Supplementary Data

Intracellular Activated Logic Nanomachines Based on Frame Nucleic Acids for Low Background Detecting of microRNAs in Living Cells

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Table of contents

1. Experimental section

1.1 Materials and reagents

The purified oligonucleotides (listed in Table S1 S2 and S3, Figure S4), were obtained from Sangon Biotech Co., Ltd. (Shanghai, China) and Jiangsu Genecefe Biotechnology Co., Ltd. (Wuxi, China). Phosphate buffer saline (PBS, pH 7.2), Roswell Park Memorial Institute (RPMI)1640, Dulbecco's modified Eagle's medium (DMEM) and Hoechst 33342 were purchased from KeyGen Biotech Co., Ltd. (Nanjing, China). Lyso-Tracker Green was bought from Invitrogen (Shanghai, China). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies. All reagents are analytical grade and require no further purification for use. All solutions were prepared through the ultrapure water which was using a Millipore Milli-Q water purification system (Billerica, MA).

Table S1. DNA sequences used to form TP-SFH.

Name	Sequence (5' to 3')
TP1	TCGCTGAGTAttttCCACCACCAAACCACATTTGttttGCATCACTGGGCACC GACACttttCGCACCGCGACTGCGAGGACttttCACAAATCTG
TP2	CACTGGTGAGttttATCAAGAAGCCGAATTGAAGttttTACTCAGCGACAGA TTTGTGttttCGCTCTTCTATACTGGCGGAttttGGTTTGCTGA
TP3	CCAGTGATGCTTTTCAACCCACAATCCCAGTGTGTTTTCTCACCAGTGT CAGCAAACCTTTTCCATGACGATGCACTACGGGTTTTGTGTCGGTGC
S1-Alexa	Alexa Fluor 488-CCTACGTCTCCAACTAACTTACGG
S1-BHQ2	BHQ2-CCTACGTCTCCAACTAACTTACGG
S1-Cy3	Cy3-CCTACGTCTCCAACTAACTTACGG
S2	CCCTTAGCTTATCAGACTGA
S2-15T	TTTTTTTTTTTTTTTTCCCTTAGCTTATCAGACTGA
S3-BHQ1	TCAACATCAGTCTGATAAGCTAAGGGCCGTAAGTTAGTTGGAGACGTA GG-BHQ1
S3-TP	CAAATGTGGTTTGGTGGTGGttttttTCAACATCAGTCTGATAAGCTAAGG GCCGTAAGTTAGTTGGAGACGTAGG
S3-TP-BHQ1	CAAATGTGGTTTGGTGGTGGttttttTCAACATCAGTCTGATAAGCTAAGG GCCGTAAGTTAGTTGGAGACGTAGG-BHQ1
S3-TP-Cy5	CAAATGTGGTTTGGTGGTGGttttttTCAACATCAGTCTGATAAGCTAAGG GCCGTAAGTTAGTTGGAGACGTAGG-Cy5
F	CCTACGTCTCCAACTAACTTACGGCCCTTAGCTTATCAGACTGA
F-TP	TCCGCCAGTATAGAAGAGCGttttttttttttttttttCCTACGTCTCCAACTAACTT ACGGCCCTTAGCTTATCAGACTGA
H(H3)	TTCCCTTCTTTTTCTTCCCTTAAGCTAAGGGCCGTTAAGGGAAG
cS	CCACCACCAAACCACATTTG
cF	CGCTCTTCTATACTGGCGGA

Table S2. Sequences used in this work.

Name	Sequence (5' to 3')
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-21 mimic	TAGCTTATCAGACTGATGTTGA
miR-21 inhibitor	TCAACATCAGTCTGATAAGCTA
miR-155	UUAAUGCUAAUCGUGAUAGGGGUU
miR-373	GAAGUGCUUCGAUUUUGGGGUGU
miR-122	UGGAGUGUGACAAUGGUGUUUG
miR-221	AGCUACAUUGUCUGCUGGGUUUC
miR-141	UAACACUGUCUGGUAAAGAUGG
Let-7a	UGAGGUAGUAGGUUGUAUAGUU

Table S3. DNA sequences used in pH nanoswitches.

Name	Sequence (5' to 3')
H_1	TTCCCTTCTTTTTCTTCCCTTGCTAAGGGCCGTAAGGGAAG
H_2	TTCCCTTCTTTTTCTTCCCTTAGCTAAGGGCCGTAAAAGGGAAG
H_3	TTCCCTTCTTTTTCTTCCCTTAAGCTAAGGGCCGTTAAGGGAAG
H_4	TTCCCTTCTTTTTCTTCCCTT CTAAGGGCCGTAAGGGAAG
H_5	TTCCCTTCTTTTTCTTCCCTTAGCTAAGGGCCGTATAAGGGAAG
H_6	TTCCCTTCTTTTTCTTCCCTTGCTAAGGGCCGTTAAGGGAAG
H-Alexa-BHQ1	BHQ1-TTCCCTTCTTTTTCTTCCCTTAAGCTAAGGGCCGTT-Alexa Fluor 488-AAGGGAAG
H_T	TTTTTTTTTTTTTCTTCCCTTAAGCTAAGGGCCGTTTTTTTTTT

1.2 Apparatus.

UV-vis absorption spectra were obtained on a UV-vis spectrophotometer (Nanodrop-2000C, USA). The fluorescence experiments were conducted on F-7000 fluorescence spectrophotometer (Hitachi Ltd., Japan). The hydrodynamic size was measured by the 90 Plus/BI-MAS equipment (Brookhaven Instruments Co., USA). Confocal fluorescence images of cells were acquired with a TCS SP8 confocal microscopy (Leica, Germany). The cell viability assay was performed using a Thermo Scientific Varioskan Flash (Thermo Fisher Scientific, U.S.A.).

1.3 Preparation of TP-SFH.

The DNA strands were dissolved in PBS (10 mM, pH 7.2), and the absorbance of oligonucleotides at 260 nm was determined by UV-vis absorption spectroscopy. The DNA framework, as the carrier, was composed of TP1, TP2 and TP3. The substrate S was composed of three DNA strands (S1, S2, S3-TP) and fuel strand FH was composed of two strands (F-TP and H). The above DNA strands were mixed in equal quantities, annealed in PBS to 95 ℃ for 5 min, and then slowly cooled down to room temperature to obtain the framework TP, substrate strand S and fuel strand FH. The probe TP-SFH was obtained by mixing TP, S and FH at the same molar ratio and incubated at room temperature for at least 3 h.

