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Supplementary Information

Single-cell Metabolic Accumulation Analysis by Microfluidic Hydrogel Microspheres Combined with Mass Spectrometry

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Materials and Methods

Reagents

SU-8 2050 negative photoresist and developer were purchased from Microchem Corporation (Newtown, MA). Sylgard 184 poly(dimethylsiloxane) (PDMS) and initiators were purchased from Dow Corning (Midland, MI). Calcium Chloride was bought from Leagene Biotechnology (Beijing, China). EDTA-Na, sodium fluorescein and 1H,1H,2H,2H-perfluoro-1-octanol (PFO) were bought from Macklin (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Gibco Corporation (New York). Sodium hydrogels, Isopropanol and 11H,1H,2H,2H-Perfluorooctyl trichlorosilane were obtained from Sigma-Aldrich (St. Louis, MO). HFE-7500 was purchased from 3M Novec 7500 Engineered Fluid (3M, St. Paul, MN) and Fluorinated surfactant (perfluorinated polyether-polyethylene glycol, PFPE-PEG) was bought from Raindance Technologies (Lexington, MA). Calcein-AM/PI Double Staining Kit was bought from Dojindo Laboratories (Japan). A549, HepG2 and HCT116 cell lines were purchased from National Infrastructure of Cell Line Resource (Beijing, China). Arachidonic acid, heptadecanoic acid, phosphate buffer (PBS, 0.01 M, pH 7.4), palmitic acid, citrulline, arginine, ornithine, lactic acid standards were purchased from Beijing Solarbio Technology Co., Ltd. (Beijing, China).

Apparatus

An inverted Nikon ECLIPSE Ts2R fluorescence microscope (Zeiss, Germany) and Fluorescence Confocal Microscopy (Zeiss, LSM-780) were applied to observe the microsphere and cell characterization. ImageJ software was utilized to analyze the obtained images. ITO glass slide was treated with oxygen plasma (PDC-32G, Harrick Plasma, Ithaca, NY, USA). The Longer LSP02-2B injection pump was applied for stable liquid delivery. The Shimadzu Cellent CM-MS with LCMS-8050 (Tokyo, Japan) was used for single-cell metabolic accumulation measurements.

Experimental

Preparation of microfluidic chips for single-cell microsphere generation and culture

The soft lithography technology was used for the production of the molds of the microfluidic chips according to our previous work.To prepare the microfluidic chip for single-cell generating, SU-8 2050 negative photoresist was first applied onto a thoroughly cleaned silicon wafer. The wafer was spun at 500 rpm for 30 s and then at 1000 rpm for 1 min, and then transferred to the heating plate to preheat the wafer at 65 °C for 3 min and 95 °C for 7 min. After the wafer cooled down to room temperature, the wafer was exposed to a UV lithography machine for 3 min. Following exposure, the silicon wafer was

post-baked at 65 °C for 1 min and 95 °C for 10 min. The wafer was developed to eliminate unexposed portions until the clean structures of the microchannels were visible. Finally, the wafer was silanized overnight to create the mold. The polydimethylsiloxane (PDMS) pre-polymer and curing agent at a 10:1 ratio were mixed, and then the mixture was poured into the mold. The filled mold was stored in a vacuum chamber to remove air bubbles using a vacuum pump. Subsequently, the mixture was baked in an oven at 75 °C for at least 2 h. The cured PDMS was peeled off, cut, punched, and bonded to with a glass slide after oxygen plasma treatment. The resulting single-cell generating chip, featuring micro-channels with a width and depth of 110 μm each, was yielded and stayed in the oven before use.

The single-microsphere culture chip was fabricated in the same way described above. Particularly, multiple coating of SU-8 2050 and exposure were used for multi-layer microfluidic chip structures. First, the silicon wafer was spun at 500 rpm for 20 s and 2500 rpm for 1 min to get the extracted channels. Then, the wafer was pre-baked and exposed for the first time. After post-baking, the silicon wafer was spun with SU-8 2050 again at 500 rpm for 20 s and 800 rpm for 1 min twice and pre-bake was conducted after each time spin. The second exposure under a UV lithography machine according to the cross markings on the two different masks was to proceed to get the culture chambers. The width and depth of the extract channels were both 50 μm, while the depth of the culture chamber and side channel were 300 μm. And the following steps were the same as the the microfluidic chip for single-cell generating.

Hydrogel preparation and cell culture

The EDTA-Ca solution was prepared firstly by combining calcium chloride solution (1 mol/L) and EDTA-Na solution (0.2 mol/L) at the ratio of 1:5. The pH of the mixture was adjusted to approximately 7 using sodium hydroxide. After filtration, the solution was calibrated to the concentration of calcium ions to reach 100 mmol/L with deionized water. DMEM medium (containing 10% fetal bovine serum) was mixed with the prepared EDTA-Ca solution at a volume ratio of 1:1. Finally, 2% (mass-volume ratio) of the sodium alginate was added and allowed complete dissolution at 37 °C for at least 2 days to form the hydrogel.

