Supporting information

Systematical analysis of different cell spheroids with a Microfluidic device using Scanning Electrochemical Microscopy and Gene expression profiling

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Experimental Section

Fabrication of Microfluidic Device

We fabricated the Top-removable microfluidic device using standard soft lithography protocol.[1] Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) was used to make the top channel and bottom microwell layer chip independently. Briefly, the silicon wafers were rinsed with acetone and isopropyl alcohol thoroughly, then blow dried and followed by baking on a hotplate (BP-2B, Jin Sheng Wei Na technology, Beijing, China) at 200°C for 2 h. We treated the wafers with O_2 plasma (PDC-MG, Mingheng Science & Technology, Chengdu, China) to get rid of any organic contaminants.

For the channel layer, the wafer was spin-coated (KW-4A, Institute of microelectronics of Chinese Academy of Science) with negative photoresist SU-2075 (Microchem, USA) at 1200 rpm for 60s to form a 200 μm thick layer. Then the photoresist was soft-baked from 55 to 65 °C for 7 min and from 80 to 95 °C for 30 min and exposed by a UV aligner (JKG-2A, Xueze, Shanghai, China) for 2 min under a high-resolution photomask (MicroCAD photo-mask, Shenzhen, China). After exposure, the mold was then post-baked from 55 to 65 °C for 5 min and then at 95 °C for 15 min to facilitate the polymerization of the SU8 photoresist. After development, the mold was then blow dried with gentle and baked on hotplate at 115 °C for 1h and then ramp up to 200 °C for 6h.

In terms of the microwell layer, SU-2075 was spin-coated at 1600 rpm for 60s to form a 100 μm thick layer and soft-baked for 5 min at 65 °C and 25min at 95 °C followed by a UV-exposure for 3 min 30 s. After the pattern was developed, it was post-baked at 115 °C for 1h and ramped up to 200 °C for 6h.

Before PDMS casting, the molds were fumigated with trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane (Sigma aldrich) for 15 min for PDMS releasing. Thereafter, 10A:1B ratio mixed PDMS (Sylgard 184, Dow Corning) was poured onto the mold with the thickness of about 3 mm, heated for 45min at 75 °C. After curing, the PDMS chip was then peeled off and cut into the appropriate size. A biopsy puncher with 3mm diameter was used for making the inlet and outlet reservoir. Subsequently, we manually assembled the channel layer and the microwell layer under an optical microscope (AZ-100, Nikon, Japan) for further spheroids culture. The height of the top channel layer and bottom microwell layer of this device was characterized by cross section imaging under a microscopy (LV-100, Nikon, Japan) as shown in figure S-1.

Cell Culture and Spheroids Formation

The human breast cancer cell line (MCF-7) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 10 % fetal bovine serum (FBS, Gibco, Thermo Fisher) and 1 % penicillin/streptomycin (Invitrogen, Thermo Fisher Scientific). The fibroblast cell line NHDF (ScienCell, California, U.S.A.) were cultured in FM medium (ScienCell) with 10% FBS. All cell lines were cultured under standard conditions at 37 °C, 5% CO_2 in a humidified incubator (Sanyo, Japan). Cells were passaged before reaching 80% confluence using 0.25% Trypsin-EDTA solution (Gibco, Invitrogen) and the cell density was determined using a hemocytometer. Before introducing into the microfluidic device, we stained MCF-7 and NHDF with Cell Tracker™ Green CMFDA and Cell Tracker™ Red CMTPX (Thermo Fisher Scientific, USA) respectively. In addition, we treated the microfluidic with 2% w/v (1 g in 100 mL) Pluronic F-127 (BASF) solution in order to prevent cell attachment. The device was vacuumed for 5 min to make sure that the microwells were filled with F-127 solution. Thereafter, we put 20 µl cell

suspension (3×10^5 /ml) into each channel of the device, permitting independent culture for different type of cell. The whole chip was then centrifuged at 1000 rpm for 3 min (5810R, Eppendorf) to facilitate cell seeding into the microwell structures. Subsequently, we washed the channel to remove the redundant cells outside the microwells. Finally, the device loaded with NHDF and MCF-7cells was placed back into the incubator and cultivated for 24 h to generate spheroids as showed in S-2. The size of cancer cell spheroid is approximately 100 μm contains 200 cells and that of fibroblast cell spheroid is around 80 μm contains 150 cells.

