Electronic Supporting Information

Three-Dimensional DNA Nanostructures for Dual-color MicroRNAs Imaging in Living Cells via Hybridization Chain Reaction

EXPERIMENTAL SECTION

Chemicals and Materials. Oligonucleotides used in this work were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. All the sequences are listed in Table S1. Lipofectamine 3000 and Opti-MEM were purchased from Invitrogen (MA, USA). HeLa cells (human cervical carcinoma cell line), MCF-10A (human mammary epithelial cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). RPMI 1640 medium, penicillin, streptomycin and 10% heat-inactivated fetal bovine serum were obtained from Thermo Scientific HyClone (MA, USA). All reagents were used as received without further purification. All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ultrapure water was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.25 MΩ.

Instruments. The fluorescence measurements were carried out at room temperature in a quartz cuvette on an FL-7000 spectrometer (Hitachi, Japan). For the Real-time fluorescence intensity study, Tecan Finite M1000 (Tecan, Switzerland) was used. Agarose gel was visualized via a Tanon 4200SF gel imaging system (Tanon Science & Technology Co., Ltd., China). The atomic force microscopy (AFM) image was performed by means of Bruker Bioscope system (Bruker, USA). All fluorescence images were acquired using an oil dipping objective (60×) on Nikon TI-E+A1 SI confocal laser scanning microscope (Japan).

Preparation and characterization of TYH probe. Before the construction of TYH probe, all oligonucleotides were solved in TE buffer and the concentrations were determined by NanoDrop ND-2000. TYH probe was prepared by simultaneous assembly of DNA units. Namely, self-assembled DNA tetrahedron (SDT) was synthesized by mixing four customized oligonucleotide strands (S-A0, S-B0, S-C0 and S-D0) in equimolar in TM buffer (20 mM Tris, 5 mM $MgCl_2$, pH=8.0), heated to 95℃ for 5 min and then immediately cooled on ice for more than 15 min. Simultaneously, Y-shaped DNA adaptors were formed and annealed with DNA hairpin probes. Subsequently, SDT was incubated with the as-prepared YH probes in 1:4 molar ratio, formed a 3D DNA architecture with four pairs of DNA hairpin probes.

Gel electrophoresis analysis. Without further purification, synthesized TYH and TYH-HCR products were dissolved in 1× PBS to give a final concentration of 0.5 μM. Ten μL of different

DNA were used for electrophoresis experiments. Electrophoresis was carried out in 0.5×Tris-borate-EDTA (TBE) buffer (45 mM Tris, 45 mM boric acid, and 5 mM EDTA, pH 8.0) at 100 V for 1 h.

AFM imaging. Atomic force microscopy of samples was observed on a Multimode 8 (Bruker/USA) using ScanAsyst mode in ambient air. A 10 μL DNA sample was placed onto the surface of freshly cleaved mica and allowed to adsorb to the mica surface for approximately 30 minutes. The mica was then rinsed in Milli-Q water and dried with compressed air.

DLS characterization. The hydrodynamic diameters of the DNA nanostructure under investigation were measured using a Zetasizer Nano ZS90 DLS system equipped with a red (633 nm) laser and an Avalanche photodiode detector (APD) (quantum efficiency > 50% at 633 nm) (Malvern Instruments Ltd., Worcestershire, England). A Kuvetten cuvette (10 mm) was used as a sample container. DLS measurements were performed at room temperature at a fixed scattering angle of 90°.

Fluorescence experiments in vitro. The ability for TYH to detect miRNA targets was performed as follows: TYH probes (250 nM) were incubated with miRNA target (100 nM) at 37 °C for 3 h in 20 μL aliquot of 1×PBS buffer, 80 μL aliquot of 1×PBS buffer were added to the sample and the fluorescence were determined by F-7000 fluorescence spectrometer. FAM fluorescence emission signal was recorded from 505 to 610 nm in 0.2 nm increment, under an excitation wavelength of 492 nm, and Cy3 fluorescence emission signal was recorded from 550 to 650 nm in 0.2 nm increment, under an excitation wavelength of 535 nm. The concentration of TYH used for the fluorescence calibration curve assays was 250 nM in 1×PBS (pH=7.4), and treated with target of a series of concentrations. The fluorescence signal without target was recorded as the background signal. All experiments were repeated at least three times.

Cell culture. HeLa cells were grown in RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum, 100 U/ml 1% penicillin and streptomycin. MCF-10A cells were cultured in DMEM/F-12 medium supplemented with 5% horse serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 mg/mL insulin, 10 ng/mL epidermal growth factor, and 1% L-glutamine. All cells were cultured in a humidified CO_2 incubator containing 5% CO_2 at 37°C.

Fluorescence imaging (Fluorescence experiments in living cells**).** For confocal imaging studies, cells were cultured on 35-mm confocal laser culture dishes with the same medium for 24 h. Then the medium was removed and the cells were washed three times using Dulbecco's PBS (DPBS, 1 mL each time). Next, 1 mL medium (DMEM/RPMI-1640) containing 100 nM TYH probes was added to cells. After culturing for another 6 hours, the medium was removed and the cells were washed three times using Dulbecco's PBS (DPBS, 1 mL each time). Then, 1 mL medium was added for fluorescence imaging. All fluorescence images were acquired using an oil dipping objective on Nikon TI-E+A1 SI confocal laser scanning microscope (Japan). Fluorescence of FAM