A549, HCT116, and HepG2 cells were cultivated in DMEM high-sugar medium in a 5% CO_2 incubator. After 48 h of incubation, the culture medium was removed. The cells were washed 1-2 times with PBS, and trypsinized for 1-2 min until most cells assumed a circular shape. Subsequently, the cell sediment was obtained after centrifugation at 1000 rpm for 3 min. Next, 250 μL of prepared hydrogel was injected into the cell sediment and mixed to create a cell-laden hydrogel, followed by tenfold dilution for the generation of single-cell microspheres.

Single-cell microsphere generating and loading into the culture chip

A syringe pump was utilized to inject cell-laden hydrogel through the inner disperse phases channel of the microfluidic chip at a rate of 20 μL/h (Inlet 1), while simultaneously injecting cell-free hydrogel

through the outer disperse phases channel at a rate of 40 μL/h (Inlet 2). The flow rate ratio between the inner and outer phases can be adjusted flexibly based on cell concentration conditions (from 1×10^6 to 8×10^6) to obtain single-cell hydrogel microspheres. About 50-100 cell microspheres were chose randomly to calculate the percentage of single-cell microspheres and and count the number of encapsulated cells under a microscope. The continuous phase channels should be infused with fluorinated oil containing 0.2% surfactant (Inlet 3) and 0.1% acetic acid (Inlet 4) at a rate of 400 μL/h to generate single-cell hydrogel microspheres. The microspheres were collected within a fluorinated oil solution containing 20% 1H, 1H, 2H, 2H-perfluorooctanol for demulsification. Subsequently, the microspheres were transferred through a cell sieve into a petri dish. Finally, the dish was placed into an incubator, maintaining a temperature of 37 °C under 5% CO_2 for the storage of cell-laden hydrogel microspheres before injecting into the culture chip.

The single-microsphere culture chip was filled with DMEM medium, ensuring the removal of air bubbles. Subsequently, a syringe was used to pump the DMEM medium with single-cell hydrogel microspheres into the inlet on the chip. Upon the side channel was closed, the DMEM medium with single-cell hydrogel microspheres could enter the culture chamber, and exit through the extraction channel. Once the microspheres were captured in the chamber, the extracted channel was sealed, completing the sequential loading of all chambers. After loading, paraffin oil was injected through the side channel to isolate the chambers. The chip was stored in a petri dish containing sterile PBS to prevent evaporation and then placed in an incubator, maintaining a temperature of 37 °C under 5% CO_2 for 24 h.

Cell viability test after single-droplet chambers culture

Following the 24 h culturing process, cell viability was assessed by staining cells using the Calcein AM/PI staining reagent. The stain work solution was introduced through the inlet, allowing it to reside in the side channel for an extended period. The dye was gradually permeated into the chamber through diffusion, and the waiting period for complete staining was approximately 30 min. A confocal microscope was utilized to capture fluorescence information.

Single-cell metabolite extraction and mass spectrometry analysis

Following a 24 h culture period, the single-microsphere culture chip was positioned vertically with the extraction channel open. A microsyringe was employed to withdraw all the culture medium (approximately 1.5 μL) from the chamber. The resulting culture medium was adjusted volume to 10 μL and transferred into a mass spectrometer vial for subsequent mass spectrometry testing using LC-MS mass spectrometry. Each experiment was repeated three times.

Conditions of mass spectrometry and liquid chromatography

The mass spectrometry system used in this study was UltiMate 3000 Rapid Separation LC system

(Dionex, Germering, Germany) tandem Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with electrospray ionization source (ESI). The Hypersil GOLD C18 column (150 mm × 2.1 mm, 1.9 μm) was used for chromatographic separation, and the column temperature was maintained at 40 °C. Mobile phase conditions consisted of water containing 0.1% formic acid as mobile phase A and acetonitrile containing 0.1% formic acid as mobile phase B. The ratio of phase A to phase B was 95:5. The gradient operated at a flow rate of 0.3 mL/min. The injection volume was 1μL. The ESI source operated sequentially in negative and positive ionization modes. Capillary voltages for negative and positive ionization modes were set at -2500 and 3500 V, respectively. The remaining parameters were as follows: capillary temperature of 300 °C, heater temperature of 250 °C. The MRM conditions for each compound are listed below (Table S1).

Data and statistical analysis

Data were repeated three times and presented as the mean±standard deviation. In the statistical analyses, t-test, one-way ANOVA test and Tukey's multiple comparison test were analyzed in GraphPad Prism 8.0.2. Ten compounds form three different cell lines were analyzed using logarithmic transformation and normalization, followed by Partial Least Squares-Discriminant Analysis (PLS-DA) using https://www.metaboanalyst.ca/faces/ModuleView.xhtml.