1.4 Gel Electrophoresis.

The EDR system was obtained by incubating the mixture of 1 μM substrate S (S1*S2*S3), 1 μM single fuel strand F and 400 nM miR-21 at 37 ℃ for 3 h. Meanwhile, different concentrations of miR-21 were added to the EDR system and incubated at 37 °C for 3 h to explore the feasibility of EDR reaction. 12% native polyacrylamide gel was used in the EDR validation experiment and 4-15% native polyacrylamide gel was used in the probe assembly experiment. The Gel electrophoresis experiment was conducted in 1×TBE buffer. During imaging, the DNA bands were stained with ethidium bromide (EB) for 10 min. The Biorad fluorescence gel imaging system was used to image and analyze the DNA bands.

1.5 Dynamic study on pH response characteristics of TP-SFH in vitro.

The H strand complementary to the part of the fuel strand F was selected, the number and sequence of complementary bases of the two were adjusted and named H_1-H_6 (listed in Table S3). Firstly, the same molar ratio of F and H_X mixed annealing to obtain FH_X. The 100 nM substrate S (S1*S2*S3) was mixed with 100 nM FH_X, and the 25 nM target miR-21 was added. The mixture was dissolved in PBS buffers with pH 5.0 and 7.2, respectively. After incubation at 37 ℃ for 3 h, the fluorescence intensity was tested.

Alexa Fluor 488 and BHQ1 were used to label the H strand (called H-Alexa-BHQ1, the sequence was listed in Table S3) and hybridized with the fuel strand F to form FH-Alexa-BHQ1 hybrid product. 100 nM H-Alexa-BHQ1 and FH-Alexa-BHQ1 were incubated in PBS buffers of different pH respectively for 1 h, and then fluorescence intensity of the above solutions was recorded by fluorescence spectrometer.

1.6 Time-Dependent Fluorescence Measurements.

In this system, miR-21 was selected as the target for verification. The TP-SFH probe assembled at 100 nM was added with 25 nM miR-21, and the fluorescence intensity curve with time at 520 nm was recorded. The cS strand hybridized with sequence complementary to the frame in S3-TP (CS), while the cF strand hybridized with the region complementary to the frame in F-TP (CF) to avoid interference from the secondary structure of the long strand DNA itself. The cS and cF strands were listed in Table S1. Similarly, 100 nM substrate strand CS (S1*S2*S3-TP*cS) and 100 nM fuel strand CF (F-TP*cF) were mixed in the solution, and 25 nM miR-21 was added. Under the same conditions as the above reaction, the fluorescence intensity curve with time was recorded.

We estimated the final concentration of all species. According to the van't Hoff equation, the Gibbs free energy change is given by

$$\Delta G = \Delta G_W^0 + \Delta G_{S1}^0 + \Delta G_{S2}^0 - \Delta G_S^0 - \Delta G_F^0 + RTlnQ \quad (1)$$

The ΔG_X^0 is the standard free energy of species X under standard conditions, R is gas constant (8.314 J/mol · K), T is the temperature (K) and Q is the reaction quotient. We used the NUPACK website to calculate ΔG_X^0 of each reactant and product and get

$$\Delta G_W^0 + \Delta G_{S1}^0 + \Delta G_{S2}^0 - \Delta G_S^0 - \Delta G_F^0 = -2.3\ kcal/mol \quad (2)$$

When the reaction is in equilibrium, $\Delta G = 0$, so we got Q is equal to 41.76. At the same time,

$$Q = \{([W]/c^0)([S1]/c^0)([S2]/c^0)\}/\{([S]/c^0)([F]/c^0)\} \quad (3)$$

The c^0 =1 M, and we used 100 nM as the initial concentration of S and F, and let the final concentration of signal strand S1 be x (unit: nM), then we could get the following equation:

$$\frac{(10^{-9}x)^3}{[10^{-9}(100-x)]^2} = 41.76$$

Using the dichotomy, the value of x is estimated to be between 99.99 and 99.999 nM, so the potential conversion rate of the system is more than 99.99% without considering the reaction time.

1.7 In vitro detection of miR-21.

Using miR-21 as the target of intracellular activated nanomachines, the sensing ability of the TP-SFH was tested. Firstly, 200 ul 100 nM TP-SFH probe was incubated in PBS buffer with pH 5.0 for 30 min. Then different concentrations of miR-21 were added to the above solutions and incubated at 37 ℃ for 2 h. The fluorescence intensity of the obtained solutions was measured and the response curve was obtained. To verify the selectivity of nanomachine TP-SFH, miR-155, miR-373, miR-122, miR-221, miR-141, let-7a were selected, incubated under the above conditions, and the fluorescence intensity was recorded.

1.8 Cell culture.

MCF-7 cells were cultured in RPMI-1640 medium containing 10 % FBS in a 5 % CO_2 environment at 37 ℃. HeLa cells and MCF-10A cells were cultured in DMEM with 10 % FBS. In the fluorescence imaging experiment, the cells were seeded in a 35 mm glass substrate dish and incubated overnight in an incubator. After cleaning with PBS, different concentrations of probe were added and the cells were cultured at 37 ℃ for different time. Living cells were stained with Hoechst 33342 and Lyso-Tracker Green according to the trade instructions.

1.9 Cytotoxicity.

MCF-7 cells were dispersed on 96-well plates with 2×10^4 cells per well and incubated at 37 ℃ with 5 % CO_2 for 24 h. After removing the original medium, TP-SFH probes with different concentrations (0, 10, 20, 40, 60, 80, 100, 120, 140, 160 nM) were added and incubated for another 10 h. Add 50 uL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution to each well and incubate them at 37 ℃ for 4 h. Then, the MTT solution was removed and 150 uL DMSO was added to each well to dissolve the remaining precipitation. Finally, the absorption intensity of each well at 490 nm was recorded.