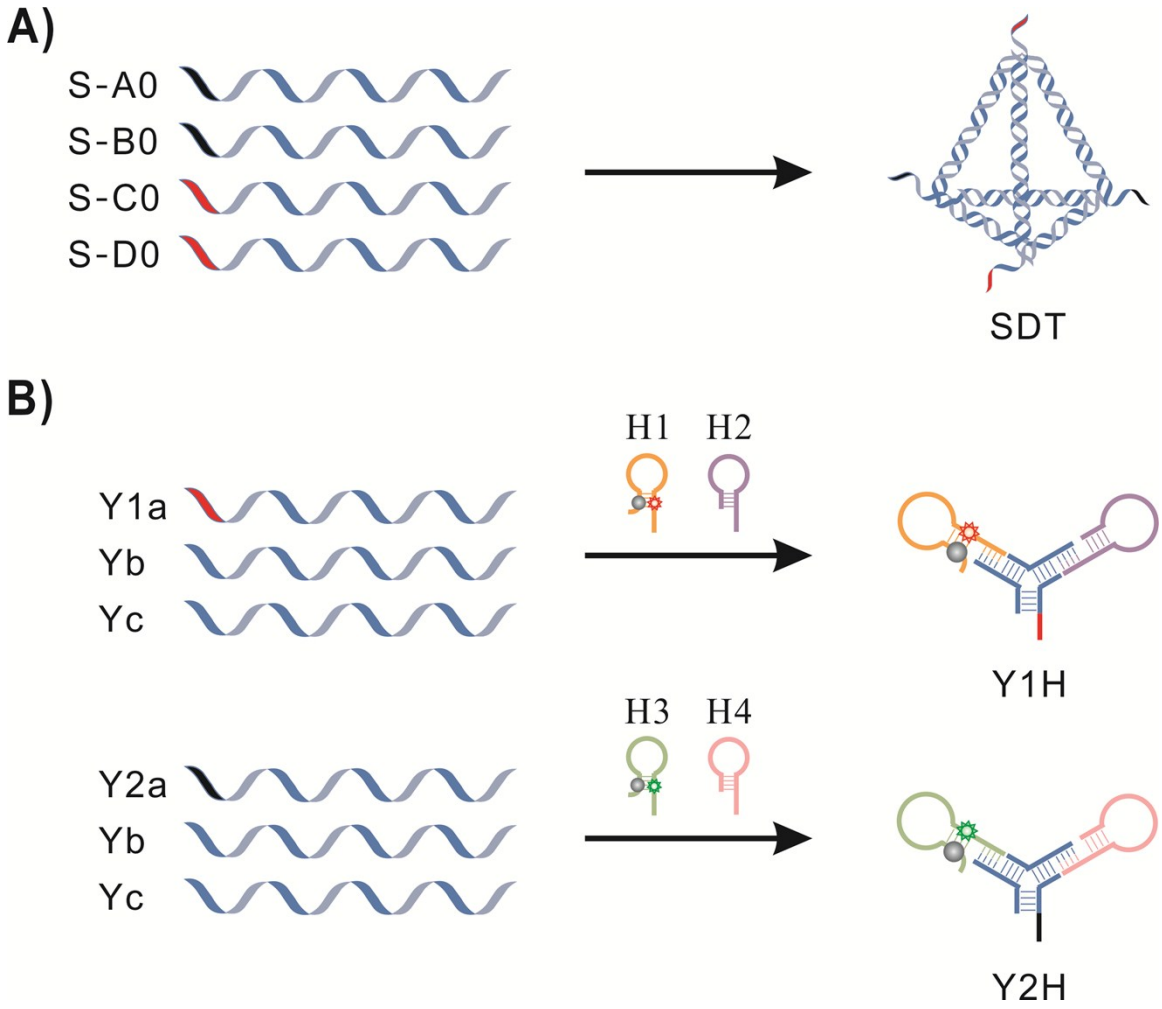
was obtained by excitation at 488 nm and collected over 510-550 nm; Cy3 was obtained by excitation at 535 nm and collected over 575-610 nm; Cy5 was obtained by excitation at 640 nm and measured over 670-720 nm;

Cytotoxicity assay (MTT). To investigate the cytotoxicity of TYH probes, MTT assay was carried out when the probes existed. HeLa cells were dispersed with replicate 96-well microtiter plates at a density of 1×10^4 cells/well. Plates were then maintained at 37°C in 5% CO_2 atmosphere for 24 h. Thereafter, the cells were treated with varying concentrations of probes (50, 100, 200 nM) for different times (6, 12, 24 h) and 100 μL MTT solutions were then added to each well for 4 h. After removing the remaining MTT solution, 150 μL DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a VersaMax Tunable Microplate Reader (VWR International, USA).

Table S1. Sequences of oligonucleotides used in this work.

Name	sequences (5'-3')
S-A0	ACATTCCTACGCCTGAATCCTTACAGCTTGCTACACGTGA AGATCCGCCATAGTG TTTTT CAGGAACGTCATGGA
S-B0	TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAAGACA TGCGAGGGTCCAATAC TTTTT CAGGAACGTCATGGA
S-C0	TTCAGGCGTAGGAATGTACTTCCCACGTAATGTCGTTTGT ATTGGACCCTCGCAT TTTTT TCCAGTGGCTCAAGC
S-D0	TCAACTGCCTGGTGATATTACGACATTACGTGGGAATCCA CTATGGCGGATCTTC TTTTT TCCAGTGGCTCAAGC
Y1a	AGGCTGATTCGGTAGTTATGCGGATCGATGCTTGAGCCA CTGGA
Y2a	AGGCTGATTCGGTAGTTATGCGGATCGATTCCATGACGTT CCTG
Yb	CATTACGGCGACTGTACCGAATCAGCCTAGACCGATGGA TGAAG
Yc	TCGATCCGCATAACCAGTCGCCGTAATGTGAAGCCACTCT GATC
Yc(Cy5)	(Cy5)TCGATCCGCATAACCAGTCGCCGTAATGTGAAGCCA CTCTGATC
H1	TCAACATCAGTCTGAT(BHQ-2)AAGCTAATCTAGCTTAGCTTAT(Cy3)CAGACTGGATCAG AGTGGCTTC
Non-labeled H1	TCAACATCAGTCTGATAAGCTAATCTAGCTTAGCTTATCA GACTGGATCAGAGTGGCTTC
H2	TAGCTTATCAGACTGATGTTGATTCAGTCTGATAAGCTAA GCTAGCTTCATCCATCGGTC
Random-H1	ATGCACTCAGTCTGAT(BHQ-2)AAGCTAATCTAGCTTAGCTTAT(Cy3)CAGACTGGATCAG AGTGGCTTC
Random-H2	TAGCTTATCAGACTGAAGTCGTATCAGTCTGATAAGCTAA GCTAGCTTCATCCATCGGTC
H3	CTAGTGGTCCTAAACAT(BHQ-1)TTCACTCAAGTGTGAAAT(FAM)GTTTAGGACGATCAGA GTGGCTTC
Non-labeled H3	CTAGTGGTCCTAAACATTTCACTCAAGTGTGAAATGTTTA GGACGATCAGAGTGGCTTC
H4	GTGAAATGTTTAGGACCACTAGAGTCCTAAACATTTCACA CTTGACTTCATCCATCGGTC
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-203	UGAGGUAGUAGGUUGUAUAGUU
miR-141	UAACACUGUCUGGUAAAGAUGG
miR-145	GUCCAGUUUUCCCAGGAAUCCCU

let-7a	UGAGGUAGUAGGUUGUAUAGUU



Scheme S1. Synthesis of self-assembled DNA tetrahedron (A) and construction of Y-H probes (B).