Data supplementation

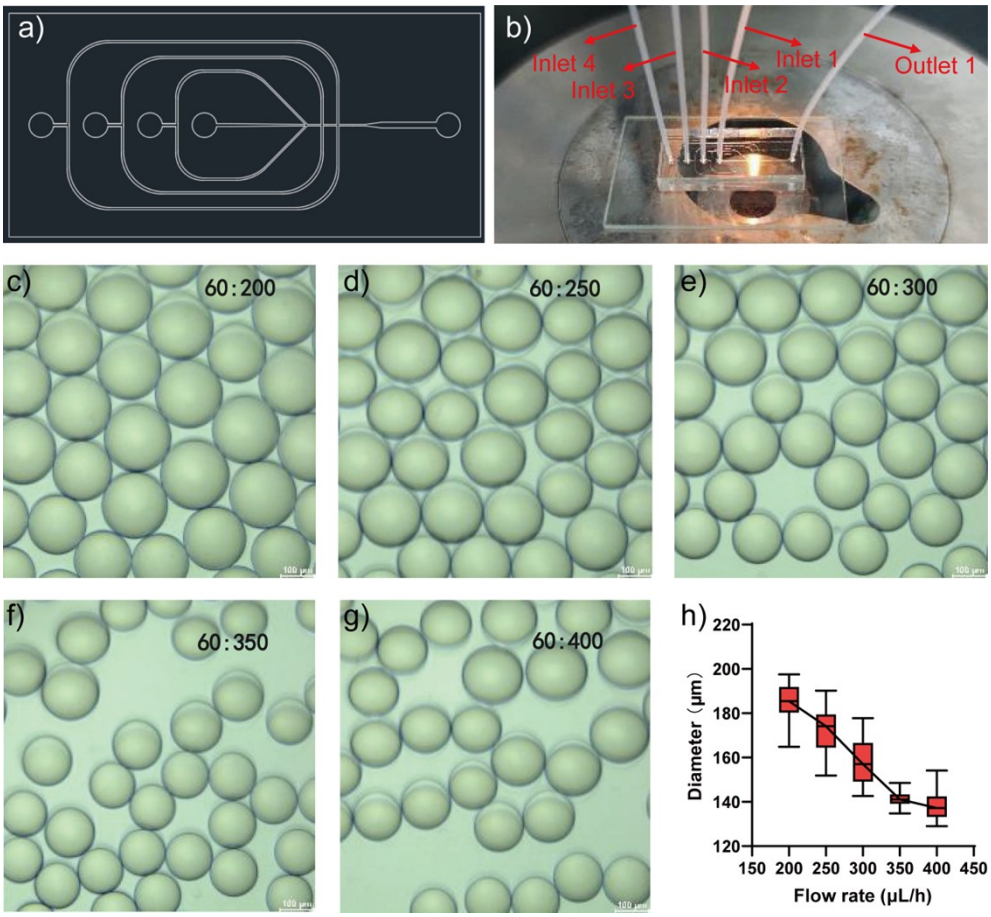


Figure S1. The droplet microfluidic device and the statistics of microsphere sizes. (a-b) The device diagram of a microfluidic device for generating hydrogel microspheres. (c-g) The size of hydrogel microspheres under microscope when flow ratio ranges from 60:200 to 60:400 (scale bar=100 μm). (h) Average diameters of hydrogel microspheres when oil flow rate ranges from 200 to 400 μL/h. (Data were presented as mean ± standard deviation (SD) (n=40)).

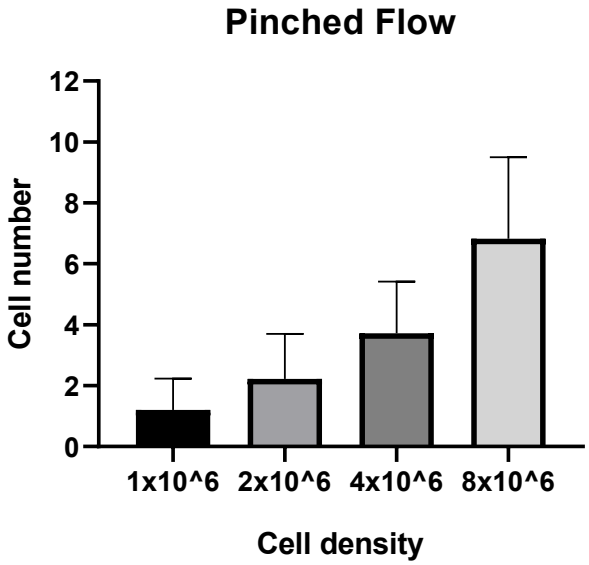


Figure S2. Cell numbers in hydrogel microspheres at different cell densities (n=100, 40, 85 and 79, respectively).

