Supporting Information For

Protein Scaffolded DNA Tetrads Enable Efficient Delivery and Ultrasensitive Imaging of miRNA through Crosslinking Hybridization Chain Reaction

Du-Juan Huang‡, Zhi-Mei Huang‡, Hu-Yan Xiao, Zhen-Kun Wu*, Li-Juan Tang, Jian-Hui Jiang*

Institute of Chemical Biology & Nanomedicine, State Key Laboratory of Chemo/Biosensing & Chemometrics, College of Chemistry & Chemical Engineering, Hunan University, Changsha 410082, China.

E-mail: jianhuijiang@hnu.edu.cn; tomwu@hnu.edu.cn

Experimental Section:

Chemicals and materials

HeLa cells (human cervical carcinoma cell line) and L-02 cells (mouse endothelial cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). HepG2 cells (Human hepatocellular liver carcinoma cell line) were purchased from Cell Bank of the Committee on Type Culture Collection of Chinese Academy of Sciences (Beijing, China). Cell culture media was obtained from Thermo Scientific HyClone (MA, USA). LysoTracker Red DND-99 was obtained from Invitrogen (Carlsbad, CA). Lipofectmine 3000, miR-21 mimic (sense strand: 5'-UAGCUUAUCAGACU GAUGUUGA-3', antisense strand: 5'-AACAUCAGUCUGAUAAGGUATT-3') and inhibitor (5'-UCAACAUCAGUCUG AUAAGCUA-3') were purchased from Thermo Fisher (MA, USA). Streptavidin from Streptomyces avidinii (SA) and Cy3-conjugated Streptavidin (SA-Cy3) were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Oligonucleotides used in this study were synthesized and purified through HPLC by Sangon Biotechnology Co., Ltd. Sequences of the synthesized oligonucleotides are given in Supporting Information Table S1.

Gel electrophoresis analysis

The different concentration ratios of H3 to SA were added to the 1x PBS buffer, forming four types of SA-DNA complexes. These samples were analyzed using 3% agarose gel electrophoresis in 0.5x TBE buffer at room temperature. The gel was stained using GelRed. Electrophoresis was performed at a constant voltage of 110 V for 90 min with a load of 10 μL of sample in each lane. After electrophoresis, the gel was visualized using a Tocan 240 gel imaging system (Tocan Biotechnol. Co., Shanghai, China).

All the HCR-related experiments were performed in 20 μL aliquot of 1×TAE-Mg_{2+} buffer (10 mM Tris, 12.5 mM $MgCl_2$, pH 7.6) incubating at 37 °C for 4 h. The HCR products were also analyzed using 2% agarose gel electrophoresis in 0.5x TBE buffer at room temperature. The gel was stained using 0.5 μg/mL GoldView and 0.5 μg/mL ethidium bromide (EB). Electrophoresis was performed at a constant voltage of 100 V for 2 h with a load of 10 μL of sample in each lane. After electrophoresis, the gel was visualized using a Tocan 240 gel imaging system (Tocan Biotechnol. Co., Shanghai, China).

In vitro fluorescence assay

To prepare DNA hairpins for DNA tetrads, the all hairpins were kept at 95 °C for 5 min, then quickly treated with ice for 5 min, and finally transferred to room temperature for 2 h before further use. 1x PBS solution was used to dissolve the hairpins. Subsequently, appropriate SA were added to form DNA tetrads. DNA tetrads was incubated with RNA target of a varied concentration in 50 μL 1×TAE-Mg_{2+} buffer at 37 °C for 4 h. Fluorescence spectra were recorded using a Fluorescence spectrometer (Jobin Yvon Horiba) with excitation at 540 nm. The slit was set to be 5 nm for both the excitation and the emission.

To evaluate its selectivity, the DNA tetrads was also used for detecting other co-existing cellular components including miR-210, miR-214, let-7a, 100 nM β-actin protein, L-02 cells lysate. Additionally, one more sample was incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Cell culture and confocal microscopy imaging

HeLa cells and L-02 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin. HepG2 cells were incubated in DMEM supplemented with 10% fetal bovine serum. All cell lines were maintained at 37 °C in a 100% humidified atmosphere containing 5% CO_2. The cell density was determined using a TC20 automated cell counter (BIO-RAD, USA).

