude products from HPLC, Y-1 and Y-2 were handled with 80% acetic acid to clear the protecting groups from the oligonucleotide bases or the DMT group. The molecular weight of the obtained pyrene-DNA conjugates was determined by a Q-Exactive electrospray ionization (ESI) mass spectrometer in Negative mode (Thermo Fisher Scientific, USA).

Table S1. Oligonucleotide sequences used in this work

Strand	Sequence (from 5' to 3')
Y-1-ssDNA	CTG ATA AGC TAC GTG CTC AAC TAT GGC TCA ACA TCA GT
Y-2-ssDNA	GCC ATA GTG GAT TGC ATC AAC ATC AGT
Y-1	Py-CTG ATA AGC TAC GTG CTC AAC TAT GGC TCA ACA TCA GT-Py
Y-2	GCC ATA GTG GAT TGC ATC AAC ATC AGT-Py
Y-3	TGC AAT CCT GAG CAC G
miR-21	UAG CUU AUC AGA CUG AUG UUG A
H1-3'-Py	CGT GTG CCT CCA AC-Py
H2 -5'-Py	Py-CGC ACC GCA GTG CG
H3	CGC ACT GCG GTG CGG TTG GAG GCA CAC G
H3 (1MS)	CGC ACT GCG GTG CGA TTG GAG GCA CAC G
H1-3'-FAM	CGT GTG CCT CCA AC-FAM
H2 -5'-BHQ	BHQ-CGC ACC GCA GTG CG
ss-DNA	CTG ATA AGC TAC GTG CTC AAC TAT GGC TCA ACA TCA GT-FAM
Single-base mismatched miR-21 (SM miR-21)	UAG CUU AUC CGA CUG AUG UUG A
Triple-base mismatched miR-21 (TM miR-21)	UAG CUU AUA CCA CUG AUG UUG A
miR-125b	UCC CUG AGA CCC UAA CUU GUG A
miR-let 7a	UGA GGU AGU AGG UUG UAU AGU U
miR-NC	UUG UAC UAC ACA AAA GUA CUG
Y-1-NC	Py-GTG TAG TAC AAC GTG CTC AAC TAT GGC CAG TAC TTT T-Py

Y-2-NC	GCC ATA GTG GAT TGC ACA GTA CTT TT-Py
Y-1-let 7a	Py-CTA CTA CCT CAC GTG CTC AAC TAT GGC AAC TAT ACA AC-Py
Y-2-let 7a	GCC ATA GTG GAT TGC AAA CTA TAC AAC-Py
miR-let 7b	UGAGGUAGUAGGUUGUGUGGUU
miR-let 7c	UGAGGUAGUAGGUUGUAUGGUU

Note: The mismatched bases are marked in red.

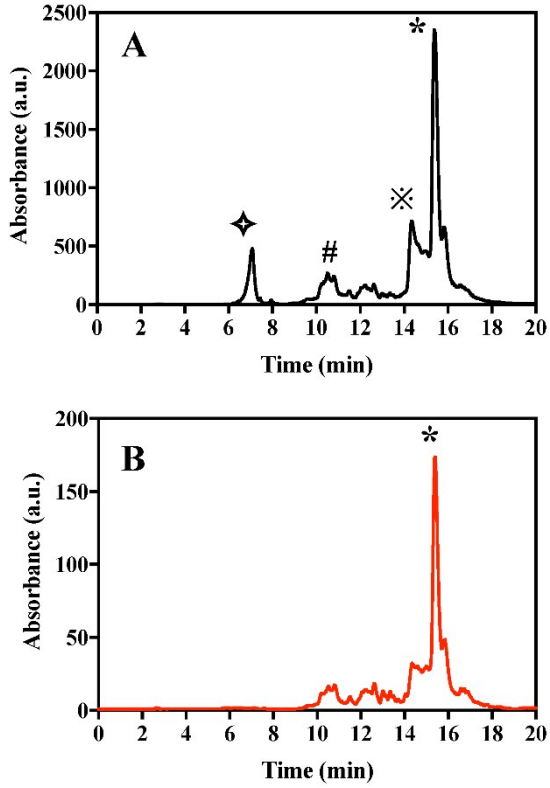

Figure S1. Reverse phase-HPLC spectra of Y-1 conjugate with absorbance at both (A) 260 nm and (B) 345 nm: the unsuccessfully synthesized DNA (✧), the unreacted alkyne-modified DNA (#), the one pyrene modified DNA (※), the dual pyrene modified DNA (*). The fraction labeled with "*" was collected.

