Supporting Information

Preparation of multicellular spheroid sections embedded with sodium carboxymethyl cellulose for mass spectrometry imaging

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Experimental methods

1. 2D cell culture. Cell lines were cultured using the following conditions: Medium for PANC-1 cells was composed of 90% Dulbecco's modified eagle medium (DMEM, VivaCell, Germany), 10% fetal bovine serum (FBS, Thermo Fisher Scientific, U.S.A.) and 1% penicillin-streptomycin (P/S, 100 U/mL, Thermo Fisher Scientific, USA). MRC-5 cells medium was composed of 90% Minimum Essential Medium containing L-glutamine (MEM, Gibco, Pittsburgh, USA), 10% FBS and P/S (100 U/mL, Thermo Fisher Scientific, U.S.A.). Both cell lines were incubated at 37°C under atmosphere containing 5% CO_2.

2. Construction and growth of heterogeneous MCTSs. PANC-1 and MRC-5 cells (Procell, China) were transferred to a 96-well ultra-low attachment plate (Corning, U.S.A.) with a cell count ratio of 1: 9 (PANC-1: MRC-5) and incubated at 37°C under atmosphere containing 5% CO_2 for three days. Medium was changed every three days until heterogeneous MCTSs were collected on day 7.

3. H&E staining. Sections on cover glass were placed in anhydrous ethanol for 2 min, immersed in hematoxylin solution (Servicebio, China) for 5 min, rinsed with running water, differentiated in 0.5% hydrochloric acid ethanol for 3 s, washed with running water until the sample turned blue, and stained with 5% eosin solution for 10 min. The cover glass was then dehydrated in 75%, 85%, 95% and 100% ethanol, immersed in xylene, mounted by neutral resin, and observed under microscope. Aperio GT450 smart digital Pathology Scanner (Leica, Germany) was applied to scan and analyze the HE stained samples.

4. Matrix coating. The samples were transferred from -80°C to -20°C, left overnight, and dried in vacuo for 30 min. 5 mg/mL 9-aminoacridine (9-AA) in MeOH/H_2O (80:20, v/v) and 20 g/L 2,5-dihydroxybenzoic acid (2,5-DHB) in MeOH/H_2O (70:30, v/v) were used as the matrix for Matrix Builder automatic matrix sprayer (Viktor, China) set at the following parameters: sprayer temperature 60°C, platform temperature 45°C, spray pressure 0.55 MPa, pump speed 0.07 mL/min, drying time

20 s, sprayer height 20 mm, scanning interval 2 mm, and 6 repeats. Slides were dried for 10 min after matrix deposition.

5. Data analysis. The raw mass spectrometry images were processed using Flex Imaging, where regions of interest (ROI) were selected, and the data from these areas were exported to Flex Analysis for ion peak identification. The acquired imaging data were imported into SCiLS Lab software, where all peaks were subjected to Root Mean Square (RMS) normalization. Segmentation maps were generated to divide the sample into proliferation and necrotic regions, enabling the evaluation of metabolic heterogeneity across different MS images and visualization of metabolite distribution. For the selected *m/z*, ion images were created, and the ion intensity values at each sampling spot within the microregions were extracted. Outlier boxplots were generated using Origin software to analyze the differences in normalized signal intensity among microregions for comparative analysis. T-test can be used to analyse the differences in between the groups. P value < 0.001 means the difference is statistically significant. All experiments were repeated three times.

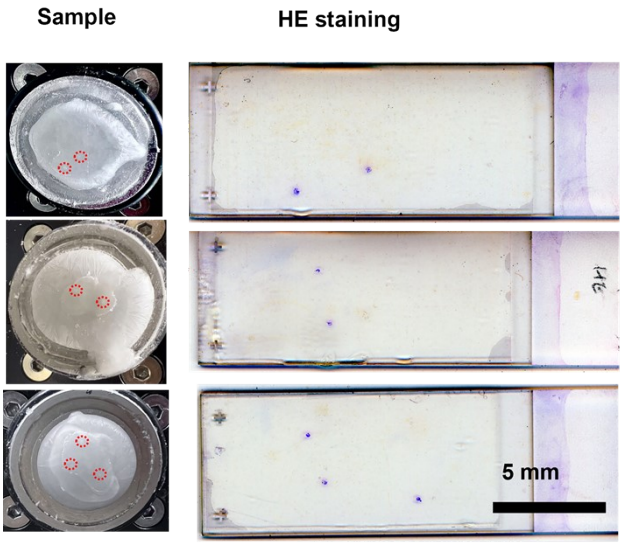

Fig. S1. High-throughput sectioning and H&E staining of heterogeneous MCTSs based on the 2% CMC embedding.