Image Capture and Cell Spheroids Release

The MCF-7 and NHDF cell spheroids were well generated with a radius of 45 μm and 40 μm respectively, after 24 h cultivation. We changed the culture medium with phosphate-buffered saline (Gibco, Thermo Fisher Scientific) and carefully peel the top channel layer off with whole device was kept in a PBS bath. After checking by microscope (AZ-100, Nikon, Japan), we found that only very few spheroids were lost after this removing process, as shown in figure S-2. Subsequently, the PBS solution was replaced with electrochemical electrolyte solution (HBSS, with 25 mM HEPES, 4.7 mM PAPP and 1mM Hexaamineruthenium (III) chloride) to perform Scanning Electrochemical Microscopy measurements (SECM). It should be noticed that the device needed to be vacuumed for 1 min to make sure the electrolyte solution was filled into the microwells.

Scanning Electrochemical Microscopy (SECM) Measurements

The SECM measurements (CH920C SECM, CH Instruments, Inc.) were performed in a three-electrode configuration using constant-height mode. A Pt disk microelectrode (CH Instruments, Inc. Shanghai, China, 25 μm in diameter) was used as the working electrode, Ag

wire and Pt wire as the quasi-reference electrode and counter electrode, respectively. The working electrode surface was well polished by using polishing cloth and solutions of alumina with different particle size (typically 1.0, 0.30 and 0.05 micron) to have a RG value (the ratio of the diameter of the glass to that of the electrode) of ~5. When we perform the SECM scanning, the working electrode with other two electrodes were put into the solution bath gently. We conducted the SECM assay in Hanks' balanced salt solution (HBSS, Thermo Fisher Scientific) contains 25 mM HEPES (Thermo Fisher Scientific, contains 138 mM NaCl, 5.3 mM KCl, 1.26 mM $CaCl_2$, 4.2 mM $NaHCO_3$, and 5.5 mM D-glucose; pH 7.5), 4.7 mM PAPP (p-aminophenyl phosphate monosodium salt), 1 mM Hexaamimineruthenium (III) chloride ($[Ru(NH_3)_6]Cl_3$) and 0.1M Potassium chloride (KCl). PAPP can firstly be catalyzed by the alkaline phosphatase (ALP), located on cell membrane, yielding the p-aminophenol (PAP), which was then oxidized into p-quinone imine (PQI) at the tip of Pt electrode at +0.3 V. Firstly, we tested the X-line scan measurement on cell spheroids (figure S-3). We prepositioned the tip over the bare region of PDMS substrate. We then monitored the approaching curve using dual-working mode. One is substrate generate-tip collect mode at +0.3 V and the other is the pure feedback mode at -0.35 V to search the start point, where the distance between probe and substrate is about 3 μm. Subsequently, the working mode was switched to +0.3 V to measure the ALP activity, and the scan rate was 30 μm/s (The increment distance was 0.6 μm and the interval time was 0.02s). To disassemble the signal of cell spheroid topography from electrochemical activity, we rescanned the same X-line at working potential of to -0.35 V (at which $[Ru(NH3)_6]^{3+}$ was reduced) right after the ALP measurement has been completed. Secondly, we performed the same procedures to gain the SECM imaging of cell spheroids with the scan rate of 400 μm/s

(The increment distance was 8 μm, with interval time of 0.02 s) on the basis of the scan area (2800 x 400 μm) as shown in figure 2. Furthermore, to figure out whether the current signal was merely aroused from the ALP catalyzed PAP diffusion, we conducted additional SECM measurement by scanning native cell-free micro pit structures with and without adding ALP (0.03 mg/ml, Sigma-Aldrich) in bulk solution as shown in figure S-3.