1.10 Cellular uptake assay.

Cells were seeded on a dish with a glass bottom and cultured overnight. After cleaning twice with PBS, the cells were cultured in culture medium containing different concentrations of probe, incubated at 37 ℃ for different time. The culture medium was then removed and the cells were stained with Hoechst 33342 for 8 min to labeled nucleus. For colocalization examination, the cells were further stained with Lyso-Tracker Green. Finally, the medium was replaced with PBS and the cells were used for fluorescence imaging on the confocal microscope.

1.11 Intracellular miR-21 detecting experiment.

After the cells were cultured in a glass dish for 12 h, 100 nM probe was incubated with the cells at 37 ℃ for 5 h. The cultured cells were cleaned three times with PBS and then the confocal fluorescence images of living cells were monitored using a Leica TCS SP8 laser scanning confocal microscope. Similarly, all controlled experiments were performed without changing the concentration of the DNA reactants unless otherwise noted. In the experiment to verify the feasibility of the probe, miR-21 mimics and miRNA-21 inhibitors were transfected into cells using lipo-3000. Firstly, the miR-21 mimics or miR-21 inhibitors (1 μL, 40 μM each) in 200 μL of Opti-MEM were gently mixed with lipo-3000 (3 μL) in 200 μL of Opti-MEM for 10 min. Then the cultured cells were transfected with the 400 μL of above mentioned Opti-MEM mixture supplied with 80 μL FBS at 37 ℃ for 4 h. Then the cells were washed with PBS for 3 times, and co-incubated with the probe for 5 h and cleaned with PBS for confocal experiment. Fluorescence images of all cells were collected using the Leica TCS-SP8 laser scanning confocal microscopy system.

1.12 qRT-PCR quantification of miR-21 expression.

Cell suspensions of MCF-7, HeLa and MCF-10A were collected, 1 mL Trizol was added, and placed at room temperature for 5 min. Then 200 μL chloroform was added, centrifuged in 4 ℃ at 12000 r/min, RNA in the upper aqueous phase was transferred into a new tube, and isopropyl alcohol was added. After shaking, let stand at -20 ℃ for 30 min. After centrifugation and cleaning with 75% ethanol for two times, 100 μL RNase-free ddH_2O was added to measure its concentration and purity. cDNA samples were then prepared by reverse transcription reaction using Vazyme reverse transcription kit according to the instructions. The PCR process was as follows :20 μL reaction solution (containing 1 μL cDNA sample, 10 μL 2X ChamQ Universal SYBR QPCR Master Mix, 0.4 μL upstream primer (10 μM), 0.4 μL downstream primers (10 μM) and 8.2 μL nuclease free water) under 95 ℃ for 2 min, then followed by 40 cycles of 95 °C for 15 s, 57 °C for 20 s and 72 °C for 15 s.

miR-21 forward primer: 5 '- ACACTCCAGCTGGGTAGCTTATCAGACTG - 3;
miR-21 reverse primer: 5 '- CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC- 3';
U6 forward primer: 5'-CTCGCTTCGGCAGCACA-3';
U6 reverse primer: 5' -AACGCTTCACGAATTTGCGT-3'.

2. Supplemental Figures

Lane	DNA
①	20 bp marker
②	miR-21
③	S1-FAM
④	S2-15T
⑤	S3-BHQ1
⑥	F
⑦	S1-FAM + S3-BHQ1
⑧	S1-FAM + S2-15T + S3-BHQ1 (S)
⑨	S+F
⑩	S+F+miR-21

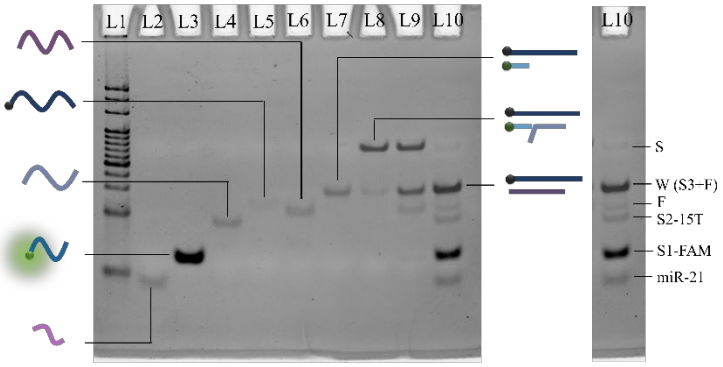


Figure S1. Native PAGE (12 %) characterization of the net EDR. The concentration of DNA used was all 1 μM. The attribution of each band in lane 10 is indicated in the figure. The W band coincides with the S1+S3 band.

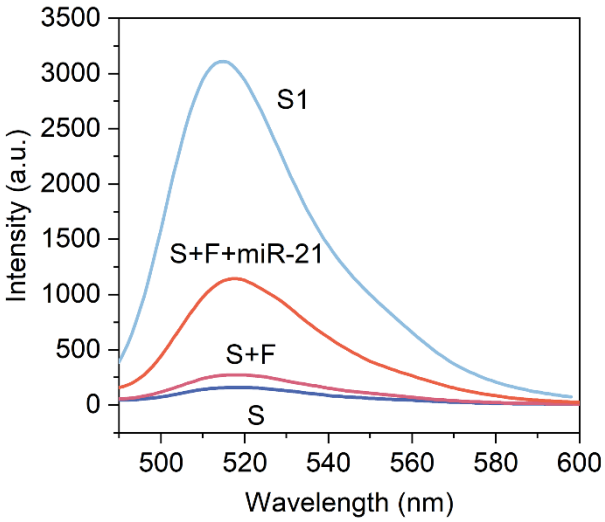


Figure S2. Feasibility exploration of the net EDR system. The fluorescence of Alexa Fluor 488 was strong when only S1 was present. When S1, S2 and S3 were annealed to get S, Alexa Fluor 488 was quenched by BHQ1 and a dark blue curve was obtained. After adding fuel strand F, a background signal is generated due to circuit leakage, pink curve. After miR-21 is added, EDR reaction is driven to occur and strong fluorescence signal is generated, as shown in the red curve in the figure. The results show that part of S1 is still not released from S. The concentration of S1, S and F is 100 nM, and the concentration of miR-21 is 25 nM. The reaction was incubated at 37 ° C for 2 h.