Fluorescence imaging of cells was performed as follows: The cells were washed three times with 1x PBS, then incubated with a fresh medium containing the given amount of DNA tetrads or Lipofectamine 3000 at 37 °C for 4 h. After washing three times with 1x PBS, the cells were incubated with 1 mL fresh medium at 37 °C before imaging.

Fluorescence imaging of lysosome escape of DNA tetrads was performed as follows: HeLa cells were incubated with 500 μL culture medium containing the given amount of DNA tetrads at 37 °C for varying time. After washing three times with 1x PBS, the cells were incubated with fresh RPMI 1640 medium containing 50 nM Lysosome traker (Lyso@tracker Red) for 10 min followed by imaging.

Fluorescence imaging of cellular uptake for DNA tetrads was performed as follows: Cells were incubated with 500 μL culture medium containing the given amount of DNA tetrads at 37 °C for 1 h. The cells were washed three times with 1x PBS before imaging. For cellular uptake experiment at 4 °C, cells were first incubated at 4 °C for 0.5 h and then grown in 500 μL culture medium containing the given amount of DNA tetrads at 4 °C for 1 h followed by imaging. For cellular uptake experiment in the presence of NaN_3, cells were first pretreated at 37 °C for 1 h with 1 mL culture medium containing

1% NaN_3 and then incubated with 500 μL culture medium containing the given amount of DNA tetrads and 1% NaN_3 at 37 °C for 1 h followed by imaging.

All fluorescence images were acquired using an oil dipping objective on Nikon TI-E+A1 SI confocal laser scanning microscope (Japan). Fluorescence of Cy3 and LysoTracker Red DND-99 was obtained by excitation at 561 nm and collected over 570-620 nm; Fluorescence of Cy5 was obtained by excitation at 640 nm and measured over 670-720 nm; Fluorescence of FRET was obtained by excitation at 561 nm and measured over 670-720 nm.

qRT-PCR quantification of miR-21 expression

Total cellular RNA was extracted from HepG2, HeLa, L-02 cells using the SanPrep Column microRNA Mini-Preps Kit (Sangon Co.Ltd., Shanghai, China) according to the indicated protocol. The cDNA samples were prepared with the PrimeScript™ II 1st strand cDNA Synthesis Kit according to the above described protocol for reverse-transcription of miRNA. The PCR process were as follows: The 20 μL reaction solution (contained 2 μL cDNA sample, 10 μL 2x SybrGreen qPCR Master Mix, 0.4 μL upstream primer (10 μM), 0.4 μL downstream primer (10 μM) and 7.2 μL nuclease-free water) staying at 95 °C for 3 min, then followed by 40 cycles of 95 °C for 7 s, 57 °C for 10 s and 72 °C for 15 s. The primers used were: miR-21 forward primer, 5’-ACACTCCAGCTGGGTAGCTTATCAGACTG-3’; miR-21 reverse primer, 5’-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC-3’; U6 forward primer, 5'-CTCGCTT CGGCAGCACA-3'; U6 reverse primer, 5' -AACGCTT CACGAATTTGCG T-3'.

Table S1. Sequences of synthesized DNA probes

Name	Sequences (5'-3')
Hairpin probe H1	Biotin-TTT TTT TTT TTT TTT TTC AAC ATC AGT CTG ATA AGC TAC T(Cy3) AA GTT AGC TTA TCA GAC TG
Hairpin probe H2	Biotin-TTT TTT TTT TTT TTT TTA GCT TAT CAG ACT GAT GTT GAC AGT CTG ATA AGC TAA CTT AG-Cy5
Hairpin probe H3	Biotin-TTT TTT TTT TTT TTT TTC AAC ATC AGT CTG ATA AGC TAC TAA GTT AGC TTA TCA GAC TG
Hairpin probe H4	Biotin-TTT TTT TTT TTT TTT TTA GCT TAT CAG ACT GAT GTT GAC AGT CTG ATA AGC TAA CTT AG-
Linear probe L1	Biotin-TTT TTT TTT TTT TTT TTC AAC ATC AGT CTG ATA AGC TAC TAA GT-Cy5
miR-21	UAG CUU AUC AGA CUG AUG UUG A
miR-210	CUG UGC GUG UGA CAG CGG CUG A
miR-214	ACA GCA GGC ACA GAC AGG CAG U
let-7a	UGA GGU AGU AGG UUG UAU AGU U

