Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Separable Nanodevice Enables Multilayer Imaging of Diverse Biomarkers for Precise Diagnosis

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Experimental Section

Chemicals and Materials

All oligonucleotides were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The stock solution of each nucleic acid (10 mM) was prepared with TE buffer containing 10 mM Tris-HCl, 1 mM EDTA, 12.5 mM $MgCl_2$ (pH 7.4). The sequences were shown in Table S1. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was purchased from Sigma Aldrich (St. Louis, MO). 40% acrylamide mixed solution, ammonium persulfate (APS), 1,2-bis(dimethylamino)-ethane (TEMED), 5×TBE buffer consisting of Tris (40 mM), acetic acid (20 mM), and EDTA (1 mM) (pH 8.3), DNA loading buffer were purchased from Sangon Bio Inc. (Shanghai, China). Phosphate buffer salt solution (PBS, 10 mM, pH 7.4), fetal bovine serum (FBS), DMEM medium and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Jiangsu Keygen Biotechnology Co., LTD. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and 4′,6-diamidino-2-phenylindole (DAPI) were ordered from Keygen Biotech Co., Ltd. (Jiangsu, Chain). Trisodium citrate, chloroauric acid ($HAuCl_4$) and sodium chloride (NaCl) were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). All the chemicals were of analytical grade and used as received without further purification. RNase-free water was used in all experiments.

Instruments

Transmission electron microscope (TEM) images were taken on JEOL-2100 Transmission electron microscope (Electron optics Laboratory, Japan). UV-vis spectroscopy was performed by UV-3600 spectrometer (Shimadzu, Japan). Fluorescence measurements were conducted on a HITACHI F-7000 fluorescence spectrometer (Shimadzu Co. Japan) equipped with a xenon lamp.

Circular Dichroism (CD) spectra were collected on a Chirascan circular spectropolarimeter (Applied Photophysics, U.K.) in the wavelength range of 220-340 nm. The dynamic light scattering (DLS) was obtained on a 90 plus (Brookhaven Instruments, USA). Polyacrylamide gel electrophoresis (PAGE) was imaged using a Bio-rad ChemiDoc XR imager. Confocal laser scanning microscopy (CLSM) imaging was performed on a Leica TCS SP8 (Germany).

Preparation of Gold Nanoparticles (AuNPs)

The AuNPs were prepared according to the sodium citrate reduction method. Briefly, 10 mL of $HAuCl_4$ (1%) was added to 290 mL of deionized water, and the mixture was heated to a boil with vigorous stirring. Then, sodium citrate tribasic dehydrate (38.8 mM) was quickly added and the color of solution changed from pale yellow to deep red. Finally, the solution was naturally cooled to room temperature and stored at 4 ℃ until use. The as-prepared AuNPs with an average diameter of 13 nm were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). The extinction coefficient of 13 nm AuNPs is 2.7×10^8 $M^{-1} \cdot cm^{-1}$ at 520 nm,[1] and the concentration of the AuNPs solution was determined to be 8.0 nM by UV-vis measurement.

Preparation of I-DNA-Au Nanodevice

15 μL of thiolated hairpin A (SH-A, 100 μM) and 15 μL of FAM-labelled strand C (FAM-C, 100 μM) were heated to 95 ℃ for 5 min and then cooled in ice to room temperature to obtain the FAM-A/C hybrid. Similarly, 15μL of thiolated hairpin B (SH-B, 100μM) and 15μL of TAMRA-labelled i-motif sequence (TAMRA-I, 100μM) were heated to 95 ℃ for 5 min and then cooled in ice to room temperature to obtain TAMRA-B/I hybrid. Subsequently, 30 μL of thiol-labeled FAM-A/C hybrid and TAMRA-B/I hybrid (50 μM each) was activated by 30 μL of tris(2-

carboxyethyl)phosphine (TCEP; 10 mM, pH 5.2) for 1 h, respectively. The activated hybrids were then mixed with 300 μL of AuNPs. After incubation for 16 h at room temperature with shocking, the resulting colloid solution was salted with 40 μL of NaCl (1 M), and the mixture was allowed to stand for 24 h. Next, the solution was purified by centrifuging for 30 min at 12000 rpm three times to obtain the I-DNA-Au nanodevice. Finally, the resulting I-DNA-Au nanodevice was redispersed in a Phosphate buffer salt solution (PBS buffer) and stored at 4 °C for further use.