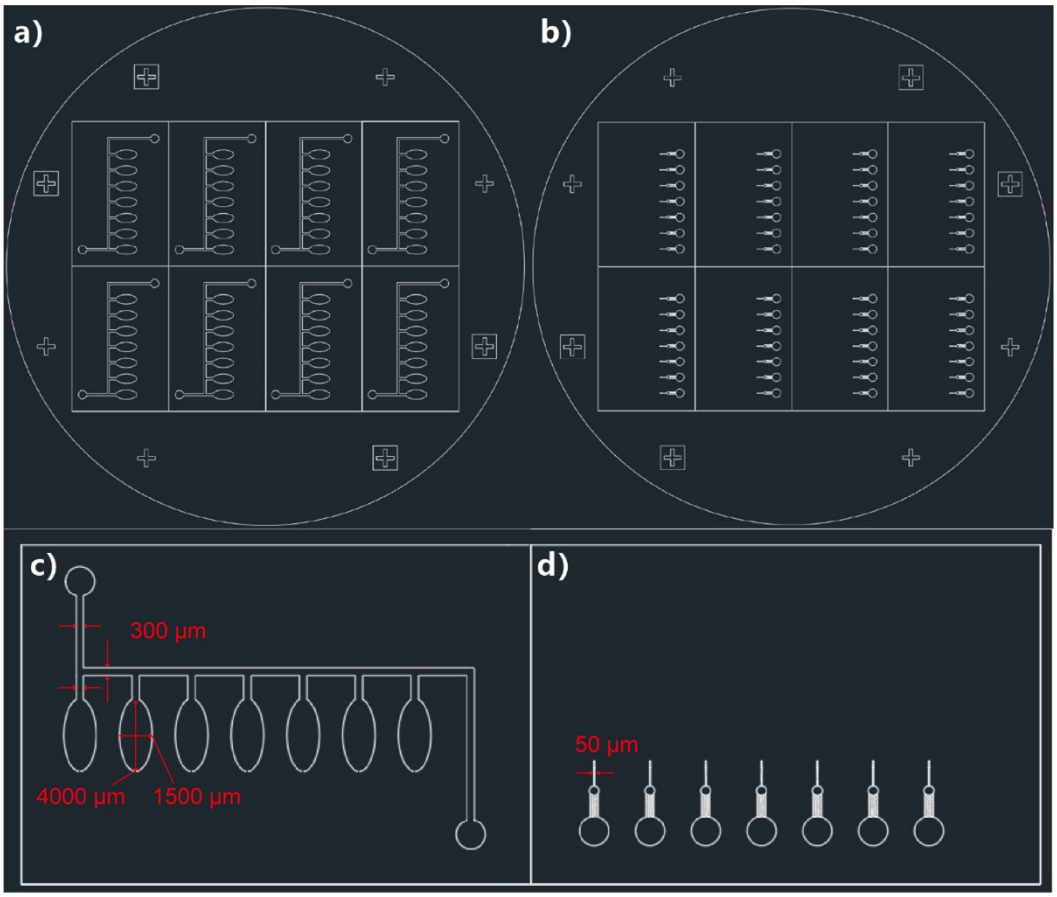
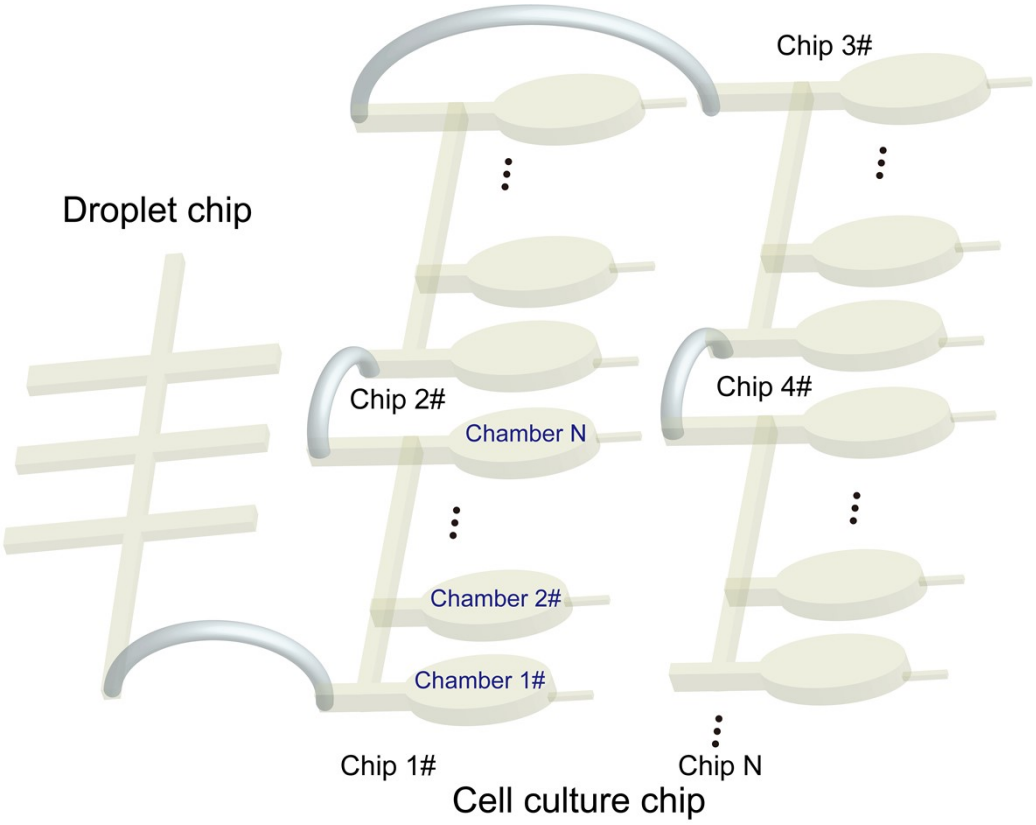


Figure S3. Mask design of the single-microsphere culture chip. Overall graphics (a, b) and individual structures (c, d) (inlet and side channel dimensions of 300 μm, metabolite extraction channel dimensions set to 50 μm).

Figure S4. Schematic diagram of increasing throughput.