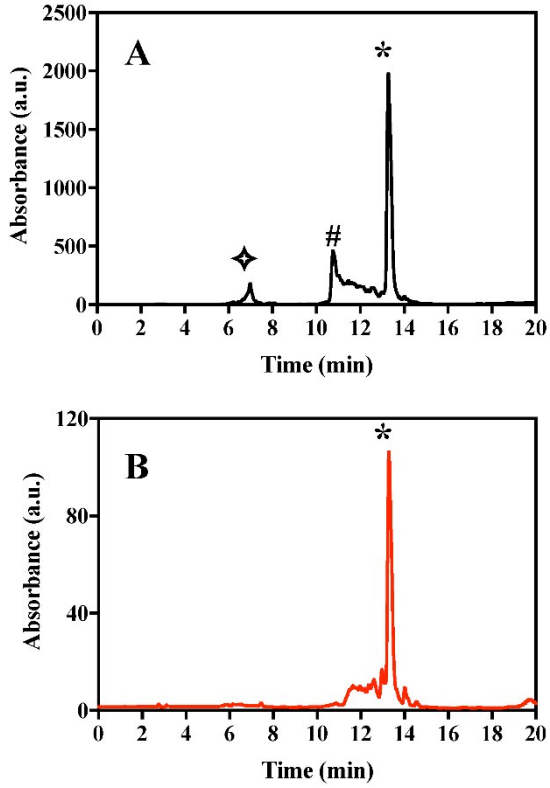

Figure S2. Reverse phase-HPLC spectra of Y-2 conjugate with absorbance at both (A) 260 nm and (B) 345 nm: the unsuccessfully synthesized DNA (✧), the unreacted alkyne-modified DNA (#), the one pyrene modified DNA (*). The fraction labeled with "*" was collected.

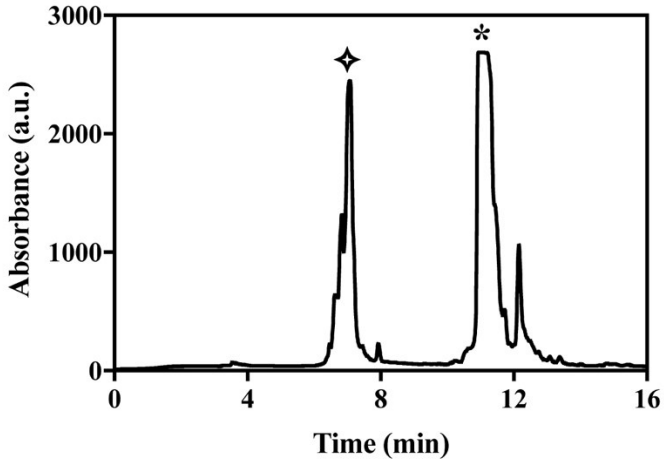

Figure S3. Reverse phase-HPLC spectra of Y-3 with absorbance at 260 nm: the unsuccessfully synthesized DNA (✧), the successfully synthesized DNA (*). The fraction labeled with "*" was collected.

The raw products of the synthesized Y-1, Y-2 and Y-3 were purified and collected via the reverse-phase HPLC (Figure S1-S3). The fractions with retention time at 15.5 min and 13.5 min correspond to Y-1 and Y-2. Y-1 with dual pyrene modification showed higher hydrophobicity compared the mono-pyrene modified Y-2, therefore a longer

retention time was observed for Y-1. While Y-3 exhibited the shortest retention time at 11 min since there is no modification of pyrene.

3. UV-Vis absorbance spectroscopy measurements

UV-vis absorbance spectra were measured via a UV-1800 spectrophotometer (SHIMADZU, Japan).

