Simultaneous detection of multiple targets involved in PI3K/AKT pathway for investigating cellular migration and invasion with a multicolor fluorescence nanoprobe

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Experimental details:

Materials. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diph-enyltetrazolium bromide (MTT), Bovine insulin and Anisomycin were purchased from Sigma Chemical Company; Tamoxifen, Hydrogen tetrachloroaurate(III) ($HAuCl_4 \cdot 4H_2O$, 99.99%), Sodium dodecylsulfate (SDS), Trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), $MgCl_2$, NaCl, DTT and DMSO were purchased from China National Pharmaceutical Group Corporation (Shanghai, China); SU11274 was purchased from Selleck Chemicals (Shanghai, China). MMP-9 (Recombinant Human Matrix Metalloproteinase-9) was purchased from ProSpec Company. MMP-9 antibody and HRP-conjugated secondary antibody were purchased from Abcam Company. Unless mentioned otherwise, cell culture products were purchased from GIBCO. The chemical reagents were all of analytical grade and used with no further purification. Sartorius ultrapure water of 18.2 MΩ cm was used throughout the experiments. DNA oligonucleotides were synthesized and HPLC-purified by Shanghai Sangon Biotech. The peptide was synthesized and further

purified by Moon Biosciences Co., Ltd. (Wuhan, China). MCF-7 and MDA-MB-231 (the human breast cancer cell lines) were bought from Nanjing KeyGEN Biotech, MCF-10A (the normal human mammary epithelial cell line) was bought from Bioleaf Biotechnology Company (Shanghai, China).

Instruments. Absorption spectra were studied on a TU-1900 UV–vis spectrometer (Purkinje General, China). Transmission electron microscopy (TEM) was taken on a JEM-100CX II electron microscope. Fluorescence spectra were recorded with Fluorescence Spectrometer (Edinburgh, FLS-920) with 1.0 cm quartz cells and a xenon lamp at the slit widths of 3.0/3.0 nm. All pH measurements were measured with a digital pH-meter (pH-3c, Shanghai LeiCi, China). Absorbance in the MTT assay was detected using microplate reader (Synergy 2, Biotek, USA). Confocal imaging were acquired with a confocal laser scanning microscopy (Leica TCS SP5, Germany) with an objective lens (×20). RT-PCR was performed on a LineGene 9620 (Bioer, Binjiang, China).

Synthesis of Oligonucleotides. All synthetic DNA oligonucleotides used to make and test miRNA and mRNA targets were from Shanghai Sangon Biotech. The sequences information of oligonucleotides are showed in Table S1. The thiol-containing oligonucleotides were reduced with (TCEP·HCl) before they were anchored on the surface of AuNPs.

MB Structure. The possible secondary structures of MBs were predicted by UNAfold on www.idtdna.com. It indicated that the two MBs had the “stem and loop” structure.

Preparation of AuNPs. The preparation of 20 nm AuNPs were as the sodium citrate reduction method reported previously.[1] All glassware used was cleaned in aqua regia (HCl/HNO_3, 3:1) newly prepared, washed with triply distilled H_2O, and used after oven drying. A 100 mL $HAuCl_4$ aqueous (0.01 %) was heated to boiling while being vigorous stirring, then trisodium citrate solution (1%, 1.8 mL) was rapidly added under stirring. The solution color changed from pale yellow to colorless and eventually to burgundy. The solution was kept boiling for another 10 min with stirring. After the heating mantle was removed, the colloid was further stirred until the prepared solution reached to room-temperature. Then it was filtrated through a Millipore membrane filter (0.45 μm). TEM images (Fig. S1) indicated the particles are 20 ± 2 nm in size (100 particles sampled). The prepared AuNPs were kept at 4 °C.

Preparation of the Nanoprobe. Equimolar MBs (labeled by Alexa Fluor 488 and Cy5) were mixed and then added to AuNPs solution (1 nM) with 25 nM final concentration for each MB and rocked overnight. SDS solution (10 %) was further added to the mixture to achieve a final concentration of 0.1%. After 12 hours, 0.1 M PBS solution (pH 7.4) was added to the mixture to yield 0.01 M phosphate final concentration with slowly increasing NaCl concentration to 0.1 M over an eight-hour period. This mixed solution was further aged for 48 h at room temperature. Then the resulted solution was centrifuged at 13000 rpm for 30 min at 4 °C and the precipitate was washed in PBS for three times to remove unbound MBs. After that, peptide was added to the nanaocarrier (1 nM) to reach a final concentration of 1500 nM peptide. The mixture was shaken softly for 48 h. After that, the solution was centrifuged

(10000 g, 20 min, 4 ºC) and resuspended in PBS for three times. Finally the nanoprobe was sterilized through 0.22 μm pore size filters and dispersed in PBS with a final concentration of 3 nM as stock solution kept at 4 °C. The nanoprobe was diluted to certain concentration for use throughout subsequent experiments. The concentration of AuNPs was confirmed by detecting their extinction at 524 nm ($\varepsilon = 2.7 \times 10^8$ L mol^{-1} cm^{-1}).