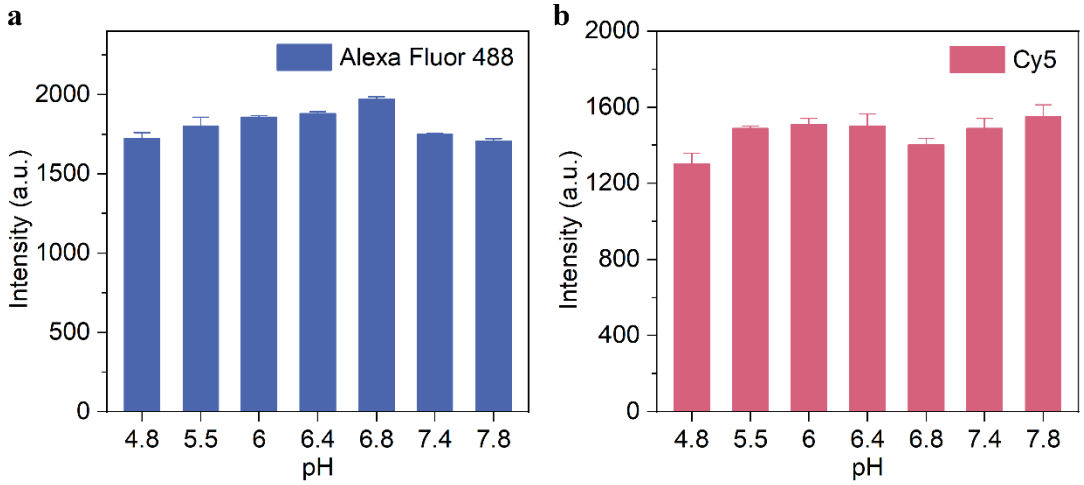


Figure S3. Fluorescence intensities of Alexa Fluor 488 and Cy5 in PBS with different pH. Error bars represent the standard deviation of three replicates.

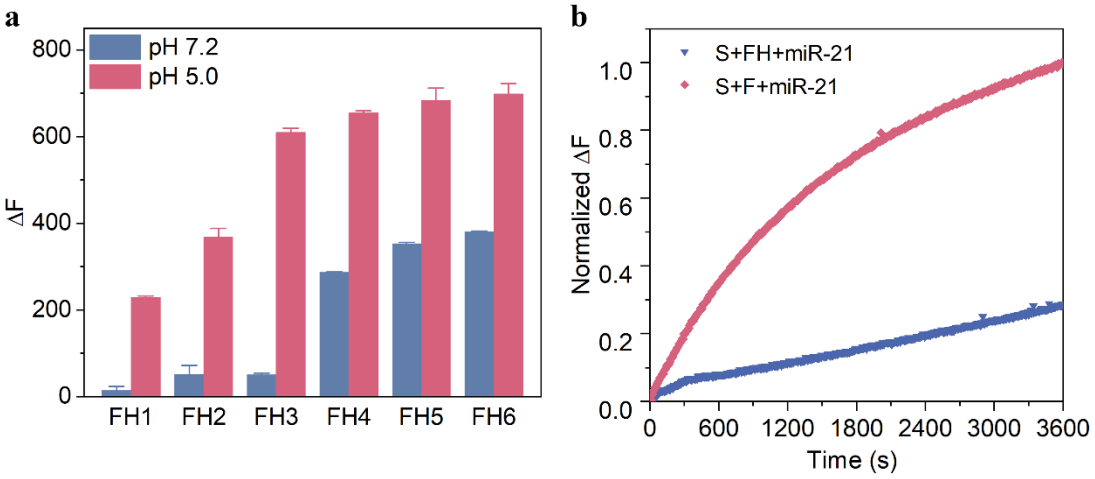


Figure S4. Optimization of pH nanoswitch. (a) When miR-21 was present, the fluorescence intensity changes of FH_X under different pH conditions. (b) Time-dependent fluorescence spectra of net EDR with and without pH nanoswitch. The solution has a pH of 7.2.

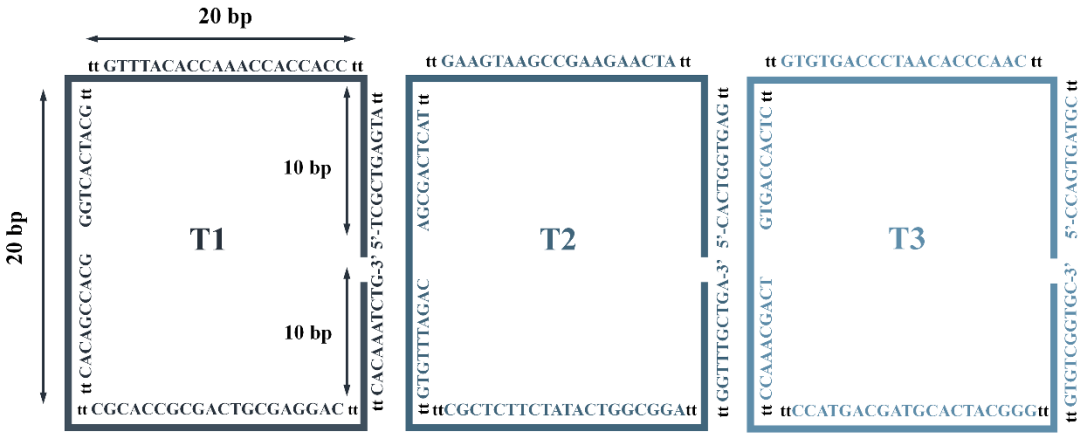
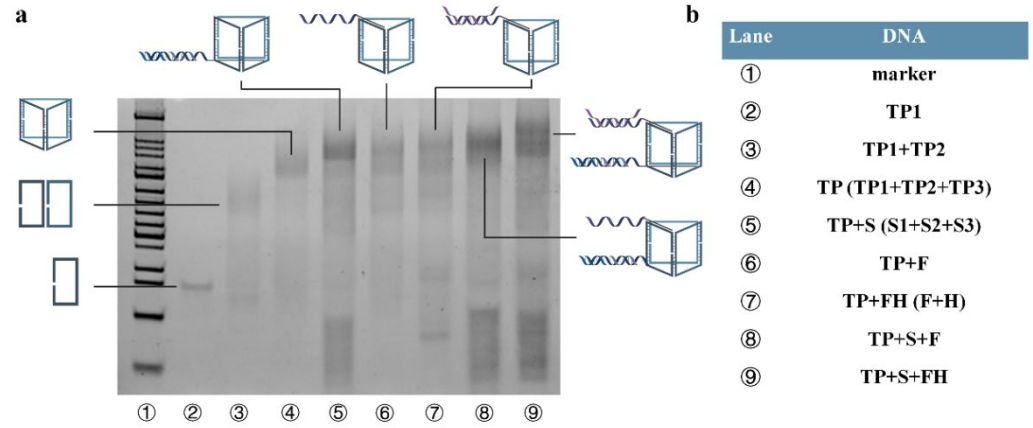
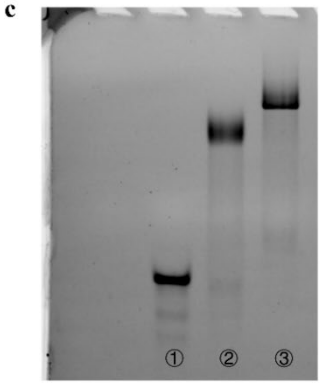


