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A fluorescence nanoprobe for detecting the effect of different oxygen and nutrient conditions on breast cancer cells migration and invasion

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Experimental Section

Materials.

3-(4,5-dimethyl-thiazol-2-yl)-2,5-diph-enyltetrazolium bromide (MTT) were purchased from Sigma. Ethylisopropylamiloride (EIPA) was purchased from J&K Scientific Ltd. All the chemical reagents were of analytical grade and used without further purification. Hydrogen tetrachloroaurate (III) ($HAuCl_4 \cdot 4H_2O$, 99.99%), Trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), Sodium dodecylsulfate (SDS), $MgCl_2$ and KCl were purchased from China National Pharmaceutical Group Corporation (Shanghai, China).All aqueous solutions were prepared using ultrapure water of 18.2 MΩ•cm^{-1}. MCF-7 cells were obtained from Procell (Wuhan, China). MDA-MB-231 cells were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences. 35 mm glass bottom petri dishes were obtained from Shengyou Biotechnology Co., Ltd (Hangzhou, China).

Instruments.

Absorption spectra were measured on a UV–Vis spectrometer (TU-1900, Purkinje General, China). Transmission electron microscopy (TEM) was carried out on a JEM-100CX Π electron microscope. Fluorescence spectra were carried out through Fluorescence Spectrometer (FLS-980, Edinburgh, UK). Absorbance was recorded in a microplate reader (Synergy 2, Biotek, USA) in the MTT assay. All pH were measured using a digital pH-meter (pH-3c, LeiCi, China). Confocal fluorescence images were accomplished with a confocal laser scanning microscopy (Leica TCS SP8, Germany). Imaging flow cytometry was accomplished on Amnis ImageStream MarkII (Merck Millipore, Seattle, WA). Centrifugation was performed on an Eppendorf 5417R Centrifuge.

Synthesis of Au NPs.

This work used the classical sodium citrate reduction method to synthesize the 13 nm Au NPs. All glassware used was pretreated with aqua regia (HNO_3/ HCl=3:1, v / v). The solution of $HAuCl_4$ (0.01%, 100 mL) was heated to boiling and refluxed 10 min. Then added trisodium citrate (1%, 3.5 mL) quickly under stirring. After a few minutes, the colors of solution turned from colorless to blue-violet and finally to burgundy. And the reaction was maintained for 15 min when the color changed into burgundy. Subsequently, stopped heating and continued stirring until it cools to room temperature. The colloidal gold solution was filtered with 0.45 nm Millipore membrane filter and stored in 4 ℃ for using after then. The size of Au NPs was measured by Transmission electron microscopy (TEM) and Dynamic light scattering (DLS). The concentration of Au NPs was calculated based on their extinction coefficient (ε= 2.7×108 L mol^{-1} cm^{-1}) at 524 nm.

DNA Functionalized Au NPs.

MB-MMP-2 (labeled with Cy5 fluorescent dye) and MB-22a (labeled with Alexa Fluor 488 fluorescent dye) were purchased by sangon biotech Ltd. At first, Mixing MB-MMP-2 and MB-22a (50 nM of final concentration) with gold nanoparticle (1 nM) solution. The mixture was slowly shaken at 180 rpm, 25 ° C. After 4 hours, 0.1% SDS solution was added to the reaction system (final concentration of SDS is 0.01%) and shaken at 25 ° C overnight. The NaCl solution (1 M) was dissolved in PBS buffer (pH = 7.4) was added to the reaction over 8 h peried and the final concentration of NaCl was 100 nM. The mixture reacted at 25 °C with shaking 180 rpm for 12 hours, and then remove it from the shaker. After aging treatment for 48 hours, the nanoprobes achieved by centrifugation (30 min, 4 ° C, 13000 rpm). The nano particle was stored at 4 ° C in the dark for next using.

Quantification of MB on the Nanoprobes.