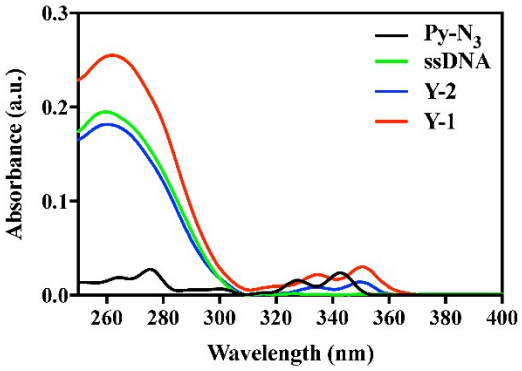

Figure S4. UV-Vis absorption spectra of Py-N_3, ssDNA, Y-1 and Y-2.

4. Characterizations of the strand of Y-1 and Y-2

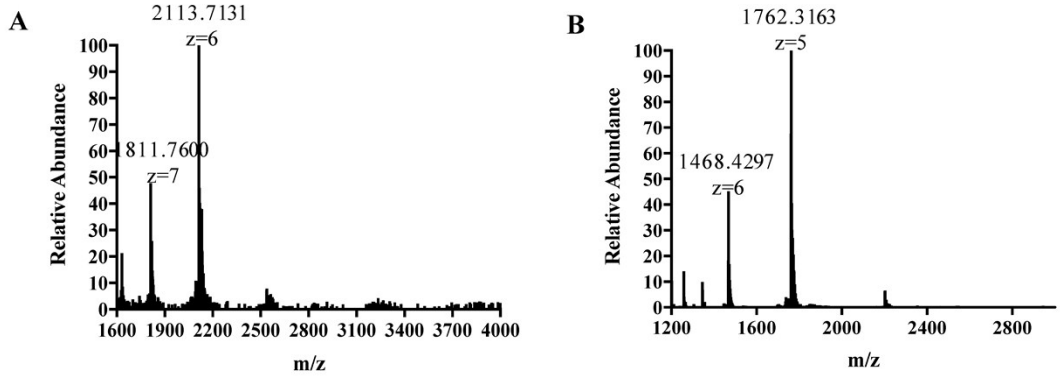

Figure S5. Electrospray ionization (ESI) MS spectrum of (A) Y-1: m/z=2113.7131, z=6 (12686 theoretical) and (B) Y-2: m/z=1762.3163, z=5 (8815.60 theoretical)

5. Preparation of Y-shaped DNA probes

The Y-shaped DNA probes (Y-Py-block) was assembled by three strands (Y-1, Y-2, Y-3). Y-1, Y-2 and Y-3 were mixed in the hybridization buffer (10 mM phosphate, 137 mM sodium chloride, 12.5 mM Mg^{2+}, pH=7.4) and the final concentration of each strand was 0.5 μM. The mixture was heat to 95 °C for 5 min and then followed by slowly cooling to the room temperature for at least 2 h.

6. Electrophoresis characterization

The electrophoresis assay of different DNA structures was investigated by 10% or 15% native polyacrylamide gel electrophoresis (PAGE). The gel was run at 100V for 90 min in 0.5 × TBE buffer (89 mM Tris Borate, 2.0 mM EDTA, 12.5mM Mg^{2+}, pH 8.3), and

stained for 15 min in a 1 × GelRed solution. Then the stained gel was imaged by the Tannon 3500 gel imaging system (Tannon, China).

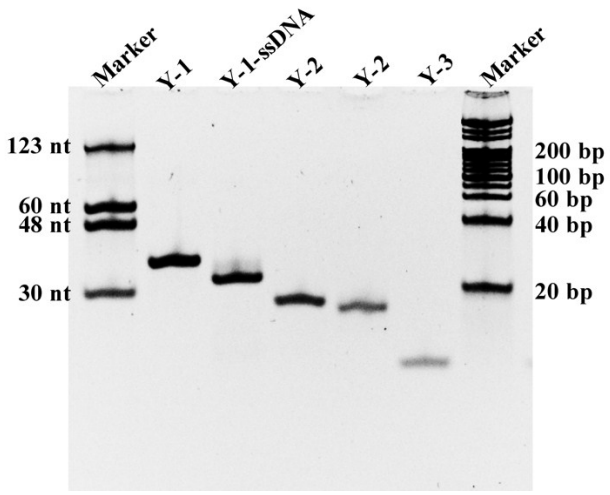

Figure S6. 10% PAGE analysis of Y-1-ssDNA, Y-2-ssDNA, Y-3 and their pyrene-functionalized sequences Y-1, Y-2. [Y-1-ssDNA] = [Y-2-ssDNA] = [Y-1] = [Y-2] = [Y-3] =1 μM