Numerical Simulation

During electrochemical scanning, the diffusion of redox compounds generated from adjacent spheroids may result in artifacts of the SECM signals. Therefore, the spatial diffusion of PAP was simulated using COMSOL Multiphysics 5.3. The model consisted of ten microwell solution (h=100 μm, diameter=100 μm, 10 microwells, 4 of them mimic high concentration of PAP in red, figure S4), which belong to two lanes on the chip in bulk solution. The distance of two channels was 450 μm and the microwells were 100 μm apart (edge to edge). Considering the back diffusion and RG value of the tip, we calculated the initial concentration of PAP with the equation of the steady-state diffusion limiting current as below:

$$I_{ss,PAP} = \varepsilon\, n_{PAP}\, \mathrm{F}\, D_{PAP}\, \mathrm{a}\, C_{PAP} \qquad [1]$$

We also approached the substrate in Ruhex solution with the same electrode, so the tip current in this system can also be obtained as below:

$$I_{ss,Ruh} = \varepsilon\, n_{Ruhex}\, \mathrm{F}\, D_{Ruhex}\, \mathrm{a}\, C_{Ruhex} \qquad [2]$$

Where ε is the saito factor which depends on the geometry of electrode, I_{PAP} is the tip current when electrode is very close to the spheroids, and the I_{Ruhex} is the steady state current obtained by, *n* is the number of electrons involved in the electrochemical reaction (n_{pap} = 2 equ./mol, n_{Ruhex}=1 equ./mol), *F* is the Faraday's constant (9.64853x10^4 coulombs/equ.), D is the diffusion coefficient (D_{PAP}=7.5×10^{-6} cm^2/s, D_{Ruhex}=7.2×10^{-6} cm^2/s), *a* is the radius of the

electrode (12.5×10^{-4} cm), C_{Ruhex} is the concentration of Ruhex in bulk solution (1 mM).[3] So the initial concentration of PAP (0.058 mM) inside the micro-pit can be obtained from equation 1 and 2.

$$\frac{I_{ss,PAP}}{I_{ss,Ruhex}} = \frac{\varepsilon\, n_{PAP}\, \mathrm{F}\, D_{PAP}\, \mathrm{a}\, C_{PAP}}{\varepsilon\, n_{Ruhex}\, \mathrm{F}\, D_{Ruhex}\, \mathrm{a}\, C_{Ruhex}} \quad [3]$$

It took about 15 minutes from the addition of PAPP to the electrochemical measurement, so we simulated the diffusion process of PAP for 15 min (as shown in figure S-4).

Deconvolution and kinetic mapping.

To gain the apparent heterogeneous rate constant and use it to reconstruct our SECM imaging, we deduced based on previous report[4].

The Vertical axis of figure 2 (i,j) and the color bar of figure 2 (f,g) represents the normalized dimensionless tip current[4] (Ni_T) described as

$$Ni_T = \frac{\mathrm{i_T}}{\mathrm{i_s}} \quad [4]$$

Where i_T is the measured tip current.

However, it should be noted that the normalized total anode signal Ni_{Tot}, which contains contributions from both cell topography and electrochemical activity, was previously described by following equantion.[5,6]

$$\mathrm{Ni_{Tot}} = Ni_s\left(1 - \frac{Ni_T^{ins}}{Ni_T^{cond}}\right) + Ni_T^{ins} \quad [5]$$

Where the Ni_T^{ins} is the normalized current over the PDMS substrate. According to the insulating approach curve (data not showed) and the analytical approximation for negative feedback current (Eq. 4),[7] the start point can be obtained that L=0.2, where the distance between tip and

substrate (d) is about 2.5 μm. Therefore, the $\mathrm{Ni}_{\mathrm{T}}^{\mathrm{cond}}$ (normalized current over a conductor) could be calculated by Eq.5. And then the heterogeneous rate constant (k_f) can be extracted by Eq.6 and Eq.7, where *RG* (=5 in our case) is the ratio of the microelectrode outer radius and metal radius, *L* is defined as the ratio of the tip to substrate distance and the radius of the metal electrode. Ni_s is kinetically controlled substrate current.