Evaluation of Numbers of Each MB and Peptide on the Nanoprobe. The numbers of each MB and peptide loaded on AuNPs was determined according to the previous Protocol of Demers et al.[2-3] The DTT was added (final concentration of 20 mM) to the nanoprobe (1 nM) and the mixture was incubated overnight at room-temperature in the dark with shaking. The released MBs and peptide were then separated via centrifugation and the fluorescence in the supernatant was detected with a fluorescence spectrometer. For the Alexa Fluor 488 labeled MB, the fluorescence intensity was obtained at 488 nm excitation and 515 nm emission; For the Cy5 labeled MB, the fluorescence intensity was obtained at 648 nm excitation and 668 nm emission; For the RhB labeled peptide, the fluorescence intensity was obtained at 554 nm excitation and 575 nm emission. The fluorescence intensity was converted to molar concentrations of two MBs and peptide by interpolation from standard linear calibration curves that were prepared with known concentrations of two MBs or peptide with identical ionic strength, buffer pH and DTT concentration (Fig. S3). By dividing molar concentrations of two MBs and peptide by the original concentration of nanoprobe, the numbers of two MBs and peptide on nanoprobe was determined.

Response Experiment. In multiplexing detection, the nanoprobe was incubated with the complementary miR-221 target, PTEN mRNA target and MMP-9 target respectively with increasing DNA targets concentrations (0, 5, 10, 15, 20, 30, 40, 50, 90, 100, 120, 150, 200 nM) or MMP-9 concentration (10^{-7}，10^{-6}，10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} μg/mL). After incubation at 37 °C for 1 h, the fluorescence intensity was measured at corresponding excitation wavelengths. All experiments were performed at least three times.

Kinetics. The nanoprobe solution (1 nM) was incubated with precisely matched targets miR-221 (200 nM), PTEN mRNA (200 nM) and MMP-9 (0.4 μg/mL), respectively and the fluorescence intensity was measured on a certain time interval (0, 5, 10, 15, 20, 30, 40, 50, 60 minutes or 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes). The fluorescence of Alexa Fluor 488 was obtained at 488 nm excitation and 515 nm emission; the fluorescence of Cy5 was obtained at 648 nm excitation and 668 nm emission; the fluorescence of RhB was obtained at 554 nm excitation and 575 nm emission.

Specificity Experiment. The precisely matched DNA targets (200 nM), MMP-9 (0.4 μg/mL) for two MBs or peptide and other targets were added to 1 nM nanoprobe at 37 °C for 1 h. The fluorescence intensity was measured at appropriate excitation and emission wavelengths. All experiments were performed at least three times.

The Stability of the Nanoprobe. For the effect of GSH on the nanoprobe, the nanoprobe (1 nM) was incubated at 37 °C for 4 h after GSH (1 mM) addition. The solution was centrifuged to remove particles and the fluorescence of the supernatant

was measured. The three excess corresponding targets were also added to the nanoprobe, respectively. The fluorescence intensities were recorded at appropriate excitation and emission wavelengths. All experiments were performed at least three times.

In this study, the stability and reproducibility of two different batches of nanoprobe were evaluated. The three corresponding targets were added to the nanoprobe (1 nM) produced on the day of preparation (0 day) at 37 °C for 1 h, respectively. And then the fluorescence intensities were recorded at appropriate excitation and emission wavelengths. The same tests were repeated after two batches of nanoprobe were stored for 14 days in dry air at 4 °C. All experiments are done in triplicate.

Cell Culture. All the cells were incubated in Dulbecco's modified Eagles medium which was supplemented with 1% antibiotics penicillin/streptomycin and 10% fetal bovine serum (FBS) and kept in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT Assay. To evaluate the cytotoxicity of the nanoprobe, a tetrazolium-based colorimetric MTT assay was performed. MCF-7 cells were seeded in replicate 96-well microtiter plates (1×10^6 cells/well in 200 μL DMEM). The culture was kept in 5% CO_2/95% air incubator at 37 °C. After cell attachment for 24 h, the initial medium was removed and the cells were cultured with 1 nM naked-AuNPs and 1 nM nanoprobe respectively for different time periods. Then MTT solution (0.5 mg mL^{-1} in PBS, 100 μL) were added to each well after being washed with PBS and further incubated for 4 h. After discarding the remaining MTT solution, DMSO (150 μL) was added to dissolve the purple formazan. The absorbance at 490 nm was recorded with a

RT 6000 microplate reader.

