Supporting Information

Photo zipper locked DNA nanomachine with internal standard for precise miRNA imaging in living cells

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Experimental Section

Materials and Reagents.

Anhydrous yttrium chloride (YCl_3) (99.9%), anhydrous gadolinium chloride ($GdCl_3$) (99.9%), anhydrous ytterbium chloride ($YbCl_3$) (99.9%), anhydrous erbium chloride ($ErCl_3$) (99.9%), oleic acid (OA), 1-octadecene (ODE), sodium hydroxide (NaOH), ammonium fluoride (NH_4F), methanol (MeOH), cyclohexene, acetone, trichloromethane ($CHCl_3$), dimethyl sulfoxide (DMSO), alendronic acid (ADA) were purchased from Aladin Ltd. (Shanghai, China). Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), glycerol phosphate disodium salt hydrate (GDSH) were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). NHS-Cy3 was purchased from Fanbo Biochemicals (Beijing, China). Maleimide-polyethylene glycol-N-hydroxy succinimide ester (Mal-PEG-NHS) was obtained from Toyongbio Inc. (Shanghai, China). RNAiso Plus Trizol reagent (TaKaRa), SYBR® Premix Ex Taq™ Ⅱ (Tli RNaseH Plus), Lipofectanmine™ 2000 (Lipo-2000) transfection reagent were purchased from Thermo Fisher Scientific Inc. (Rockford, USA). MTT cell proliferation and cytotoxicity assay kit and all cell lines (HeLa, MCF-7, HEK-293, MDA-MB-231) were obtained from KeyGen Biotech. Co. Ltd. (Nanjing, China). The miRNAs were purchased from GenePharma Co. Ltd. (Shanghai, China). All of the DNA oligonucleotides were synthesized and purified by Sangon Biotech Co. Ltd. (Shanghai, China) with sequences listed below:

Name	Sequence(5'to 3')
S-DNA-BHQ2	HS-TTTTTTCCACCACATTGAAATTGCACTATrAGGAAGAGATTTACGAGGCGGTGGTGG-BHQ2
S-DNA	HS-TTTTTTCCACCACATTGAAATTGCACTATrAGGA

	AGAGATTTACGAGGCGGTGGTGG
S-DNA-BHQ1	HS-TTTTTTCCACCACATTGAAATTGCACTATrAGGAAGAGATTTACGAGGCGGTGGTGG-BHQ1
S-DNA-FAM	HS-TTTTTTCCACCACATTGAAATTGCACTATrAGGAAGAGATTTACGAGGCGGTGGTGG-FAM
F1	GGAAGAGATTTACGAGGCGGTGGTGG
F2	TTTTTTCCACCACATTGAAATTGCACTATA
DNAzyme walker	HS-$(T)_{40}$TCAGACTGATGTTGATCTCTTCTCCGAGCCGGTCGAAATAGT
photo zipper	AAGAGATCAACATCAGTCTGATAAGCTAPC-LinkerGGGCAGACTAGCTTA
photo zipper-FAM	FAM-AAGAGATCAACATCAGTCTGATAAGCTAPC-LinkerGGGCAGACTAGCTTA
AL	AAGAGATCAACATCAGTCTGATAAGCTA
miRNA 21	UAGCUUAUCAGACUGAUGUUGA
miRNA 141	UAACACUGUCUGGUAAAGAUGG
MiRNA 199-a	ACAGUAGUCUGCACAUUGGUUA
1-mismatchated miRNA 21	UAGCUUAUCAGACCGAUGUUGA
3-mismatchated miRNA 21	UAGCUAAUCAGACCGAUGUAGA

Apparatus.

Transmission electron microscopic (TEM) images were carried out on JEM-2800 transmission electron microscope (JEOL Ltd., Japan). Absorption spectra were measured with an UV-3600 UV-vis-NIR spectrophotometer (Shimadzu Company, Japan). Fluorescence spectra were recorded on FluoroMax-4 spectrofluorophotometer (HITACHI, Japan) equipped with an external continuous-wave laser (980 nm) as the excitation source. FTIR spectra were measured with a Magan-IR spectrometer 500 (Nicolet) with the KBr pellet technique. Zeta potential analysis was performed on Nano-Z Zetasizer (Malvern, UK). The gel electrophoresis was conducted on PowerPac™ Basic electrophoresis analyzer (Bio-Rad, USA) and imaged with Biorad ChemDoc XRS (Bio-Rad, USA). Unlocking of the photo-zipper was achieved via a UV lamp (LUYOR-365, China). The cell images were recorded on TCS SP5 confocal laser scanning microscope (CLSM) (Leica, Germany). Numbers of cells were determined with Countess® II FL Automated Cell Counter (Thermo Fisher Scientific, USA). Real-time reverse transcription polymerase chain reaction was carried out using a CFX96 touch qRT-PCR detection system (Bio-Rad, USA).