miR-21 is the initiator for HCR reaction between probes H1 and H2. H3 and H4 is a non-labeled version of probe H1 and H2.

Table S2. Comparison of detection limit for miRNA detection

Method	**Target**	**Detection limit**	**Ref**
DNAzyme Based Amplification	miRNA	100 pM	S1
DNAzyme Based Amplification	miRNA	25 pM	S2
ATP-fueled DNA Nanomachine	miRNA	100 pM	S3
Entropy-driven DNA Nanomachine	miRNA	8 pM	S4
FRET Based DNA Tetrahedron Nanotweezer	miRNA	330 pM	S5
Crosslinking Hybridization Chain Reaction	miRNA	6 pM	The work

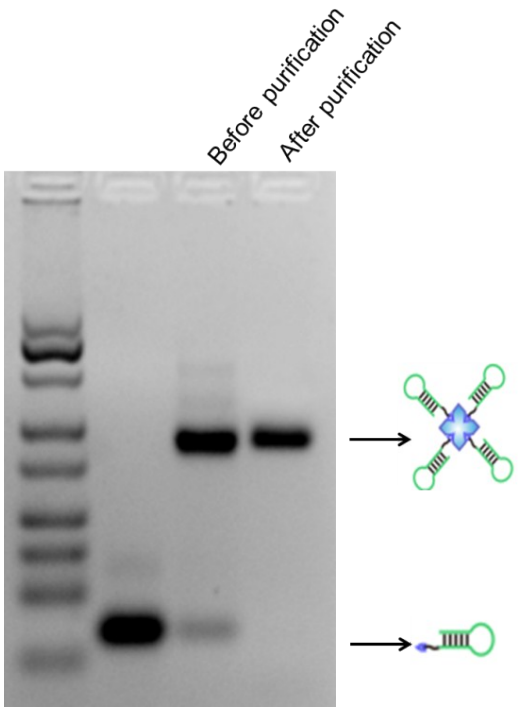

Figure S1. 3% Agarose gel to compare the purity of the DNA nanotetrads before and after agarose gel purification.

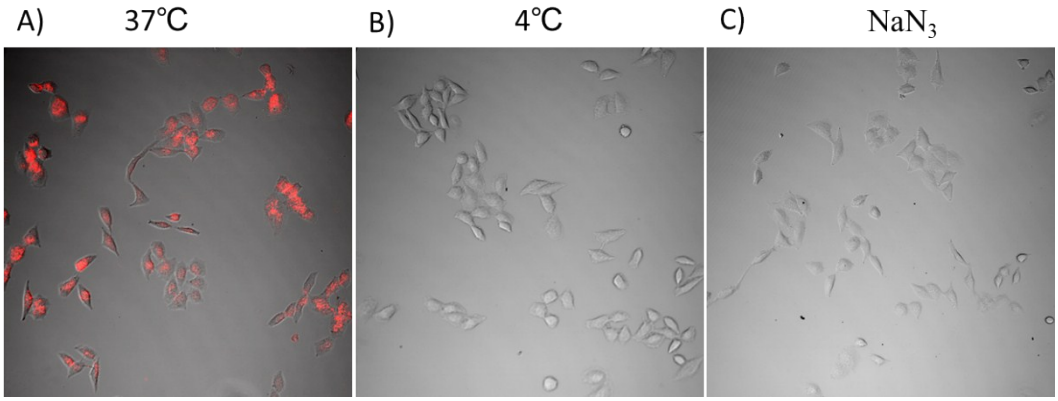

Figure S2. (A) HeLa cells incubated with DNA nanotetrads at 37 ^{o}C, (B) HeLa cells incubated with DNA nanotetrads at 4 ^{o}C, (C) HeLa cells pretreated using NaN_3 followed by incubation with DNA nanotetrads.