Native PAGE Characterization

The 12% PAGE was first prepared by 40% acrylamide mixed solution (2.4 mL), 5×TBE buffer (1.6 mL), ultrapure water (4 mL), ammonium persulfate (APS, 56 μL), 1,2-bis(dimethylamino)-ethane (TEMED, 8 μL). Then, the A/C hybrid and B/I hybrid was analyzed by this PAGE in 1×TBE buffer at 150 V for 40 min, after staining with ethidium bromide (EB) and water elutreaction, the resulting gel was imaged by BIO-RAD ChemiDoc XRs.

Fluorescence Measurements

For the response of pH in the buffer solution, 100 μL of PBS buffer with different pHs containing 2 nM nanodevice were incubated at 4 °C for 15 min and then the fluorescence spectra of TAMRA were collected from 550 to 700 nm with 535 nm excitation. For the detection of miR-125a in the buffer solution, different concentrations of miR-125a were added to 100 μL of PBS buffer containing 2 nM nanodevice. After incubation for 2 h at 37 °C, the fluorescence spectra of FAM were collected from 500 to 700 nm with 488 nm excitation. All the fluorescence measurements were repeated three times.

Calculation of Detection Limit.

The detection limit was calculated according to the literature as follows.[2]

The calibration curve is plotted as:

$$Y = a + bX \qquad (1)$$

where a and b are the variables obtained via a least-squares linear regression of fluorescence intensity (named Y) versus the logarithm of miR-125a concentrations (logC, named X)

The linear regression equation can be expressed as $Y=3165.68+276.67LogC$ ($R^2=0.9986$), the a is corresponding to 3165.68, the b is corresponding to 276.67.

The $b > 0$,

$$A = LogC_0 + 3SD \qquad (2)$$

$$LOD = 10^{\frac{A-a}{b}} \qquad (3)$$

where SD is the standard deviation and C_0 is the fluorescence intensity of the blank sample (that is without miR-125a)

In this assay, SD = 33.075, C_0 = 199, thus, A = 101.52. Bring the A, a and b values into the formula (3), the LOD was calculated to be 8.5 pM.

Cell Culture

The human breast cancer cells (MDA-MB-231 cells), human breast cancer cells (MCF-7 cells) and human normal liver cells (L02 cells) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cells were respectively cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FBS and penicillin streptomycin (100 U/mL) at 37 °C in a humidified 5% CO_2 incubator. The cells at the end of the log phase were selected for the following experiments.

Cytotoxicity Assay

The cytotoxicity of nanodevice was assessed by the MTT assay. MDA-MB-231 cells, MCF-7

cells and L02 cells (1×10^5 cells for each well) were cultured in 96-well plate and incubated at 37 °C for 24 h to reach 85% confluency, respectively. After incubation with various concentrations of nanodevice for 24 h, the cells were washed with PBS buffer for three times and then 10 % (v/v) of MTT/DMEM medium were added to each well with incubation at 37 °C for 4 h. Finally, DMSO was added and recorded the absorbance at 490 nm.