The number of beacons modified on the surface of the gold nanoparticles was determined according to a fluorescence quantification method. Gold nanoparticles (1 nM) and 56 mL of DTT (0.1 M) were mixed well in water and incubated at 37 ° C for 24 hours in the dark. The MBs modified on nanoparticles are competed by DTT. The MBs detached from the nanoprobes and distributed in the supernatant was acquired by centrifugation (30 min, 13,000 rpm). The fluorescence intensity of Cy5 and Alexa Fluor 488 in MBs was detected by fluorescence spectrometer. The fluorescence of Cy5 dye was excited at 643 nm and measured at 667 nm, and the fluorescence of Alexa Fluor 488 was excited at 490 nm and measured at 520 nm. Standard curves were prepared by collecting the fluorescence of Cy5 and Alexa Fluor 488 dyes at a concentration gradient of 10 nM-100 nM, respectively. Repeat the experiment three times.

Kinetics.

The nanoprobe solution (1 nM) was incubated with Target DNA (200 nM) at 37 °C and the fluorescence intensity was measured on a certain time interval (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes). The fluorescence of Alexa Flour 488 and Cy5 were obtained *via* the same way described above.

Specificity Experiment.

To study the specificity of the nanoprobes, the Target-MMP-2 and Target-RAB22a (200 nM) and other DNAs were added to 1 nM nanoprobes at 37 °C for 1 h. The Alexa Flour 488 fluorescence and Cy5 fluorescence were measured at appropriate excitation and emission wavelengths. All experiments were performed at least three times.

Cell Culture.

The MCF-7 cells were incubated in 1640 medium and MDA-MB-231 cells were treated with Dulbecco's modified Eagles medium (DMEM). All the mediums were supplemented with 1% antibiotics penicillin/streptomycin and 10% fetal bovine serum (FBS). All the cells kept in a humidified atmosphere of 5% CO_2 at 37 °C. All the cells were cultured in hypoxia.

MTT

To verify the cytotoxicity of the nanomaterials in both MCF-7 and MAD-MB-231 cells, a tetrazolium-based MTT expriment was designed. MCF-7 and MAD-MB-231 cells were dispersed in replicate 96-well microtiter plates (1×10^6 cells/well), respectively. The plates were kept in a 37 °C incubator with 5% CO_2 for 24 h. Then the initial medium was discarded and the cells were incubated with PBS, 1 nM Au NPs and 1 nM nanoprobe, respectively, for 4, 12 and 24 h. After being washed with PBS, the MTT solution (0.5 mg / mL in PBS, 150 μL) was added to each well and further cultured 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 detected with a RT 6000 microplate reader.

Fluorescence Imaging Assay.

The changes of cell invasion and migration progressions were evaluated *via* the fluorescence imaging of nanoprobes. Two cancer cell lines: MCF-7 and MDA-MB-231 cells were chosen and separated into two groups. Two groups of cells were cultured under normoxic or hypoxic conditions. Next, the cells were cultured with the nanoprobes for 4 h at 37 °C. After washing cells twice with PBS to discard the excess nanoprobes, fresh medium was added to the cells and incubated for 2 h. The cells were examined by confocal laser scanning microscopy (CLSM) to acquire the fluorescence intensity change.

In the experiment of identifying the effects of nutrients on changing cell behaviors, MCF-7 and MDA-MB-231 cells were chosen and divided into 10 groups, respectively. Five groups of MCF-7 or MDA-MB-231 cells were pretreated with DMEM containing 0%, 0.5%, 1%, 5% and 10% FBS under normoxia condition, respectively. The other five groups of MCF-7 or MDA-MB-231 cells were pretreated with DMEM containing 0%, 0.5%, 1%, 5% and 10% FBS under hypoxia condition, respectively. Finally, the 20 groups of cells were incubated with the nanoprobes for 4 h and then examined by CLSM to record the fluorescent signal change.

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 MDA-MB-231 cells were treated as in the same way cell migration assay, 2×104 cells were planted into the upper compartment and further incubated for 24 h. Then DMEM containing 0%, 0.5%, 1%, 5% and 10% FBS under hypoxia or normoxia condition was added into the 24-well plates and further incubated for 48 h. After that, the noninvasive cells on the upper surface of the membrane were removed with a cotton-tipped swab. Then the invasive cells 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. All the experiments were performed in triplicate.