$$Ni_{\mathrm{ins}} = \frac{\left(\frac{2.08}{\mathrm{RG}^{0.358}}\right)\left(\mathrm{L} - \frac{0.145}{\mathrm{RG}}\right) + 1.585}{\left(\frac{2.08}{\mathrm{RG}^{0.358}}\right)(\mathrm{L} + 0.0023\mathrm{RG}) + 1.57 + \frac{\ln(\mathrm{RG})}{\mathrm{L}} + \left(\frac{2}{\pi \mathrm{RG}}\right)\ln\left(1 + \frac{\pi \mathrm{RG}}{2\mathrm{L}}\right)} \quad [6]$$

$$\mathrm{Ni}_{\mathrm{cond}} = \frac{0.78377}{\left(\mathrm{L} + \frac{1}{\Lambda}\right)} + 0.3315 * \exp\left(-\frac{1.0672}{L}\right) + 0.68 \quad [7]$$

$$\mathrm{Ni}_{\mathrm{s}} = \frac{0.78377}{\left(\mathrm{L} + \frac{1}{\Lambda}\right)} + \frac{0.3315 * \exp\left(-\frac{1.0672}{L}\right) + 1.585}{1 + \frac{\left(\frac{11}{\Lambda \mathrm{L}}\right) + 7.3}{110 - 40L}} \quad [8]$$

$$\Lambda = \mathrm{k_f} * \frac{\mathrm{d}}{\mathrm{D}} \quad [9]$$

Gene Expression Analysis

To compare the gene expression patterns between 3D tumor and stromal cell spheroid, we performed qPCR analysis on a single cell spheroid that has been used for SECM characterization. The spheroids in microwells were carefully washed by PBS. Consequently, the wanted MCF-7 and NHDF cell spheroids were manually retrieved from microwell array chip using mouth-pipetting with 100 μm diameter capillary needle which was made by a

micropipette puller (PC-10, Narishige, Japan) and each recovered spheroid were put into a 0.2 ml PCR tube directly (Video S-2).[8] Consequently, we applied RNeasy Kit (Qiagen, Germany) according with the manufacturer's protocol for cell lysing and RNA extracting. The RNA product was then converted to cDNA by using Reverse Transcription Kit (Qiagen, Germany), followed by 20 cycles PCR pre-amplification of the target genes (GAPDH, SOX2, ALP, MUC-1, EPCAM, ESR1, TGF-β, ALDH1a2, CD133, SNCG, CDH2, SDF-1, CD44, PDGFR, FN1, α-SMA, VIM, TM4SF-1). The samples were diluted 20 times with nuclease free H_2O (Invitrogen, ThermoFisher) before qPCR assay using a real time PCR system (q225, Kubo Technology, Beijing, China). The expression level of each gene was calculated by the -ΔΔCt method (As showed in figure 2l) and GAPDH was used for normalization.[8]

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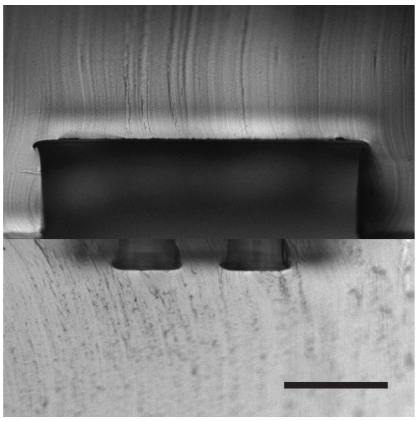

Figure S-1. The micrograph of cross section view of the microfluidic device. The diameter and height of microwell is 100 μm. The width of the channel is 600 μm and the height is 190 μm. The distance between neighbor channels is 450 μm. Scar bar is 200 μm.

