Electronic Supplementary Information

In situ monitoring of functional activity of extracellular matrix stiffness-dependent multidrug resistance protein 1 using scanning electrochemical microscopy

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1. Experimental section

Chemicals and materials. Ferrocenecarboxylic acid (FcCOOH) and acrylamide (Acr) were obtained from Aladdin Reagent Co., Ltd. (China). Ferrocenemethanol (FcCH2OH), hexaammineruthenium(III) chloride ([Ru(NH3)6]Cl3), N,N methylene-bis-acrylamide (MBA), ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), N-lacetyl-L-cysteine (NAC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and TWEEN®20 were all obtained from Sigma Aldrich Co., Ltd. (U.S.A.). Acrylamide (Acr) and phosphate buffered saline (PBS) were purchased from MP Biomedicals Co., Ltd. (U.S.A). Leibovitz's L-15 and Radio immunoprecipitation assay (RIPA) lysis buffer were obtained from Solarbio Science & Technology Co., Ltd. (China). Bicinchoninic acid (BCA) protein assay kit was obtained from Beyotime Biotechnology Co., Ltd. (China). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corp (U.S.A.). Goat anti-Rabbit IgG HRP conjugated antibody was purchased from InCellGene LLC (U.S.A.). RNAiso Plus was purchased from Takara Bio Inc. (Japan). Collagen (type I) was purchased from Dow Corning Corp (U.S.A.). DMEM (with high glucose), penicillin/streptomycin, and trypsin were obtained from Gibco Life Technologies Corp (U.S.A.). N-Sulfosuccinimidyl-6-(4-azido-2-nitrophenylamino) hexanoate (sulfo-SANPAH) was purchased from Proteochem Inc (U.S.A.). Fetal bovine serum (FBS), DAPI and CellMask Plasma Membrane Stains and RevertAid First Strand cDNA Synthesis Kit were purchased from Thermo Fisher Scientific Inc (U.S.A.). ThiolTracker™ Violet and Calcein-AM were obtained from Molecular Probes Inc. Vincristine (VCR) was obtained from Selleck Chemicals LLC (U.S.A.). MK 571 sodium salt, anti-MRP1 antibody, and goat anti-Rabbit IgG H&L (Alexa Fluor® 488) were obtained from Abcam PLC (U.K.). Cell Counting Kit-8 was obtained from Dojindo Laboratories (Japan). All the chemicals were used as-received without further purification. The aqueous solutions used in this work were all obtained from a Milli-Q reagent system (Millipore Corp., resistivity >18.2 MΩ).

Preparation and characterization of PA gels. The PA gels with three stiffnesses were prepared by varying the mixing ratios of 50% (w/v) acrylamide (Acr), 1.25% (w/v) MBA, 10% (w/v) APS and 1% (w/v) TEMED. The ratios of acrylamide (%)/MBA(%) were adjusted to 6/0.06, 10/0.10 and 10/0.30, and the volumes of APS and TEMED were kept at 1/100 and 1/1000 of the total volume to obtain the PA gels with stiffness around 2 kPa, 17 kPa and 26 kPa, respectively. Then the mixed polymer solution (50 μL) was sandwiched between the glass slides of the glass bottom dishes (NEST, 35 mm) and the glass coverslips, which were functionalized with 2% 3-(trimethoxysilyl) propyl methacrylate and dichloromethylsilane (DCMS) in advance to ensure the attachment of PA gels to glass slides and easy detachment of glass coverslips subsequent to polymerization. After polymerization for 45 minutes under room temperature, the glass coverslip was removed and the obtained PA gels were rinsed with PBS three times. Next, each as-prepared PA gel was treated with 300 μL sulfo-SANPAH under 365 nm ultraviolet (UV) irradiation for 10 minutes and washed with 50 mM HEPES buffer (pH 8.0) three times to enable collagen type I to conjugate to PA gels. Finally, the as-prepared PA gels were irradiated by UV light for 30 minutes for sterilization and then coated with collagen type I for 12 hours for cell adhesion. The stiffness of the prepared PA gels was characterized by a Piuma Nanoindenter (Optics 11, Netherlands).