CLSM Analysis

The MDA-MB-231 cells with miR-125a overexpression were used as target cells, while the MCF-7 cells and L02 cells were used as negative control cells. To test the fluorescent response of nanodevice as pH sensor in living cells, the three group of MDA-MB-231 cells, MCF-7 cells and L02 cells (1×10^5 cells) were incubated with nanodevice (0.2 nM) for 20 min at 37 ℃ in PBS buffer with different pHs to form the pH-sensitive i-motif structure and induce the nanodevice separation. Then, these treated cells were incubated at 4 ℃ for another 15 min to make the released cholesterol modified i-motif structure anchor to the cell membrane. Finally, washing twice with 1 mL of PBS (10 mM, pH 7.4) and adding fresh medium for CLSM observation. The excitation wavelength and emission filters of TAMRA was set to 535 nm and (570 ± 20) nm, respectively.

To investigate the expression level of miR-125a in different cells, MDA-MB-231 cells, MCF-7 cells and L02 cells (1×10^5 cells for each) were incubated with nanodevice (0.2 nM) were first treated as above procedure. Subsequently, the PBS was removed and added fresh mediums to each well. After incubation for another 8 h at 37 ℃, the mediums were removed and 100 μL DAPI and 100 μL PBS were added into cells to stain the nuclei for 10 min at 37 ℃. Finally, the results were observed by CLSM. The excitation wavelength and emission filters: FAM, 488 nm laser line

excitation, emission BP (520 ± 20) nm; DAPI, 405 nm laser line excitation, emission BP (430 ± 20) nm.

Table S1. All the DNA oligonucleotides sequences used in this work.

Oligonucleotides	Sequences (5'-3')
A-HS	GGCTCCCAAGAACCTCACCTGTGAGGTTCTTGCCATTCCGTAAGTCGTTTT-HS
FAM-C	FAM-CGACTTACGGAATGG
SH-B	SH-TTTTGGGGAAGGGGCGACTTACGGAATGGCAAGAACCTCACAGGTGAGGTTC
Cholesterol-I-TAMRA	Cholesterol-TTTTTTCCCCTTCCCCTTCCCCTTCCCC-TAMRA
miR-125a	ACAGGUGAGGUUCUUGGGAGCC
miR-125b	UCCCUGAGACCCUAACUUGTGA
miR-21	TAGCUUAUCAGACUGAUGUUGA
Single-base mismatched miR-125a	ACAGGUGAGGUACUUGGGAGCC

A is the mismatched base of miR-125a.

Additional Results and Discussion

Optimization of pH Response Conditions.

In order to screen the appropriate fluorophore and the reaction buffer for pH response, the effects of different pHs and reaction buffer on the fluorescence intensity of various fluorophores, including Cy5, FAM, Rhodamine 6G and TAMRA, were explored. As shown in Figure S1, in the PBS buffer, the change of fluorescence intensity of TAMRA with pH was the least. Similarly, the fluorescence intensity of TAMRA in PBS buffer showed the minimal change compared with in HEPES buffer and PB bufffer. Therefore, the TAMRA in PBS buffer was the optimal experiment condition for the subsequent experiment.

