Electronic Supplementary Information (ESI)

Hairpin-Fuelled Catalytic Nanobeacons for Amplified MicroRNA Imaging in Live Cells

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Experimental Section

Chemicals and Reagents. 3-(4,5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide (MTT) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl) were obtained from Sigma Chemical Company. DNase I endonuclease was obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Trisodium citrate($C_6H_5Na_3O_7·2H_2O$) was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Chloroauric acid ($HAuCl_4·4H_2O$) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). The lipofectamine-3000 was purchased from Invitrogen (Thermo Fisher Scientific, USA). All the chemicals were of analytical grade and used without further purification. All cell lines were purchased from Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Sartorius ultrapure water (18.2 MΩ cm) was used throughout the experiments. DNA oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and purified by high-performance liquid chromatography (HPLC). The sequences of the oligonucleotides are described in Table S1.

Apparatus. The transmission electron microscopy (TEM) work was performed with a JEM-2100 transmission electron microscope (JEOL Ltd., Japan). The UV−vis absorption spectra were measured with a Biospec-nano UV−vis spectrophotometer (Japan). The fluorescence spectra were recorded on a Hitachi F-7000 fluorescence spectrometer (Japan). The cells were visualized under an Olympus IX-70 inverted microscope with an Olympus FluoView 500 confocal scanning system. Flow cytometry data were collected with a BD FACS Calibur Flow Cytometer (USA).

Preparation of AuNPs. AuNPs were prepared using the sodium citrate reduction method that has been previously reported[1]. All glassware was cleaned in aqua regia (HCl/HNO_3, 3:1), rinsed with H_2O, and ovendried before the experiments. Then 100 mL $HAuCl_4$ (0.01%) was heated to boiling with vigorous stirring, after that 2.0 mL trisodium citrate (1%) was added under stirring. The color of the solution turned from pale yellow to colorless and finally to burgundy. Boiling was continued for an additional 10 min. The colloid was stirred until the solution reached room temperature. Then, it was filtered through a 0.45 μm Millipore membrane filter. The prepared AuNPs were stored at 4 °C.Transmission electron microscopy (TEM) performed with a JEM-2100 transmission electron microscope was used to determine the size and monodispersity of the resulting nanoparticle solutions.[4] For TEM analysis, the AuNPs were immobilized onto a holey carbon TEM grid. followed by wicking the solution away. The grid was subsequently dried under vacuum and imaged.

Preparation of Nanobeacons. According to our previous protocol[2]. Thiolated H1 were reduced by Tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl) for 1 h. The solution was added to gold colloids at a concentration of 3 μM of oligonucleotide per 1 mL of 10 nM colloid. After 16 h, phosphate buffer (PB; 100 mM Na_2HPO_4 and 100 mM NaH_2PO_4, pH = 7.4) was added to the mixture to achieve a 0.01 M phosphate concentration, and NaCl solution (2.0 M) were added to the mixture over

an 8 h period to achieve a final sodium chloride concentration of 0.3 M. The solution was centrifuged (13000 rpm, 30 min) and resuspended in phosphate buffered saline (PBS; 137 mM NaCl,10 mM Phosphate, 2.72 mM KCl, pH = 7.4) three times to produce the purified AuNPs used in all subsequent experiments.

Quantitation of H1 Loaded on Each AuNP. The H1 loaded on AuNPs were quantified according to the published protocol[3]. The mercaptoethanol (ME) was added (final concentration 20 mM) to the nanobeacons solution (2 nM). After being incubated overnight with shaking at 25 °C. Released FAM-labeled H1 were separated via centrifugation and the fluorescence was measured with a fluorescence spectrometer. The fluorescence with a 488 nm excitation wavelength was converted to molar concentrations of DNAs by interpolation from a standard linear calibration curve that was prepared with known concentrations of FAM-labeled H1 with identical buffer pH, ionic strength, and ME concentrations. By comparing to the standard curve generated with the same conditions, the concentration of FAM-labeled H1 was determined 75.39 nM while the concentration of AuNPs was 2 nM, so the average number of FAM-labeled H1 per gold nanoparticle surface is about 37.