7. The high sensitivity of the pyrene-based DNA probes

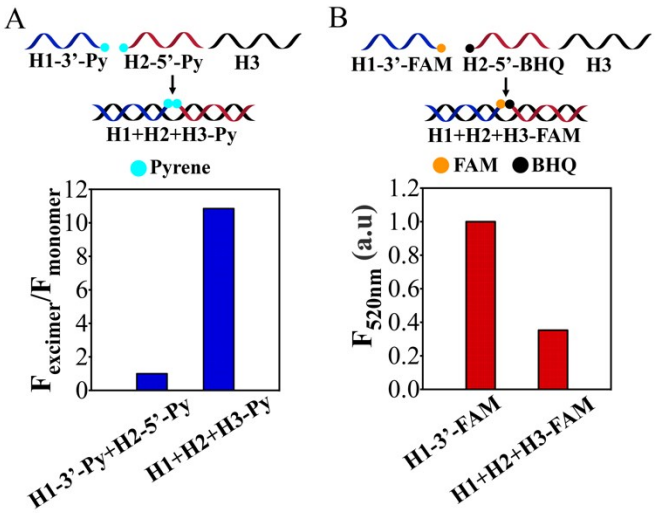

Figure S7. (A) schematic illustration of pyrene-modified DNA strands and its ratiometric response. (B) schematic illustration of FAM/BHQ-modified DNA strands and its responses in single-wavelength signal.

8. *In vitro* miRNA detection

The Y-shaped probes were prepared by the above-mentioned methods in advance. The experiments were performed in 100 μL of the hybridization solution containing 0.1 μM Y-shaped probes and different concentrations of target miR-21 at 37 °C for 30 min. Fluorescence emission spectra were recorded by an Agilent Cary Eclipse Fluorescence Spectrophotometer (Agilent Co., USA), and with the following settings: the excitation

wavelength was 350 nm, the excitation and emission slits were set for 5.0 and 5.0 nm, respectively. The fluorescence intensity at 400 nm and 480 nm was used for data analysis.

9. Real-time monitoring of the target-triggered assembly

The experiments were performed in 100 μL of the hybridization solution containing 0.1 μM Y-shaped probes and 20 nM miR-21. From the start of the addition of target, the fluorescence of the mixture was recorded every 20 seconds at 37 °C. Fluorescence emission spectra were measured by a BioTek Cytation 3 (Molecular Devices, USA), and the fluorescence intensity at 400 nm and 480 nm was used for data analysis.

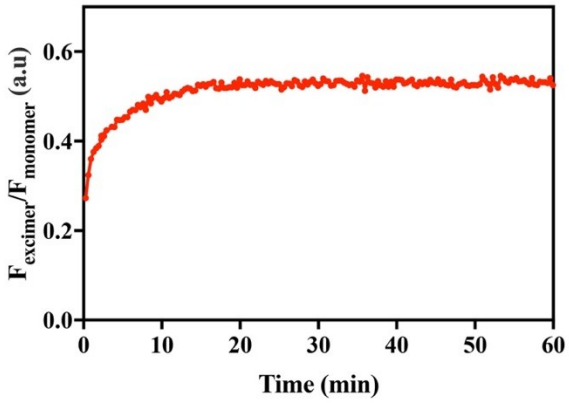

Figure S8. Real-time monitoring of the change of the pyrene excimer-to-monomer emission ratio in the presence of the target miR-21. $F_{excimer}$ is the fluorescence emission intensity of pyrene excimer at 480 nm in the presence of miR-21, and $F_{monomer}$ is the fluorescence emission intensity of pyrene monomer at 400 nm. [Y-Py-block1] = [miR-21] =0.1 μM

10. Cell culture

MCF-7 cells and HeLa cells were cultured in DMEM medium containing 10% FBS, 100 mg/mL penicillin and 100 mg/mL streptomycin in a humidity incubator (5% CO_2, 37 °C).