Table S1. DNA sequences employed in this work.

	Sequence
MB-MMP-2	5’-Cy5-AGCTACAAAATACTTGGGTTA AAAGTAGCTAAAAAA-(CH2)3-SH-3’
MB-RAB-22a	5’-Alexa Fluor 488-AGCTACGGGGGATAT TATTGTTAGTAGCTAAAAAA-(CH2)3-SH-3’
T-MMP2	TAACAATAATATCCCCC
T-RAB-22a	TTTTAACCCAAGTATTTT
Mis-MMP-2	TAACAATATTATCCCCC
Mis-RAB-22a	TTTTAACCGAAGTATTTT

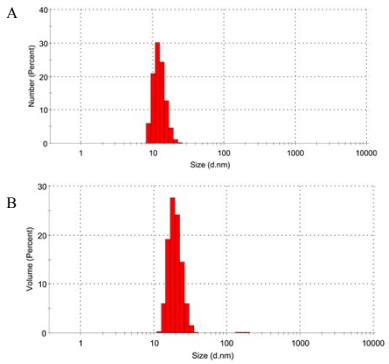

Figure S1, Hydrodynamic diameters acquired from DLS of Au NPs (A) and nanoprobes (B).

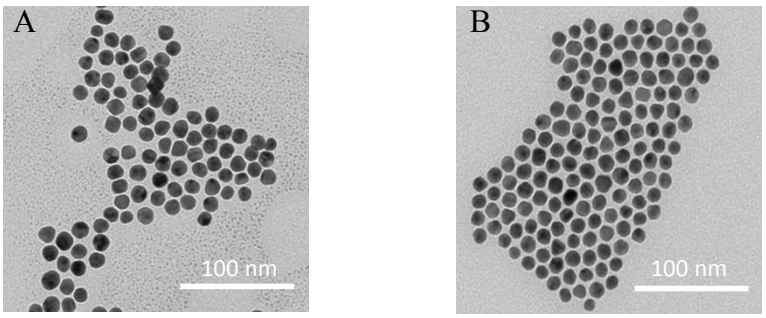

Figure S2. TEM images of Au NPs (A) and nanoprobes (B).

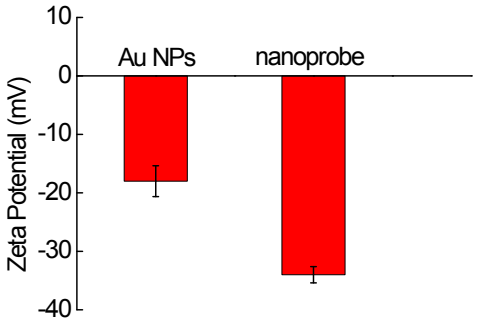

Figure S3. Zeta potentials of Au NPs and nanoprobes.

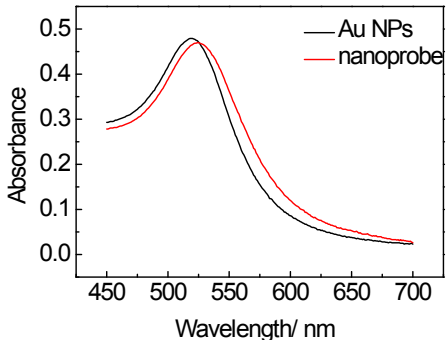

Figure S4. UV-Vis spectra for Au NPs and nanoprobes.

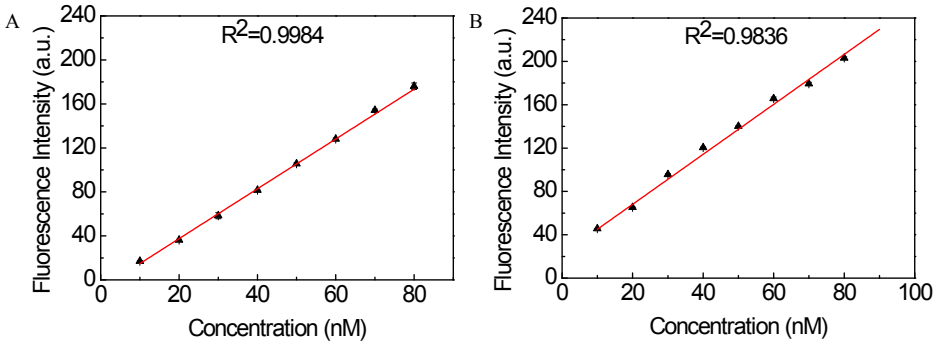

Figure S5. Standard linear calibration curves of dyes Cy5 (A) and Alexa Flour 488 (B).