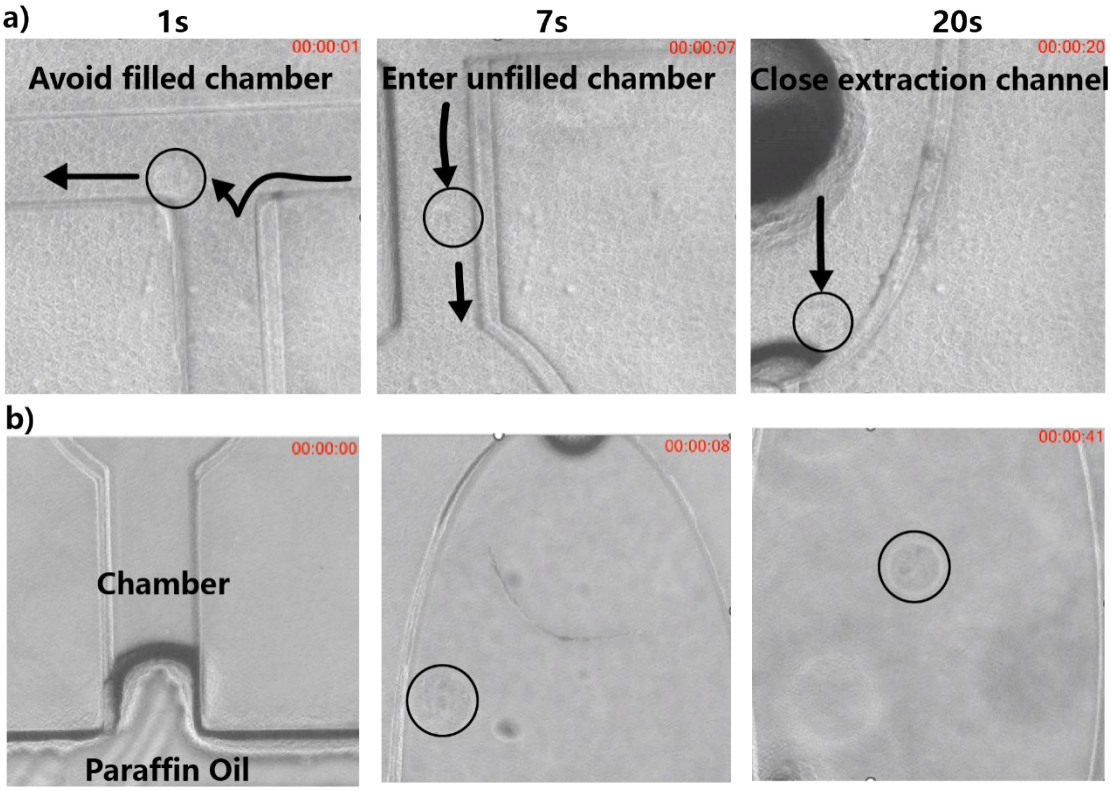


Figure S5. Photomicrograph of microfluidic chip loading for single-microsphere isolation culture. (a) Hydrogel microspheres during loading: microspheres avoided the chamber already filled with microspheres and entered the chamber unfilled with microspheres thus blocking the extraction channel. (b) Photomicrograph of the chamber after loading.

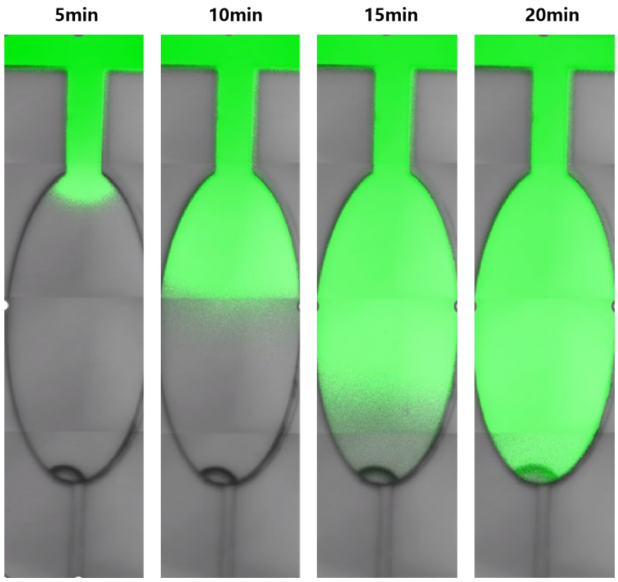


Figure S6. Diffusion of sodium fluorescein over time in single-microsphere culture chips.

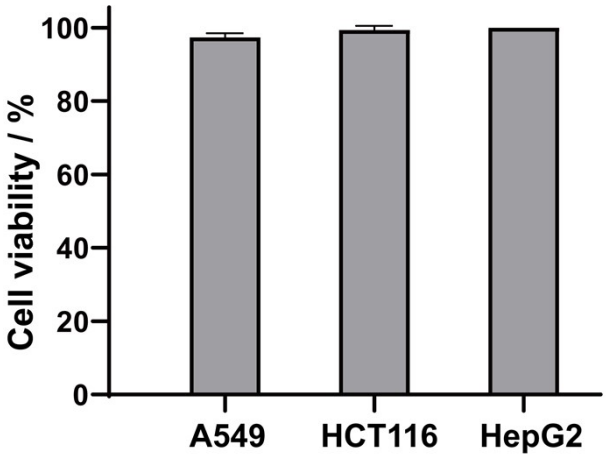


Figure S7. Viability of single cells cultured on the culture chip (n=100).

