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Direct Transfer of Multicellular Tumor Spheroids Grown in Agarose Microarrays for High-Throughput Mass Spectrometry Imaging Analysis

Yuan Liu¹, Jillian Johnson¹, Hua Zhang¹, Penghsuan Huang², Lingjun Li^{1,2,3,*}

¹School of Pharmacy, University of Wisconsin-Madison, Madison, WI

²Department of Chemistry, University of Wisconsin-Madison, Madison, WI

³Lachman Institute for Pharmaceutical Development, School of Pharmacy, University of Wisconsin-Madison, Madison, WI

Abstract

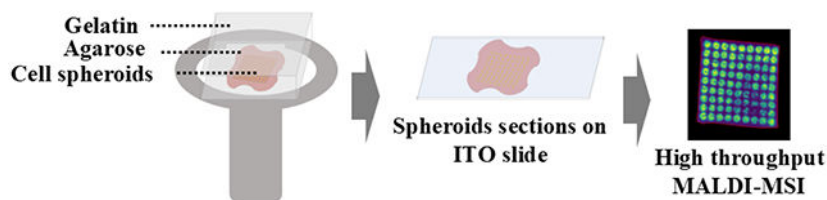
Multicellular tumor spheroids (MCTSs) play an important role in biological studies and cancer research. There is an emerging research interest in molecular profiling and drug distribution of MCTSs by leveraging the superior sensitivity and molecular specificity of mass spectrometry imaging (MSI). Current methods for sample preparation of MCTSs can suffer from low throughput, as MCTSs are typically individually transferred from cell culture into an MSI embedding media and sectioned individually, or sometimes, a few spheroids are placed in a small block of embedding media in preparation for MSI. Here, we developed a method to minimize the sample preparation steps needed to create high-throughput MCTS frozen sections for MSI. Agarose-based microarrays created from Microtissues[®] molds were used during MCTS culturing, after which the entire MCTS agarose microarray was taken out of the cell culture well and then directly embedded in 5% gelatin, without the need for a transfer step for each individual MCTS into the embedding media. This method enables rapid profiling of up to 81 MCTSs for larger MCTSs (500–800 μm) or up to 256 MCTSs for smaller MCTSs (200–300 μm) in a single section, remarkably improving the throughput possible for MSI MCTS workflows. Notably, sectioning MCTSs together in the agarose microarray also improves MCTS visualization during sectioning, such that staining each MCTS section to ensure the presence of the MCTSs within the embedding media is not necessary during the sectioning process. The method described here provides a more direct, convenient strategy to achieve high throughput sections. MSI MCTS sectioning throughput is an important advancement for both pharmaceutical testing of MCTS; the direct transfer 3D cell cultures grown within cell-culture compatible polymer scaffolding is also critical for expanding MSI for the characterization of microfluidic and complex *in vitro* models, where agarose is readily utilized as a non-adhesive 3D cell culture scaffold.

*Corresponding authors: Dr. Lingjun Li, lingjun.li@wisc.edu, Mailing address: School of Pharmacy, University of Wisconsin-Madison, 777 Highland Ave, Madison, WI 53705, United States, Fax: +1 608-262-5345.

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Graphical Abstract



Keywords

Multicellular tumor spheroid microarrays; High throughput; MALDI MS imaging; Mass spectrometry; Agarose Microtissues[®] microwells; 3D cell culture

Introduction

Two-dimensional (2D) cell culture is widely used in cancer research and drug development. However, tissues and organs are three dimensional (3D) *in vivo*. Compared to 2D cell culture, 3D cell culture models are more physiologically relevant in cell morphology and signaling and show more relevant drug metabolism related to *in vivo* systems (1). Among various 3D culture systems, including organoids that recapitulate organ-specific architecture, multicellular tumor spheroids (MCTSs) have gained emerging attention in cancer research and drug evaluation due to their ability to bridge the gap between traditional cell culture and animal experiments while enabling the study of cellular heterogeneity in drug exposure and response (2). In this study, we utilized MCTSs as our primary 3D culture model. To study MCTSs, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) is a powerful tool for analyzing biomolecules in a spatially resolved manner (2–4). MALDI MSI can be used to visualize the spatial distribution of biomolecules within MCTS (5–8). This technique also enables analysis of drug penetration, metabolism, and endogenous pharmacodynamic changes within MCTS (9–13). The MCTS have also been used in numerous biological studies, including evaluation of cancer cell response to drug treatment (14, 15) and plays an important role in understanding *in vitro* to *in vivo* translation gap in pharmaceutical development.