Agarose Gel Electrophoresis. In the gel electrophoresis assay, a sample containing 10 μL of each reaction sample, 2 μL 6 × Loading buffer, and 2 μL of SYBR Gold was subjected to the 2% agarose gel electrophoresis. 1 μM H1 and 1 μM H2 were incubated with 100 nM miR-21 in TNaK buffer reacted for 3 h at 37 °C. The electrophoresis was conducted in 1 × TBE buffer at constant voltage of 80 V for 1 h.

Fluorescence Analysis. A series of miR-21 at different concentrations were incubated with nanobeacons (2 nM) and H2 (300 nM) in TNaK buffer (20mM Tris-HCl, 140 mM NaCl, 5mM KCl, pH 7.4) at 37 °C reacted 4 h, the fluorescence of FAM used the maximal excitation wavelength at 488 nm, and collected between 510 and 650 nm. The slit width was set to 5 nm either the excitation or the emission light path. For selectivity test, a certain concentration of miR-200b, miR-429, miR-21, and let-7d stock solution were added into the probes with a final concentration of 100 nM.

Cell Culture. HeLa cells, HepG2 cells, and L02 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U/mL 1% antibiotics penicillin/streptomycin and maintained at 37 °C in a 100% humidified atmosphere containing 5% CO_2.

Nuclease Assay. Two groups of nanobeacons (2 nM in buffer) were placed in a 96-well fluorescence microplate at 37 °C. 1.5 μL of DNase I in assay buffer (2 U/L) was added to one group of nanobeacons. The last untreated group was served as control. The prepared four groups of solution were monitored for a period of 60 min.

Transfection of nanobeacons and H2 into live cells. Using HeLa cells as model, HeLa cells (1×10^5 cells) was seeded into a 6-well cell culture plate (Corning, USA) and incubated for 24 h at 37 °C in a humidified 5% CO_2 containing atmosphere. Transfection assays were performed according to the manufacturer's protocol. The reaction mixture was prepared separately as a part A solution and part B solution. The part A solution contained 125 μL Opti-MEM and certain concentrations of probes including 2nM nanobeacons and 300 nM H2. The part B solution contained of 125 μL Opti-MEM and 3.5 μL lipofectamine-3000. First, the A and B solutions were

incubated separately for 10 min at room temperature. Subsequently, A and B were mixed and incubated for another 15 min at room temperature and 1000 μL DMEM was added to the mixture. Then, the mixture was added into each well and incubated at 37 °C in a humidified incubator for 4 h.

Cell Viability Assay. MTT assays were carried out to evaluate the potential cytotoxicity of the proposed probes and lipofectamine to the cells. HeLa cells were seeded in a 96-well plate at a density of 1×10^5 cells per well in a total volume of 300 μL. The plates were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. After overnight incubation, the original incubating medium was discarded. Fresh media containing lipofectamine-3000 and nanobeacons (0, 2, and 5 nM) were added to the HeLa cells and incubated for 6, 12, 18, and 24 h, respectively, 0.1 mL of MTT solution (0.5 $mg \cdot mL^{-1}$ in PBS) was added to each well with incubation at 37 °C for 4 h. Finally, 150 μL of DMSO was added to dissolve the precipitated formazan violet crystals. The cell viability was determined by measuring the absorbance at 490 nm by a multidetection microplate reader.

Confocal Fluorescence Imaging. HeLa, HepG2 and L02 cells were cultured in RPMI-1640 medium supplemented for 24 h. Cells were transfected with nanobeacons (2 nM) and H2 (300 nM) for 4 h or various time (for time optimization), and then the cells were washed three times with PBS (pH = 7.4) before imaging. All cells were observed under an Olympus IX-70 inverted microscope with an Olympus FluoView 500 confocal scanning system, and a 515 nm (±10 nm) bandpass filter was used for fluorescence detection. The Hoechst dye was excited with a 405 nm laser line and detected with a 460 nm (±10 nm) bandpass filter. The FAM fluorescence image was recorded in green channel with 488 nm excitation.