Synthesis of core-shell UCNPs $NaYF_4$:Yb,Er,Gd@$NaYF_4$

To prepare the UCNPs shell precursor $NaYF_4$, YCl_3 (0.20 mmol, 0.0392 g) was mixed with 2 mL OA and 6 mL ODE, degassed in vacuum at 150 ^oC for 1 h and cooled down to 45 ^oC. 2 mL methanol containing NH_4F (1.00 mmol, 0.0371g) and NaOH (0.63 mmol, 0.0248 g) were dropwisely added in the above prepared solution, stirred at 45 ^oC for 0.5 h, and heated to 110 ^oC for complete remove of methanol.

Core UCNPs $NaYF_4$:Yb,Er,Gd was prepared by mixing YCl_3 (0.70 mmol, 0.1367 g), $YbCl_3$ (0.18 mmol, 0.0503 g), $ErCl_3$ (0.02 mmol, 0.0055 g), and $GdCl_3$ (0.10 mmol, 0.0264 g) in a three-necked flask with subsequent addition of 15 mL ODE and 6 mL

OA. The mixture was degassed in vacuum at 150 °C for 1 h, cooled down to 45 °C, dropwisely added with 10 mL methanol containing NH_4F (4.00 mmol, 0.1482 g) and NaOH (2.50 mmol, 0.0992 g) and stirred at 45 °C for 1 h. After complete evaporation of methanol at 110 °C, the reaction mixture was heated to 300 °C gradually and kept under nitrogen atmosphere for 90 min. The above prepared shell precursor $NaYF_4$ was then hot injected into the core $NaYF_4$:Yb,Er,Gd and stirred at 300 °C for 0.5 h. After naturally cooled down to room temperature, the resulted $NaYF_4$:Yb,Er,Gd@$NaYF_4$ (UCNPs) was precipitated with acetone, washed with cyclohexane, and redispersed in trichloromethane or cyclohexane for future use.

Modification of UCNPs with ADA

UCNPs (200 mg) dispersed trichloromethane solution (10 mL) was mixed with 4 mL ethanol and 6 mL ADA aqueous solution (0.1 M), and pH of mixture solution was adjusted to 2~3 with 1 M HCl. After 2 hours stirring reaction for ADA ligand exchange with UCNPs surface OA, the upper layer fluid containing prepared ADA functionalized UCNPs (UCNPs-ADA) was transferred to a new centrifuge tube, washed twice with water, and re-dispersed in aqueous solution. Aqueous solution containing 100 μM GDSH was then reacted with as-prepared UCNPs-ADA for 12 h to prevent DNA strand nonspecific adsorption in following surface functionalization step.

Preparation of UCNPs-Cy3/PEG-Mal

NHS-Cy3 and NHS-PEG-Mal were covalently conjugated to UCNPs-ADA via amidation with their surface NH_2 groups. NHS-Cy3 and NHS-PEG-Mal were mixed in various molar ratios (0, 1:20, 1:15, 1:10, 1:7, 1:5), dispersed in DMSO with total molar concentration of 300 μM, and added into 2.5 mL DMSO containing UCNPs-ADA (2.0 mg/mL) respectively. After 24 h stirring, the as-obtained UCNPs-Cy3/PEG-Mal were centrifuged, washed with 0.01M PBS for three times, and redispersed in 5 mL 0.01M PBS.