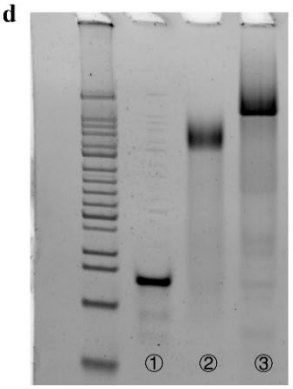
Figure S5. Structure and sequence information of the DNA TP.



b

Lane	DNA
①	marker
②	TP1
③	TP1+TP2
④	TP (TP1+TP2+TP3)
⑤	TP+S (S1+S2+S3)
⑥	TP+F
⑦	TP+FH (F+H)
⑧	TP+S+F
⑨	TP+S+FH





e

		Gray value	Yield
Blank	-	583	-
①	TP2	23381	-
②	TP (TP1+TP2+TP3)	20944	89.31 %
③	TP-SFH (TP+S+FH)	21489	91.70 %

Figure S6. (a) Native PAGE (4 - 15%) characterization of the construction of TP-SFH, and the information for each lane is listed in (b), and the band fluorescence came from EB. (c) Gel electrophoresis, FAM was modified on TP2, and band fluorescence came from FAM. (d) Gel electrophoretic diagram obtained by EB staining of (c). Band fluorescence came from FAM and EB. (e) The information for each lane of (c) and (d). Image-J was used to extract the gray values of the three bands in (c). The concentration of TP is 1 μM, and the concentration of S and FH is 1.2 μM.

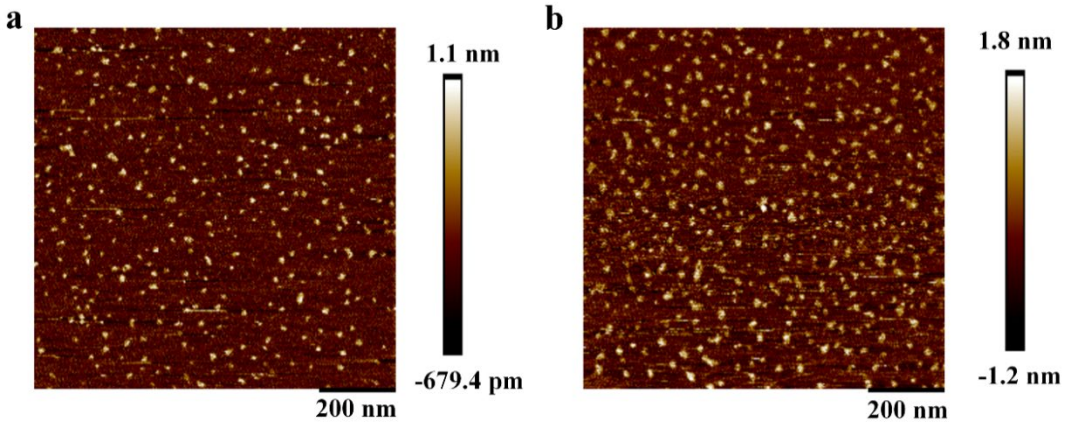


Figure S7. AFM images of the TP (a) and TP-SFH (b) with the concentration of 25 nM.

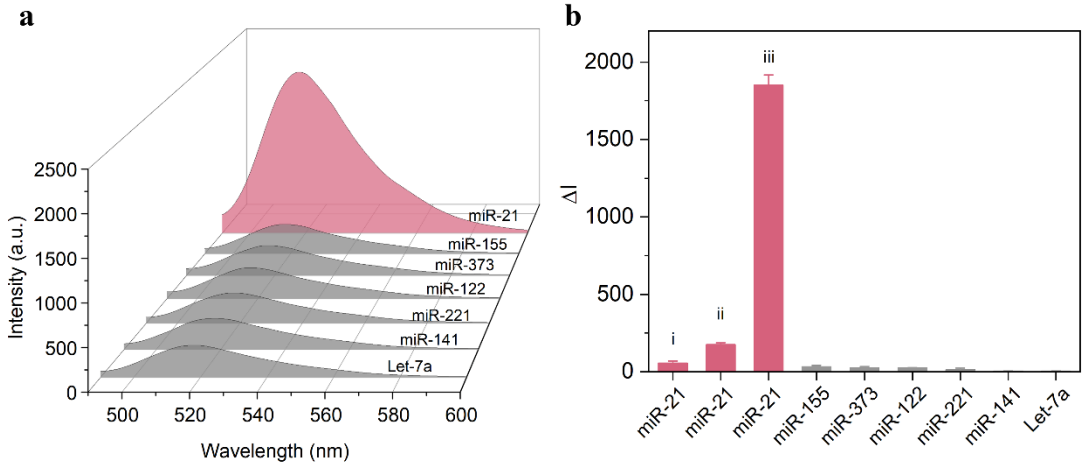


Figure S8. Fluorescence spectra (a) and intensity statistics (b) of miR-21, miR-155, miR-373, miR-122, miR-221, miR-141 and Let-7a on TP-SFH nanomachines. (i) is the ΔI when 0.1 nM miR-21 is added. (ii) is the ΔI when 0.5 nM miR-21 is added. (iii) is the ΔI when 25 nM miR-21 is added. The concentration of other miRNA was 25 nM.