Cell culture and treatment with vincristine (VCR). MCF-7 cells and MDA-MB-231 cells (American Type Culture Collection (ATCC), U.S.A.) were cultured with DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin in an incubator (5% CO_2, 37°C). For all the experimental groups, MCF-7 and MDA-MB-231 cells in the logarithmic growth phase were digested with trypsin and seeded on PA gels at a density of 1×10^4 cm^{-2}. Then, the cells were incubated for 24 h at 37°C and 5% CO_2. For the two cell groups treated with vincristine, the two cells culture media were replenished with fresh medium containing vincristine at half inhibition concentrations (IC_{50}), and then the cells were further cultured for 24 hours before SECM experiments.

Measurement of intracellular GSH levels. After culturing the MCF-7 and MDA-MB-231 cells on the PA gels with three stiffnesses for 24 hours, the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) twice and then labeled with ThiolTracker™ Violet fluorescent probe (20 μM) (Invitrogen, T10095) for 30 minutes. For the cell groups treated with NAC (a GSH synthesis accelerator), the medium was replenished with 1 mL of D-PBS solution containing 5 mM NAC before adding the GSH dye. The control groups were replenished with the same volume of D-PBS solution and incubated for 30 minutes. Confocal microscopy (Olympus, FV3000, Japan) was utilized to capture the fluorescent images of cells and ImageJ was used to quantify the expression levels of intracellular GSH in cells. Each experiment was performed for three replicates.

Characterization of the functional activity and expression of MRP1 in cells. The efflux activity of MRP1 in MCF-7 and MDA-MB-231 cells was quantified by a calcein-AM assay. First, MCF-7 cells were cultured in an incubator (5% CO_2, 37°C) for 24 hours, digested with trypsin and seeded on PA gels with different stiffnesses at a density of 2×10^4 cells cm^{-2}. After 24 hours, the medium was replenished with medium containing 2 μM Calcein-AM, and then the cells were incubated in the dark for 15 minutes. After rinsing the cells with PBS, 1 mL of medium was added again. Inverted fluorescence microscope (Olympus, IX-53, Japan) was utilized to capture the fluorescent images of the cells, in which the time was set as t = 0. The Calcein-AM assay procedure for MDA-MB-231 cells was the same as that for MCF-7 cells. Then, MK571, a specific inhibitor of MRP1, was added to the medium of the control groups at concentrations of 50 μM for MCF-7 cells and 20 μM for MDA-MB-231 cells. The fluorescence intensities of the groups with/without MK571 were recorded at 30 minutes and 8 hours, respectively. The functional activities of MRP1 in cells were calculated based on the following equation.

$$\text{Calcein efflux rate (\%)} = \frac{FI_0 - FI_t}{FI_t} \times 100\% \quad \text{(S(1))}$$

where FI_0 is the fluorescence intensity value at $t = 0$, and FI_t is the fluorescence intensity value at $t = 30$ minutes and 8 hours, respectively.

To characterize the MRP1 expression on MCF-7 and MDA-MB-231 cells, the cells were first fixed on PA gels using anhydrous methanol at -20°C for 10 minutes and washed with PBS three times. Then, 1% BSA in PBST (1×PBS, 0.1% Tween-20) was used to block cells. After 1 hour, the cells were incubated with anti-MRP1 primary antibody at a dilution of 1:50for another 16 hours at 4°C. Then the cells were washed with PBS three times and stained with Alexa Fluor 488-conjugated secondary antibody at a dilution of 1:1000 for 1.5 hours. Confocal microscopy (Olympus, FV3000, Japan) was used to capture fluorescence images of two cell lines and ImageJ (NIH, U.S.A.) was used to quantify the fluorescence intensities of the MRP1 expression levels in the cells.

Western blot analysis. MCF-7 and MDA-MB-231 cells were firstly cultured on the PA gels at a density of 1×10^6 cm^{-2} in six -well plates and incubated in an incubator (5% CO_2, 37°C) for 24 hours and then lysed by RIPA lysis buffer. The total protein concentrations of the MCF-7 and MDA-MB-231 cells were determined using a BCA protein assay kit. Then the protein samples of the two cells were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Thereafter, the PVDF membranes were blocked with 1.5% BSA for 2 hours and then incubated with anti-MRP1 primary antibody. After 24 hours, membranes were stained with HRP-conjugated secondary antibody for 1 hour at room temperature. Finally, the membranes were visualized and measured using a chemiluminescence imaging system (Clinx, 3300 mini, China).

qRT-PCR analysis. MCF-7 and MDA-MB-231 cells were firstly cultured on the PA gels at a density of 1×10^6 cm^{-2} in six-well plates and incubated in an incubator (5% CO_2, 37°C). After 24 hours, the total RNA of the MCF-7 and MDA-MB-231cells was extracted using RNAiso Plus and reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit. Then the FastStart Universal SYBR Green Master (Vazyme, cat no: Q111) was used for qRT-PCR and the total of 40 cycles were performed on Applied Biosystems qRT-PCR system (7500 fast, U.S.A.). The relative

amounts of cDNA of MCF-7 and MDA-MB-231 cells were analyzed using the $2^{-\Delta\Delta CT}$ method. The primers used for the qRT-PCR experiments were as follows.