Preparation of PZ-DNA nanomachine

DNAzyme walker was silenced with photo zipper firstly. To make sure complete blocking, 6 μL DNAzyme walkers (100 μM) were added with three times molar excess of photo zipper and diluted with 0.01M PBS buffer (pH=7.4) to make total volume of 30 μL. The mixture solution was annealed by heating to 75 ^{o}C with subsequent cooling down to 4 ^{o}C at a rate of 1.2 ^{o}C/min. 20 μM BHQ2 labelled hairpin structured substrate DNA strand (S-DNA-BHQ2) was also annealed by heating to 95 ^{o}C for 5 min with subsequent cooling down to 25 ^{o}C a rate of 1.0 ^{o}C/min. P-DNA walker (10 μM) and S-DNA-BHQ2 (10 μM) were mixed at different molar ratios with total volume of 200 μL, added with 30 μL above-prepared UCNPs-Cy3/PEG-Mal (1.0 mg/mL) and 70 μL 0.01 M PBS to make final volume of 300 μL and reacted for 24 h, the as-obtained PZ-DNA nanomachines were centrifuged, washed with 0.01 M PBS for three times and re-dispersed in 300 μL 0.01 M PBS and stored at 4 ^{o}C.

To measure the number of S-DNA-BHQ2 and P-DNA walker in each UCNPs, FAM labelled S-DNA (S-DNA-FAM) and P-DNA walker were reacted with UCNPs-Cy3/PEG-Mal according to the same procedure, and the fluorescence intensities of FAM was recorded at 519 nm, the concentration of S-DNA-FAM on UCNPs could be calculated according to the standard curve. Combined with the concentration of UCNPs, the average number of S-DNA-BHQ2 per UCNP was derived to 133. Based on the molar ratio of 10:1 for S-DNA-BHQ2 and P-DNA walker in the reaction, the number of P-DNA walker per UCNP was calculated as 13.

Polyacrylamide Gel Electrophoresis (PAGE) analysis

10% native polyacrylamide gel was prepared using 1×TBE buffer. The loading sample was prepared by mixing 7 μL DNA sample, 1.5 μL 6×loading buffer and 1.5 μL UltraPowerTM dye, and placed for 3 min before injected into polyacrylamide gel. To verify the activation of miRNA 21 responsive a*+b on P-DNA walker, 2 μL P-DNA walker (10 μM) was irradiated with UV lamp (7 mW/cm^2) for 5min, then mixed with 1.5 μL miRNA 21 (10 μM), diluted with 0.01 M PBS to make total volume of 20 μL, and incubated at 37 ^{o}C for 30 min reaction. The gel electrophoresis was run at 80

V for 115 min in 1×TBE buffer, and scanned with a Molecular Imager Gel Doc XR.

To verify the reaction feasibility of DNAzyme, 30 μL S-DNA (10 μM) and 15 μL DNAzyme walker (10 μM) were diluted with 25 mM Tris-acetate buffer (pH=8.0, 200 mM NaCl) to total volume of 190 μL, incubated at 37 ^{o}C for 10 min, and added with 10 μL Mn^{2+} (10 mM) for 30 min reaction. The gel electrophoresis was run at 100 V for 95 min in 1×TBE buffer, and scanned with a Molecular Imager Gel Doc XR.

In Vitro Detection of miRNA 21

5 μL miRNA 21 of various concentrations were mixed with 100 μL PZ-DNA nanomachine (50 μg/mL), and diluted with 25 mM Tris-acetate buffer (pH=8.0, 200 mM NaCl) to total volume of 190 μL. After irradiated with UV lamp (7 mW/cm^2) for 5min, the reaction mixture was incubated at 37 ^{o}C for 20 min, added with 10 μL Mn^{2+} (10 mM) and incubated for another 1.5 h. The intensities of Cy3 fluorescence recovery was measured at 580 nm and compared with internal standard luminance intensity measured at 658 nm with an excitation wavelength of 980 nm. To verify the protection of photo zipper DNA strand, control experiment was also performed with the same procedure in the absence of UV irradiation.

Cell Culture

HeLa cells, MDA-MB-231 cells, HEK-293 cells and MCF-7 cells (KeyGEN Biotech, Nanjing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 U/mL penicillin-streptomycin. All cells were maintained at 37 ^{o}C in a humidified incubator containing 5 % CO_2 and 95 % air. Cell numbers were determined with Countess® II FL Automated Cell Counter (Thermo Fisher Scientific, USA.).