In MALDI-MSI analysis of MCTSs, sample preparation is a critical step. Currently, gelatin embedding is widely used for MCTS sample embedding and sectioning (16, 17). However, the limited transparency and color similarity between frozen gelatin and MCTS introduces a challenge when locating the MCTS sections in gelatin embedding media during cryosectioning, especially for small (<200µm) MCTSs. Therefore, hematoxylin and eosin staining (H&E staining) or cresyl violet staining techniques are often used to facilitate the visualization of MCTS or organoids after mounting a tissue section on the slide (18). The addition of a staining step to visualize the MCTS during sectioning inevitably increases the time to create the tissue sections, to make sure that the MCTS are captured within the embedding media and able to visualize them once mounted on the slide. Another method intended to address the visualization issue uses ice-coated MCTS (6), in which MCTS could

be visualized in contrast to the ice during cryosectioning. However, a noted limitation in this work was that only one spheroid at a time was sectioned using the ice-embedding method.

Previously, a strategy for high-throughput organoid sectioning using a gelatin-based microarray has been described (5). Although dozens of organoids could be sectioned and analyzed via MSI, the sample preparation involved transferring samples out of Matrigel and then into gelatin-based microarray is an additional step required before sectioning the organoids. There have also been significant efforts to expand the types of embedding media used for MSI for 3D cell culture systems beyond gelatin. Gelatin has been seen as the primary embedding media for MSI because it produces minimal mass spectral interference, compared to the conventionally available media for frozen sections, TissueTek's Optimal Cutting Temperature (O.C.T.) compound, which is made of polyethylene glycol, ionizes very easily and can interfere with MSI analysis (19). Some of additional MSI-compatible embedding materials have expanded from using just gelatin into gelatin-carboxymethylcellulose mixtures or gelatin-agarose mixtures to better support tissue structures during sectioning or even replacing gelatin with more niche polymers – including pHPMA (poly[N-(2-hydroxypropyl)methacrylate amide] and hydroxy-propyl methyl cellulose /polyvinylpyrrolidone (HPMC-PVP) (20–22), which both allow MSI to be more easily incorporated with multi-modal imaging modalities pipelines within histology and microscopy. Gelatin, while MSI compatible, can cause background H&E staining, however pHPMA and HPMC-PVP do not produce this same background interference in conventionally paired imaging modalities (i.e. H&E, autofluorescence, etc.). However, while new embedding media are being used for MCTS MSI workflows, the key issue that limits the adaptation of MSI of MCTS in the pharmaceutical industry is the low throughput of the MCTS sectioning for the assay throughput. There has been some interesting work for MCTS MSI platforms using paper-based cultures for MCTSs intended for achieving high throughput drug screening (23). This strategy greatly improved the MSI throughput for MCTSs; however, since the MCTS are not actually sectioned in this sample preparation method, information regarding the spatial distribution in the z-axis plane of the MCTSs is not covered in the analysis for each MCTS (12). In this proof-of-principle study, we developed a streamlined sample preparation strategy for sectioning MCTSs using non-adhesive microwells, enabling high-throughput MSI analysis.

Materials and Methods

Chemicals and materials.