MRP1	forward: 5'-AGTGACCTCTGGTCCTTAAACAAGG-3'
	reversed: 5'-GAGGTAGAGAGCAAGGATGACTTGC-3'
GAPDH	forward: 5'-CGACGACTTCTCCCGCCGCTACCGC-3'
	reversed: 5'-CCGCATGCTGGGGCCGTACAGTTCC-3'

Measurements of drug-dose response and cell viability of VCR. MCF-7 and MDA-MB-231 cells were cultured on the PA gels with three different stiffnesses at a density of 2×10^4 cm^{-2} and incubated (5% CO_2, 37°C) for 4 hours to enable full adherence of cells to the PA gel surface. Thereafter, the MCF-7 and MDA-MB-231 cells were replenished with 1 mL of prepared medium containing different concentrations of vincristine and then incubated for 24 hours. To assess the VCR sensitivity of the cells, the concentrations of VCR added to the cell culture varied from 0.1 nM to 50 μM. For the control groups, the cells were replenished with the same volume medium without VCR. Each experiment was carried out for three replicates.

A Cell Counting Kit-8 (CCK8) was used to characterize the cell viability. Generally, after removing the medium containing VCR, fresh medium containing CCK8 solution (10% V/V) was added to the petri dish. Then, the MCF-7 or MDA-MB-231 cells were incubated in the dark for another 4 hours. 90 μL of the supernatant medium was transferred from each Petri dish into 96-well plates Then, the absorbance of the MCF-7 and MDA-MB-231 cells after treatment with VCR at 450 nm was characterized using a microplate reader (Tecan, Spark® 10M, Switzerland). The cell viabilities were calculated using Eq. (S(2)).

$$\text{Cell viability (\%)} = \left(\frac{OD_{treatment} - OD_{blank}}{OD_{control} - OD_{blank}}\right) \times 100\% \quad \text{(S(2))}$$

where $OD_{treatment}$ is the absorbance of the cells treated with VCR, $OD_{control}$ is the absorbance of the nontreated cells, and OD_{blank} is the absorbance of medium. GraphPad

Prism 6 software was used to treat the dose-response curves and calculate the half inhibition concentration (IC_{50}).

2. Stiffness of PA gels

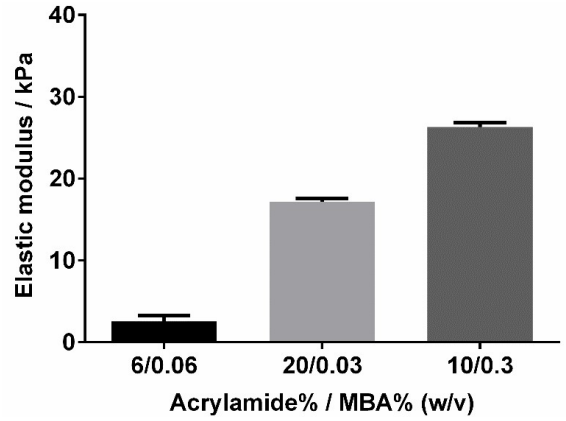

Fig. S1 The elastic modulus of the as-prepared PA gels characterized by a nanoindenter.

3. 2D axial SECM geometry simulation model

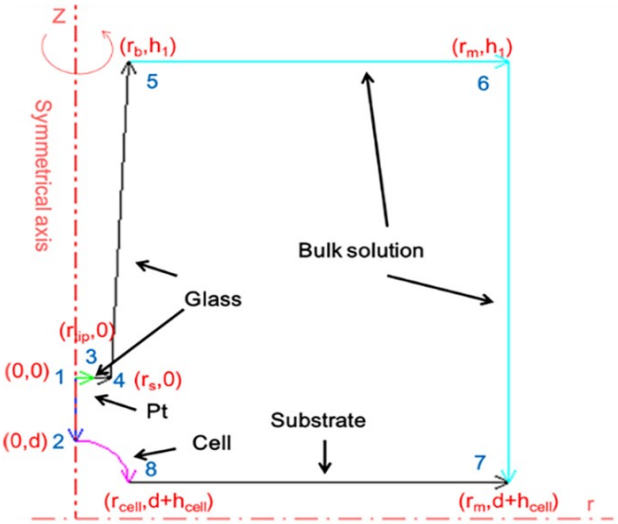

Fig. S2 An illustration of the 2D axial SECM geometry simulation for the cell.