Confocal Fluorescence Imaging

HeLa Cells (~1x10^4) were seeded in a confocal dish for 24 h incubation at 37 ^{o}C and washed twice with PBS. 200 μL opti-MEM containing PZ-DNA nanomachine

(50 μg/mL) was added to each well with 9 h incubation at 37 °C to allow cell uptake of the DNA nanomachine. After washed with 25 mM Tris-acetate buffer (pH=8.0, 125 mM NaCl) for three times to remove excess nanomachine. Cells were subsequently treated with 25 mM Tris-acetate buffer (pH=8.0) containing 125 mM NaCl and 5 mM $MnCl_2$, irradiated with UV lamp for 5 min and incubated at 37 °C in 5% CO_2 for 2 h. After washed twice with PBS, cells were imaged by TCS SP5 confocal laser scanning microscope CLSM with 63x oil objective. Cy3 fluorescence recovery was visualized from 555 to 605 nm and UCNP luminance as internal standard was visualized from 640 nm to 670 nm under 980 nm excitation. All images were digitized and analyzed with Leica Application Suite Advanced Fluorescence (LAS-AF) software package, which integrates intracellular fluorescence intensities of Cy3 and UCNPs at 580 nm and 658 nm respectively, and averages the resulted intensities from 20 randomly selected cells. For the intracellular imaging of HEK-293, MDA-MB-231 and MCF-7 cells, the cells were treated according to the same procedure above, Cy3 fluorescence and UCNPs fluorescence were collected according to the same intensity extraction procedure above.

To modulate intracellular expression of miRNA 21, HeLa cells were incubated with opti-MEM containing Lipofectanmine® 2000 and 300 nM synthetic miRNA 21 mimic or miRNA 21 inhibitor respectively for 24 h, and imaged with CLSM according to the same procedure as above described. The transfection process was operated according to Lipofectanmine® 2000 DNA Transfection Reagent Protocol.

qRT-PCR quantification of intracellular miRNA 21

Total miRNAs were extracted from HeLa, MCF-7, MDA-MB-231 and HEK-293 cells respectively using RNAiso Plus Trizol reagent (TaKaRa, China). cDNA was prepared following operation instrument, which was detected with real time PCR to calculate intracellular miRNA 21 level. Standard curve was constructed with cycle threshold (Ct) values for synthetic miRNA 21 of various concentrations, and the expression levels of cellular extracted miRNA 21 were calculated from standard curve.

MTT Assay

MTT assays were performed to investigate the cytotoxicity of PZ-DNA nanomachine. HeLa cells (200 μL medium/1x10^4 cells/well) were seeded in a 96-well plate and maintained at 37 ^oC for 24 h. After removed the medium and washed HeLa cells twice with PBS, series concentrations of PZ-DNA nanomachine were incubated with HeLa cells for 24 h. The cells were then washed twice with PBS, mixed with 50 μL MTT solution (5 mg/mL) and cultured for 4 h. After removing the remained MTT solution, 150 μL DMSO was added to dissolve the formazan crystal precipitates, and the 96-well plate was vibrated slightly for 30 min. The optical density (OD) was measured at wavelength of 490 nm via Bio-Rad microplate reader.

HeLa cells were exposed under UV irradiation (7 mW/cm^2) for 5 min, incubated at 37 ^oC for 3h, and MTT assay was performed according to above procedure to test the phototoxicity of UV irradiation to cells.