Flow Cytometry Assay. HeLa, HepG2 and L02 cells were cultured separately with fresh media consisting of lipofectamine-3000 with nanobeacons (2 nM), and lipofectamine-3000 with nanobeacons (2 nM)/H2 (300 nM) in culture media for 4 h. Then the cells were washed with PBS (pH = 7.4) three times and detached from culture plate by Trypsin-EDTA solution. Next, the suspended cell solution was centrifuged at 2000 rpm for 4 min and washed with PBS three times. Finally, the cells were resuspended in PBS for flow cytometric analysis on a BD Calibur Flow Cytometer (USA) under 488 nm excitation.

qRT-PCR. Total cellular RNAs was extracted from HeLa cells, MCF-7 cells, HepG2 cells and L02 cells respectively, using Trizol reagent (Sangon Co. Ltd., Shanghai, China) according to the manufacturer's instructions. The cDNA samples were prepared by using the reverse transcription (RT) reaction with AMV First Strand cDNA Synthesis Kit (BBI, Toronto, Canada). qPCR analysis of miRNA was performed with SG Fast qPCR Master Mix (2×; BBI), according to the indicated protocol on a LightCycler480 Software Setup (Roche). The relative expression of miR-21 was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in this experiment were described in Table 1. All qRT-PCR reactions were performed in triplicate.

Supporting Tables:

Table S1. Oligonucleotide sequences used in this work.

names	Sequence (from 5′ to 3′)
H1	HS–TTTTTTTTTTTCAACATCAGTCTGATAAGCTACATTGGATGCTCTAGCTTATCAGACTG–FAM
H2	TAAGCTAGAGCATCCAATGTAGCTTATCAGACTGCATTGGATGCTC
miR-21	UAGCUUAUCAGACUGAUGUUGA
smiRNA-21	UAGCUUAUCACACUGAUGUUGA
tmiRNA-21	UCGCUUAUCGGACUGAUCUUGA
miR-429	UAAUACUGUCUGGUAAAACCGU
let-7d	AGAGGUAGUAGGUUGCAUAGUU
miR-200b	UAAUACUGCCUGGUAAUGAUGA
miR-21 forward	ACACTCCAGCTGGGTAGCTTATCAGACTGA
miR-21 reverse	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC
U6 forward	CTCGCTTCGGCAGCACA
U6 reverse	AACGCTTCACGAATTTGCGT

The red bases were mismatched bases of targets.

Supporting Figures:

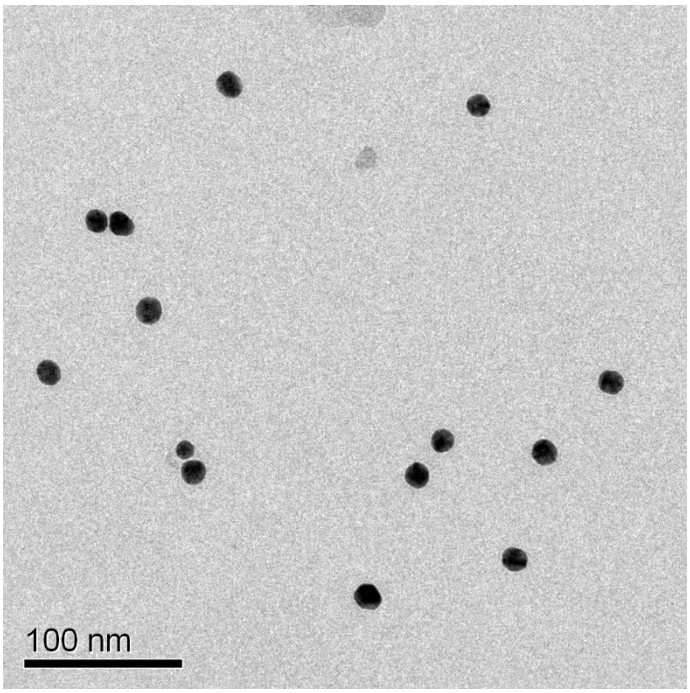

Figure S1. TEM images of AuNPs.