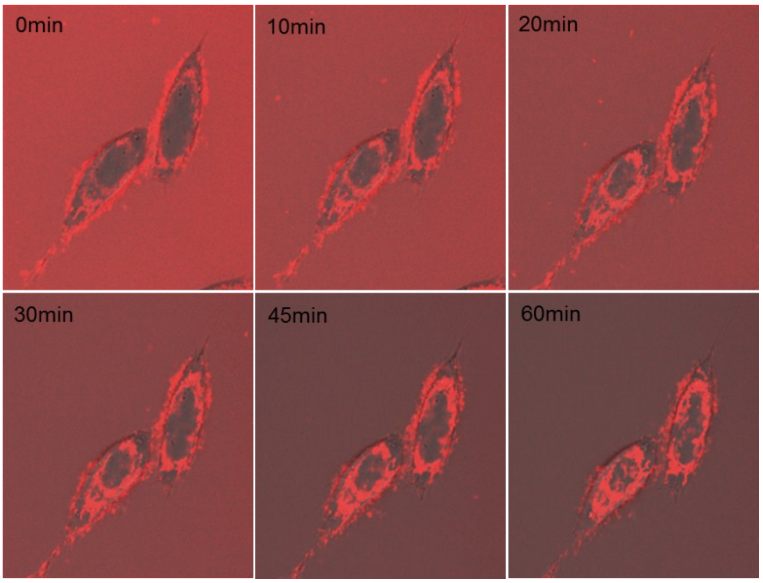

Figure S3. Real-time studies were performed to monitor uptake of DNA tetrads in live cells. The 0 min label represents the starting point of release.

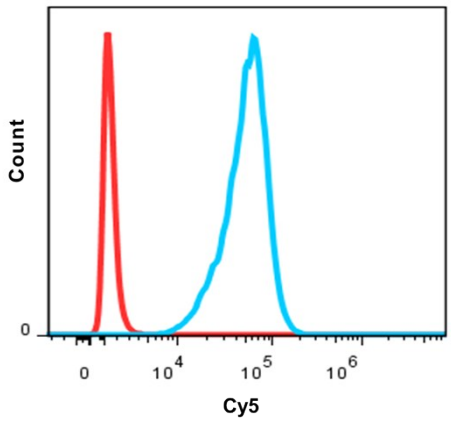

Figure S4. Flow cytometric assay for HeLa cells incubated with DNA tetrads carrying linear probes. The red curve is obtained from cells not incubated with DNA tetrads. The blue curve represents cells incubated with DNA tetrads carrying L1.

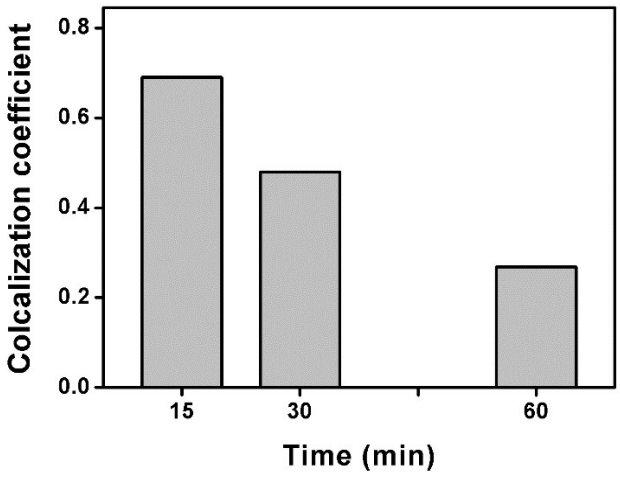

Figure S5. Lysosomes colocalization coefficient analysis of DNA nanotetrads with varying incubation time.