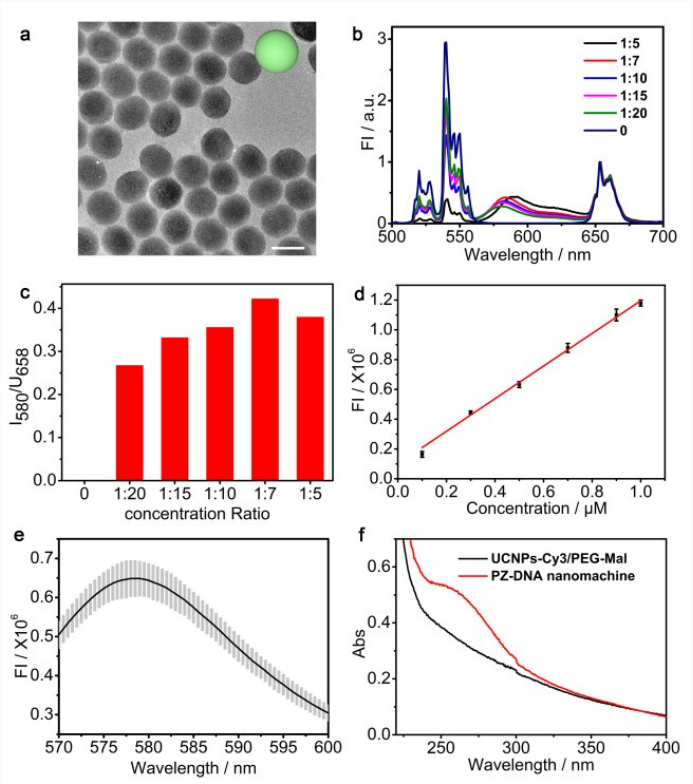

Fig. S1 (a) TEM image of UCNPs $NaYF_4$:Yb,Er,Gd. (scale bar: 20 nm) (b) Normalized upconversion luminance spectra and (c) corresponding fluorescence intensity ratios of Cy3 fluorescence at 580 nm over UCNPs luminance at 658 nm (I_{580}/U_{658}) for UCNPs-Cy3/PEG-Mal with different modification concentration ratios of Cy3 over NHS-PEG-Mal from 1:20 to 1:5. (d) Standard calibration curve of Cy3 fluorescence intensity versus concentrations from 0.1 to 1 μM. The error bars indicate means ± S.D. (n=3). (e) The fluorescence spectrum of UCNPs-Cy3/PEG-Mal (50 μg/mL) under 545 nm excitation. The light gray region indicates the standard deviation (n=3) (f) UV-Vis spectra of UCNPs-Cy3/PEG-Mal and PZ-DNA nanomachine.

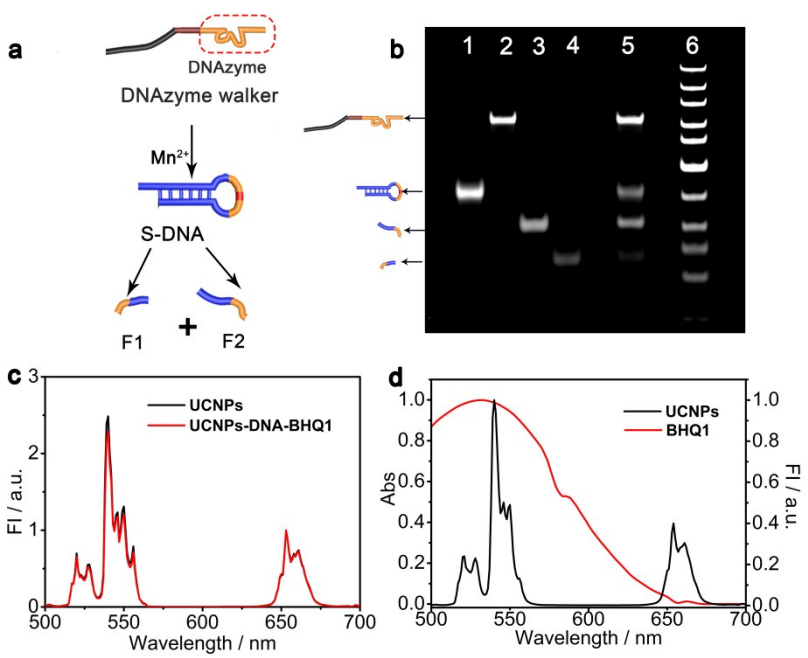

Fig. S2 (a) Schematic illustration and (b) Gel electrophoresis assay characterization of DNAzyme catalytic cleavage reaction. Lane 1: S-DNA, lane 2: DNAzyme walker, lane3: F2, lane4: F1, lane5: mixture of S-DNA and DNAzyme walker, lane6: 300-bp DNA ladder markers. (c) Normalized upconversion luminance spectra of UCNPs and UCNPs-DNA-BHQ1 (d) Normalized fluorescence emission spectra of UCNPs under 980 nm excitation and normalized absorption spectra of BHQ1.