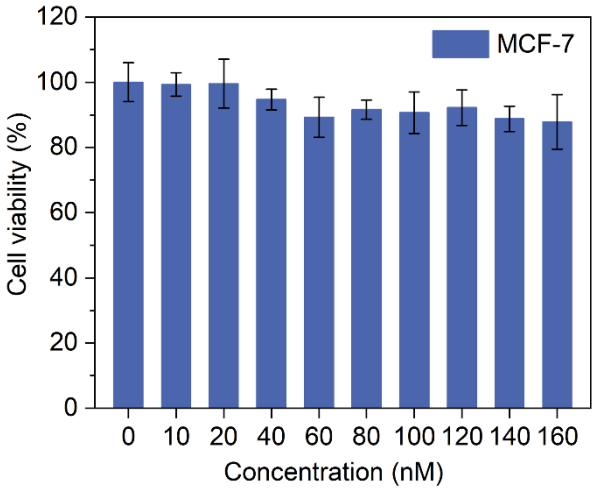


Figure S9. Cell viability of MCF-7 cells incubated with TP-SFH nanomachines with different concentrations for 10 h.

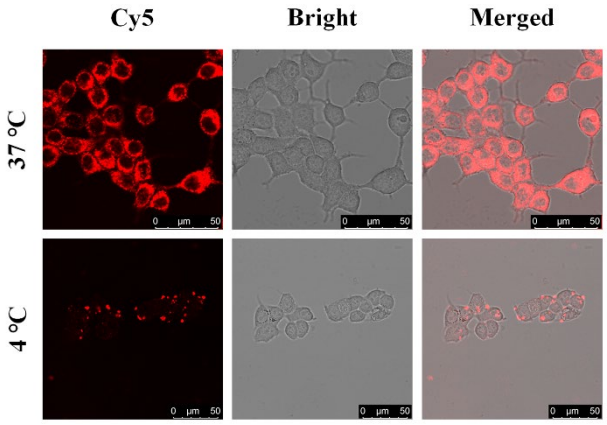


Figure S10. CLSM images of MCF-7 cells after incubation with TP-ON at 37 °C and 4 °C.

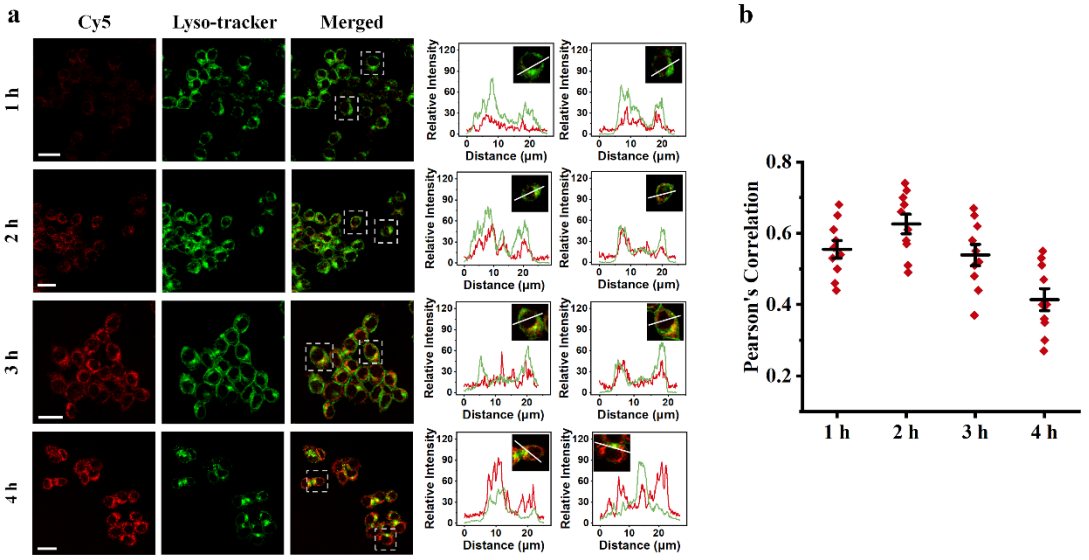


Figure S11. (a) CLSM images of MCF-7 cells after incubation with TP-ON at 37 °C for 1-4 h and stained by Lyso-tracker. Yellow fluorescence signal demonstrates the colocalization of the red Cy5 fluorescence signal and the green Lyso-tracker fluorescence signal. Scale bars: 25 μm. (b) Pearson's correlation of TP-ON and lysosome at different incubation time of 10 cells.

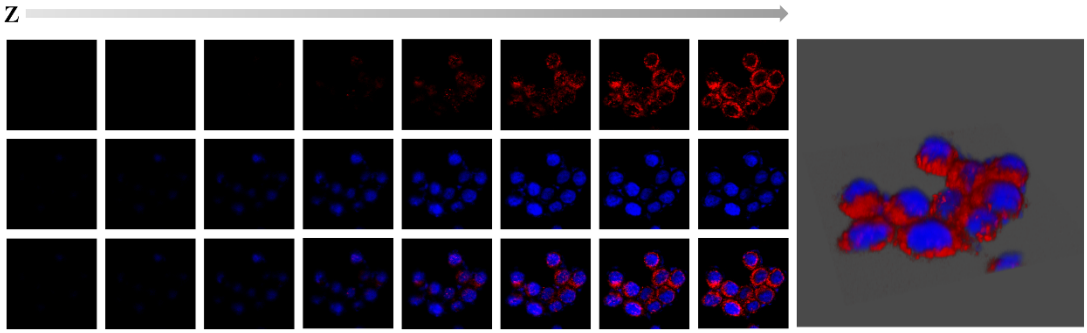


Figure S12. Fluorescence intensity distribution of TP-ON in MCF-7 cells.

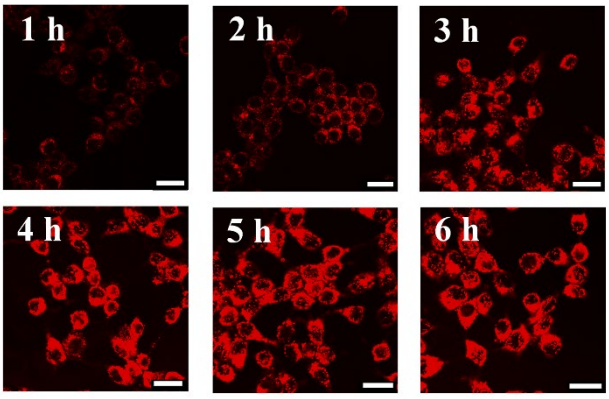


Figure S13. CLSM images of MCF-7 cells treated with the TP-ON at one-hour intervals. All scale bars correspond to 25 μm.