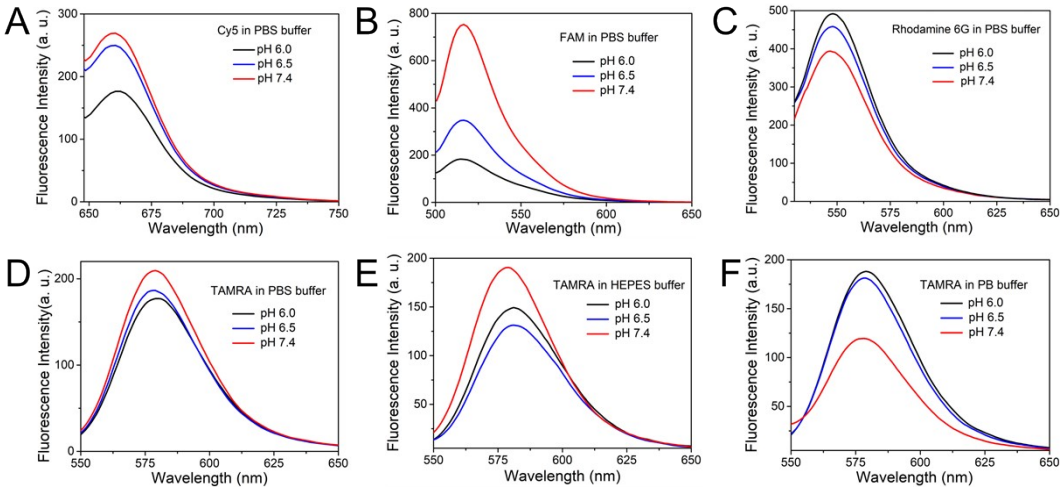

Figure S1. (A-C) Fluorescence spectra of the different fluorophore (Cy5, FAM and Rhodamine 6G) in PBS buffer with different pHs (6.0, 6.5, 7.4). (E, F) Fluorescence spectra of the TAMRA in different buffer (PBS buffer, HEPES buffer and PB bufffer) with different pHs (6.0, 6.5, 7.4).

Characterization of i-motif Structure.

The circular dichroism (CD) spectra was used to characterize the form of the pH-responsive i-motif structure. Briefly, the strand I and the B/I hybrid were diluted by PBS of pH 7.4 and pH 6.0 to the final concentration of 5μM and then incubated for 1 h at 37 ℃ for CD measurements. The CD spectra ranging from 220 nm to 300 nm were recorded. The background spectra of corresponding buffers were subtracted from the samples. As displayed in Figure S2A, the positive band near 280 nm and the negative band near 240 nm of strand I were red-shifted to 290 nm and 263 nm, respectively, as the pH of PBS buffer changes from 7.4 to 6.0, which is consistent with the CD characteristic bands of i-motif structure.[3] The CD spectrum of the B/I hybrid displayed a negative peak at 240 nm and a positive peak at 280 nm (pH 7.4), which are characteristic from double-stranded DNA structure, indicating that the successful hybridization of hairpin B and I.[4] In the PBS buffer of pH 6.0, the positive band of the B/I hybrid was red-shifted to 290 nm as well.

All of these results demonstrate that the pH-responsive i-motif structure has been successfully formed in the PBS buffer with low pH.

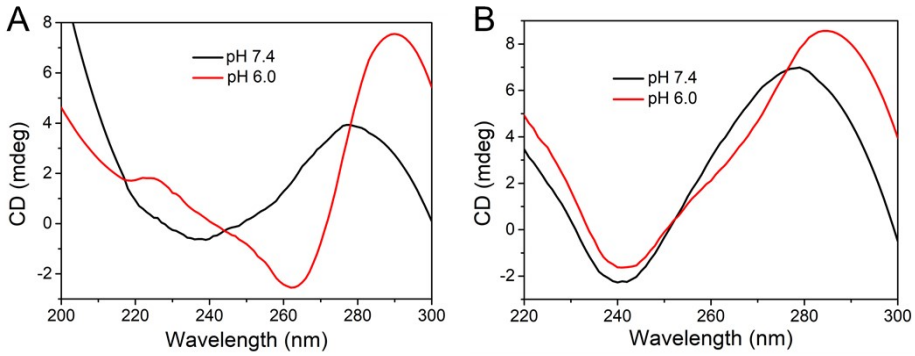

Figure S2. CD spectra of the (A) strand I and (B) B/I hybrid in PBS buffer at pH 6.0 and 7.4, respectively.

Characterization of Nanodevice.