Confocal Fluorescence Imaging. In the experiment for simultaneously imaging miRNA, mRNA and protease involved in the signaling pathways, MCF-7 cells were seeded on chamber slides for 24 h in an incubator at 37 °C and 5% CO_2. One group of MCF-7 cells was treated with anisomycin (10 μM) for 1 h. Then, the cells were cultured with DMEM at 37 °C for 24 h after washing with PBS for three times. The other group of MCF-7 cells was treated with SU11274 (2.5 μM) for 24 h. Untreated MCF-7 cells were maintained in DMEM for 24 h serving as control. The nanoprobe (1 nM) was added to the three groups of MCF-7 cells for 4 h. The fluorescence images of cells treated with nanoprobe were examined by confocal laser scanning microscopy (CLSM) with appropriate laser transmitters. In the experiment for the effect of miRNA loaded in exosomes secreted from MDA-MB-231 on the expression levels of miRNA, mRNA and MMP in MCF-10A, the medium collected from the MDA-MB-231 cells was introduced to MCF-10A cells cultured in DMEM for 2 days (at 37 °C, 5% CO_2) and the same procedure was repeated three times. After six days, the MCF-10A cells were treated with the nanoprobe (1 nM) for 4 h at 37 °C, and then observed by CLSM. MCF-10A cells were cultured in DMEM alone serving as control.

RT-PCR. Total RNA was extracted from sorted cells with the RNA simple Total RNA Kit (Tiangen, China). cDNA synthesis was performed using an FastQuant RT Kit (Tiangen). RT-PCR was carried out with SuperReal PreMix Plus (Tiangen) on an ABI PRISM 7000 sequence detection system. Primer sequences used were as follows: PTEN forward, 5′-TCTAGAGACTCTGATCCAGA GAATGAACC -3′; PTEN

reverse, 5′-TCTAGAGTTGCCACAAGTGCAAAGGGGTAGGATGTG -3′; GAPDH forward, 5′-GGGAAACTGTGGCGTGAT-3′; and GAPDH reverse, 5′-GAGTGGGTGTCGCTGTTGA-3′. Relative expression of PTEN mRNA was expressed as the ratio of PTEN/GAPDH mRNA levels. MicroRNA was isolated from the cell line using the miRcute miRNA Isolation Kit (Tiangen). cDNA synthesis was generated using miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen) in accordance with manufacturer's instructions. RT-PCR of miRNAs was carried out with miRcute miRNA qPCR Detection Kit (Tiangen) on an ABI PRISM 7000 RT-PCR instrument. The primers used in microRNA amplification experiment were as follows: MiR-221 forward, 5′-TGAATGCAGTAGGCAGTTGTG -3′; U6 forward, 5′-ACACTCCAGCTGGGTCGTGAAGCGTTC -3′. The reverse primers were provided by the miRcute miRNA qPCR detection kit. Relative level of miRNA was calculated from the quantity of miRNA PCR products and the quantity of U6 PCR products.

Western Blot Analysis. MCF-7 cells were pretreated with anisomycin or SU11274 for different incubation times. Then cells were washed with PBS and cellular proteins were extracted in a lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1% Triton-100, 50 mM NaF, 5 mM $NaVO_3$, 100 mM PMSF and protease inhibitor cocktail). Protein concentration was determined with BCA Protein Assay Kit (Beyotime, China). Equal amounts of proteins (100 μg) were separated by gel electrophoresis and electransferred onto a nitrocellulose membrane (Bio-Rad) which was blocked with 5% nonfat dry milk in TBST buffer for 2 h at room temperature. Membrane was incubated overnight at 4 °C with the primary

antibodies. Subsequently, membrane was incubated with secondary antibodies conjugated with horseradish peroxidase (HuaAn Biotech, China) for 1 h. Finally, the blot was washed and bound antibodies were detected with the enhanced chemiluminescence reagent according to the manufacturer's instructions (Amersham Biosciences).