Chemicals including acetonitrile (ACN), formic acid (FA), methanol, ethanol, trifluoroacetic acid (TFA), and HPLC grade water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Indium tin oxide (ITO)- coated glass slides (25 mm × 75 mm × 1 mm) were purchased from Delta Technologies (Loveland, CO, USA). Agarose was purchased from National Diagnostics (AquaPor LE GTAC Agarose, Atlanta, GA, USA). Microtissues[®] micro-molds (Catalogue #12–81 and #12–256) were used to generate agarose-based 3D Petri Dishes[®] with outer dimensions of 12 mm × 12 mm (L × W) and inner culture area dimensions of 9 mm × 9 mm. Base molds with dimensions of 18 × 18 × 5 mm (L × W × H) or bigger size were used.

Cell culture and MCTS growth.

The pancreatic cancer cell line PANC-1 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was maintained in DMEM (11965, Gibco, Life Technologies, USA) containing 10% fetal bovine serum (FBS) (Gibco, Origin: Mexico) and 1% penicillin-streptomycin solution (Gibco, Life Technologies Corporation, Grand Island, NY, USA). Cells were cultured in a 37 °C moisture incubator filled with 5% CO₂. Cells suspensions were prepared by trypsinization at 70%–90% confluence using 0.25% trypsin EDTA solution (Gibco, Life Technologies Corporation, Grand Island, NY, USA). The cell suspension was centrifuged at 300 xg for 5 minutes and the medium was discarded. MCTS were prepared by adding PANC-1 cell suspension to 1% agarose-based 3D petri dishes generated using Microtissues® 3D Petri Dish micro-mold (Sigma-Aldrich, St. Louis, MO, USA). The spheroid formation process follows the established protocols from Microtissues® (detailed protocols available at <https://www.microtissues.com/protocols>). To prepare the agarose-based petri dishes, we first created a 1% agarose solution by combining agarose with water at a 1:100 (w/w) ratio, followed by heating and vortexing until complete dissolution. We then added 500 µL of the liquefied 1% agarose solution to each micro-mold placed on wet ice for cooling and solidification. Using sterile spoons, we transferred the solidified 3D petri dishes to 6-well plates, with one dish per well. For spheroid formation targeting a nominal diameter of 400 µm, we prepared a concentrated cell suspension of 648,000 cells in 190 µL media. This suspension was carefully pipetted into each 3D petri dish (#12–81) using a 1000 µL pipette. Following seeding, the plates were incubated in a 37°C humidified incubator for 20 minutes to allow initial cell settling. Finally, we slowly added cell culture media to provide nutrients for spheroid growth. Medium was exchanged by half every 2 days during cell culture. The growth duration required for MCTS to reach 600–800 µm in diameter depends on the initial seeding density. Using MicroTissues® 3D Petri Dish micro-molds (#12–81), PANC1 cells seeded at 8,000 cells/well (or 648,000 cells per dish) typically achieve this target diameter after 7 days of culture. Lower initial seeding densities require extended culture periods to reach equivalent spheroid dimensions, while higher seeding densities reach the target size more rapidly. More details about how to prepare the 3D Petri Dish and how to seed cells can be found in the protocol of the product.

Preparation of frozen sections of MCTS.

Gelatin solution (50 mg/mL or 5%) in a 50 mL tube was prepared in HPLC grade water, vortexed vigorously, and placed in a water bath at 60 °C for dissolving. Gelatin solution was cooled down before use. Cell culture medium was removed and MCTS together with 3D petri dishes were washed with PBS 3 times. MCTS carried by 3D petri dish were carefully placed onto plastic disposable base molds and gelatin solution were added into the molds to cover the 3D petri dish. Then, MCTS covered by gelatin solution were placed in a – 20 °C freezer immediately. MCTS were incubated at –20 °C for at least 3 hours before cryosection or stored at – 80 °C for future use. Carboxymethylcellulose (CMC, EMD Millipore Corp., Burlington, MA, USA) embedded MCTS arrays were prepared as the procedures described above with a 2% (w/w) solution.