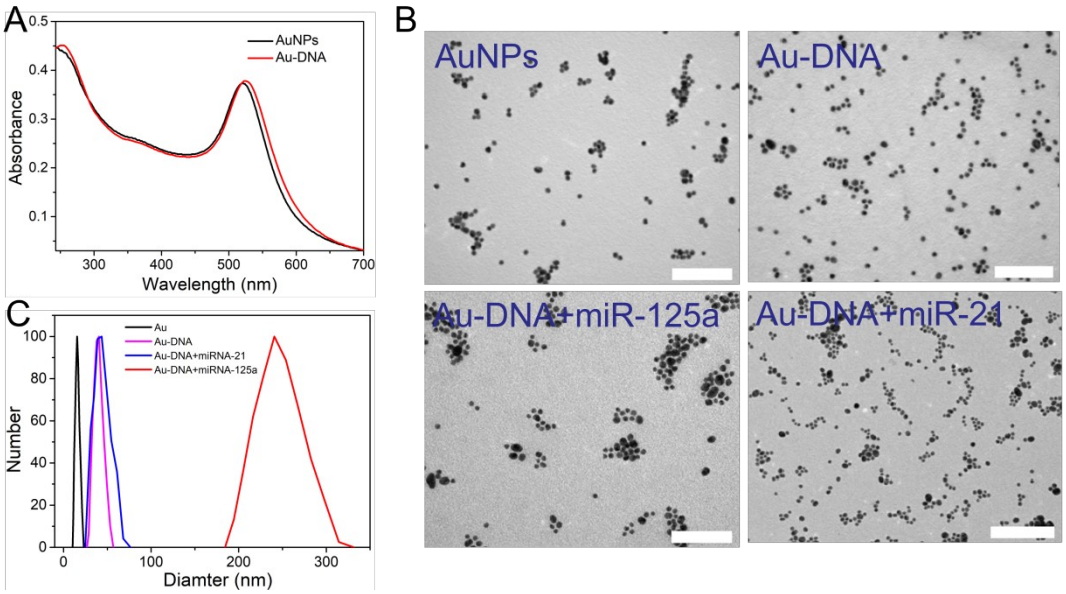

Figure S3. (A) UV-Vis absorption spectra of AuNPs, Au-DNA. (B) TEM images of AuNPs, Au-DNA, Au-DNA+miR-125a and Au-DNA+miR-21, respectively. Scale bar: 150 nm. (C) The DLS results of AuNPs, Au-DNA, Au-DNA + miR-21 and AuDNA + miR-125a, respectively.

Optimization of the LCHA Reaction Time.

The reaction time of the like-catalytic hairpin assembly (LCHA) reaction has been optimized. As displayed in Figure S4, the fluorescence signal of FAM gradually increased from 1 h to 3 h and tended to level off with the extension of reaction time, indicating the completion of the LCHA reaction at this time point, which is identified as the optimized reaction time of LCHA.

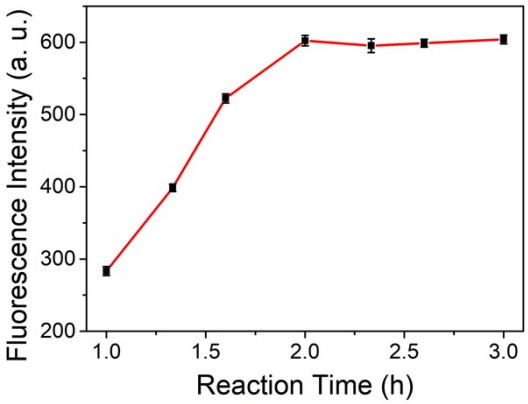

Figure S4. Influences of the LCHA reaction time on the fluorescence signal intensity (miR-125a, 0.1 nM). Error bars represent standard deviations from three repeated measurements.

Selectivity of Nanodevice.

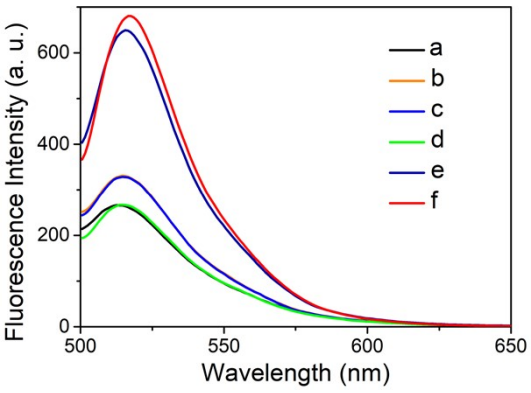

Figure S5. Fluorescence spectra of the nanodevice in response to different species, including (a) blank, (b) miR-125b, (c) miR-21, (d) single-base mismatched miR-125a, (e) the mixture of these

four kinds of miRNAs and (f) miR-125a. The concentration of miR-125a is 0.1 nM, while the other interferent miRNAs are 10 nM.