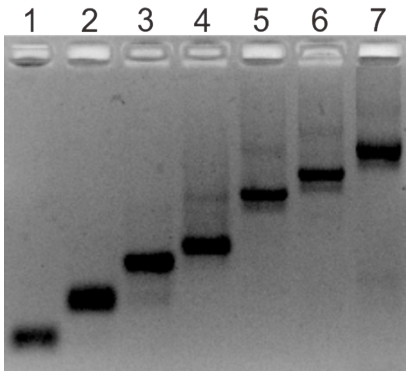


Fig. S1 Agarose gel analysis of the synthesis of TYH probe. Lane M: marker, lane 1: S-A0, lane 2: S-A0+S-B0, lane 3: S-A0+S-B0+S-C0, lane 4: S-A0+S-B0+S-C0+S-D0 (SDT), lane 5: SDT+Y (TYN), lane 6: SDT+Y+H1 (TYH1); lane 7: SDT+Y+H1+H2 (TYH). The concentrations of DNA used in gel electrophoresis were 1, 0.5, 0.4, 0.3, 0.15, 0.1, 0.1 μM respectively.

With the addition of DNA strands from lane 1 to lane 6, a significant reduction of electrophoretic mobility could be observed, which was attributed to the increased molecular size and more complex spatial construction. The result indicated that the DNA nanostructure has been successfully formed.

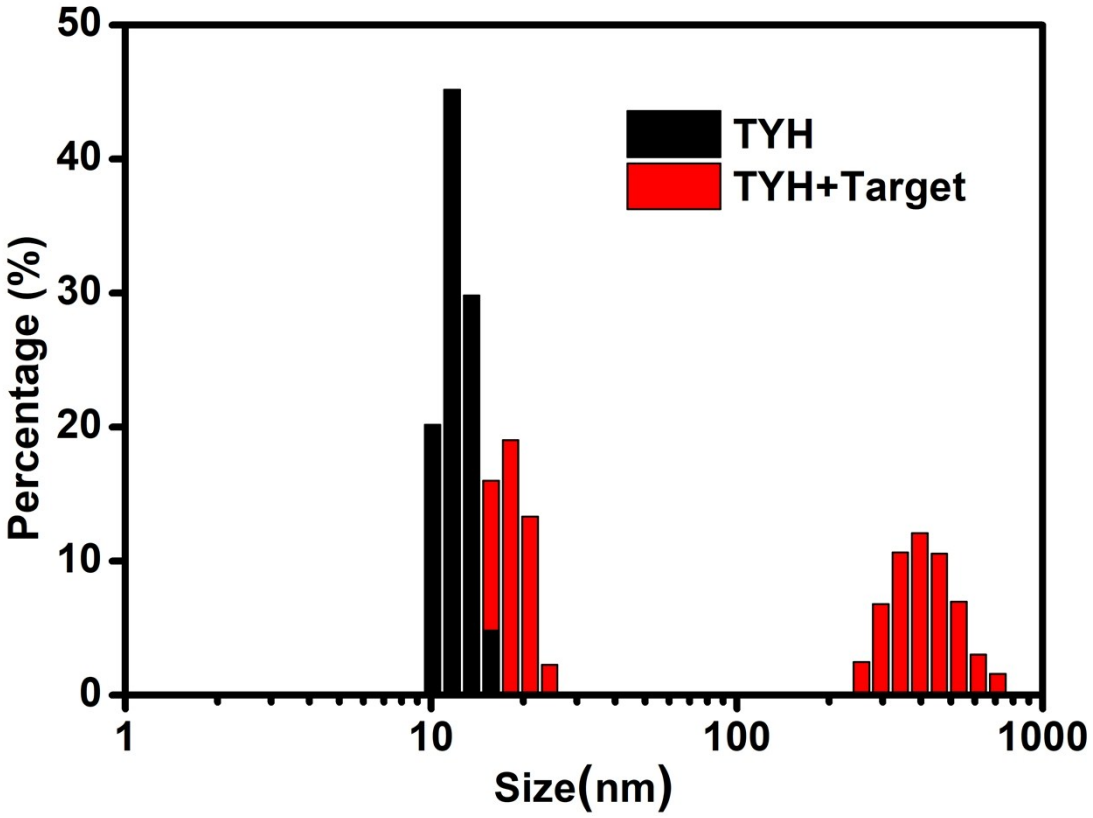


Fig. S2 DLS analysis for TYH probes (black) and TYH probes response to target miRNA (red). The nominal concentration of TYH probes was 100 nM.

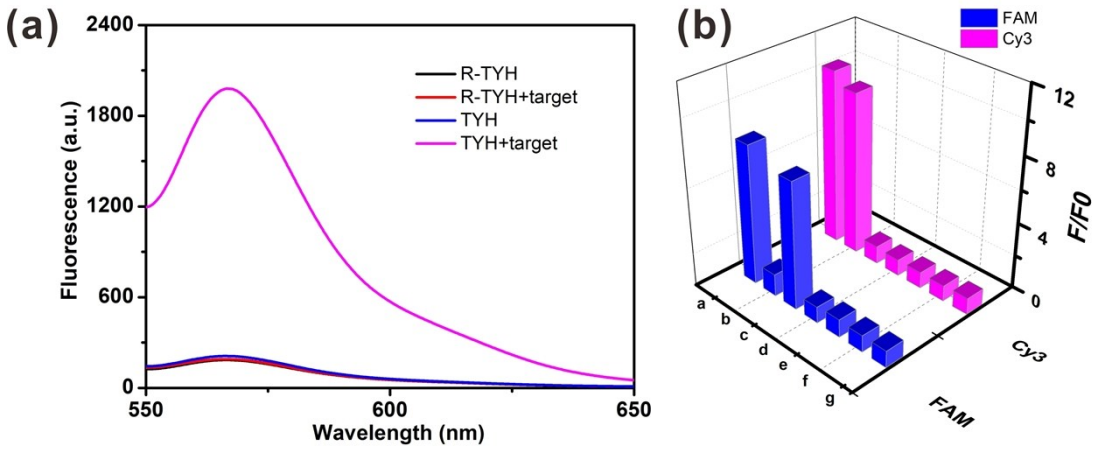


Fig. S3 (a) Fluorescence spectral responses obtained from reactions of nanoprobes with random sequence replaced hairpin probes (R-TYH) (black), R-TYH with 100 nM miR-21 (red), TYH (blue), and TYH with 100 nM target miRNA (violet). (b) Specificity evaluation of the TYDH probes for the target 100 nM miRNA-21 and 100 nM miR-203 against non-complementary miRNAs with the concentration of 200 nM. (a) TYDH probes+miR-21+miR-203; (b) TYDH probes+ miR-21; (c) TYDH probes+miR-203; (d) TYDH probes+miR-145; (e) TYDH probes+miR-141; (f) TYDH probes+let-7a; (g) TYDH probes.

Only the fluorescence of Cy3 was recovered in the present of the miR-21, while robust fluorescence enhancement of FAM was observed in the present of miRNA-203. Negligible fluorescence enhancement can be detected when non-complementary miRNAs were incubated with nanostructure. These results testified that TYH strategy was selective to the HCR reaction between target miRNA and its specific hairpin probes and can be applied in simultaneous detection of multiple target miRNAs.