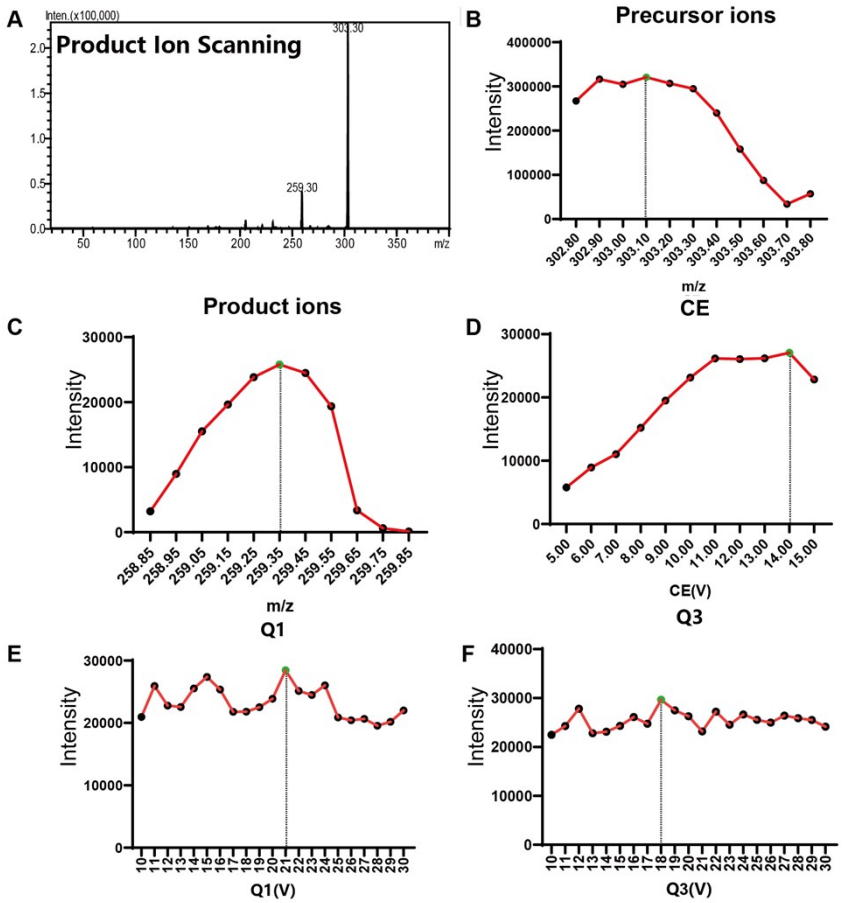


Figure S8. Mass spectrometry condition optimization process. (a) Product ion scanning. (b) Precursor ion optimization. (c) Product ion optimization. (d) Collision voltage optimization. (e) Q1 Prerod voltage optimization. (f) Q3 Prerod voltage optimization.

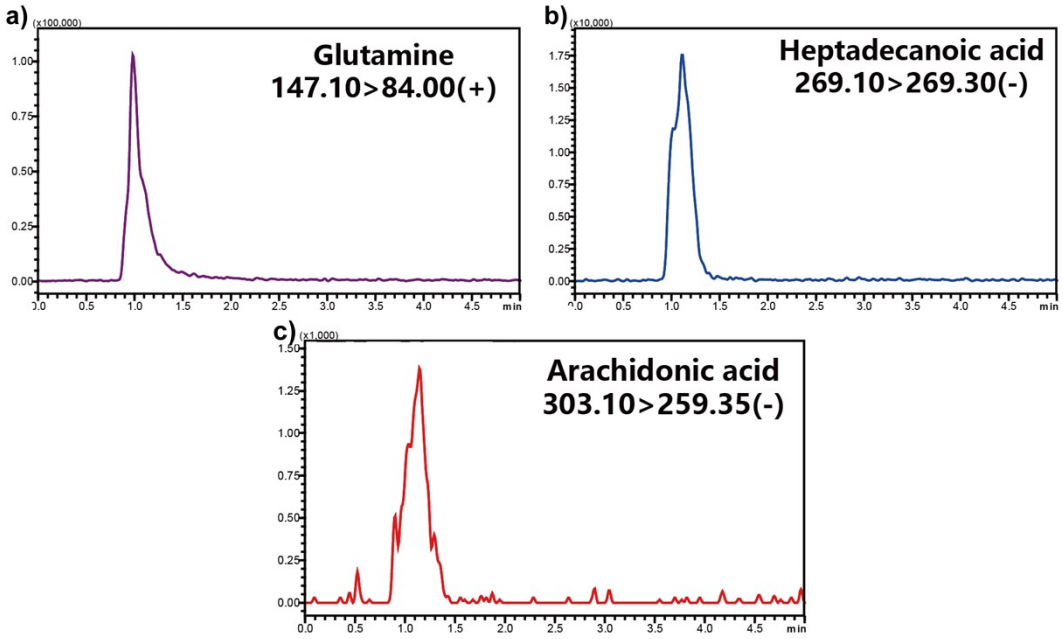


Figure S9. Chromatograms of single-cell metabolites samples. (a)-(c) for glutamine, heptadecanoic acid, and arachidonic acid, respectively.