The procedures of the sectioning MCTS together with agarose-based petri dishes were provided below. The 2% CMC block on a cryostat support was sliced and marked with an

oil pen to prepare a surface paralleled with the moving path of the cutting blade (Fig. 1a). MCTS microarrays were placed on the base mold and covered by 5% gelatin solution and then frozen at -20°C (Fig. 1b). It is vital that the bottom of the microarray is parallel to the bottom of the base mold, guaranteeing simultaneous sectioning of MCTS in the same z-axis in subsequent procedures. The frozen sample was taken out of the mold and mounted on CMC block by adding water surrounding the sample and the tissue holder was placed back to the cryostat and secured with the oil pen markers aligned (Fig. 1c). When the frozen sample was mounted on CMC block, the sealed end was used for mounting. It is worth noting that when release cryostat chuck or put the chuck back on the cryostat stage, only loosen the chuck release knob, but do not adjust the orientation knob. Otherwise, the cutting surface of the CMC block may no longer be parallel with the moving path of the blade. Then, MCTS were cut into slices and transferred to regular glasses or ITO-coated glasses (Fig. 1d). It is important to note that the critical part of this procedure is the alignment of the array in the cryostat – a very careful alignment of z-axis is required for sectioning through same z-axis planes of the 81 spheroids. To optimize sectioning quality and enhance success rates, we recommend using new cryostat blades and ensuring the anti-roll plate is free from any damage or unevenness. The sections on the slides were quickly dried in a vacuum after sectioning prior to applying the MALDI matrix. The sections can also be stored at -80°C for further use. Matrix was sprayed on MCTS using TM-sprayer (Fig. 1e) before MALDI MSI analysis (Fig. 1f).

MALDI-MSI analysis.

Application of matrices were performed based on previous studies with some modifications (24, 25). In brief, DHB (40 mg/mL in 50% MeOH and 0.1% TFA) was deposited via the TM sprayer system using the following conditions: a nozzle temperature of 80°C , a gas pressure of 10 psi, 16 passes, a moving velocity of 1250 mm/min, a drying time of 30 s, and a flow rate of 0.1 mL/min.

MALDI-MSI experiments were performed on a timsTOF Flex mass spectrometer (Bruker Scientific, LLC, Bremen, Germany) coupled with a SmartBeam 3D 10 kHz frequency tripled Nd:YAG laser (355 nm). The laser settings used were 50 μm of diameter circular spot size, with 200 shots per pixel and a raster step size of 50 μm for MCTS section imaging. The laser power was set to 70%. The MSI data were collected over a mass range of m/z 400–1300 for positive ion detection mode. MSI images were analyzed using SCiLS Lab Pro (Bruker Scientific, LLC, Bremen, Germany) with data normalized to total ion count (TIC). After acquisition of MSI data, H&E staining was performed on MCTS sections. Lipids in spheroids were identified based on accurate mass matching from using a 50 μm diameter circular size laser spotting to match conventional lipidomic analysis of PANC-1 cell culture lipid extracts via high-performance liquid chromatography (HPLC)-ESI-MS/MS (26). Representative lipid identities were confirmed using MALDI-MS/MS with the similar instrumental parameters used to acquire the MSI except for parameters mentioned below: mass range was fixed at m/z 100–1350, energy ramping mobility values were set at 0.55–1.90 $\text{V}\cdot\text{s}/\text{cm}^2$, the laser energy was set at 60%, isolation windows were fixed for 1 Da, collision energy for MS/MS bb CID were set ranging from 30eV to 70eV to acquire desired MS2 spectra.

Results and Discussion

Optimization of sectioning method for MCTS.