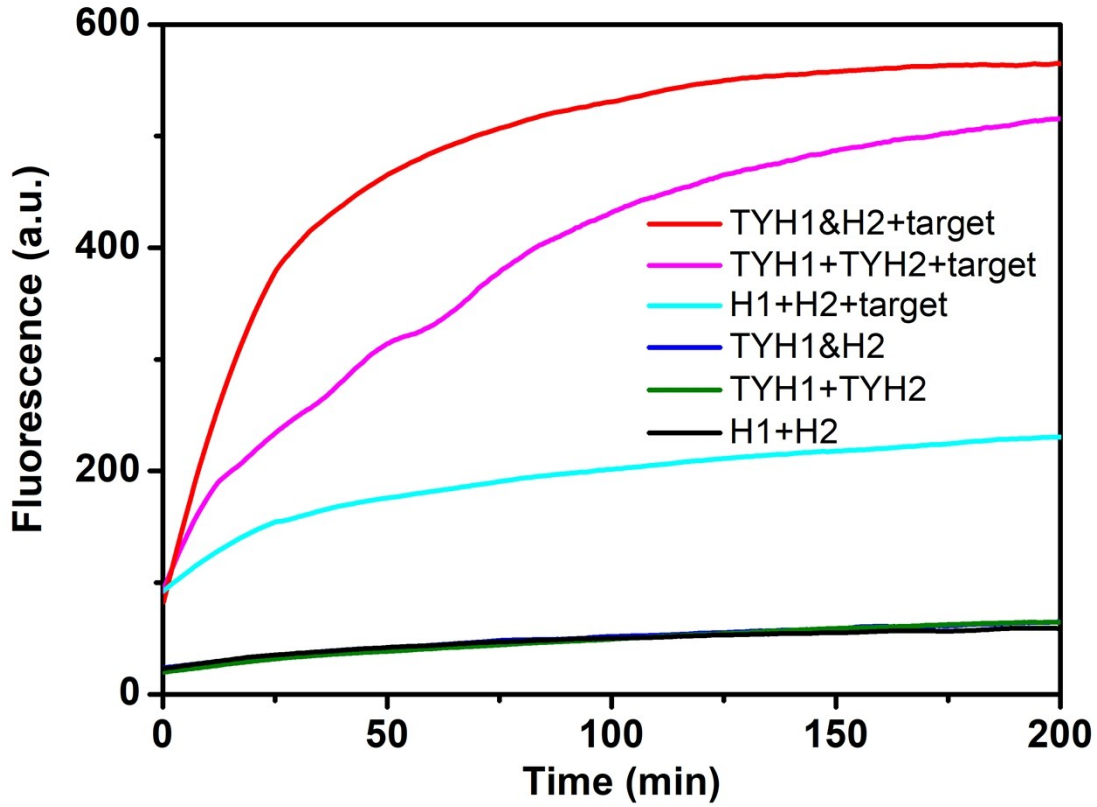


Fig. S4 Real-time fluorescence signals for 250 nM TYH probes in response to 100 nM RNA target (red), TYH only (blue), 250 nM TYH1 (without H2) and TYH2 (without H1) probes in response to 100 nM RNA target (purple), TYH1 and TYH2 only (green), HCR reaction of 1 μM H1 and H2 with 100 nM RNA target (cyan), and H1 and H2 only (black).

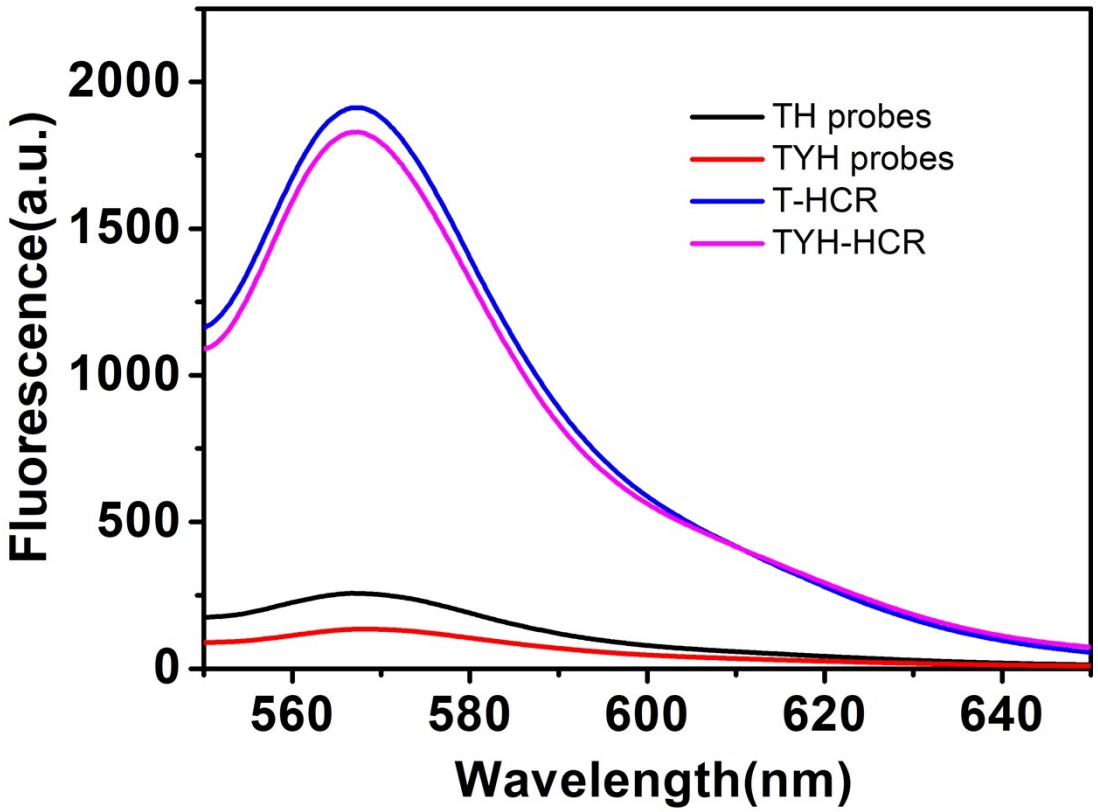


Fig. S5 Fluorescence spectral responses obtained from reactions of 500 nM TH (black), 250 nM TYH (red), 500 nM TH and 100 nM target miRNA (blue), and 250 nM TYH plus 100 nM target miRNA (purple).Signal to background ratio (S/N) of T-HCR was 7.4 and S/N of TYH-HCR was 9.7.