11. The stability assay of Y-2 strands

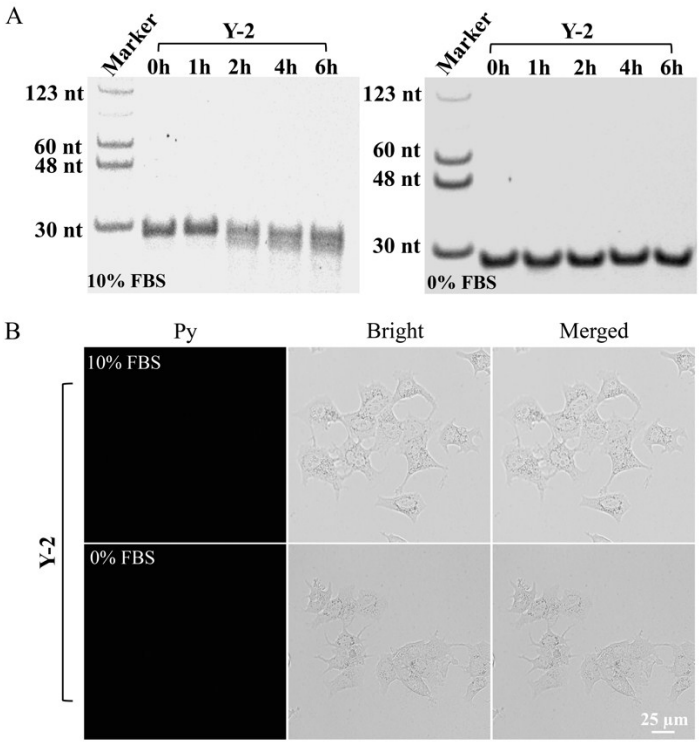

Figure S9. (A) Y-2 strands were incubated in 10% FBS medium or FBS-free medium, and then analyzed with 10% PAGE electrophoresis. (B) The confocal fluorescence images of MCF-7 cells incubated with Y-2 in 10% FBS medium and FBS-free medium, respectively.

12. The stability assay of the ss-DNA, ds-DNA, Y-Py-block1 and target-triggered assembly structure

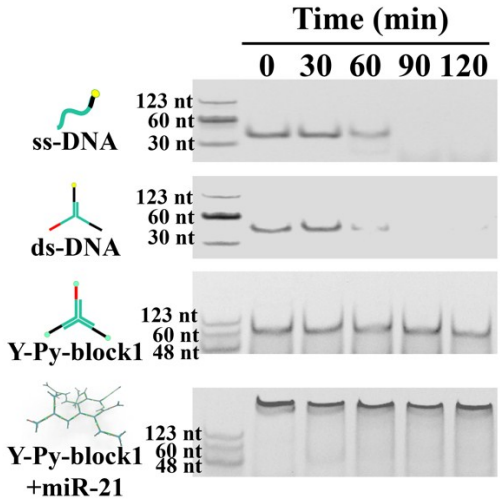

Figure S10. 10% PAGE characterization of the stability of the ss-DNA, ds-DNA, Y-Py-block1 and target-triggered assembly structure (Y-Py-block1 + miR-21) in the presence of 3U/mL DNase I.

13. MTT Assay

For the evaluation of the cytotoxicity of the pyrene-based Y-shaped probe, 200 μL MCF-7 cells and HeLa cells were pre-seeded into a 96-well plate (5000 cells/well) and cultured for 24 h. Then removed the medium and the cells were cultured with 200 μL new medium with different concentrations of Y-shaped probe (20 μM, 50 μM, 100 μM and 200 μM) for another 24 h. After a wash with 1 × PBS buffer (three times), 100 μL MTT solution (5 mg/mL in PBS) was added to each well and incubated for 3 h. And finally, the medium was removed and the formazan products were dissolved with 150 μL DMSO. The absorbance of each cell was measured at 490 nm for the calculation of the cell viability.

14. Live Cell miRNA Imaging

MCF-7 cells and HeLa cells were pre-cultured for 24 h. Then 200 μL Opti-MEM (serum-free medium) containing 200 nM Y-Py-block probes was added to cells and incubated for 6 h. After the incubation, the cells were washed with 1 × PBS buffer (three times) and the cellular fluorescent images were taken under a Leica SP8 confocal microscope (Leica, Germany). The laser transmitters were: 405 nm excitation, 450-520 nm emission for Pyrene. 488 nm excitation, 500-550 nm emission for FAM.