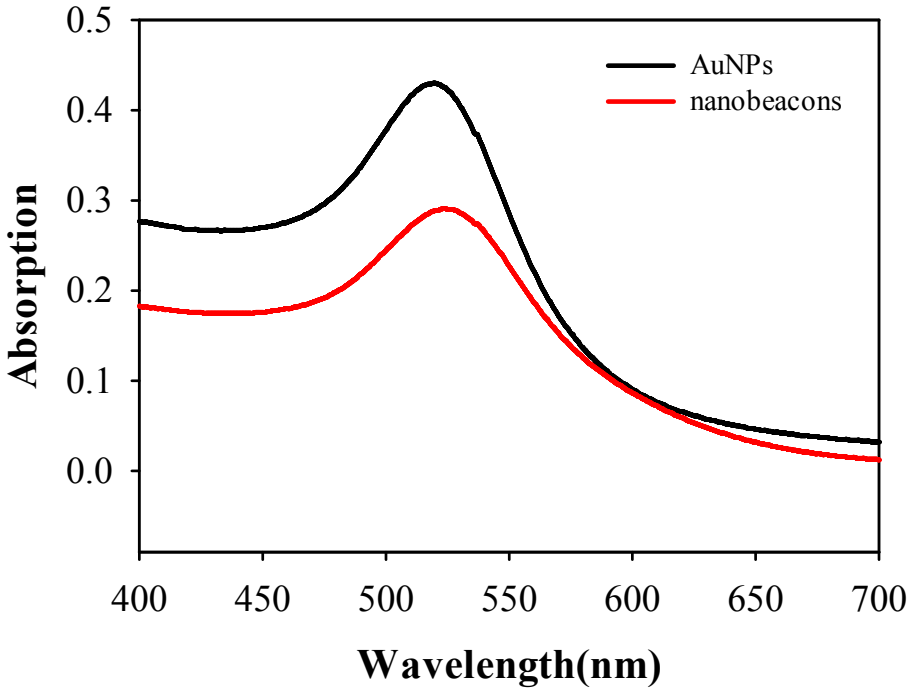

Figure S2. Absorption spectra of AuNPs and nanobeacons. The maximum absorption of the AuNPs was at 519 nm and that it was red-shifted to 524 nm after modification.

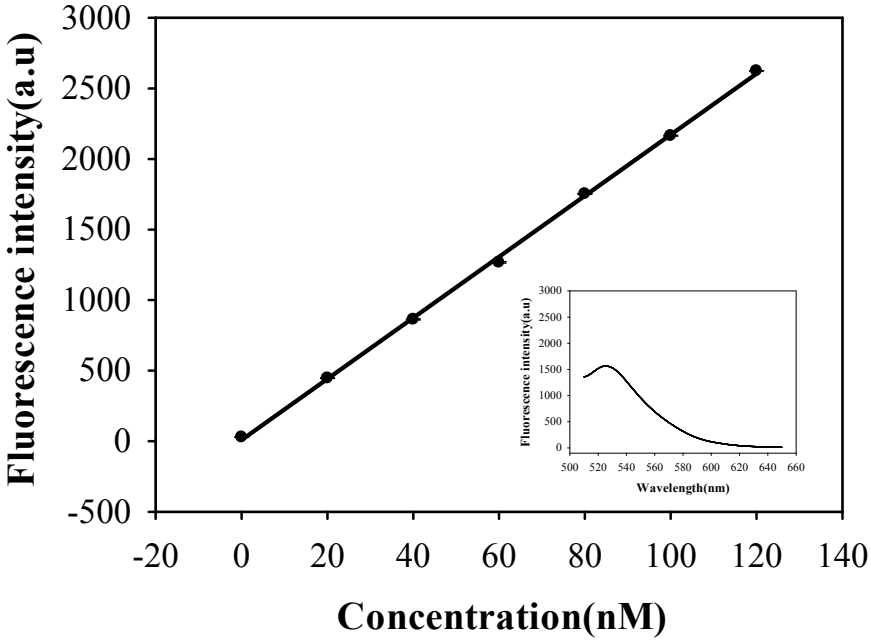

Figure S3. Evaluation of the amount of H1 on each AuNP. The standard linear calibration curve of fluorescence signal against the concentration of FAM-labeled H1. Error bars were estimated from three replicate measurements. Inset: the fluorescence intensity of non-quenched H1.