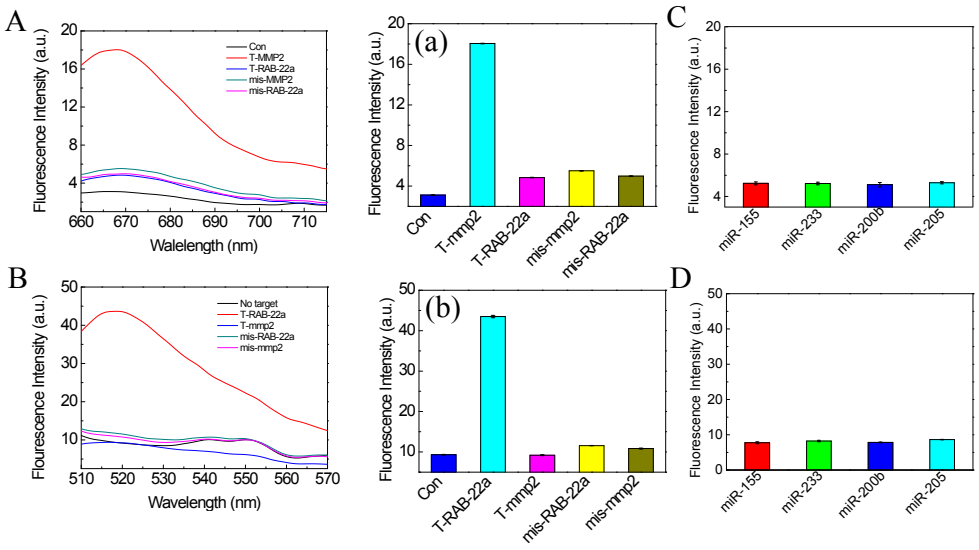

Figure S6. Specificity tests of the nanoprobes against different DNA targets (A, a; B, b). The fluorescence recovery experiment with the addition of four miRNAs (C, D).

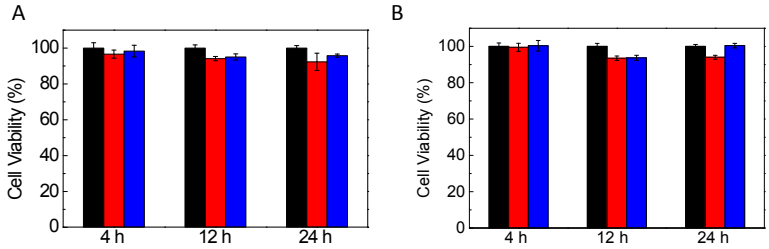

Figure S7. Growth inhibition assay (MTT). MCF-7 (A) and MDA-MB-123 (B) cells were treated with PBS (black) , Au NPs (red) and nanoprobes (blue) for 4h, 12h and 24h, respectively.

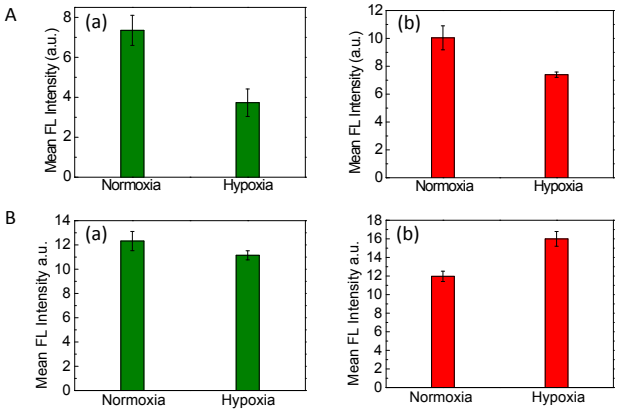

Figure S8. Quantitative fluorescence intensities of RAB-22a (A) and MMP-2 (B) in MCF-7 cells (A) and in MDA-MB-231 cells (B).