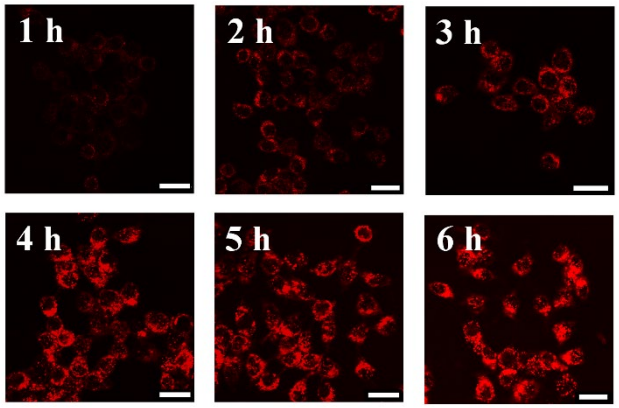


Figure S14. CLSM images of MCF-7 cells treated with the TP-SFH at one-hour intervals. All scale bars correspond to 25 μm.

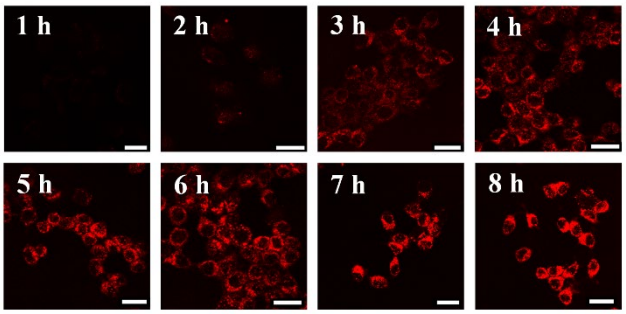


Figure S15. CLSM images of MCF-7 cells treated with the TP-S & TP-FH at one-hour intervals. All scale bars correspond to 25 μm.

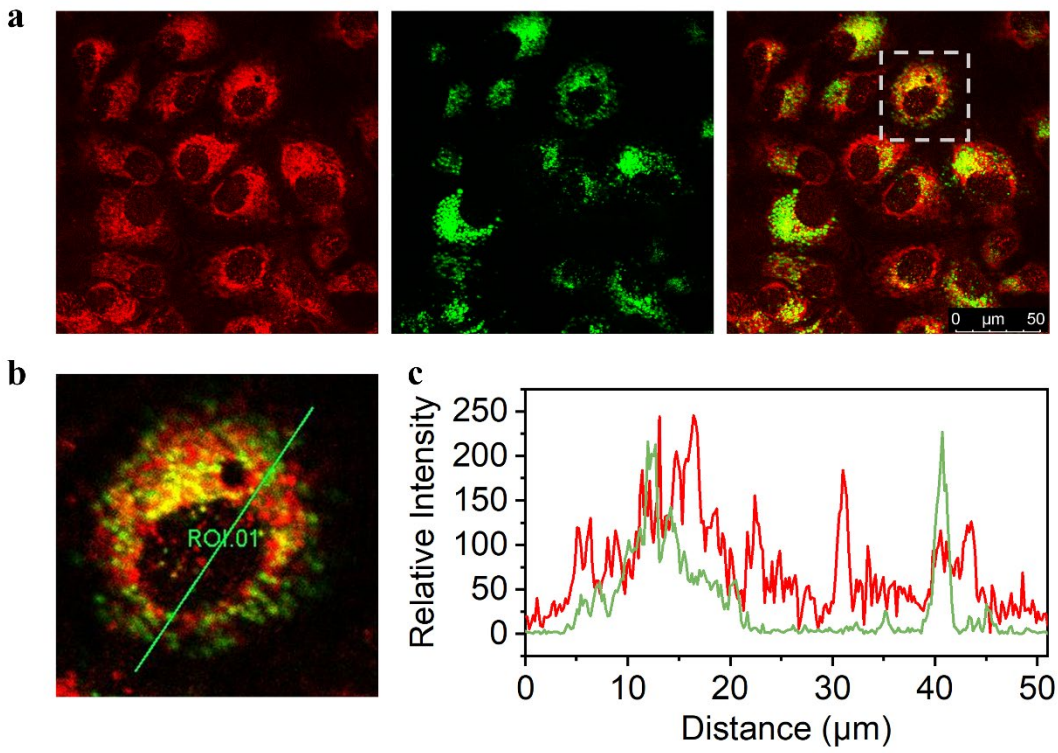


Figure S16. (a) CLSM images of MCF-7 cells after incubation with TP-SFH at 37 °C for 5 h and stained by Lyso-tracker. (b) A magnification of a single cell. (c) The fluorescence intensity profile analysis of Lyso-tracker and Cy5 across the arrowed line in (b).