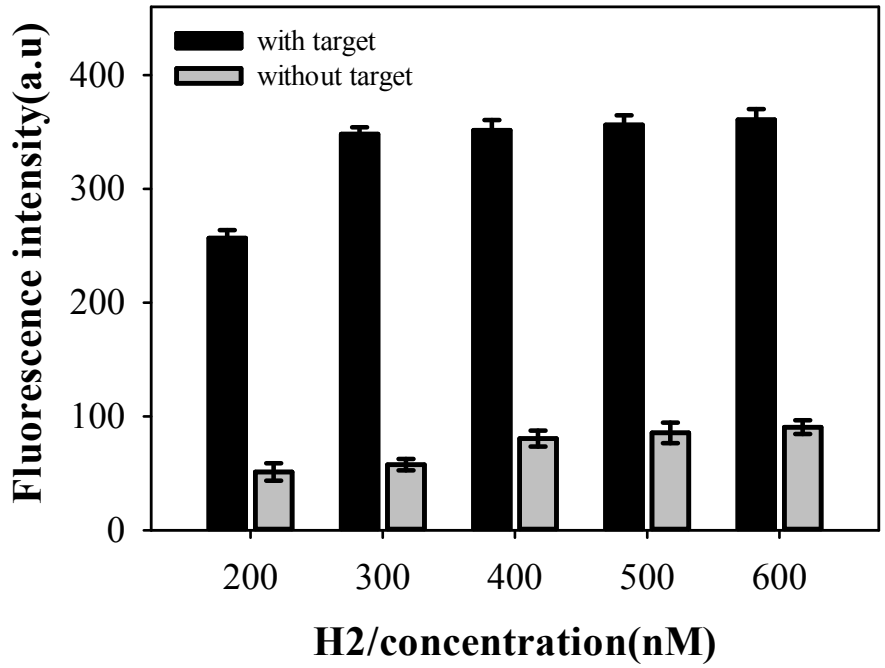

Figure S4. Optimization of H2 concentration. Various concentrations of H2 were added into the nanobeacons (2 nM) and miR-21 (50 nM) reacted 4 h at 37 °C. The result showed that the optimal concentration of H2 was found to be 300 nM. Error bars represent variations between three measurements.