We monitored MCTS formation through brightfield microscopy, which revealed progressive cell aggregation into well-defined three-dimensional structures within the microwells. The microscopy observations demonstrated consistent patterns of cellular organization characteristic of MCTS development, including compact structure formation and defined boundaries. Once the MCTS achieved their target dimensions, we proceeded with harvesting them for sectioning procedures. To section all the MCTS grown on agarose-based microarrays, sample embedding methods were optimized. We tried different potential embedding materials including water, CMC, and gelatin (6, 17, 27). During the initial testing, we tried ice, 2% CMC and 10% gelatin frozen blocks. We found that 2% CMC and 10% gelatin were section-able, while we were not able to get intact ice sections (Fig. 2a). The ice section tended to break, and it would be difficult to transfer all the MCTSs onto glass slides. Then we tried both CMC and gelatin for further optimization. We tried 1% CMC, 2% CMC, CMC and gelatin mixture (1% of each), 2% gelatin, 5% gelatin and 10% gelatin for embedding and sectioning. We found that it was possible to get intact MCTS sections using 1% CMC or 2% CMC but it often caused distortion of the sections. The mixture of CMC and gelatin (1% of each), and 2% gelatin worked better than 1% CMC or 2% CMC in terms of avoiding deformation, while 5% gelatin and 10% gelatin worked the best for assisting sectioning (Fig. 2). Gelatin solution of 10% was more susceptible to folding after sectioning when we tried to transfer the section to the slide. We conducted at least triplicate tests for each embedding condition, which revealed that 5% gelatin provided optimal results for obtaining intact sections and successful transfers to glass slides. Therefore, 5% gelatin was selected as the embedding media for MCTS experiments for MSI. Based on testing these conditions, it seems that the embedding media needed to have good frozen sectioning properties on its own, and then also have a similar enough deformation and stiffness to the 1% agarose microwells to be able to remain adhered during sectioning.

For tissue samples or MCTS embedding, we noticed that there were two slightly different ways in sample preparation from the literature. One method was that fresh samples were frozen and then embedded in embedding solution and frozen again (5). The other way was that fresh samples were immersed directly in embedding materials and then frozen sequentially (6). For the comparison between methods in preparing intact MCTS sections, we performed at least duplicate tests for each condition. We found that the microarray tended to deform when we froze the MCTS before embedding (Supplementary Fig. S1). This result suggests that embedding MCTS before freezing works better for maintaining MCTS shape during sectioning.

Preparation of MCTS sections with different sizes

We grew PANC-1 MCTSs in agarose-based microarray (Microtissues, #12–81) and the MCTSs were sliced when they reached a diameter between 600~800 μm (Fig. 3a). The sliced MCTSs were mounted on glass slides/ITO-coated glass slides. It can be difficult to locate individual MCTS embedded in gelatin because the color of frozen gelatin is

similar to the color of the frozen MCTS, making it difficult to see the position of the MCTSs. To mitigate this issue, it is possible to add a drop or two of food coloring into the gelatin to improve the contrast.(22) Despite this, when MCTSs are embedded in agarose, the agarose is still almost transparent when frozen, and therefore could be easily distinguished from the MCTSs. With this method, MCTSs could be easily located by eye after sectioning, especially after mounting the array onto the slide. The location of MCTSs could be further confirmed by H&E staining, or cresyl violet staining. We used H&E staining that demonstrated that MCTS sections obtained by our sectioning method maintained the morphology during sectioning following transfer to an ITO coated slide (Fig. 3b). We further applied this strategy to section smaller MCTSs (300 – 400 μm) and found that it also worked well as shown in microscope images for H&E staining (Fig. 3b) and scanned images for all the MCTSs (Fig. 3c). This demonstrates that within the agarose wells, MCTSs do not need to be maintained at the largest size to ensure good sectioning quality.