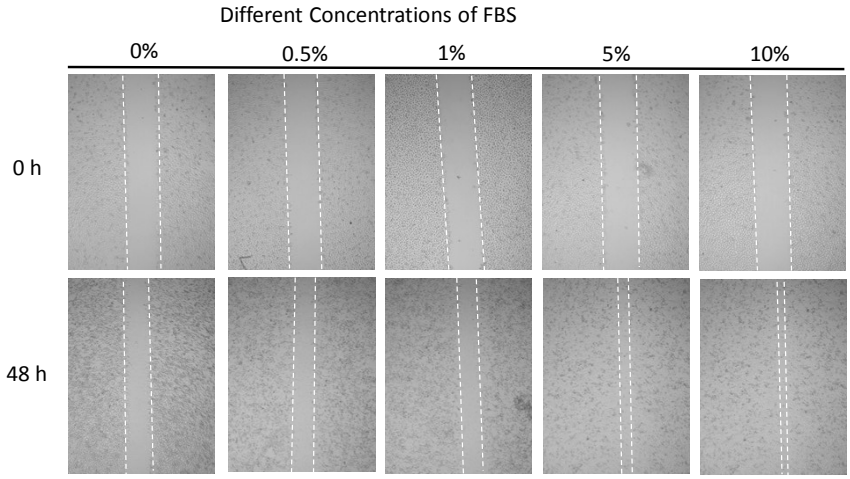

Figure S9. Representative images of wound-healing assay in MCF-7 cells were captured at 0 h and 48 h after wounding with different FBS and normoxia treatment.

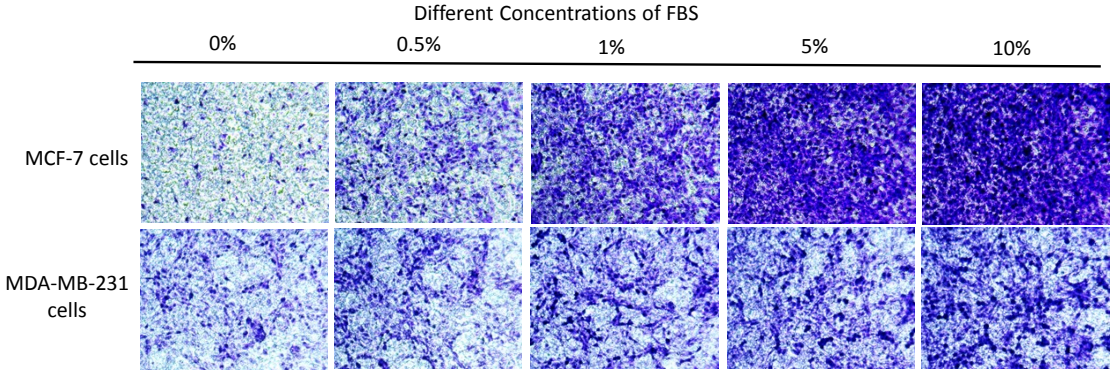

Figure S10. The invasive cells were fixed, stained and photographed under normoxia condition.

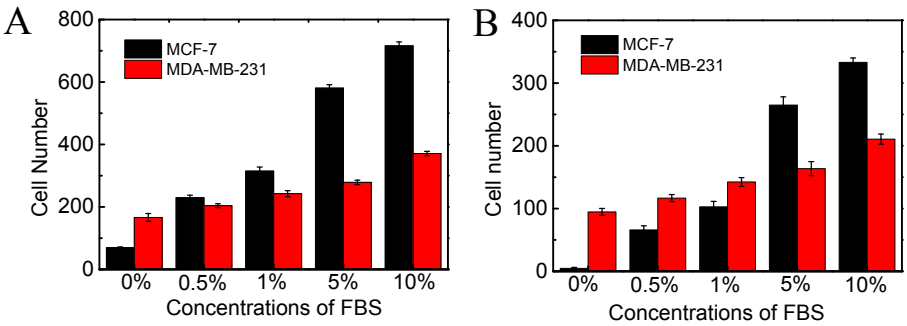

Figure S11. The quantitative results of invading cells in Figure S10 (A) and Figure S16 (B).