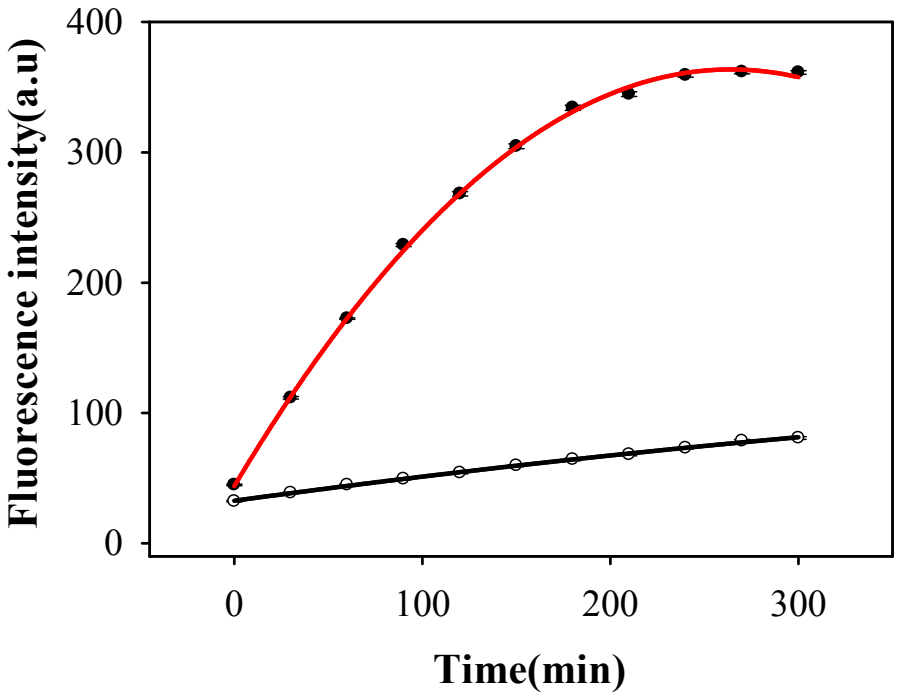

Figure S5. Optimization of reaction time. Time-dependent fluorescence response of the hairpin-fuelled catalytic nanobeacons to miRNA-21 at the concentration of 50 nM (red line) and 0 nM (black line), respectively. The data error bars indicate means ± SD (n=3).

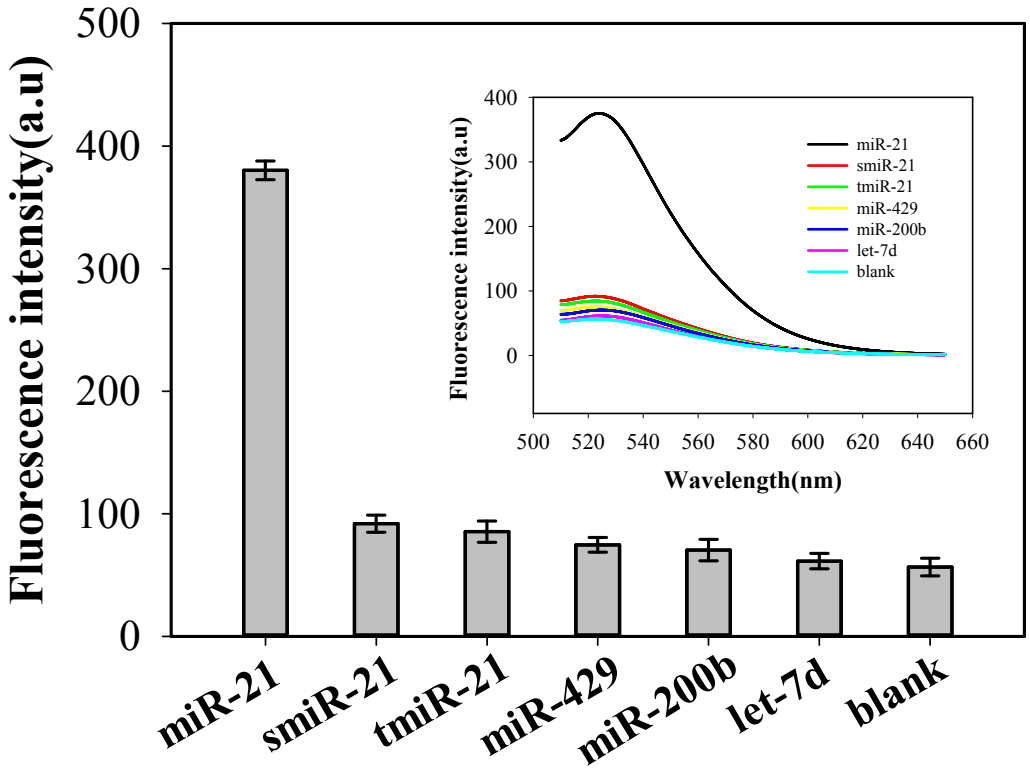

Figure S6. Specificity of the hairpin-fuelled catalytic nanobeacons. a certain concentration of miR-21, smir-21, tmiR-21, miR-429, miR-429 and let-7d stock solution were added into the hairpin-fuelled catalytic nanobeacons with a final concentration of 50 nM reacted 4 h at 37 °C. Error bars were estimated from three replicate measurements.