Cell Migration Assay. MCF-7 cells were pretreated with drugs and further cultured in DMEM medium during the migration assay. MCF-10A cells were cultured with MDA-MB-231 medium for six days and further incubated in the medium from MDA-MB-231 cells during the migration assay. The cells were seeded into 60-mm Petri culture dishes according to earlier reports. After reaching confluence cells were wounded by dragging a 10 μl sterile pipette tip across the monolayer. The cells were washed with PBS to remove cellular debris or dead cells and allowed to migrate for 48 h. Cell migration images were taken at time 0, 12, 24 and 48 h post-wounding using an inverted microscope. The ratio of wound healing was determined as restored area percentage by measuring the area at each time point and considering 0 h as a blank. The measurements were repeated three times.

Cell Invasion Assay. Cell invasion potential in vitro was evaluated using Matrigel-coated invasion chambers with an 8-μm pore size in 24-well plates (BD Biosciences). After MCF-7 or MCF-10A cells were treated as cell migration assay, 2×10^4 cells which were trypsinized were added into the upper compartment and further incubated for 24 h. The noninvasive cells on the upper surface of the membrane were removed with a cotton-tipped swab. Then the invasive cells which adhered to the lower surface

of the membrane were fixed with 4% paraformaldehyde and stained with 2% crystal violet before counting the number of invaded cells under microscope. Cells were photographed under microscope from ten random fields. Cristal violet-stained cells were decolorized in acetic acid and absorbance were measured at 595 nm with a microplate reader. All the experiments were performed in triplicate.

Supplementary Table:

Table S1. DNA sequences information and peptide applied in this work.

Oligonucleotide	Sequence
MB1	5'-Cy5-AGCTACGAAACCCAGCAGACAATGTAGCTAAAA

	AA -$(CH_2)_3$-SH-3’
MB2	5’-Alexa Fluor 488- AGCTCTCTCATCTCCCTCGCCTGAGCT
	AAAAAA -$(CH_2)_3$-SH-3’
peptide	RhB-Gly-Pro-Leu-Gly-Leu-Ala-Gly-Gly-Cys
miR-221 perfectly matched target	5’-AGCTACATTGTCTGCTGGGTTTC-3’
miR-221 single-base mismatched target	5’-AGCTACATTGTGTGCTGGGTTTC-3’
PTEN mRNA perfectly matched target	5’-AGGCGAGGGAGATGAGA-3’
PTEN mRNA single-base mismatched target	5’-AGGCGAGGCAGATGAGA-3’

[a]Underlined letters represent the stem sequence; [b]Letters in red represent the mismatched site.

Supplementary Figures:

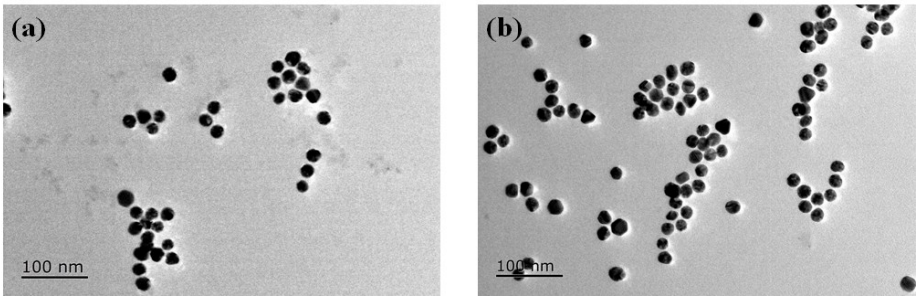


Fig. S1 TEM images of AuNPs (a) and the nanoprobe (b). Scale bars: 100 nm.

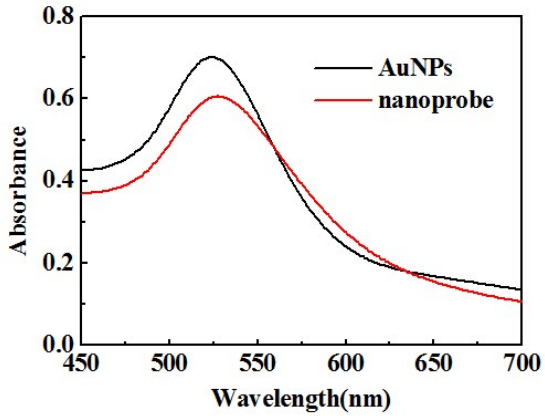
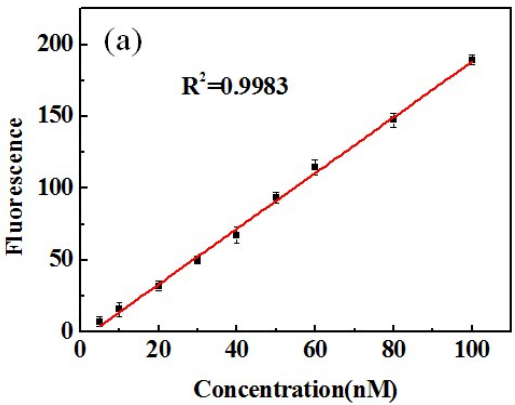


Fig. S2 UV/Vis spectra of AuNPs and the nanoprobe.