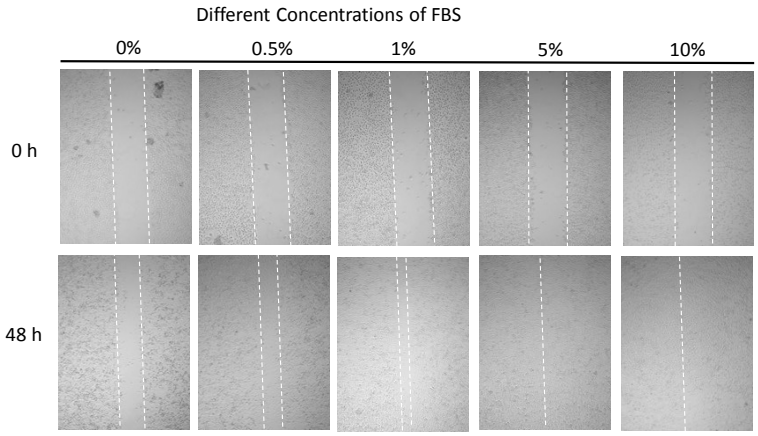

Figure S12. Representative images of wound-healing assay in MDA-MB-231 cells were captured at 0 h and 48 h after wounding with different FBS and normoxia treatment.

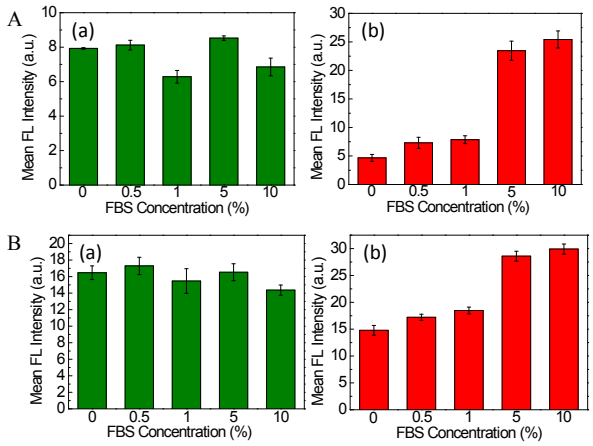

Figure S13, Quantitative fluorescence intensities of RAB-22a (a) and MMP-2 (b) in MCF-7 (A) and MAD-MB-231 (B) cells as the change of FBS concentrations for Figure 3.

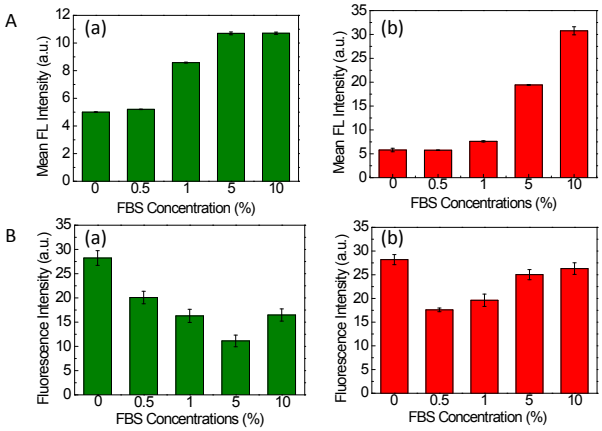

Figure S14, Quantitative fluorescence intensities of RAB-22a (a) and MMP-2 (b) in MCF-7 (A) and MAD-MB-231 (B) cells as the change of FBS concentrations for figure 4.