15. Flow Cytometry

MCF-7 cells were cultured in a 24-well plate for 24 h. After washing the cells with 1 × PBS buffer, the medium was changed to Opti-MEM having 200 nM ss-DNA, ds-DNA, Y-DNA probes, respectively. After incubation of 6 h, cells were washed three times with 1 × PBS buffer and digested by trypsin. Followed by a thorough wash with 1 × PBS buffer (three times), then flow cytometry was performed using a BD FACSCanto SORP (Becton Dickinson and Company, USA) and data were analyzed by Flowjo software (Tree Star, USA).

16. The cell permeability assay between ss-DNA, ds-DNA and Y-DNA

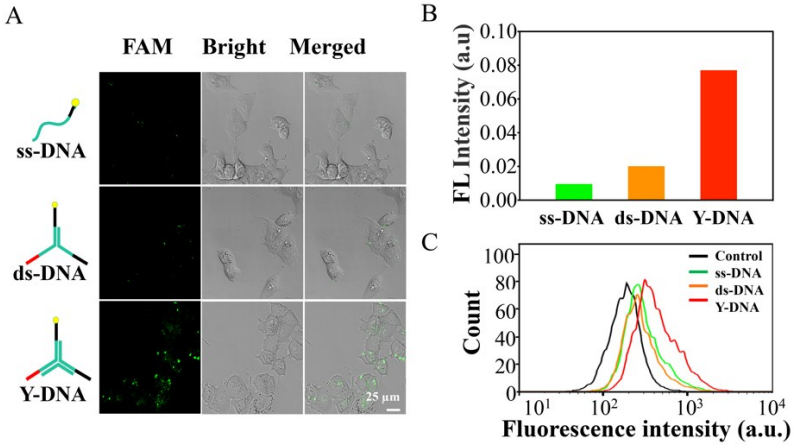

Figure S11. (A) The confocal fluorescence images of MCF-7 cells incubated with FAM modified ss-DNA, ds-DNA and Y-DNA, respectively. (B) Mean fluorescence intensity that derived from the (A). (C) Flow cytometry results of MCF-7 cells treated with the FAM modified ss-DNA, ds-DNA and Y-DNA.

17. The universality of the proposed method

In order to prove the universality of this method, Y-Py-block2 was designed and synthesized for the detection of miR-let7a (Previous probe for the detection of miR-21 is named as Y-Py-block1 in the revised version). Y-Py-block2 was constructed by three DNA strands (Y-1-let7a, Y-2-let7a and Y-3), which has three extended sticky ends to recognize miR-let7a. Strands Y-1-let7a and Y-2-let7a were characterized and identified by ESI-MS as shown in Figure S12. The successful self-assembly of Y-Py-block2 by Y-1-let7a, Y-2-let7a and Y-3 was confirmed by 15% native PAGE analysis (Figure S13). The band in lane 4 (Y-Py-block2) migrated to the similar position as that of the lane 5 (Y-Py-block1), which proved the self-assembly of Y-shape nanostructure. Besides, Y-Py-block2 would self-assemble into a large dendrimer structure through recognizing miR-let7a as show in lane 6.

Thereafter, Y-Py-block2 probe was applied for intracellular miR-let 7a detection. As shown in Figure S14A, MCF-7 cells emitted a bright fluorescence in the cytoplasm, demonstrating that Y-Py-block2 probe could be applied for intracellular detection of miRNAs. For A549 cells, very weak fluorescence was observed because of the low expression of miR-let 7a in this cell line (Figure S14B). All these results indicate that Y-Py-block2 probe can detect miR-let 7a with high sensitivity and selectivity, which also verifies the universality of the proposed strategy.

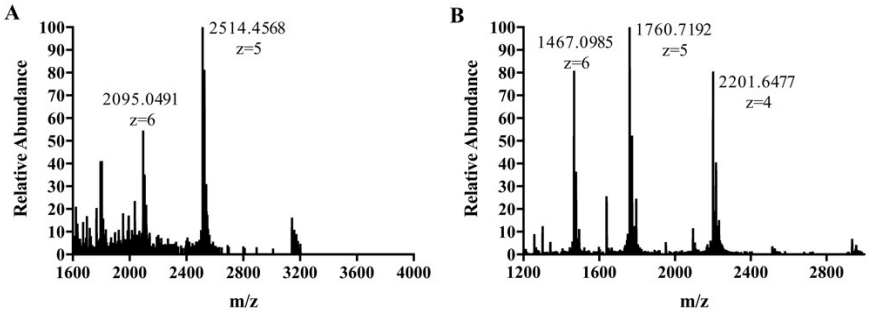

Figure S12. ESI-MS spectrum of (A) Y-1-let7a: m/z=2514.4568, z=5 (12577.3 theoretical) and (B) Y-2-let7a: m/z=1760.7192, z=5 (8809.8 theoretical).