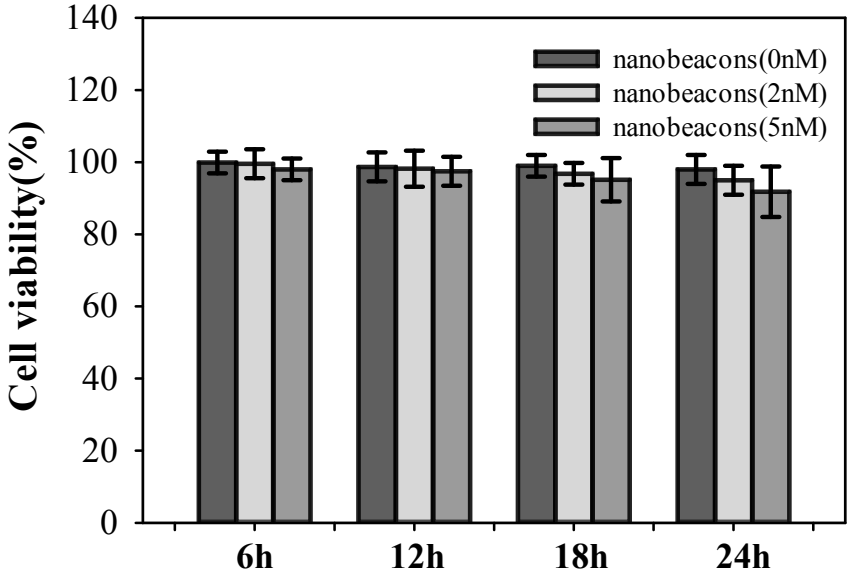

Figure S7. Growth inhibition assay (MTT): HeLa cells were incubated with nanobeacons (0 nM, 2 nM and 5 nM) for 6 h, 12 h, 18 h and 24 h, respectively. Error bars represent variations between three measurements.

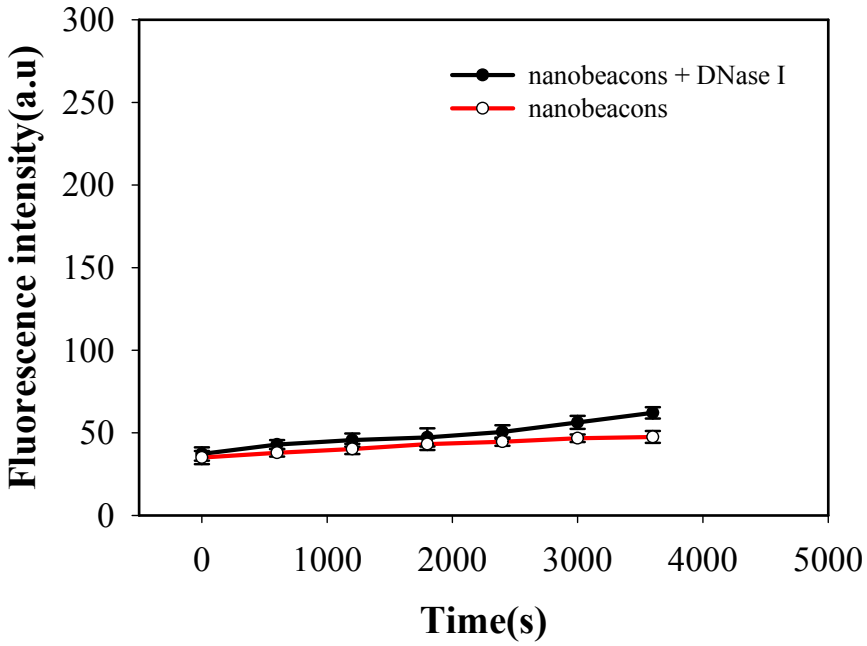

Figure S8. Studies of nuclease stability of the nanobeacons. Error bars represent variations between three measurements.