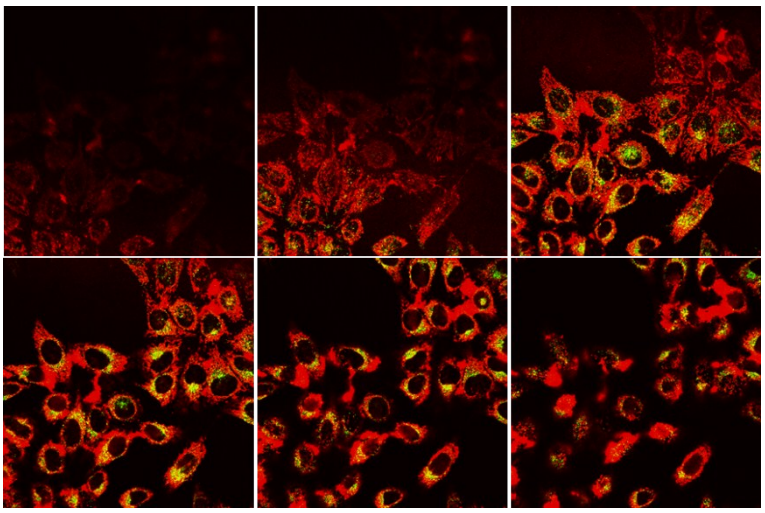

Figure S6. Z-stack images of HeLa cells incubated with DNA nanotetrads for 60 min followed by Lyso Tracker Red DND-99 staining.

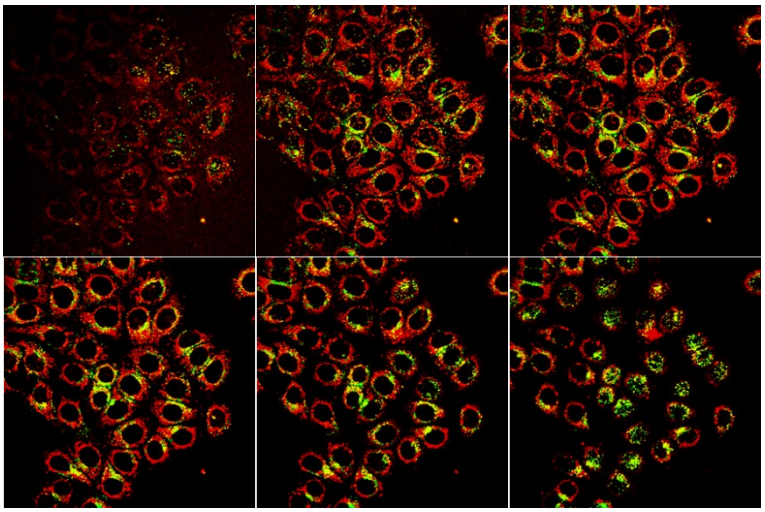

Figure S7. Z-stack images of MCF-7 cells incubated with DNA nanotetrads for 60 min followed by Lyso Tracker Red DND-99 staining.