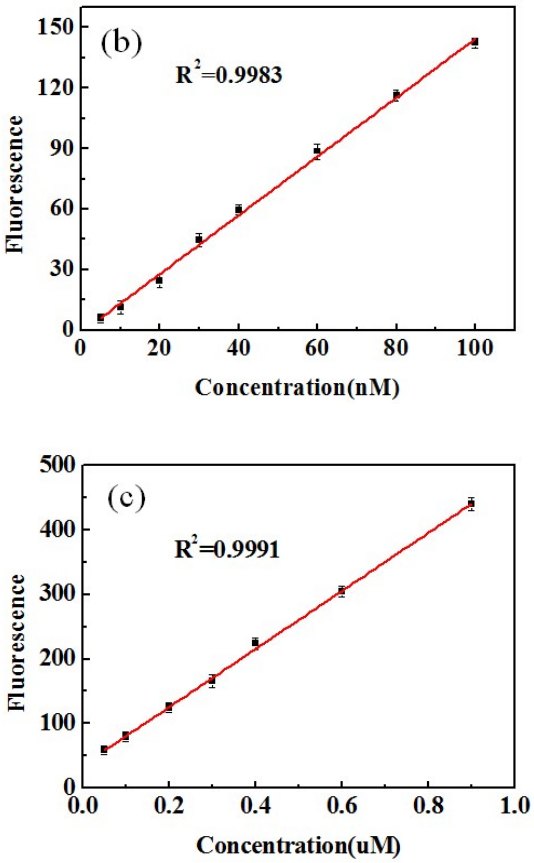
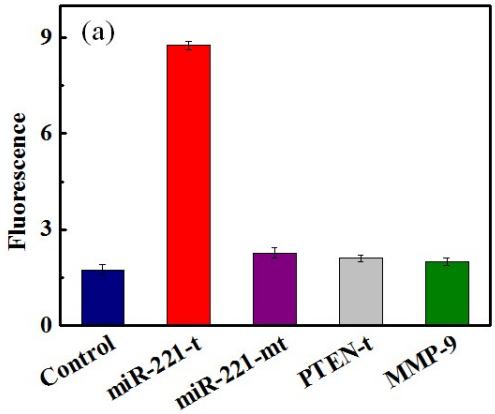


Fig. S3 Standard linear calibration curves of fluorescent dyes. (a) Cy5, (b) Alexa Fluor 488 and (c) RhB.



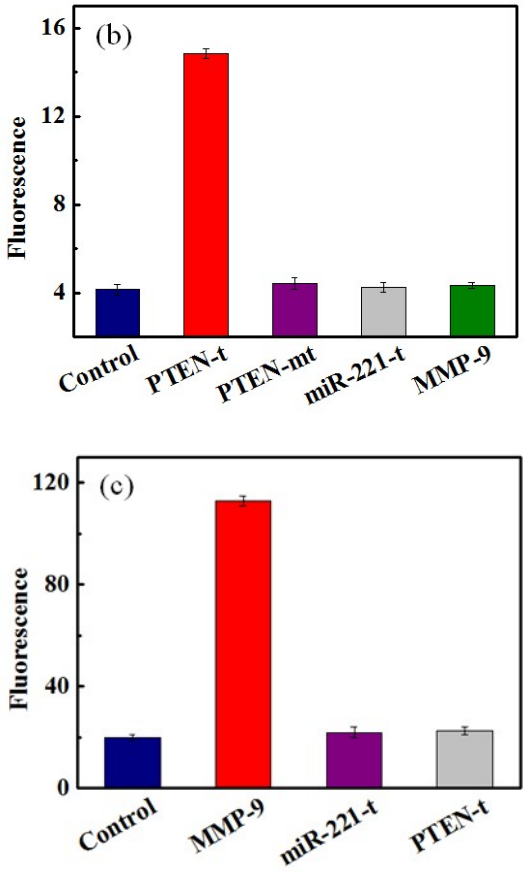


Fig. S4 Specificity test of the nanoprobe against several DNA targets and MMP target. The fluorescence signals were monitored when the nanoprobe was mixed with the perfectly matched target, single-base mismatched target and the other two targets. MiR-221-t, miR-221-mt, PTEN-t and PTEN-mt stand for miR-221 target, miR-221 single-base mismatched target, PTEN target, PTEN single-base mismatched target, respectively.