Table S1. Definition of the boundary conditions in SECM 2D simulation model

Boundary		Defination	Equation
***z* axes**	**(1→2)**	$\frac{\partial c(r,z)}{\partial r} = 0$ ， $0<z<d$， $r = 0$	S(3)
Pt probe surface (1→3)		$c(r,z) = 0$ ， $0<r<r_{tip}$	S(4)

Glass of probe	**(3→4)**	$\frac{\partial c(r,z)}{\partial z} = 0$, $r_{tip}<r<r_s$, $z = 0$	S(5)
Glass of probe	**(4→5)**	$\frac{\partial c(r,z)}{\partial z} = 0$, $\frac{\partial c(r,z)}{\partial r} = 0$, $r_s<r<r_b$	S(6)
Bulk solution	**(5→6)**	$c(r,z) = c_0$, $r_b<r<r_m$, $z = h_1$	S(7)
Bulk solution	**(6→7)**	$c(r,z) = c_0$, $h_1<z< d + h_{cell}$, $r = r_m$	S(8)
Cell surface	**(2→8)**	$D\frac{\partial c(r,z)}{\partial z} = k_f[c_0\text{-}c(r,\text{-}d)]$, $0<r<r_{cell}$, $z = d$	S(9)
Substrate	**(7→8)**	$\frac{\partial c(r,z)}{\partial z} = 0$, $r_{cell}<r<r_m$, $z = d + h_{cell}$	S(10)

4. Characterization of cell topography and probe-cell surface distances in SECM measurements

To determine the highest point of the cells, the SECM probe was firstly approached to the surface of the PA gel next to the cell. As shown in **Fig. S3**, when the tip of the SECM probe touched the PA gel surface, an "inflection" point of the probe current-distance curve was observed. Then the probe was lifted about 25 μm up and the line scanning along *x* and *y* directions was performed. The highest point of the cell was consequentially determined according to the obtained cell profile.

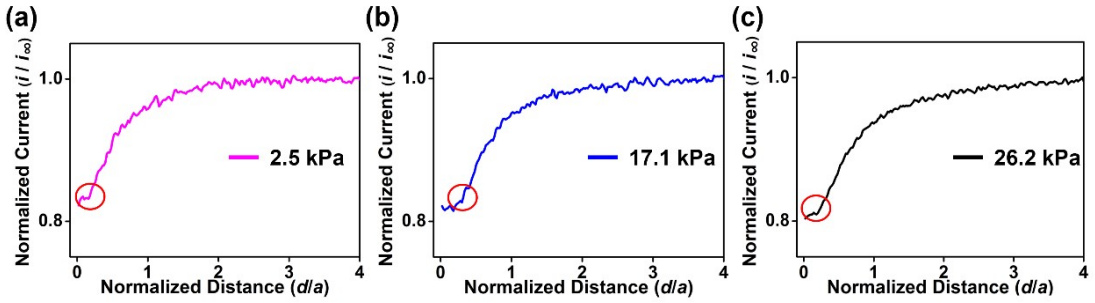

Fig. S3 SECM approach curves to the PA gels with the stiffnesses of (a) 2.5, (b) 17.1 and (c) 26.2 kPa, respectively.

To verify whether the cell topography change affects the approach curves in the theoretical model, the theoretical models with different cell heights and widths were

tested while maintaining k constant (k = 1.70×10^{-5} m·s^{-1}). As illustrated in **Fig. S4**, the changes in cell height (**Fig. S4a**) and diameter (**Fig. S4b**) in the model have significant impacts on the SECM simulation approach curves. The widths and heights of the MCF-7 and MDA-MB-231 cell on the PA gels with three stiffnesses were characterized using the inverted optical microscopy and SECM, respectively. The coordination of the labelled points used in the COMSOL simulations was set according to the type and size of the breast cancer cells (MCF-7 and MDA-MB-231) from **Fig. S4**.