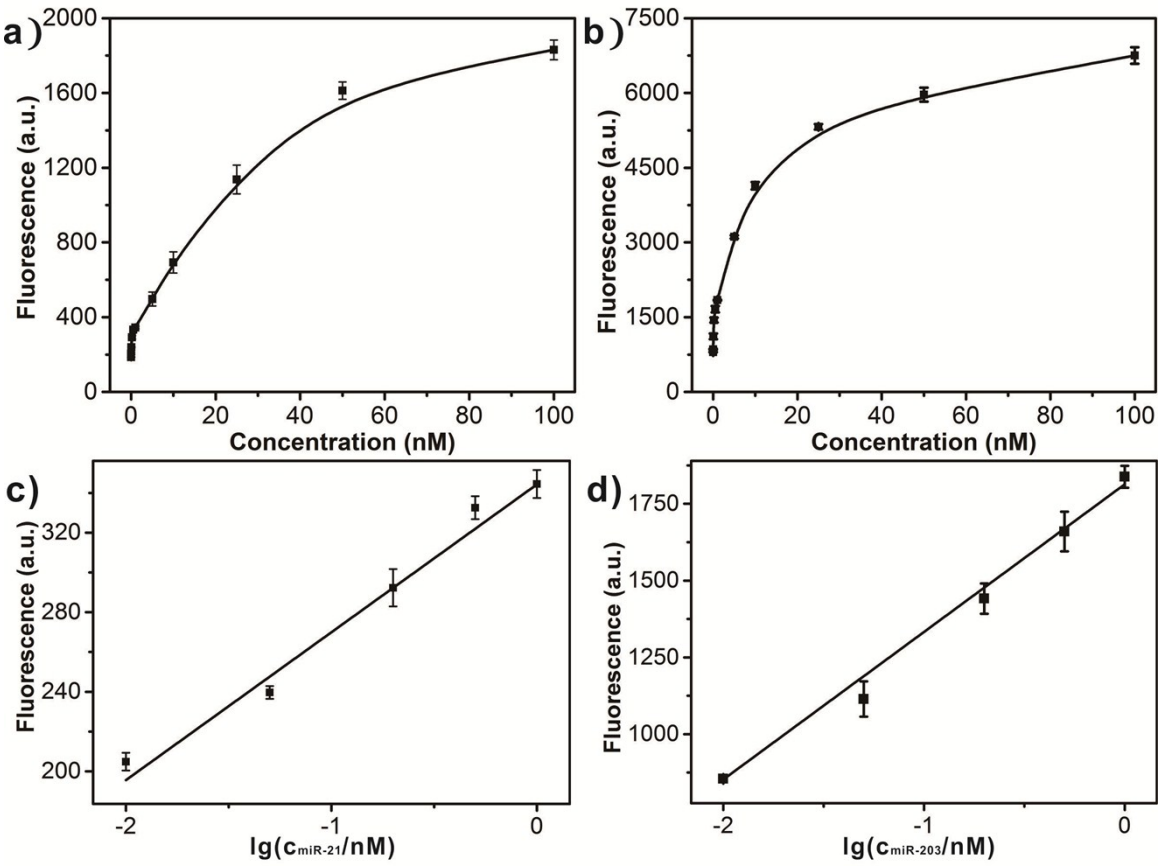


Fig. S6 Plot of fluorescence peak intensities versus miR-21 (a) and miR-203 (b) concentrations. Plot of fluorescence peak intensities versus logarithmic concentrations of miR-21 (c) and miR-203 (d). Error bars indicated standard deviations across three repetitive assays. The excitation/Emission wavelength used in the assay: 535/567 nm for miR-21 and 492/522 nm for miR-203.

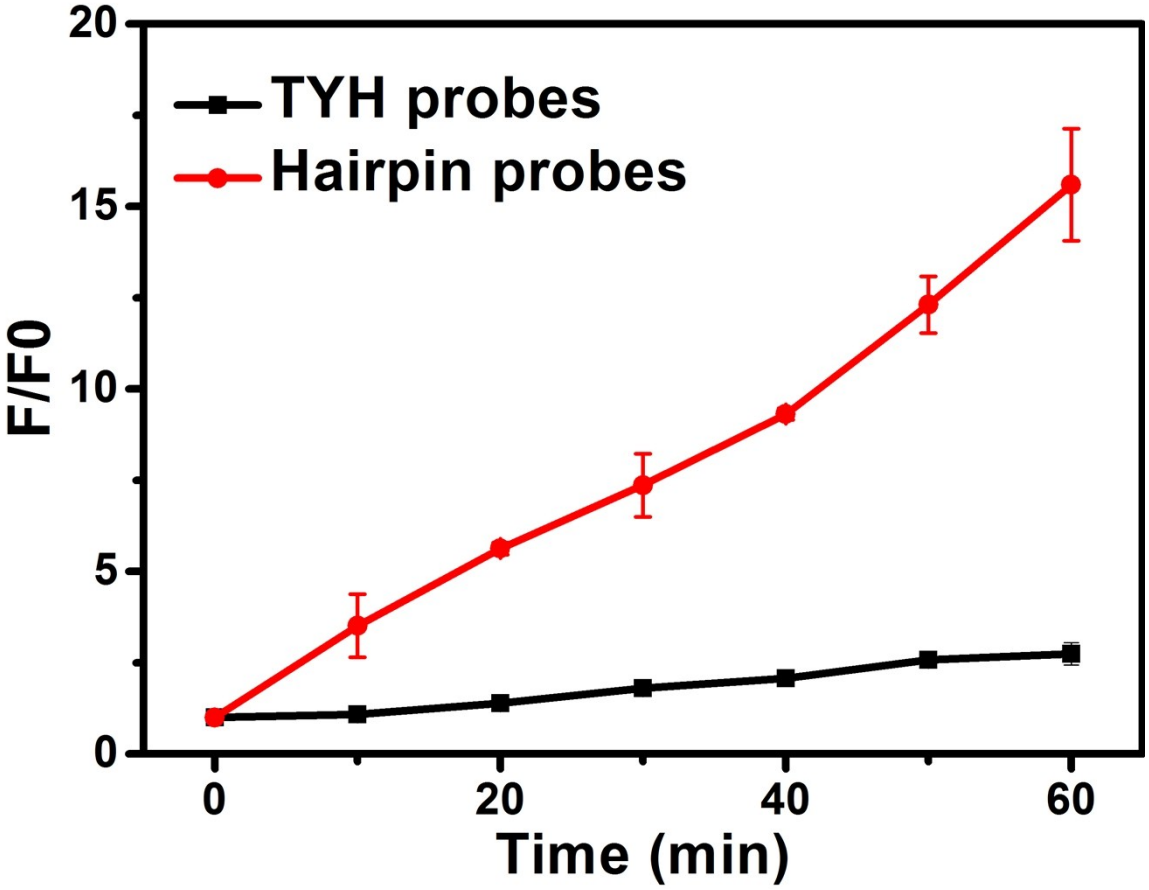


Fig. S7 Studies of nuclease (Exo III) digestion resistant ability between TYH probes and DNA probes. 250 nM TYH probes and 1 μM hairpin probes were treated by 4.0 U/mL Exo III for 0, 10, 20, 30, 40, 50, 60 min, and the ratio of fluorescence after Exo III digestion (F) to initial fluorescence (F0) were measured to estimate the nuclease resistant ability of TYH probes and DNA probes.