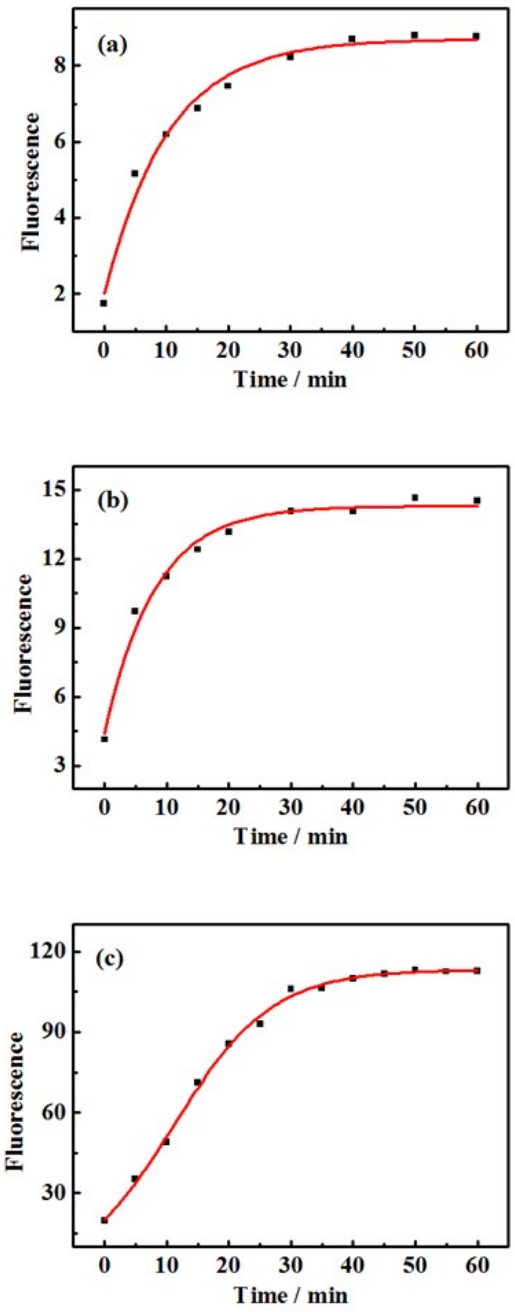


Fig. S5 Kinetic studies of the nanoprobe. The nanoprobe (1 nM) was treated with: (a) miR-221 target, (b) PTEN mRNA target and (c) MMP-9. The target concentrations of miR-221 and PTEN mRNA are 200 nM and the MMP-9 activity is 0.4 μg mL^{-1}.

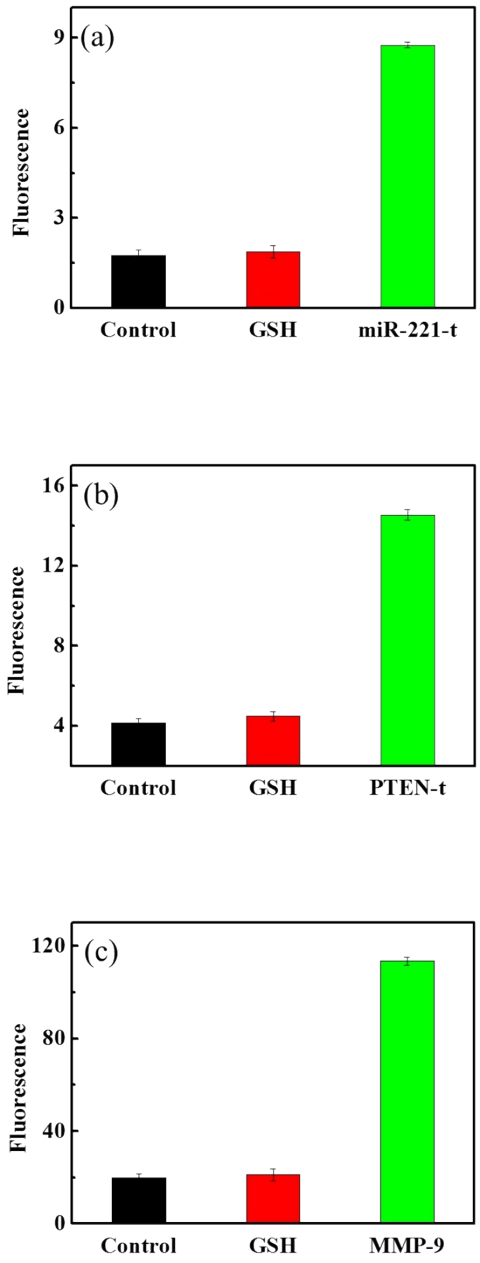


Fig. S6 The effect of GSH on nanoprobe stability. The fluorescent intensity of the nanoprobe in response to 1 mM GSH and three corresponding targets: (a) miR-221, (b) PTEN mRNA, (c) MMP-9.