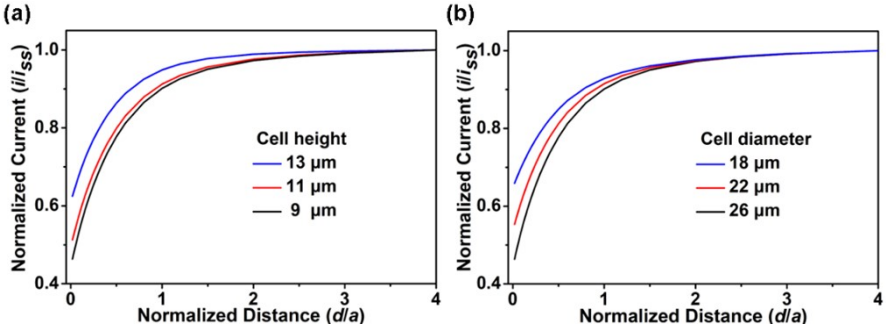

Fig. S4 Simulated SECM approach curves towards cells with (a) different heights (diameter = 13 μm) and (b) different widths (height = 9 μm).

For cell height characterization, the approach curves to the cell surface and surface of the PA gel next to the cell were recorded. The height of the cell was then obtained by the difference in the absolute distances of the two approach curves. From the results in **Fig. S5**, it can be seen that the heights and diameters of MCF-7 and MDA-MB-231 cells on the PA gels with three stiffnesses were significantly different, and all the values followed a normal distribution. Therefore, theoretical models considering the average cell heights and diameters of MCF-7 and MDA-MB-231 cells on PA gels at each stiffness were built and used in this work.

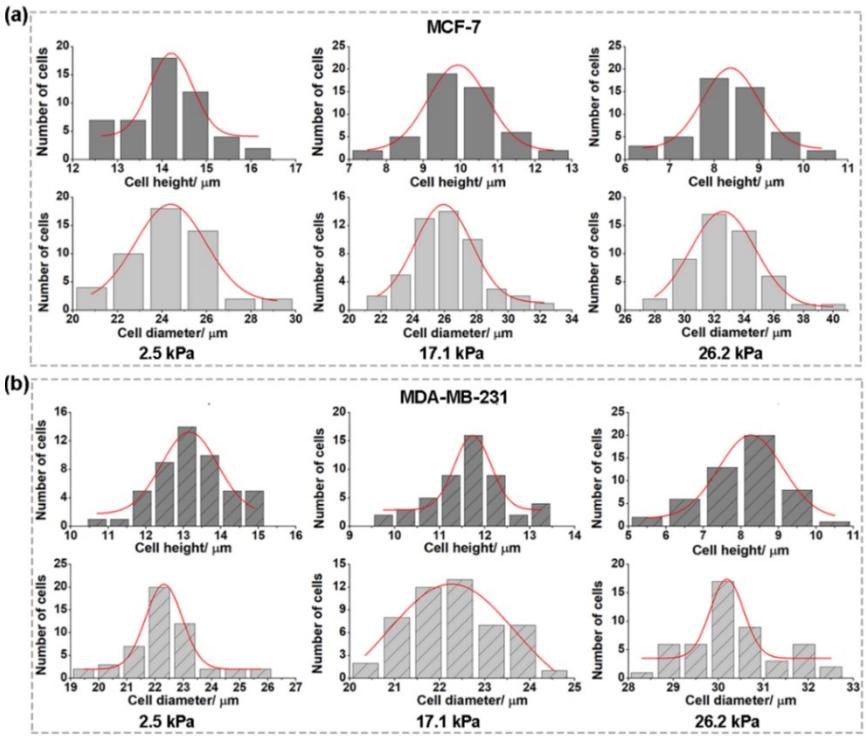

Fig. S5 Distribution of cell heights and diameters of (a) MCF-7 cells and (b) MDA-MB-231 cells cultured on PA gels with stiffnesses of 2.5, 17.1 and 26.2 kPa, respectively.