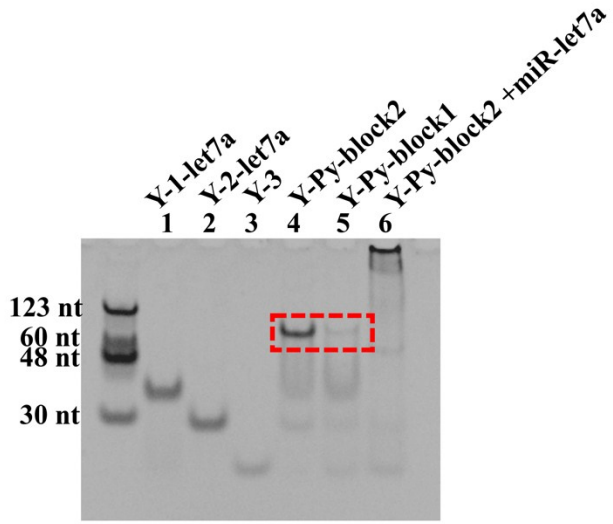

Figure S13. Electrophoresis characterization by 15% Native PAGE. (Lane 1: Y-1-let7a; lane 2: Y-2-let7a; lane 3: Y-3; lane 4: Y-Py-block2; lane 5: Y-Py-block1; lane 6: Y-Py-block2+miR-let7a. [Y-1-let7a] = [Y-2-let7a] = [Y-3] = [Y-Py-block1] = [Y-Py-block2] =0.5 μM, [miR-let 7a] =0.1 μM.

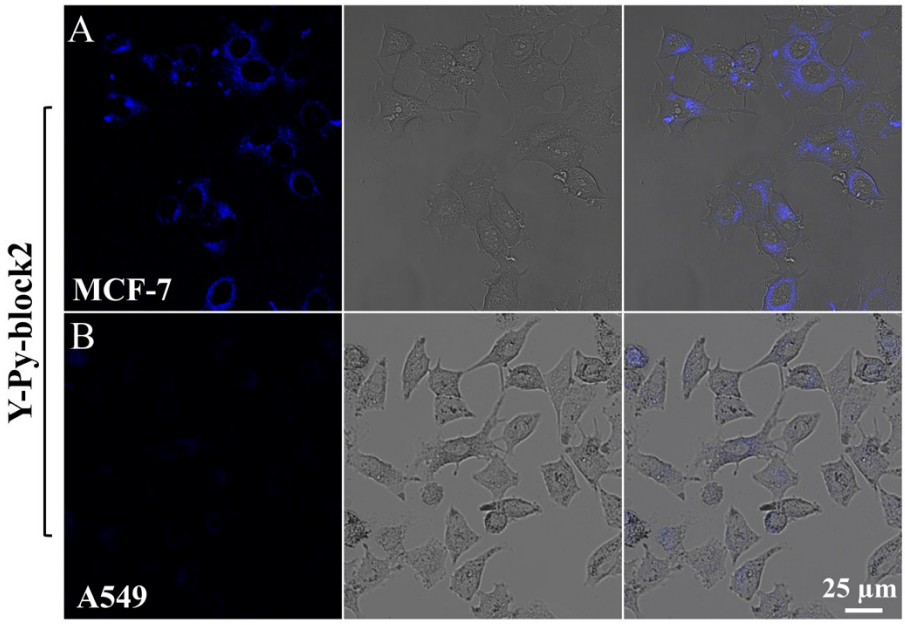

Figure S14. The confocal fluorescence images of MCF-7 cells (A) and A549 cells (B) incubated with Y-Py-block2.