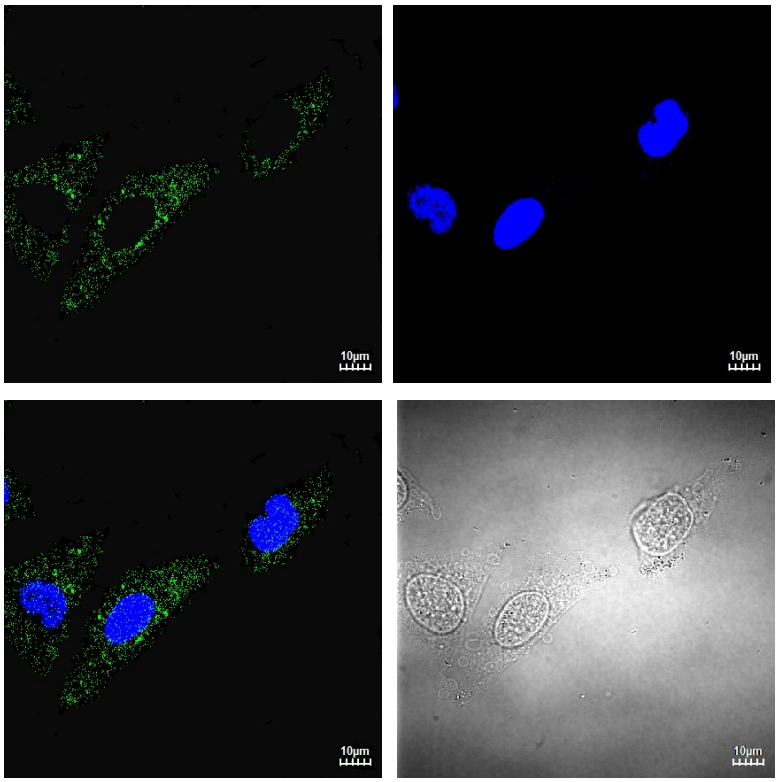

Figure S9. Fluorescence colocalization analysis. The cell nucleus was stained with hoechst 33342. The merge image shows co-localization of both FAM and hoechst in cytoplasm of HeLa cells after incubated with various probes for 4 h. The FAM fluorescence emission channel was collected under an exictation laser at 488 nm. Excitation of hoechst 33342 was carried out at λ = 405 nm and emissions was collected in the blue channel. Scale bar is 10 μm.

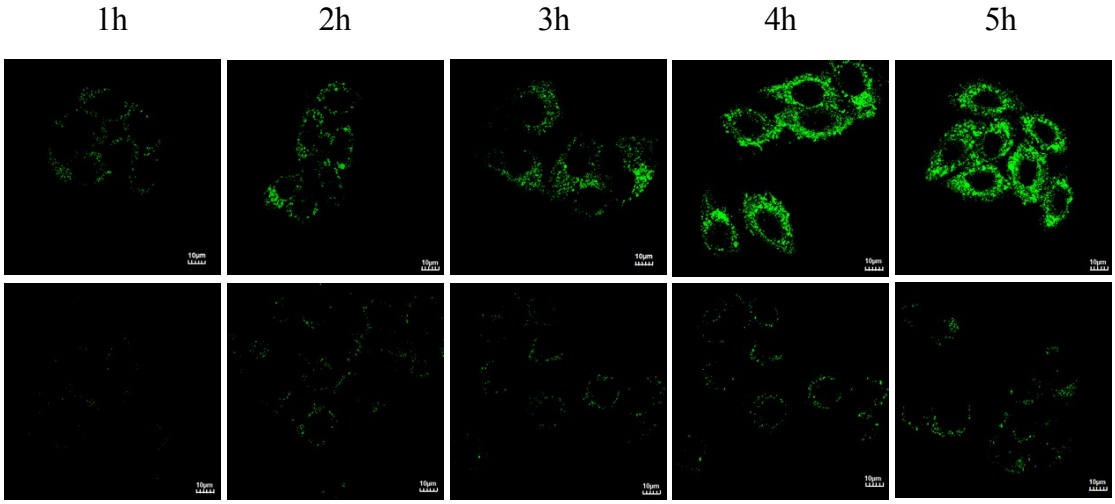

Figure S10. The optimization of cellular incubation time. The HeLa cells and L02 cells were treated with lipofectamine-3000 with nanobeacons (2 nM)/H2 (300 nM) for 1, 2, 3, 4 and 5h at 37 °C, respectively. The FAM fluorescence emission channels were collected under an excitation laser at 488 nm. Scale bar is 10 μm.

References

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