High throughput MALDI MS imaging of MCTS

With successful sectioning of the MCTS agarose arrays, we performed MALDI-MSI of MCTS sections at 50 μm spatial resolution in positive ionization mode. The results showed that we could observe abundant molecular signals from the MCTS samples across a mass range of m/z 400–1000 (Fig. 4a). The 50 μm spatial resolution was selected to balance analytical sensitivity, spatial resolution, and total acquisition time for high-throughput imaging of MCTS arrays. This resolution enables efficient analysis of molecular distributions across spheroids while maintaining sufficient pixel density (6–16 pixels per spheroid diameter) for reliable spatial mapping. However, the optimal spatial resolution should be adjusted based on specific experimental requirements and research objectives. For detailed subcellular analysis, higher spatial resolution may be necessary, while broader molecular screening studies might benefit from lower resolution to increase throughput. Researchers should consider factors such as MCTS size, desired molecular detail, acquisition time constraints, and biological questions being addressed when selecting imaging parameters. MSI images showcase high throughput visualization of metabolites across the MCTS arrays (Fig. 4d–p). The identities of lipids in MCTS sections were searched in LIPID MAPS with accurate mass matching within 10 ppm mass error tolerance and on tissue MALDI MS/MS spectra were acquired to confirm the identifications and structures of some lipids (Supplementary Fig. S2). In addition, the lipids identified shown in Supplementary Table S1 were further confirmed with LC-MS/MS analysis of the PANC1 cells according to our previous study (26). Furthermore, we tried smaller MCTS (size of 300–400 μm) for MSI, generating similar lipid distributions as the larger MCTS (Supplementary Fig. S3). Simultaneous analysis reduces the time of sample preparation significantly and can reduce batch effect impact in a dataset. Something to note is that regardless of the size of the MCTS mold, when sectioning through the MCTSs, there will be variations in the cross-sectional area of the MCTSs in different z-axis planes— especially because using the Microtissues[®] agarose mold, the agarose with gelatin can contort during the freezing process and all MCTS will not always form perfect spheres with the exact same size. Some cell types are more prone to a more sphere morphology with equal x, y, and z radii, while other cell types may form an oblate spheroid geometry with a shorter

z-radius. Additionally, the alignment of the entire array for achieving the same z-axis for all MCTSs in the array during cryosectioning is really challenging due to the manual nature of sectioning and aligning the chuck in the cryostat. This positional variation affects molecular analysis in several ways. First, the interpretation of molecular gradients depends heavily on the sectioning plane. A section through the MCTS center will show the full radial distribution of molecules from core to periphery, while sections closer to the poles may present compressed or incomplete molecular gradients. This becomes particularly important when studying drug penetration or analyzing spatial molecular organization. Therefore, it is very important during MCTS studies using the microarrays to capture scanned images of multiple sections of the arrays to understand the z-axis depth for each spheroid in the array selected for MSI. On the other hand, the z-axis position and uniformity of MCTS cross-sections can be systematically controlled and documented when sectioning is performed. For MCTSs (e.g., with a z-axis depth of 400 μm) sectioned at defined thickness (e.g., 16 μm): (1) The first section containing MCTS tissue is labeled as section #1; (2) The last section containing MCTS tissue would be section #25; (3) The center section (#13) represents the MCTS equatorial plane; (4) Section numbers relative to #13 indicate precise distance from the MCTS center. Through documenting section thickness and recording the section numbers for each section, we can roughly determine the z-axis positions of the MCTS section. Our analysis of MCTS heterogeneity/variability and MCTS coverage is shown in Supplementary Figures S4–S6, which uses unsupervised statistical analyses to examine the MCTS heterogeneity across the array. Through careful examination of both H&E staining and MALDI MSI data, we achieved a successful imaging rate of approximately 85% (69 out of 81 MCTSs) in our 9×9 array sections. While some MCTSs in the lower right corner showed inconsistent z-axis alignment due to deformation of the Microtissues mold after repeated use, this affected only a small portion of the array. In addition, our data from a representative 16×16 array (256 theoretical positions) in Supplementary Figure S3 showed successful imaging of approximately 220 MCTSs, representing an 86% success rate. While MCTS size can influence sectioning success rates, with larger MCTS generally being more amenable to sectioning, our optimized methodology achieved comparable yields across both array formats. The high yield of viable MCTS demonstrates that many MCTSs are sectionable at a single time, although there is room for further optimization to achieve even more consistent results across the entire array.