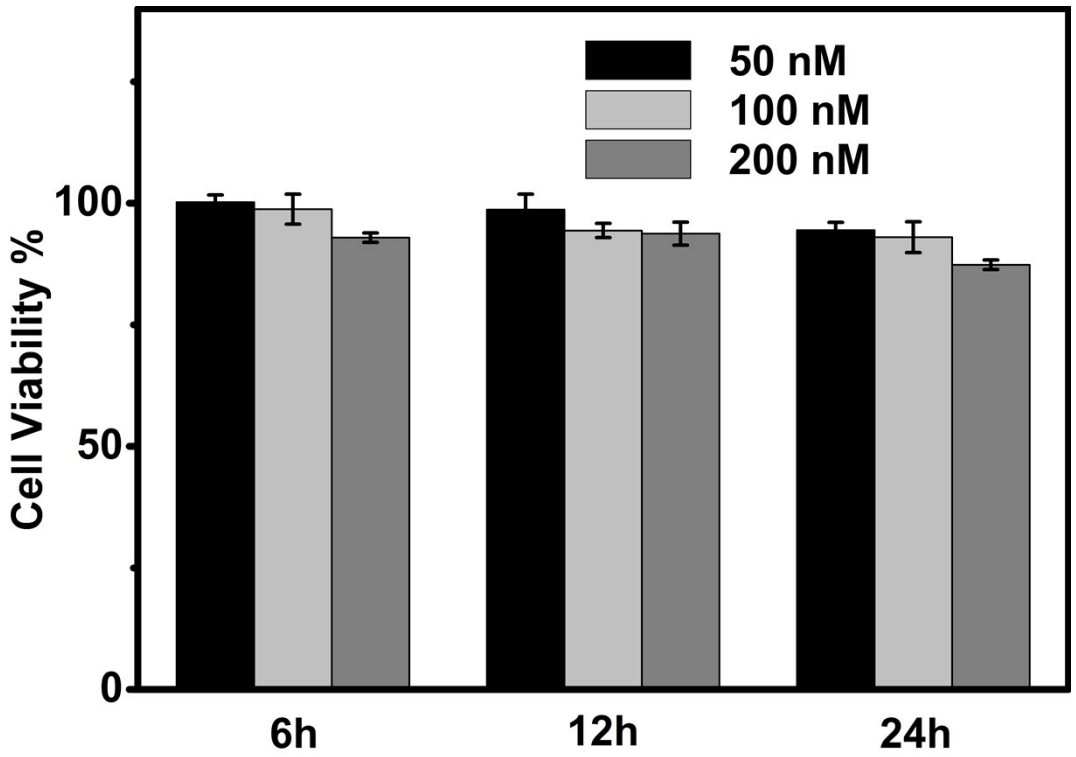


Fig. S8 Cell proliferation assay for cytotoxicity of TYH probes. The cell viability values (%) were determined by incubating HeLa cells with TYH probes of varying concentrations (0, 50, 100, 200 nM) for 6, 12, 24 h.

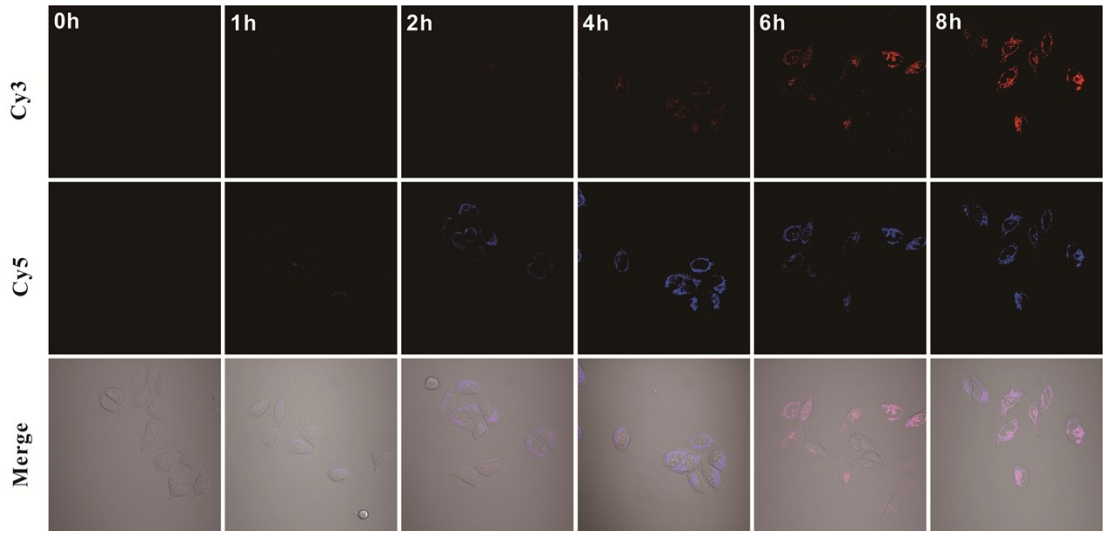


Fig. S9 Optimization of incubation time for TYH probes in living cells. HeLa cells were incubated with 100 nM probes for different time (0, 1, 2, 4, 6, 8h). Cy3 channel is for the fluorescent signal of miR-21 and Cy5 channel is for tracking the probes.

The fluorescence imaging data showed that the tracking signals of Cy5 fluorescence in HeLa cells were gradually increased and finally reached a ceiling with the incubation time up to 4 h, indicating that the internalization of TYH probes were accomplished in 4 h. Meanwhile, the Cy3 fluorescence started to lighten the cytoplasm after 2 h and finally reached a plateau after 6h, demonstrated that intracellular miRNA-generated TYH-HCR can be finished in 6 h. It suggested that 6 h incubation time should be appropriate for this research.