Stability of Nanodevice.

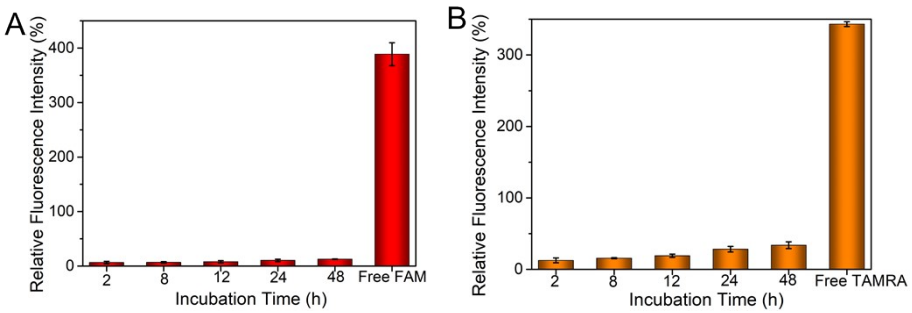

Figure S6.The fluorescence spectra of (A) FAM-labelled nanodevice and (B) TAMRA-labelled nanodevice after incubation with 10% FBS at 37 ° C for different times (2, 8, 12, 24 and 48 h).

Cytotoxicity Study of Nanodevice.

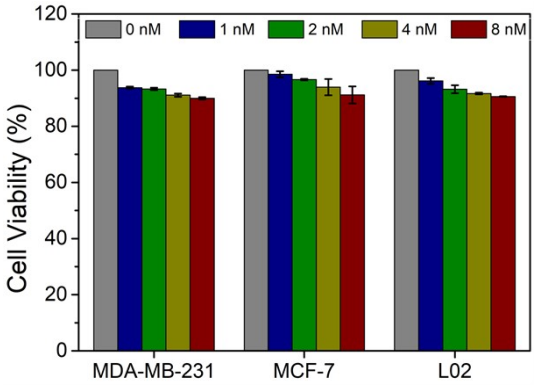

Figure S7. MTT assay of MDA-MB-231 cells, MCF-7 cells and L02 cells treated with different concentrations of nanodevice (0, 1, 2, 4, and 8 nM). The error bars represent the standard deviations from three repetitive measurements.

The Anchoring Property of Nanodevice.

The anchoring property of cholesterol on cell membrane is investigated by CLSM. As displayed in Figure S8A, when the MDA-MB-231 cells treated with cholesterol-modified nanodevice, the clear TAMRA fluorescence (red) can be observed on the cell membrane. Whereas, the cells treated with the cholesterol-unmodified nanodevice, it is difficult to observe the red fluorescence on the cell membrane because the released TAMRA-i-motif DNA mainly existed in the extracellular environment. This result indicates that the cholesterol-modified nanodevice has excellent anchoring performance on the cell membrane, thus establishing the feasibility of nanodevice for extracellular pH sensing.

In addition, the release of TAMRA-labelled i-motif DNA with and without cholesterol modified is monitored by fluorescence spectra as well. As shown in Figure S8B, the cholesterol-unmodified i-motif DNA has the similar fluorescence intensity with that cholesterol-modified, indicating the modification of cholesterol has no effect on the fluorescence of TAMRA.