5. Measurement of IC_{50} values of VCR of MCF-7 and MDA-MB 231 cells on PA gels

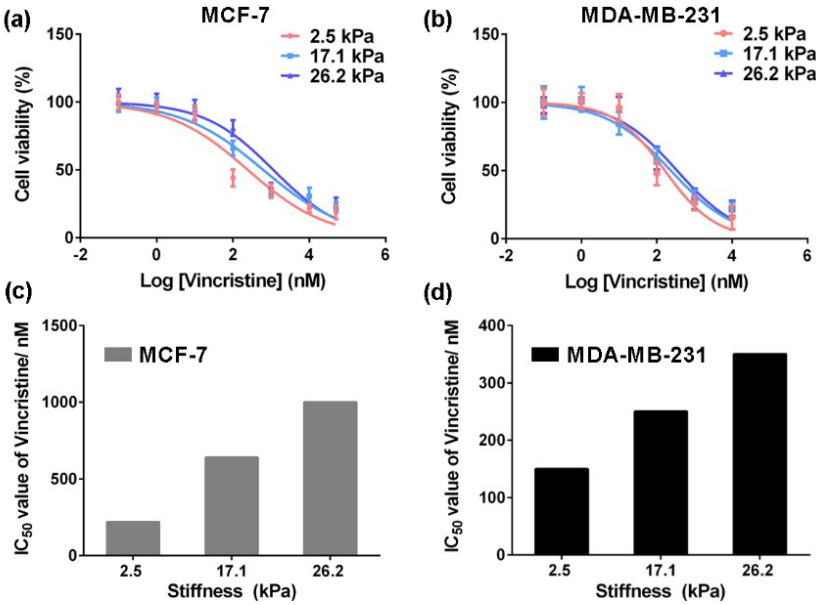

Fig. S6 Cell viability of MCF-7 and MDA-MB-231 cells upon exposure to vincristine (VCR). (a, b) Dose response curves and (c, d) IC50 values of VCR of (a, c) MCF-7 cells and (b, d) MDA-MB-231 cells cultured on the PA gels with stiffnesses of 2.5 kPa,

17.1 kPa and 26.2 kPa, respectively.

6. Characterization of the cell membrane permeability of MCF-7 and MDA-MB 231 cells on PA gels with/without VCR treatment by SECM

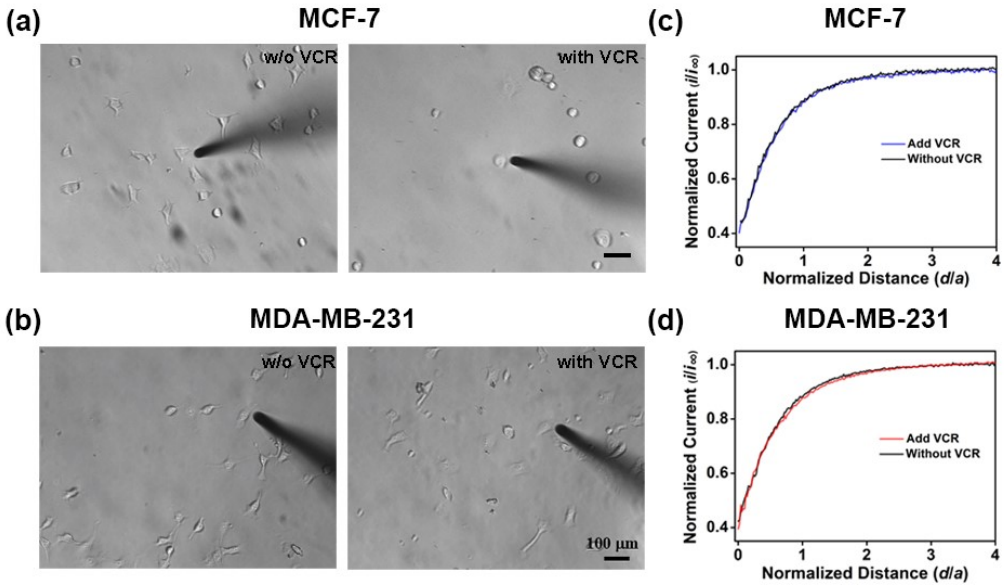

Fig. S7 Optical microscope images of (a) MCF-7 cells and (b) MDA-MB-231 cells on PA gels with 26.2 kPa stiffness with or without VCR treatment. (b) Representative SECM approach curves for (c) MCF-7 cells and (d) MDA-MB-231 cells cultured on a PA gel with 26.2 kPa stiffness treated with or without VCR for 24 h. SECM experiments were performed in L15 cell culture medium containing 0.5 mM $[Ru(NH_3)_6]Cl_3$ (E_{probe} = -0.35 V vs. Ag/AgCl RE), the probe/cell distances were set to 5 μm for SECM 2D scanning experiments.