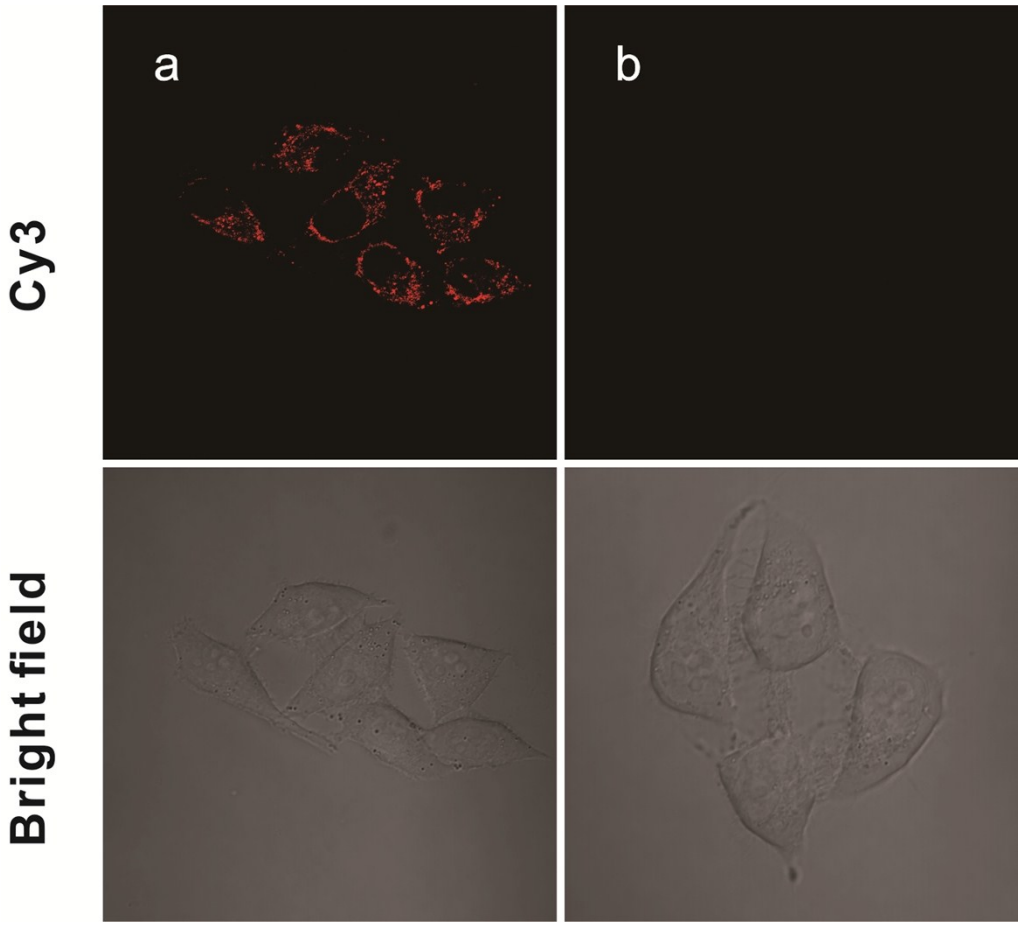


Fig. S10 Intracellular fluorescence imaging of miR-21 in HeLa cells incubated with TYH (a) and R-TYH (b).

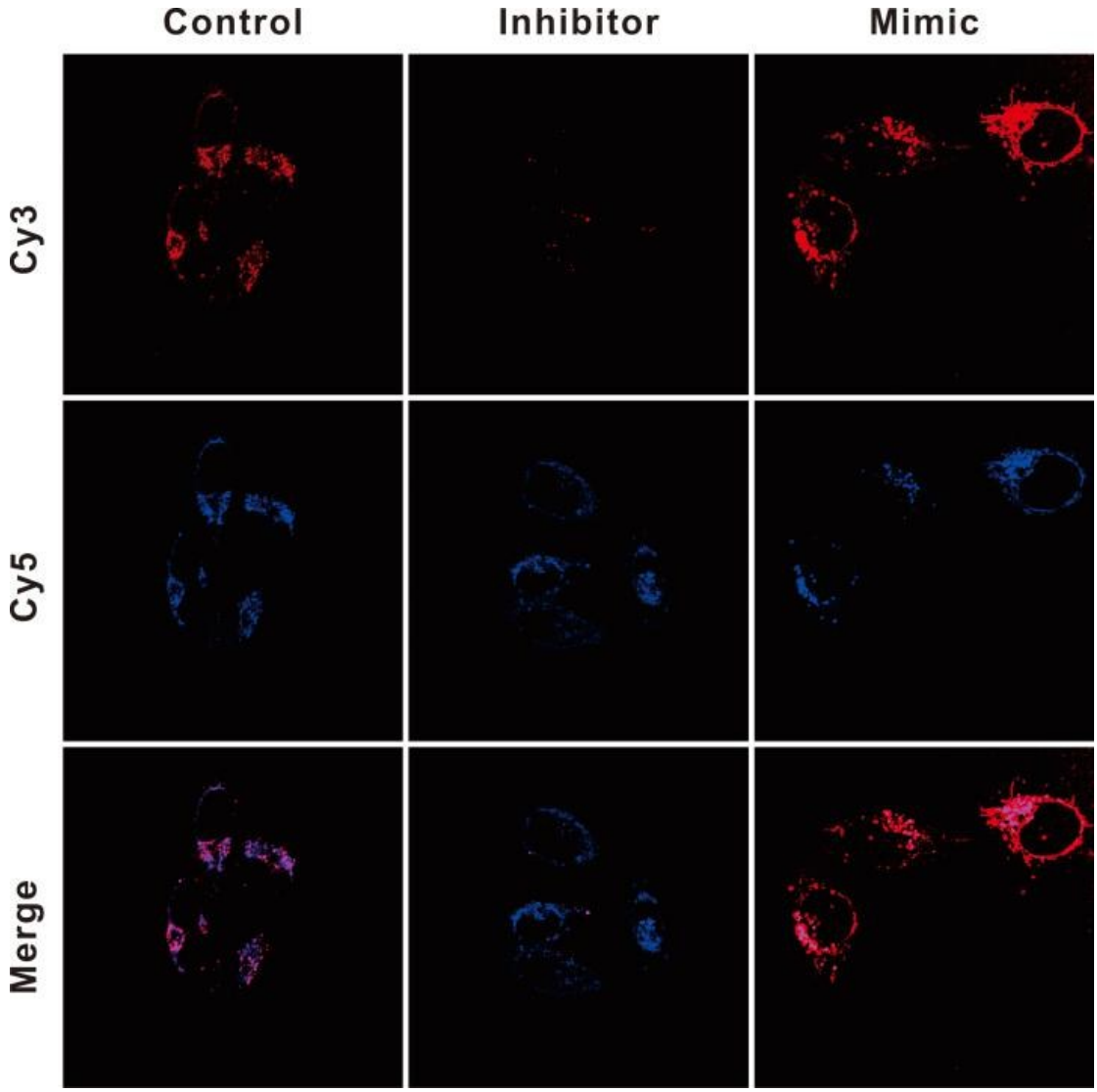


Fig. S11 Fluorescence images of different expression levels of miR-21 in HeLa cells (left), treated with 200 nM miR-21 inhibitor (middle) and 200 nM miR-21 mimic (right).Blue fluorescence indicates the existence of intracellular TYH and miR-21 were represented by red fluorescence.