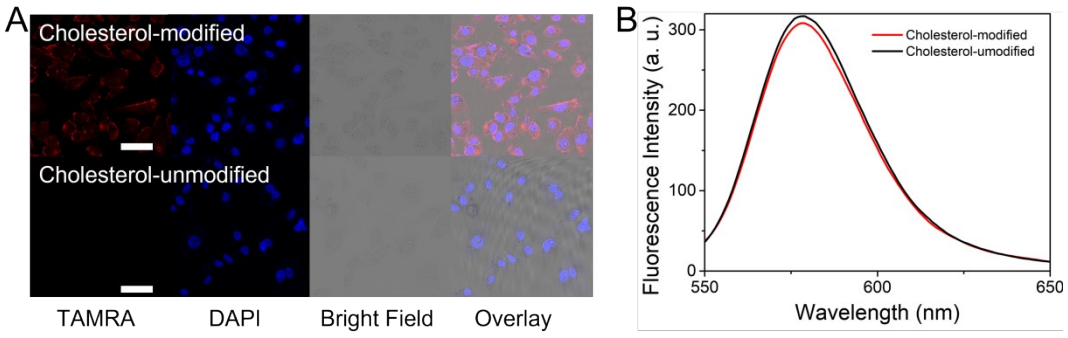

Figure S8. (A) CLSM images of MDA-MB-231 cells treated with or without cholesteryl-modified nanodevice in PBS at pH 6.5 for the assay of anchoring property. Scale bar: 50 μm. (B) Fluorescence spectra of the TAMRA-labelled i-motif DNA with and without cholesterol modified in PBS at pH 6.5.

Monitoring of miR-125a expression levels in MDA-MB-231 Cells.

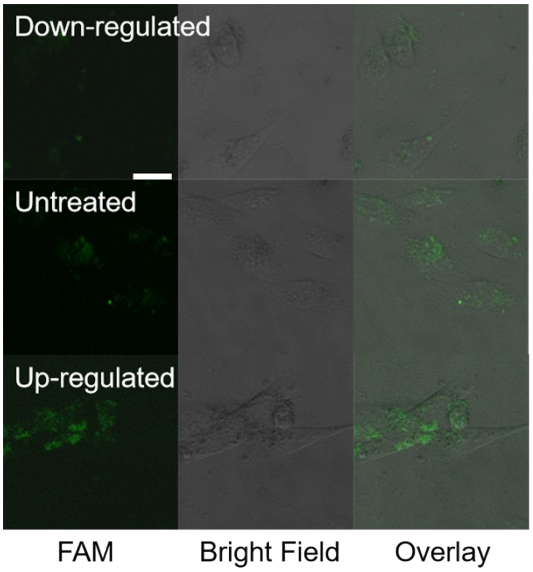

Figure S9. CLSM analysis of the expression levels of miR-125a in MDA-MB-231 cells treated with miR-125a and the antisense miR-125a to up-regulate and down-regulate the miR-125a expression, respectively. An untreated group is used as a control. Scale bar: 20 μm.

Simultaneous Imaging of Nanodevice in MCF-7 Cells and L02 cells.