One advantage to sectioning MCTSs in an array format is having replicates of the same biological treatment available for MSI on the same slide, which gives researchers the opportunity to evaluate the variability and reproducibility in the dataset. In MSI datasets, there is an overwhelming abundance of ways to statistically evaluate the variability and heterogeneity of MCTS systems; however, how this is measured depends on the research question in the study and the specific endpoints of the related biological assays. Some unsupervised statistical algorithms, including principal component analysis (PCA), bisecting *k*-means, *k*-means, UMAP, and other algorithms help to capture information on the variability/heterogeneity that exists within a dataset, however the parameters that contribute to how the data is separated – including filtered lists, confirmed lipid identifications, number of iterations, will also play a significant role in the statistical separation of the spheroids in a high-throughput array. In the supplemental section (Supplementary Figures S4–S6),

we examine *k*-means, bisecting *k*-means, and PCA algorithms using all individual spectra (TIC normalized) with $n=5$ iteration shown for all analyses to capture information on the heterogeneity that exists within our section of the MCTS microarray. When looking at the entire array in all 3 analyses, the largest variation in the data is caused by if a MCTS is able to be sectioned at a specific (x,y) coordinate in the microarray grid (the ability to distinguish MCTS tissue vs. background gelatin and matrix signal, noting where MCTS are missing from the array). Beyond that, it appears that possibly the z-axis alignment in the array during sectioning or deformation in the agarose during freezing is contributing to the MCTS around the outside of the array (the edges of the array) being most similar in the *k*-means and bisecting *k*-means data. Therefore, any steps to mitigate the influence of these freezing and z-axis sectioning alignment artefacts will play an important factor in evaluating heterogeneity within a single section. For sectioning embedded MCTS in a microarray, it is extremely important to perform careful documentation of the z-axis plane of the MCTS for the 3-dimensional shape during sectioning, by noting when the MCTS first are sectioned. This will put the context of the MCTS depth of each MCTS when examining the MCTS heterogeneity. Within a single MCTS, it is important to note that the natural biological variability is present, which is the existence of an outer proliferating region, a middle quiescent region, and a necrotic core that forms at distances $>200\mu\text{m}$ from the available oxygen in the MCTS (28). The bisecting *k*-means and a *k*-means algorithms of an individual MCTS reveal these different regions of an individual MCTS in the array (Supplementary Fig. S4c and Supplementary Fig. S5c), however these biological regions present in the single MCTS are not shown as being responsible for capturing the higher percentage of the variation shown in the entire array, or even as significant variation just in the top outer row of the spheroid array. While these unsupervised analyses can show MCTS variation in the microarray section, our method's ability to efficiently analyze larger numbers of biological MCTS enables more comprehensive studies of MCTS tissue heterogeneity and reproducibility. The high-throughput analysis provides important advantages for pharmaceutical testing, including increased statistical power for evaluating drug responses across multiple MCTS, rapid assessment of treatment effects through parallel analysis. This capability is particularly valuable for drug development workflows, where understanding both biological variability and treatment effects across many samples is critical. As the field of 3D cell culture continues to advance in pharmaceutical research, methods that enable efficient, reproducible analysis of multiple spheroids will become increasingly important.

Sample preparation for sectioning many MCTS or organoids together requires multiple steps, making the process laborious for implementation. As shown in a previous study, organoids grown in Matrigel were recovered by centrifugation and then transferred via pipette to sectionable gelatin microwells (5). These steps could damage 3D cell culture structure, depending on the MCTS cell line, or could impact more fragile organoid structures, such as budding or hollow organoids, during transfer. To mitigate this, a wide bore pipette is recommended or coating the pipette tip with 0.1% bovine serum albumin to minimize interactions or binding to the pipette tip, however depending on the MCTS/organoid sample, it remains an on-going issue for sample preparation. Additionally, it can be difficult to fill all the wells in microwells in a gelatin mold with MCTSs, or on the

other hand, it is possible to get multiple MCTSs stacked in a single well, thus not fully utilizing all the microwells in the arrays and limiting the throughput of the technology. Because MCTSs must be transferred into the microwell array, it can be difficult to match any variability displayed with live microscopy to chemical variability observed with MSI. Because the array format is conserved during the transfer, any live cell imaging, such as confocal microscopy during MCTSs growth done in the agarose array, can then be correlated with the MSI based on the position of the MCTSs in the array.