18. The high specificity and selectivity of the proposed method

As for the high specificity and selectivity of this method, there are two important reasons. (1) The recognition of miRNA of pyrene-based DNA probe is based on two kinds of interactions; except base-pairing of nucleic acid hybridization, π-π interaction of pyrene molecules also plays an important role (as shown in Figure S15A). The similar effect was reported by Wengel et al. that the sensing sensitivity of pyrene-based DNA probe was affected by the position of mismatch.[1] The position that would

more affect the formation of pyrene excimer could be more sensitively detected by the probe. It is different from the fluorophore/quencher pair-based probe which is only controlled by distance-dependent FRET, pyrene-based DNA probe required a proper local conformation for pyrene π-π stacking, which make the probe more sensitive to mis-matches. (2) As Y-Py-block is a three-dimensional (3D) DNA self-assembly structure, which shows higher cell uptake, more resistance to enzyme degradation, and higher spatial resolution for imaging. In addition, due to the higher steric hindrance and electrostatic repulsion, the self-assembly of Y-Py-block in the presence of microRNA has to overcome a higher energy barrier compared with the pyrene-modified single-stranded DNA (as shown in Figure S15B). This effect also makes Y-Py-block probe more sensitive to mismatches in the targets. According to our result, for detection of the target and the one mismatch interference, Y-Py-block1 showed about 3-time difference while the pyrene-modified single-stranded DNA probes showed about 2-time difference (as shown in Figure S15C-D).

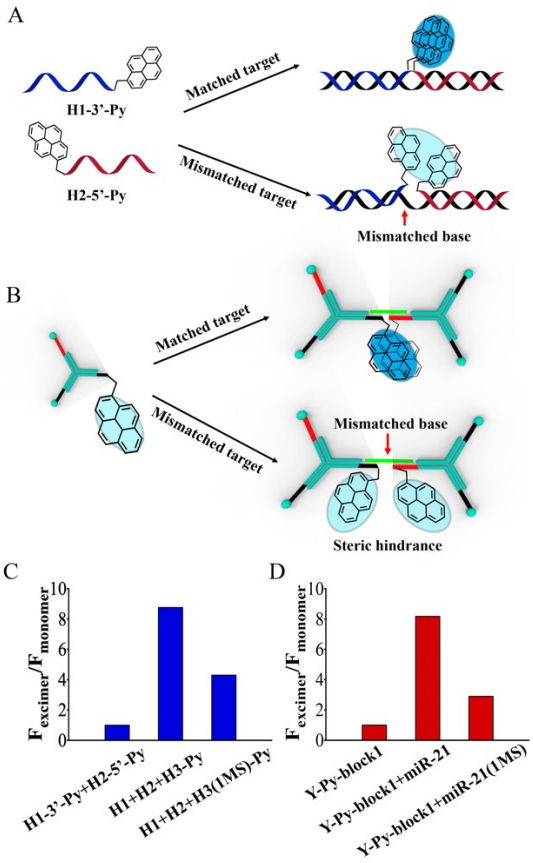

Figure S15. (A) Schematic representation of the ternary complex constructed by fully matched target or mismatched target. (B) Schematic representation of the Y-shaped DNA probes in response to fully matched target or mismatched target. (C) Ratiometric response of the fully matched target and mismatched target of the ternary complex. (D) Ratiometric response of the fully matched target and mismatched target of the Y-Py-block1.

Table S2. The summary of recent miRNA analytical methods in comparison with our strategy.

Detection method	Target	Detection limit	Specificity	Universality	Reference
Colorimetry	miR-21	3.2 nM	miR-125b/ miR-29a	No mention	2
Fluorescent biosensor	miRNA	1.7 nM	one-base mismatch	No mention	3
Fluorescent biosensor	miR-21	3 nM	one-base mismatch	No mention	4
Fluorescent biosensor	miR-21	330 pM	miR-155/ miR-125b/ miR-145/ miR-10b	No mention	5
Fluorescent biosensor	miR-21	300 pM	miR-let 7f/ miR-222/ miR-141	No mention	6
DNAzyme based amplification	miR-21	100 pM	one-base mismatch	No mention	7
DNA tetrahedrons based fluorescent biosensor	miR-21	620 pM	miR-155/ miR-141/ miR-let 7a/ miR-199a	miR-155	8
Pyrene-labelled Y-shape DNA nanoprobe based fluorescent biosensor	miR-21	200 pM	one-base mismatch	miR-let 7a	This work

Reference:

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