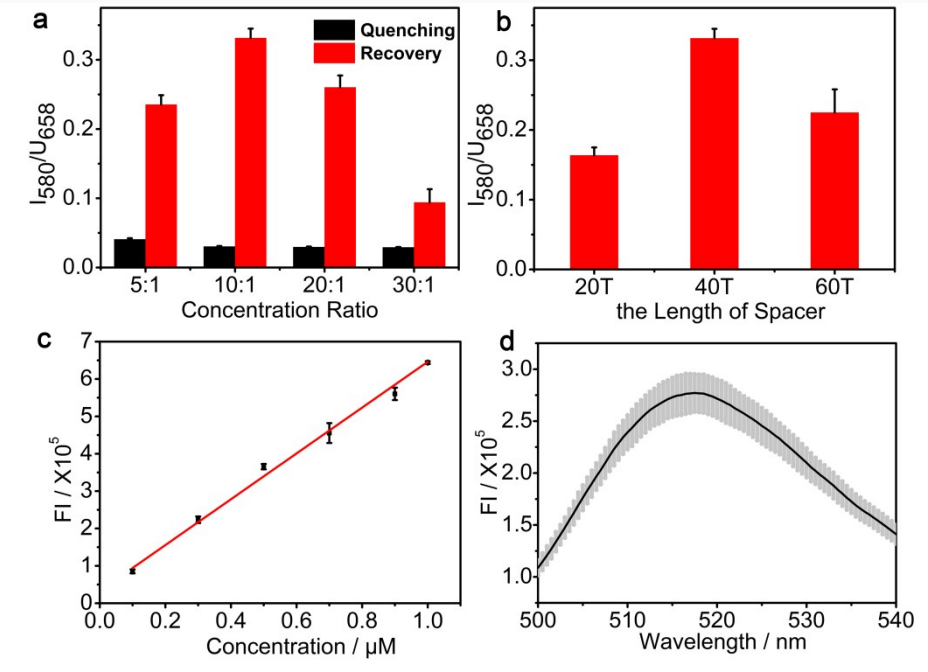

Fig. S3 Fluorescence intensity ratio of Cy3 fluorescence at 580 nm over UCNPs luminance at 658 nm (I_{580}/U_{658}) (a) for UCNPs functionalized with different molar ratios of S-DNA-BHQ2/DNAzyme walker from 5:1 to 30:1 before (Quenching) and after (Recovery) DNAzyme catalytic reaction, and (b) for UCNPs functionalized with 10:1 S-DNA-BHQ2/DNAzyme at different poly T length from 20T to 60T after DNAzyme catalytic reaction. (c) Standard calibration curve of S-DNA-FAM fluorescence intensity versus concentrations from 0.1 to 1 μM. The error bars indicate means ± S.D. (n=3). (d) The fluorescence spectrum of UCNPs-Cy3/S-DNA-FAM/DNAzyme walker (50 μg/mL) under 485 nm excitation. The light gray region indicates the standard deviation (n=3).

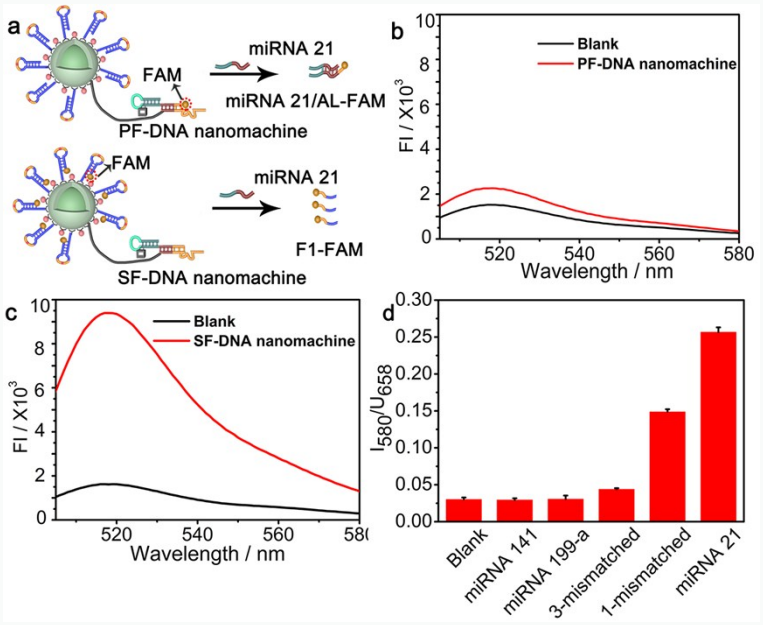

Fig. S4 (a) Schematic illustrations and FAM fluorescence spectra in response to 2 nM miRNA 21 of (b) PF-DNA nanomachine and (c) SF-DNA nanomachine. (d) Luminance intensities ratio for Cy3 at 580 nm over UCNPs at 658 nm (I_{580}/U_{658}) of PZ-DNA nanomachine in absence (blank) and present of 2 nM miRNA 21 and nonspecific miRNAs of miRNA 141, miRNA 199-a, three-base mismatched miRNA 21 (3-mismatched), and single-base mismatched miRNA 21 (1-mismatched). The error bars indicate means ± S.D. (n=3).