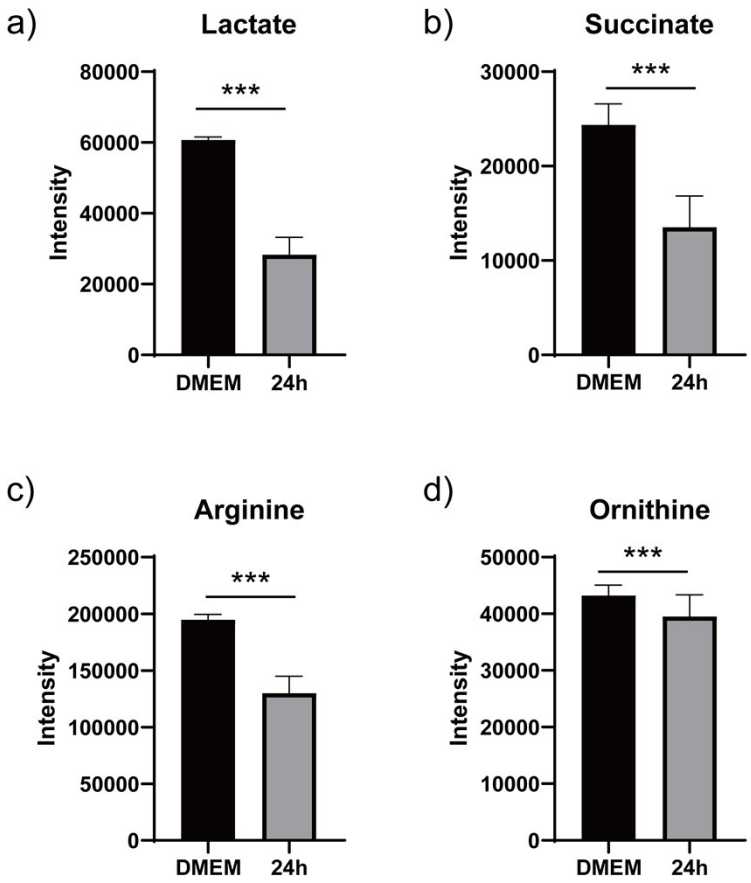


Figure S10. Pre-experimental procedure of cellular metabolites. (a)-(d) Changes in metabolite (lactate, succinate, arginine and ornithine) content with incubation time in pre-experiments. Data above were presented as mean ± standard deviation (SD) (n=3). ***P < 0.01, compared to the control group (DMEM).

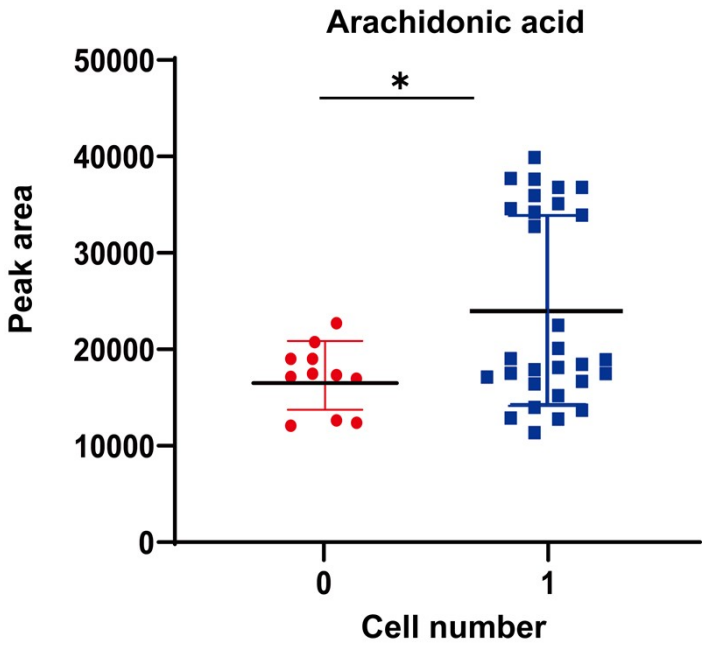


Figure S11. Metabolic accumulation of arachidonic acid from A549 cultured on the chip (*P < 0.05).

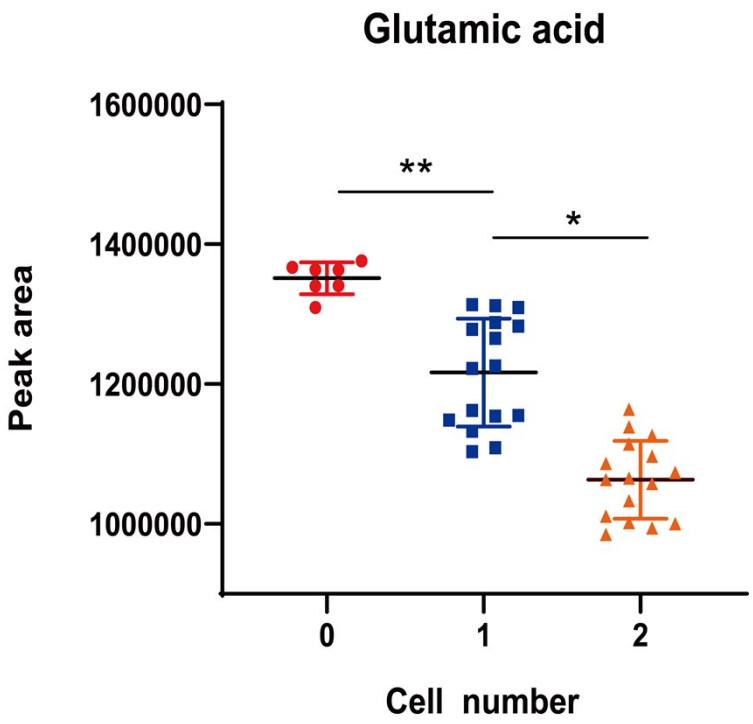


Figure S12. Metabolic accumulation of glutamic acid from A549 cultured on the chip (**P＜0.01, *P＜0.05).