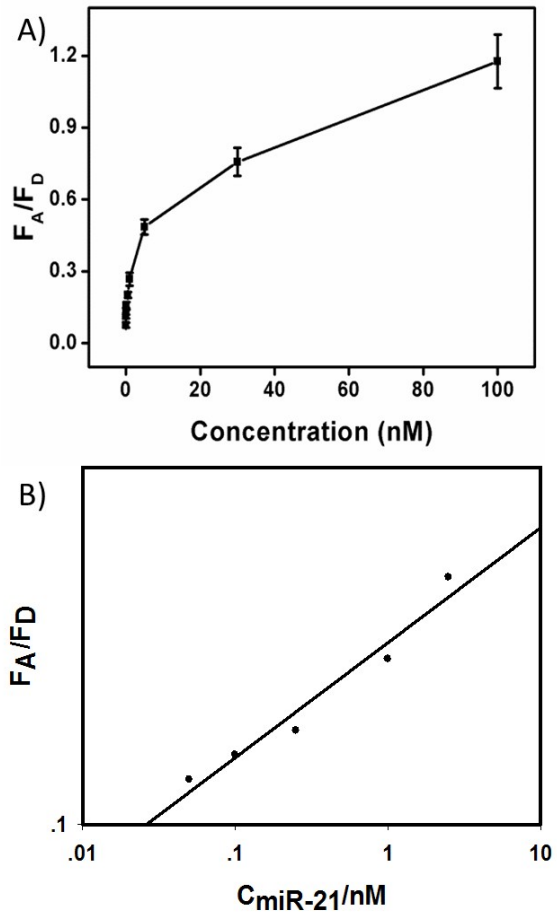

Figure S8. A) Plot of fluorescence ratio (ex/em at 540/662 nm) versus miR-21 concentrations. B) Linear relationship between F_A/F_D versus miR-21 concentrations in logarithmic scale.

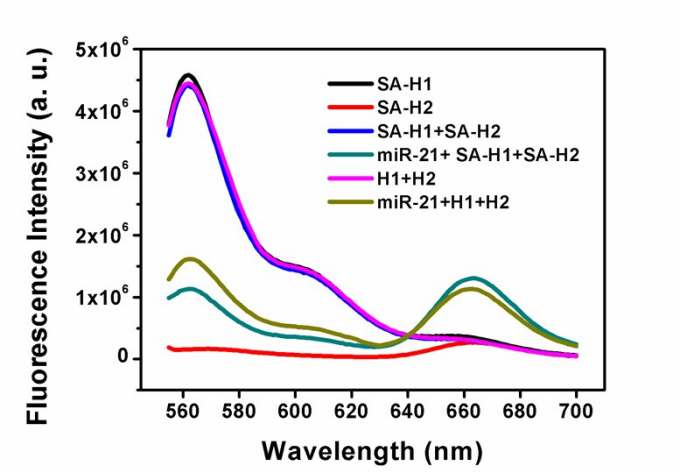

Figure S9. Fluorescence spectral responses obtained by incubating DNA tetrads carrying H1 (black), DNA tetrads carrying H2 (red), DNA tetrads carrying H1 and H2 (blue), DNA tetrads carrying H1 and H2 with target RNA (green), H1 and H2 (pink), H1 and H2 with target RNA (brown).

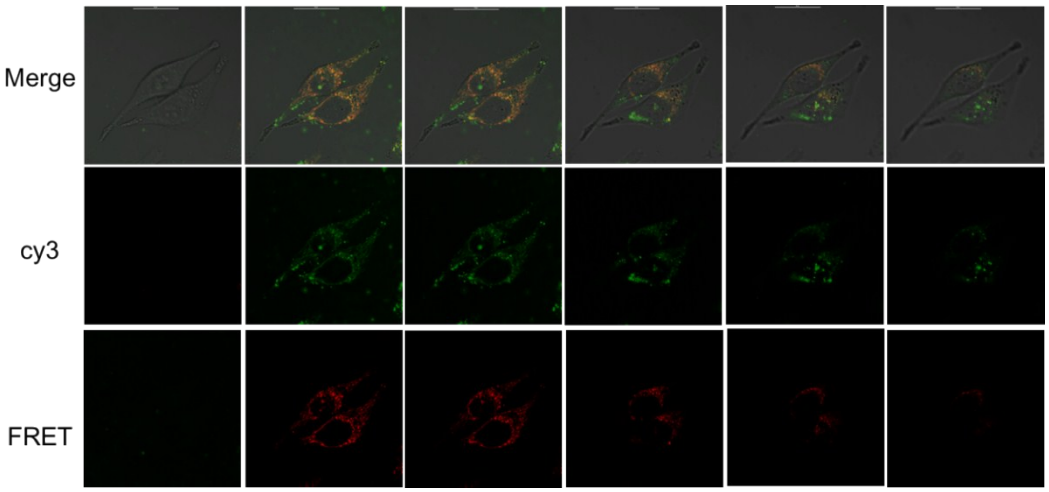

Figure S10. Z-stack images of HeLa cells with DNA tetrads carrying H1 and H2.