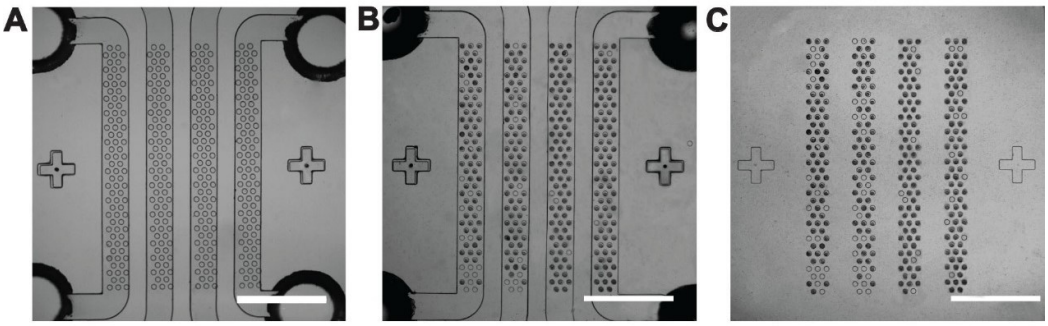

Figure S-2. Optical micrographs of the microfluidic device before (A) and after (B) cell spheroids generated. (C) The micrograph after the channel chip has been peeled off. Only a few cell spheroids were lost in this process. Scale bar is 2000 μm.

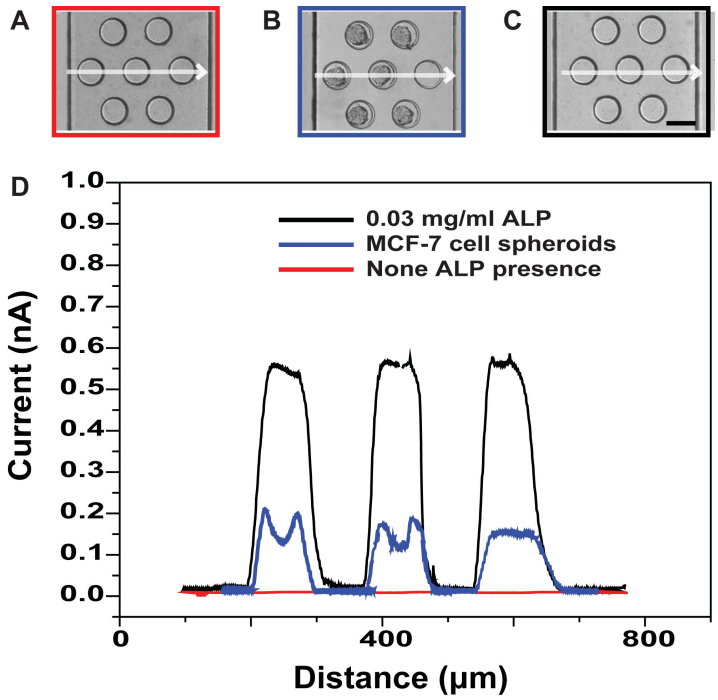

Figure S-3. One-line scan measurement based-SECM in microfluidic device with different conditions. (A) The micrograph of microfluidic device before removal of top channel and the SECM line scan trajectory is indicated by white arrow. Solution condition is standard SECM working solution (HBSS + 25 mM HEPES + 0.1 M KCl + 4.7mM PAPP), without ALP. The corresponding SECM line scan signal is indentified in red line in (D). (B) Micrograph of MCF-7 cell spheroids in microfluidic device before removal of channel and line scan direction and trajectory is indicated by white arrow. The SECM was performed in working buffer. The corresponding line scan is indentified in blue line in (D). (C) The micrograph of microfluidic wells before top layer removed and corresponding SECM scan route is indicated by white arrow. The one line scanning was carried out in SECM working buffer with 0.03 mg/ml ALP. Scale bar is 100 μm. (D) SECM measurement of ALP generated PAP. One line scan was performed to characterize anode electrochemical signal under different conditions. Colored lines indicate the corresponding signal for line scan across micro pits structures indicated in (A), red line, (B), blue line, and (C) black line.