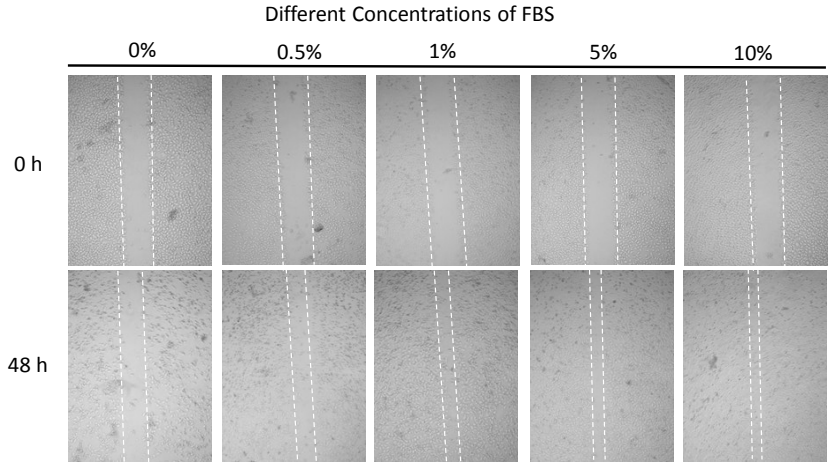

Figure S15. Representative images of wound-healing assay in MCF-7 cells were captured at 0 h and 48 h after wounding with different FBS and hypoxia treatment.

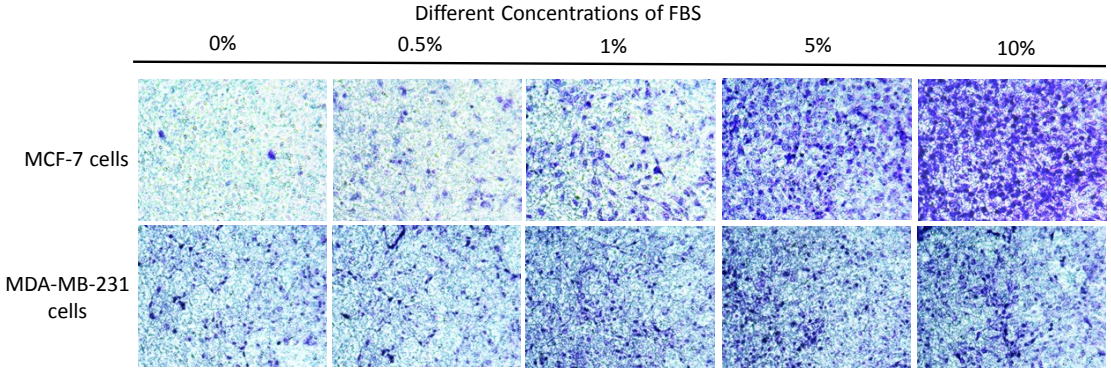

Figure S16. The invasive cells were fixed, stained and photographed under hypoxic conditions.

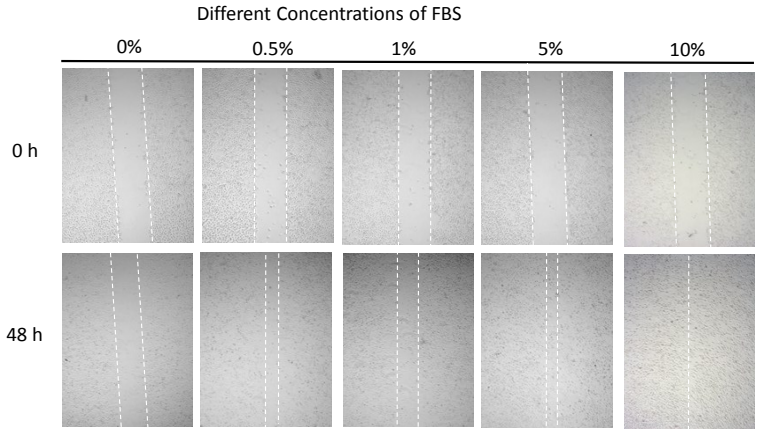

Figure S17. Representative images of wound-healing assay in MDA-MB-231 cells were captured at 0 h and 48 h after wounding with different FBS and hypoxia treatment.