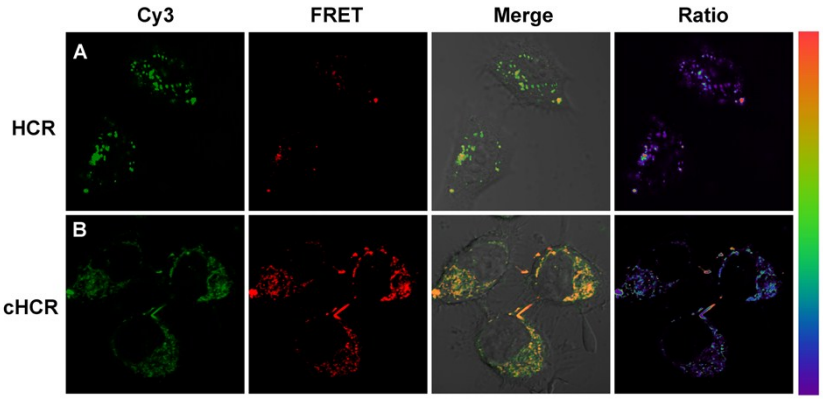

Figure S11. Fluorescence imaging of HeLa cells (A) transfected with dimmer probes of H1 and H2 using Lipofectamine 3000, (B) treated with DNA tetrads carrying H1 and H2.

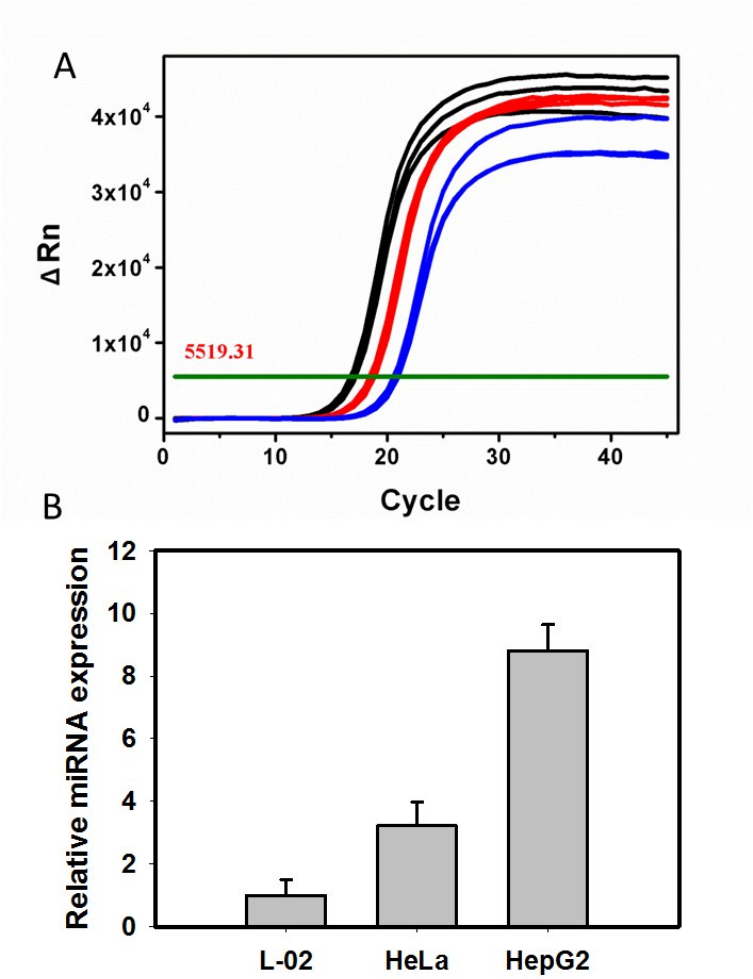

Figure S12. Expression analysis of miR-21 in L-02 (blue), HeLa (red) and HepG2 (black) cells. (A) Real-time fluorescence curves in qRT-PCR analysis, (B) Relative expression levels for miRNA-21.

Reference:

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S2. Y. Yang, J. Huang, X. Yang, X. He, K. Quan, N. Xie, M. Ou and K. Wang, *Anal Chem*, 2017, **89**, 5850-5856.

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