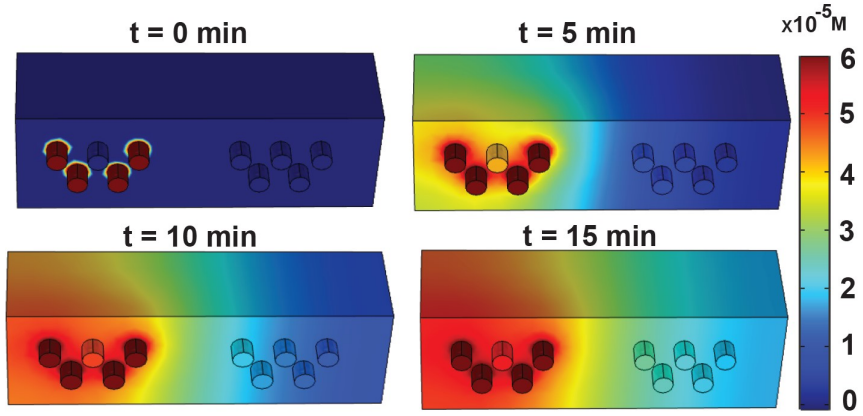

Figure S-4. Simulation of diffusion process of PAP (the product of the enzymic catalytic reaction) at different time points in the microwell chip. (Comosol Multiphysics 5.3a). The diffusion coefficient of the PAP was 7.5×10-10 m2/s. The color bar represents the concentration of PAP.

Supplementary Table S1: Primers for quantitative PCR.

Genes	Primer Sequence (5’-3’)
EPCAM-Forward:	CGCAGCTCAGGAAGAATGTG
EPCAM-Reverse:	TGAAGTACACTGGCATTGACG
Sox2-Forward	TGGACAGTTACGCGCACAT
Sox2-Reverse	CGAGTAGGACATGCTGTAGGT
CDH2-Forward	CCTGACACTGGTGGCACTAC
CDH2-Reverse	GCTGGGGTCAGAGGTGTATC
FN1-Forward	GCCAAAGCTTTACTACTGTGGA
FN1-Reverse	TTTCCCCCGAAGGTGTCTTA
MUC-1-Forward	AGTGCTTACAGTTGTTACGGGT
MUC-1-Reverse	CTCAGTAGAGCTGGGCACTG
ALDH1A2-Forward	TGGAAAATCTGGCCAAGCCT
ALDH1A2-Reverse	TGACAGAGGAGCTCAGTGGA
CD133-Forward	AGCAATGAAGAACTGGTCGGA
CD133-Reverse	GGGGGAATGCCTACATCTGG
VIM -Forward	CGGGAGAAATTGCAGGAGGA
VIM -Reverse	AAGGTCAAGACGTGCCAGAG
CD44-Forward	AGAGCTGGCCAAGTCTTCAC
CD44-Reverse	GCTTCCAGAGTTACGCCCTT
TGF-β-Forward	TGCACCATGCTTTGGCTTTC
TGF-β-Reverse	CTGGCTGGCTCAGCAACTAT
PDGFRA -Forward	TAAGTGCGAAGACTGAGCCA
PDGFRA -Reverse	AGAAGTTTCATCCGGCCTCA
SNCG -Forward	TGTTGTACAGAGCGTGACCT
SNCG -Reverse	GATGGCCTCAAGTCTCCTT
ESR-1-Forward	CGTCGCCTCTAACCTCGGG
ESR-1-Reverse	AGCTCGTTCCCTTGGATCTG
ALPL -Forward	CTTTATAAGGCGGCGGGGG
ALPL -Reverse	AGAGATGCAATCGACGTGGG
GAPDH -Forward	ATGGGTGTGAACCATGAGAAG
GAPDH -Reverse	AGTTGTCATGGATGACCTTGG
SDF-1-Forward	CTAGTCAAGTGCGTCCACGA
SDF-1-Reverse	GGACACACCACAGCACAAAC
ACTA2 (α-SMA)-Forward	GTGTGTGACAATGGCTCTGG
ACTA2 (α-SMA)-Reverse	TGGTGATGATGCCATGTTCT
TM4SF1-Forward	CAGGAATGGGCTGAGAGTGG
TM4SF1-Reverse	GATGCATCGTGCACACTTCC