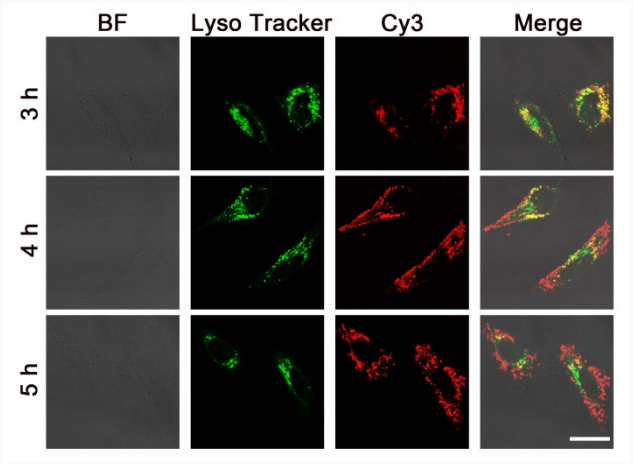

Fig. S5 Time-dependent CLSM images of HeLa cells incubated with PZ-DNA nanomachine (in the absence of quencher BHQ2) and Lysotracker Green. Emission was collected by green channel (Lysotracker Green) at 505-535 nm with 488 nm excitation and red channel (Cy3) at 555-605 nm with 980 nm excitation. (scale bar: 20 μm).

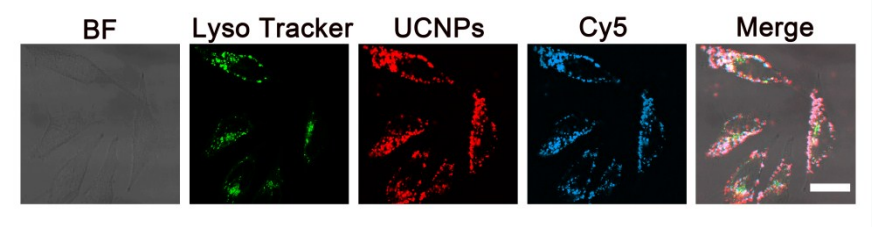

Fig. S6 CLSM images of HeLa cells incubated with PZ-DNA nanomachine (S-DNA-Cy5 as component) for 5 h. Emission was collected by green channel (Lysotracker Green) at 505-535 nm with 488 nm excitation, red channel (UCNPs) at 520-560 nm with 980 nm excitation and blue channel (Cy5) at 650-700 nm with 633 nm excitation. (scale bar: 20 μm).

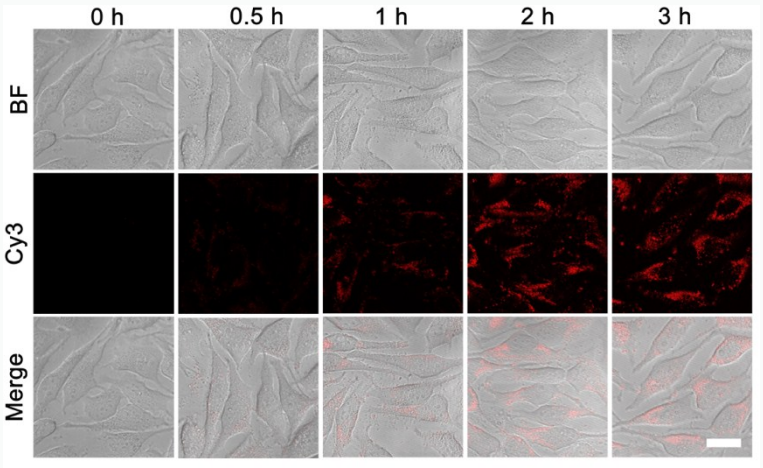

Fig. S7 Cy3 fluorescence recovery in HeLa cells in response to intracellular miRNA 21 at different times after photo activation. (scale bar: 20 μm).