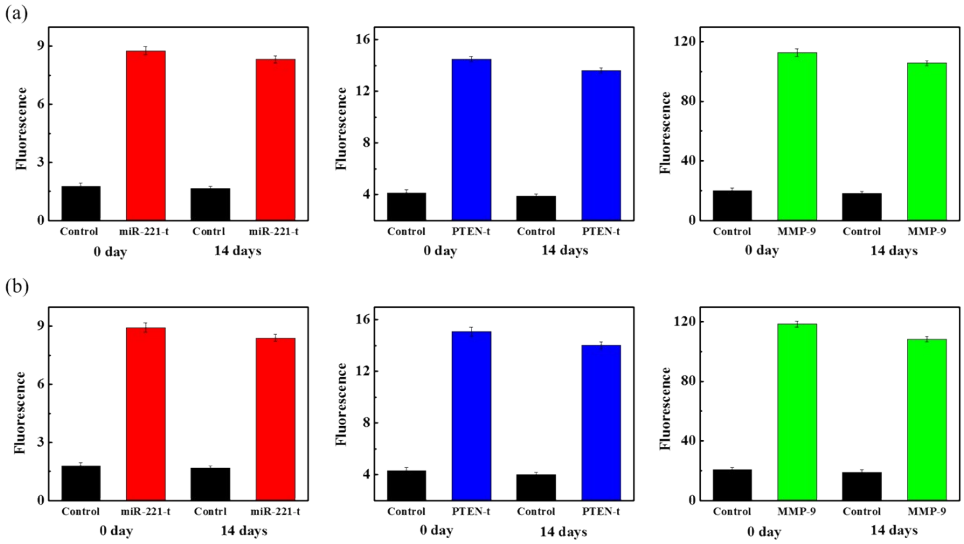


Fig. S7 The stability and reproducibility of the nanoprobe. The three targets were added to the nanoprobe at 37 °C for 1 h, respectively, after two different batches of nanoprobe (a, b) were stored in dry air at 4 °C for 0 day and 14 days. The fluorescence was measured with 648 nm, 488 nm and 554 nm excitation wavelengths, respectively. The three corresponding targets are miR-221 target (left), PTEN mRNA target (middle) and MMP-9 (right). All experiments are done in triplicate.

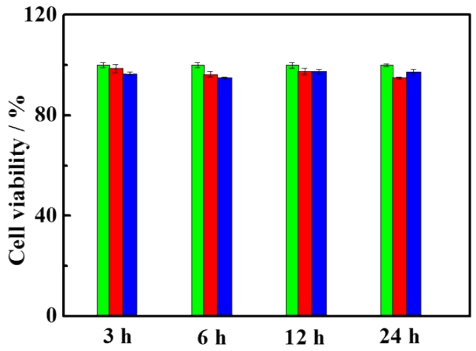


Fig. S8 Growth inhibition assay (MTT). MCF-7 cells were incubated with naked AuNPs (1 nM), and nanoprobe (1 nM) for 3 h, 6 h, 12 h, 24 h. Green bars represent for the control, red bars represent for the naked AuNPs, blue bars represent for the nanoprobe.

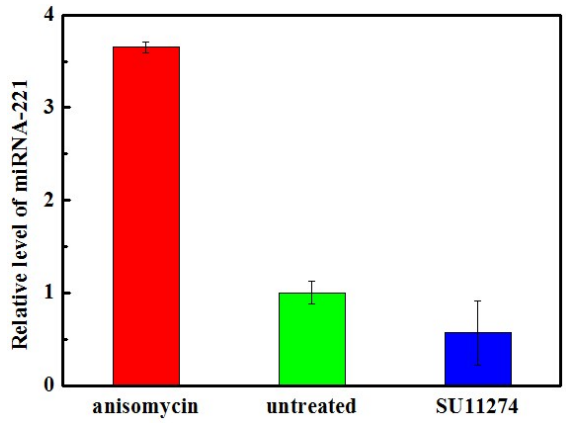


Fig. S9 RT-PCR of miR-221 in MCF-7 cells. MCF-7 cells were treated with anisomycin for 1 h or with SU11274 for 24 h. The relative level of miR-221was calculated from the quantity of their PCR products relative to the quantity of GAPDH PCR products and normalized to the expression level in untreated MCF-7 cells, respectively.