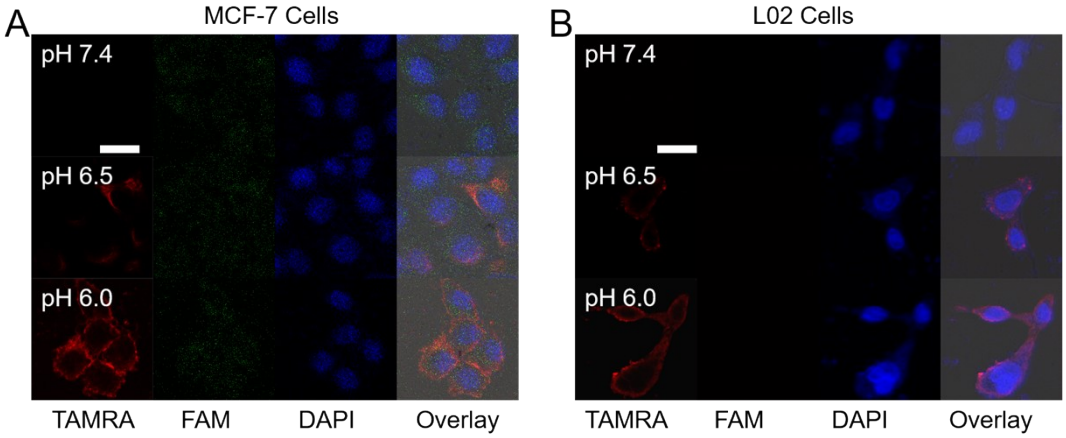

Figure S10. CLSM images of MCF-7 cells and L02 cells treated with nanodevice in mediums at different pHs for simultaneous imaging. Scale bar: 20 μm.

The Rearrangement of Electrophoresis Image

The rearrangement process of electrophoresis image are displayed in Figure S11. These two electrophoresis images are the condition optimization experiment and the repeated experiment of DNA-Au nanodevice probes. The optimal reaction temperature of DNA probes is 37 ℃ and the results have the excellent reproducibility. The electrophoresis image of Figure 1A in the manuscript is obtained by rearrangement the optimal results of these two electrophoresis data. The lanes 1, 2, 3, 4, Control (Ct), 5 of Figure 1A in the manuscript are from the lanes 1-6 in PAGE 1 (blue dashed outline), the lanes 6, 7, 8 of Figure 1A in the manuscript are from the lanes 8-10 in PAGE 2 (red dashed outline). Notably, both the "lane Ct" (that is the A/B hybrid obtained by annealing) and "lane 8" (that is the A/B hybrid obtained in 37 ℃) are used as the control, we still keep the "lane Ct" in the electrophoresis data to ensure the credibility of the data.

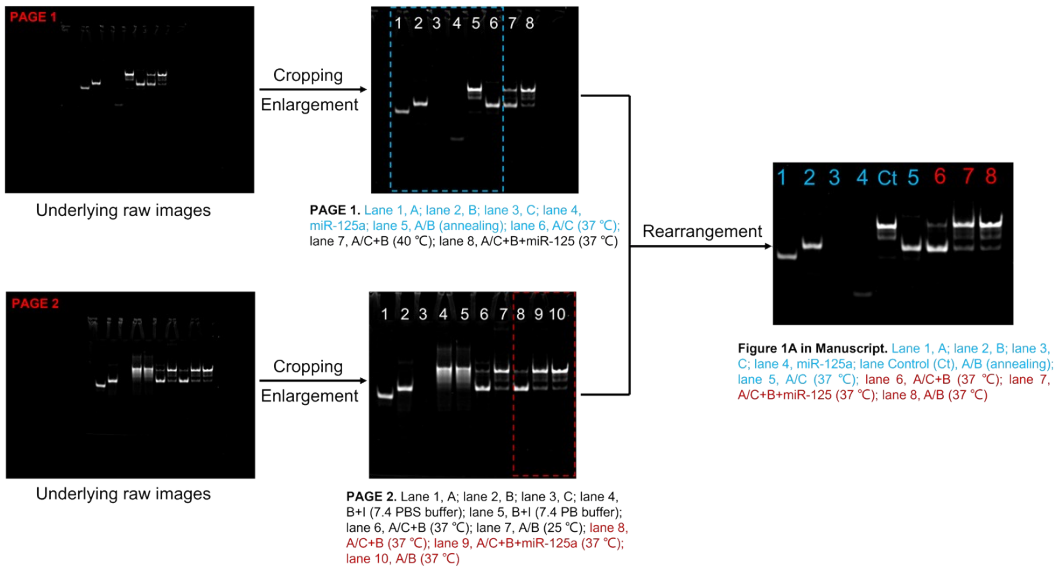

Figure S11. Schematic diagram of the rearrangement process for electrophoresis image.

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