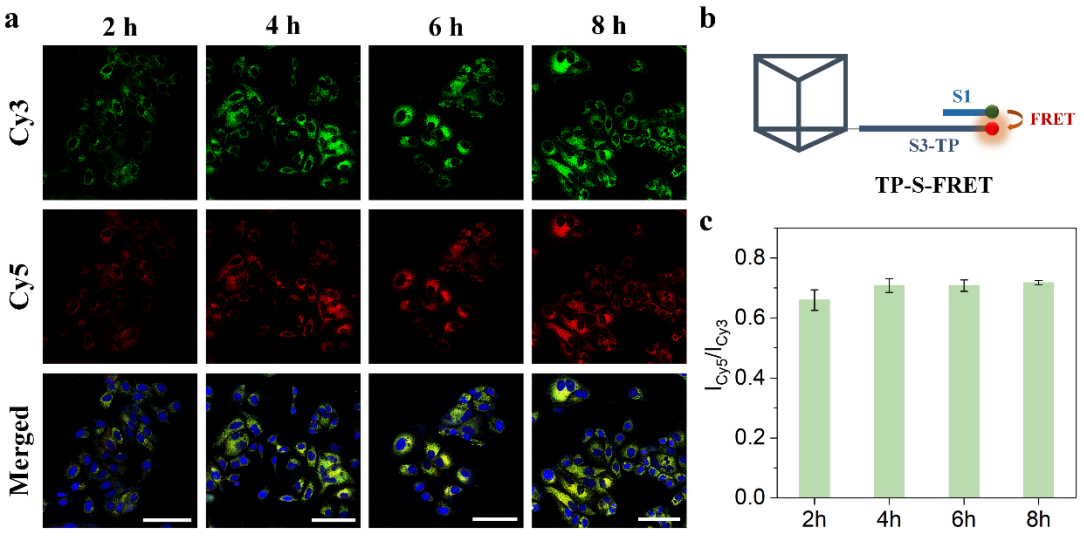


Figure S17. (a) CLSM images of MCF-7 cells after incubation with TP-S-FRET at 37 °C for 2, 4, 6 and 8 h. Five strands of TP1, TP2, TP3, S1, and S3-TP were annealed to obtain a TP-S-FRET probe. Cy3 and Cy5 are used to generate FRET signals. (b) Diagram of probe TP-S-FRET. (c) FRET intensity of each group of cells at different incubation time. The parallel experiment consisted of three groups. The scale bar was 100 μm.

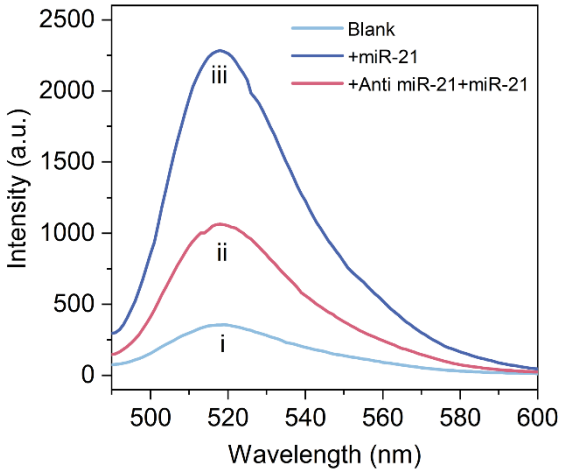


Figure S18. miR-21 inhibitor down-regulates miR-21. The probe used was 100 nM TP-SFH. (i) no target was added. (ii) 25 nM miR-21 was added, and (iii) the same amount of miR-21 and miR-21 inhibitor were incubated at 37 ℃ for 12 h, and the fluorescence spectra was obtained by adding 25 nM pretreated target. All experiments were incubated in PBS buffer with pH 5.0 for 30 min. Then 25 nM miR-21 or preteated miR-21 were added to the above solutions and incubated at 37 ℃ for 2 h.

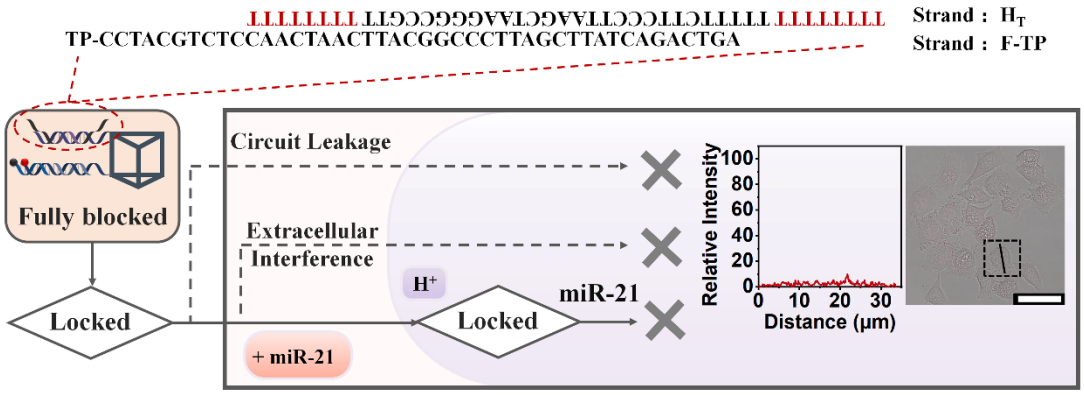


Figure S19. Response flow diagrams and CLSM images of completely blocked nanomachines. The scale bar was 50 μm.

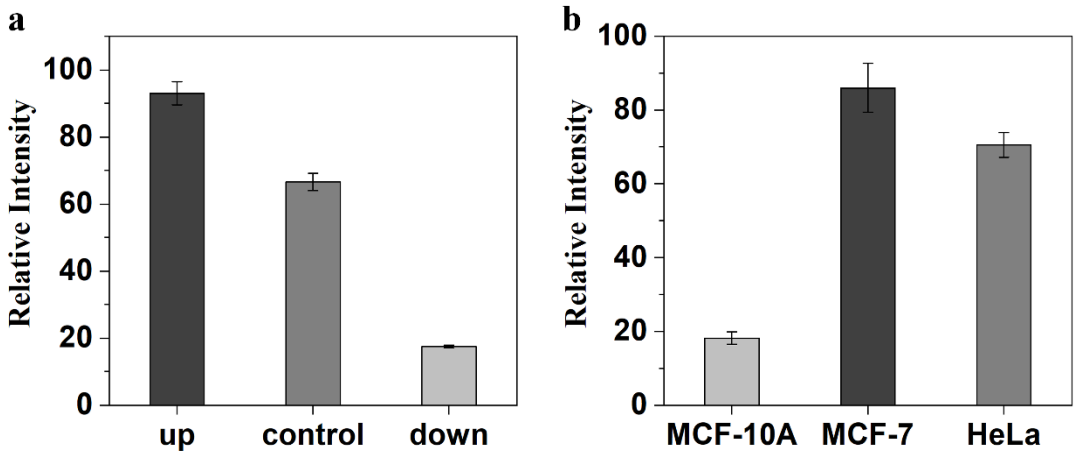


Figure S20. (a) Fluorescence intensity of MCF-7 cells treated with miR-21 mimics, RPMI-1640, and miR-21 inhibitors. The concentration of TP-SFH is 100 nM. Each group of cells was detected three times in parallel. (b) After incubation with TP-SFH, the mean fluorescence intensity analysis of the three cells. Each group of cells was detected three times in parallel.