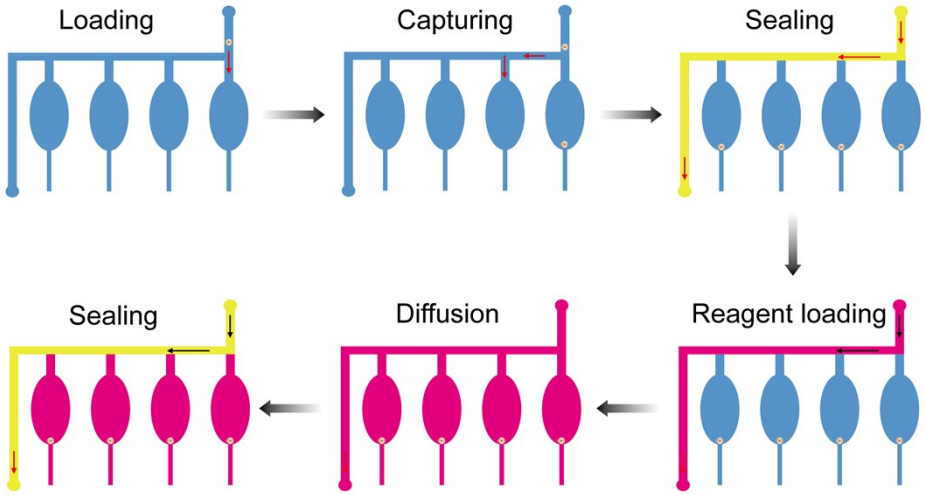


Figure S13. Schematic diagram of repeating metabolite collections.

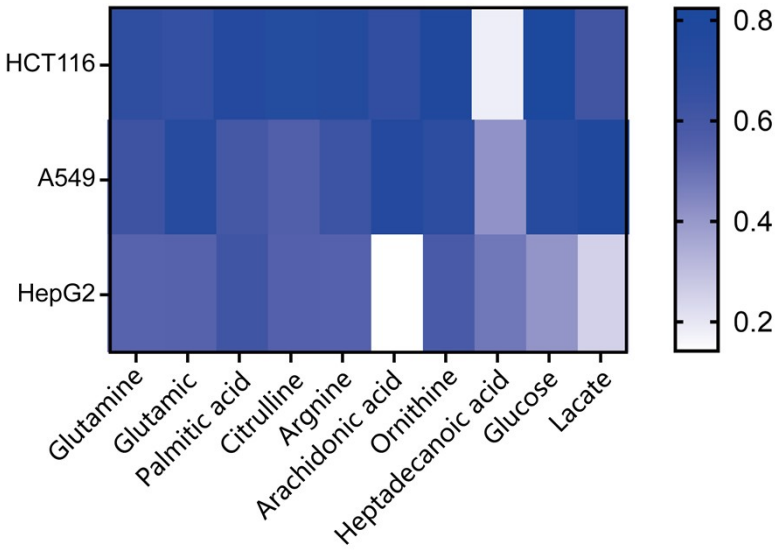


Figure S14. Heatmap of metabolic accumulation differences among HCT116 cells (n=41), A549 cells (n=47) and HepG2 cells (n=35). The intensity of all MS peaks were processed by Linear Normalization.

Table S1 MRM conditions of single-cell metabolite.

Ionisation mode	Compound	Formula	Precursor Ion m/z	Product Ion m/z	Dwell Time (ms)	Q1 Prerod (V)	CE (V)	Q3 Prerod (V)
-	Lacate	$C_3H_6O_3$	89.05	43.0	10	10	13	13
+	Argnine	$C_6H_{14}N_4O_2$	175.2	60.1	10	-19	-16	-24
+	Ornithine	$C_5H_{12}N_2O_2$	133.1	116.05	10	-10	-15	-24
+	Citrulline	$C_6H_{13}N_3O_3$	176.1	159.05	10	-12	-14	-18
-	Glucose	$C_6H_{12}O_6$	179.15	89.05	10	13	8	28
+	Glutamine	$C_5H_{10}N_2O_3$	147.1	84	10	-12	-18	-15
+	Glutamic	$C_5H_9NO_4$	148.1	84.1	10	-12	-18	-17
+	Palmitic acid	$C_{16}H_{32}O_2$	256.75	179	10	-11	-13	-18
-	Arachidonic acid	$C_{20}H_{32}O_2$	303.1	259.35	10	21	14	18
-	Heptadeca-noic acid	$C_{17}H_{34}O_2$	269.1	269.3	20	13	14	21