HeLa cells were treated with chemically modified single-stranded RNAs which enable suppress the expression level of miRNA-21, and miRNA-21 level was down-regulated. Meanwhile, another group of HeLa cells were treated with miRNA-21 mimic, chemically modified double-stranded RNAs mimicking miRNA-21 and enabling up-regulation of miR-21. Fluorescence image with increased brightness was obtained, indicating that the miRNA was up-regulated obviously.

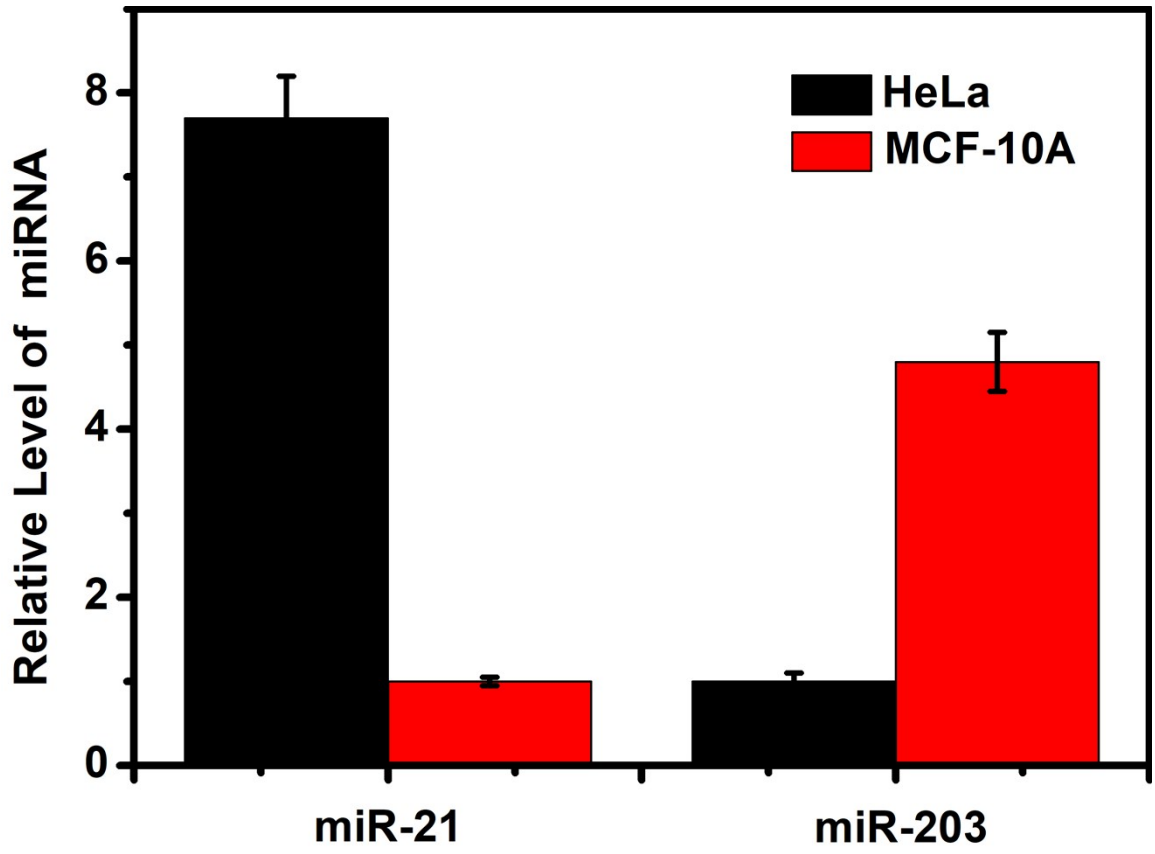


Fig. S12 Relative expression levels for miRNAs in HeLa and MCF-10A cells by qRT-PCR analysis.

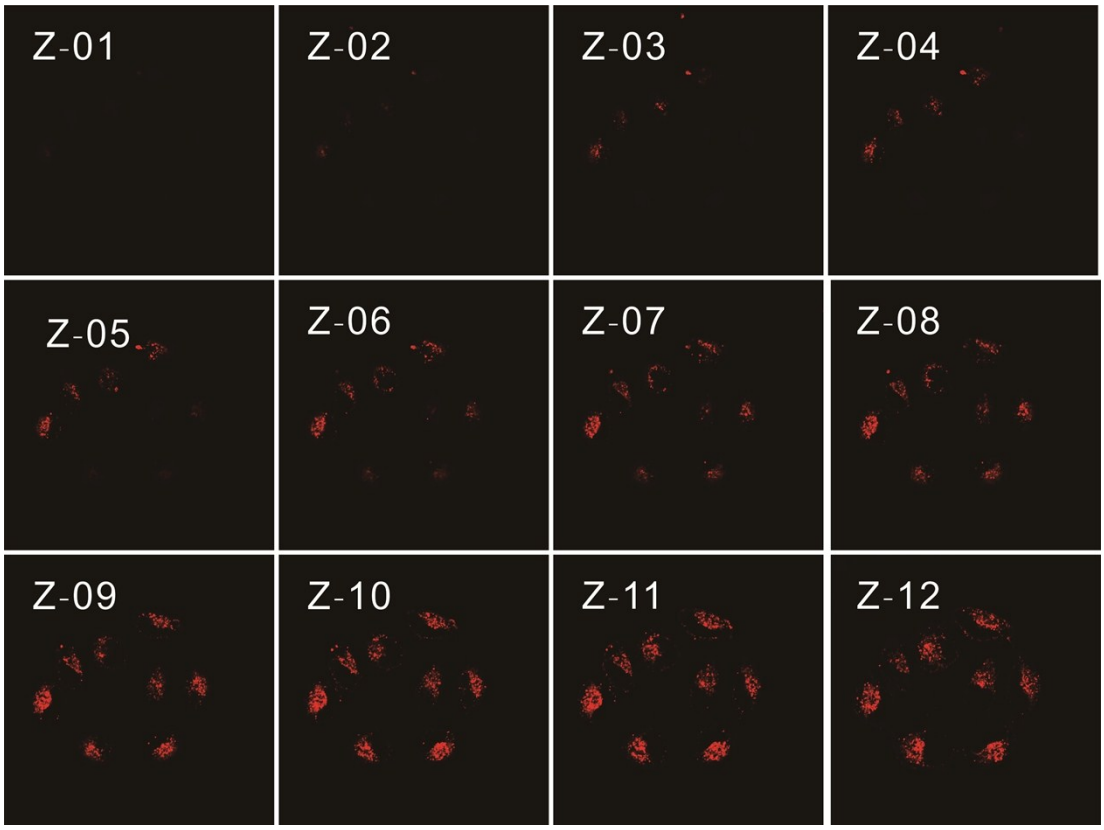


Fig. S13 Z-stacks analysis of target miR-21 in HeLa cells using TYH-HCR.