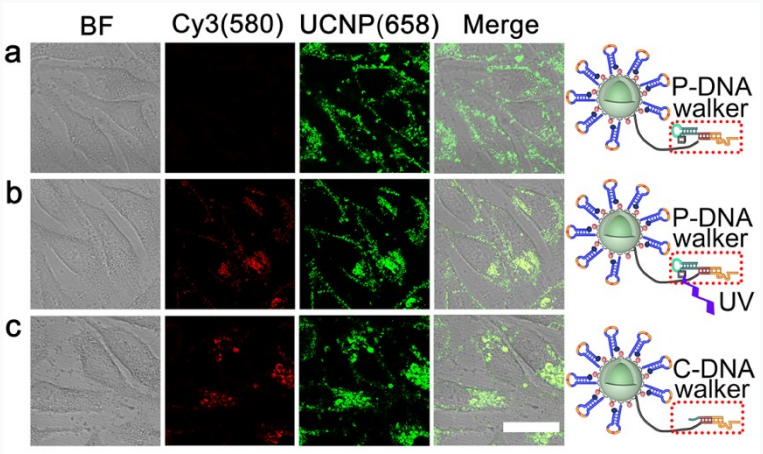

Fig. S8 CLSM images of HeLa cells incubated with (a) PZ-DNA nanomachine, (b) photo activated PZ-DNA nanomachine, and (c) unprotected PZ-DNA nanomachine. (scale bar: 20 μm).

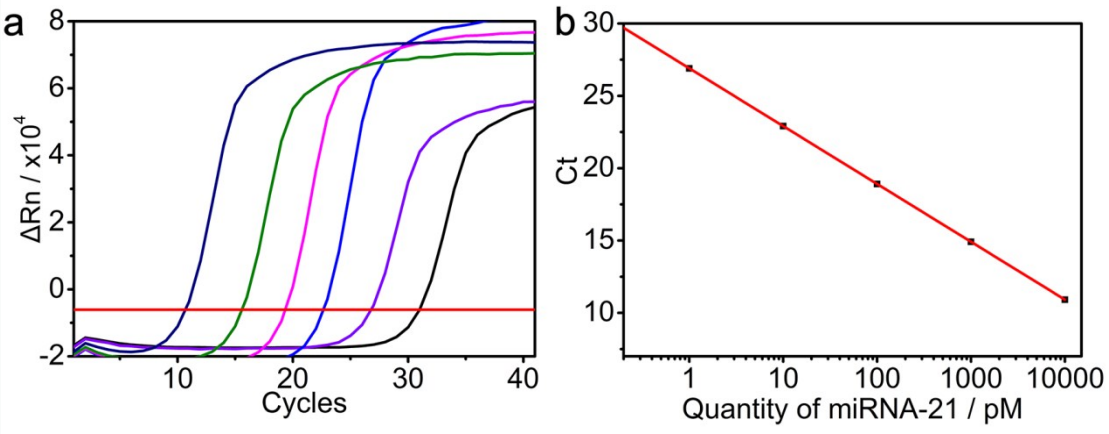

Fig. S9 (a)Amplification curves of miRNA 21 input ranging from 1 pM to 10 nM in qRT-PCR. (b) Standard curve of miRNA 21.

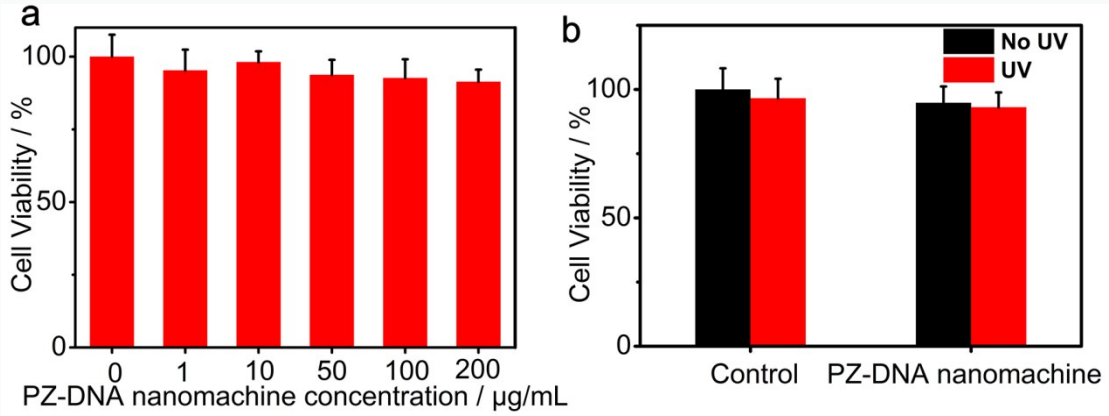

Fig. S10 MTT assays of HeLa cells. (a) Cytotoxicity of PZ-DNA nanomachine with different concentrations. (b) Cell viability of HeLa cells incubated with PBS (control) and 50 μg/mL PZ-DNA nanomachines with or without 5 min UV (7 mW/cm^2) irradiation. The error bars indicate means ± S.D. (n=3).