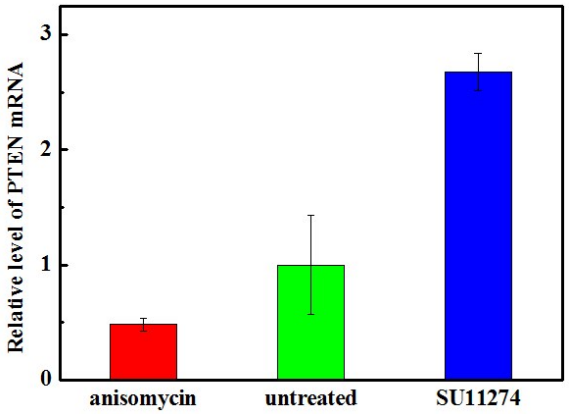


Fig. S10 Effect of the variation in miR-221 expression in MCF-7 cells on the level of PTEN mRNA. MCF-7 cells were treated with anisomycin or SU11274 to induce or reduce the level of miR-221. The relative level of PTEN mRNA was calculated from the quantity of their PCR products relative to the quantity of GAPDH PCR products and normalized to the expression level in untreated MCF-7 cells, respectively.

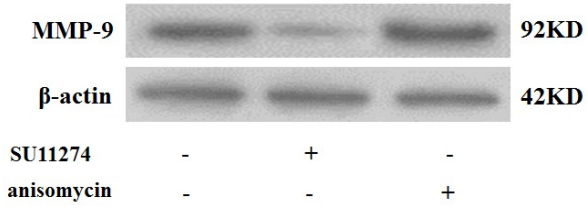


Fig. S11 Effect of the variation in miR-221 expression in MCF-7 cells on the level of MMP-9. MCF-7 cells were treated with anisomycin or SU11274 to induce or reduce the abundance of miR-221. MMP-9 protein was extracted from cell lysate and subjected to western blot analysis. β-actin served as the loading control.

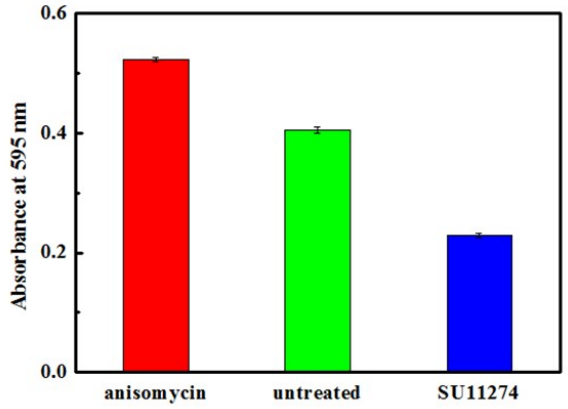


Fig. S12 Analysis of the number of invasive MCF-7 cells by measuring the absorbance at 595 nm after drug treatment. All experiments are done in triplicate.

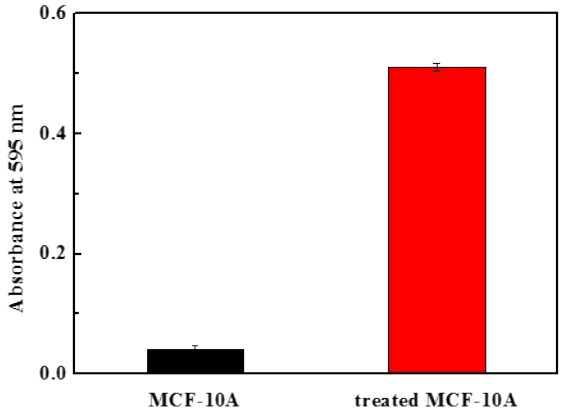


Fig. S13 Analysis of the number of invasive cells by measuring the absorbance at 595 nm with or without MDA-MB-231 medium treatment. All experiments are done in triplicate.

Reference

(1) Grabar, K. C.; Freeman, R. G.; Hommer, M. B.; Natan, M. J. Anal. Chem. 1995, 67, 735−743.

(2) Pan, W.; Zhang, T.; Yang, H.; Diao, W.; Li, N.; Tang, B. Anal. Chem. 2013, 85, 10581-10588.

(3) Qiao, G.; Gao, Y.; Li, N.; Yu, Z.; Zhuo, L.; Tang, B. Chem. Eur. J. 2011, 17, 11210-11215.