In this study, we grew MCTSs in agarose-based microwells directly, and MCTSs could be embedded in embedding media without centrifugation or manual transferring of MCTSs, keeping them undisturbed while largely reducing the steps involved in the sample preparation procedures. Furthermore, to section MCTSs all together, we cut CMC blocks to generate a surface that is parallel with the path of the blade for sectioning. This step would provide a surface that allowed all the MCTS to be sectioned simultaneously as much as possible. Through careful optimization of the embedding media and procedure, we achieved a success rate of 80% or higher in the sectioning step for the microarray. From these sections, we selected those containing the most MCTS with similar sizes and successfully completed at least duplicate runs of both H&E staining and MALDI MSI for MCTSs from two types of Microtissues[®] molds. We have also noted that while sectioning and transferring presented the main technical challenges, once intact sections were obtained, we achieved high success rates with both MALDI MSI and H&E staining. It is worth mentioning that, thus far, this high throughput MSI of MCTSs strategy has only been demonstrated for MCTSs grown on agarose microarrays. For MCTS formation, agarose acts as a non-adhesive surface for cells; by seeding cells in small agarose microwells, the cells will adhere to each other, instead of the microwell, to form the MCTS. Following culture, the entire agarose microwell array can be directly embedded in optimized gelatin solution for cryosectioning. The method utilizes readily available materials and commercially available Microtissues[®] molds that accommodate various MCTSs sizes and geometries (rods, honeycombs, toroids), making it easily adaptable for labs without specialized microfluidic fabrication expertise. This approach significantly impacts the broader 3D cell culture community by demonstrating that a cell culture-compatible non-adhesive material can be directly integrated with MSI analysis. Additionally, incorporating advanced microfabrication techniques could enable generation of diverse 3D architectures optimized for specific biological applications.

However, this approach also has its limitations -- certain MCTS/organoids are required to grow in Matrigel or other specific materials, therefore, transferring of MCTS/organoids from these materials to microwells would be inevitable. While it may be possible to seed MCTS in Matrigel within agarose wells, this application requires further investigation beyond the current study. Additionally, incorporating advanced microfabrication techniques, could enable generation of diverse 3D architectures optimized for specific biological applications (29, 30). This transfer method can further expand the application of this strategy in 3D cell culture sample preparation and high-throughput MSI. With increasing studies supporting that MCTS model shows great potential in anti-cancer treatment for precision medicine (31, 32), the method presented here shows significant promise to advance the field of cancer

research and cell development research, through the implementation of an adaptable, and throughput- and user-friendly approach.

Conclusion

In summary, we developed a method enabling high-throughput analysis of MCTSs grown in agarose microarray using mass spectrometry imaging without having to transfer the MCTSs. With optimized embedding materials for the microarray, our method enabled sectioning of large quantities of MCTS simultaneously and maintained the visibility of small MCTS by eye during cryosectioning. Increasing the number of biological replicates in MALDI MS-based analysis may help increase the accuracy and precision of the results in molecular profiling of MCTS because in different MCTS models, different cellular compositions or cell structures can result in spatial heterogeneity within an individual MCTS. By not having to transfer the MCTS with a pipette or other measures, and directly embedding an agarose mold in gelatin, it is easier to preserve a MCTS microarray or other complex geometries that could be possibly made from scaffolding materials in microfluidic fabrications. Our method ultimately reduces the sample preparation time and steps and offers an attractive alternative for sample preparation enabling high-throughput MSI. Beyond MCTSs, this approach holds potential for preparing frozen tissue sections of other biological specimens, such as organoids, embryos, or other small tissues. Building on this foundation, future studies will explore more in-depth applications, such as molecular profiling and drug distribution analyses, to fully leverage the capabilities of this method in understanding tumor biology and therapeutic responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability:

MALDI MSI data generated in this study have been deposited in Zenodo under accession code <https://doi.org/10.5281/zenodo.15028243>. The intermediate pre-processing results of the MSI study are available upon request from the corresponding author L.L.

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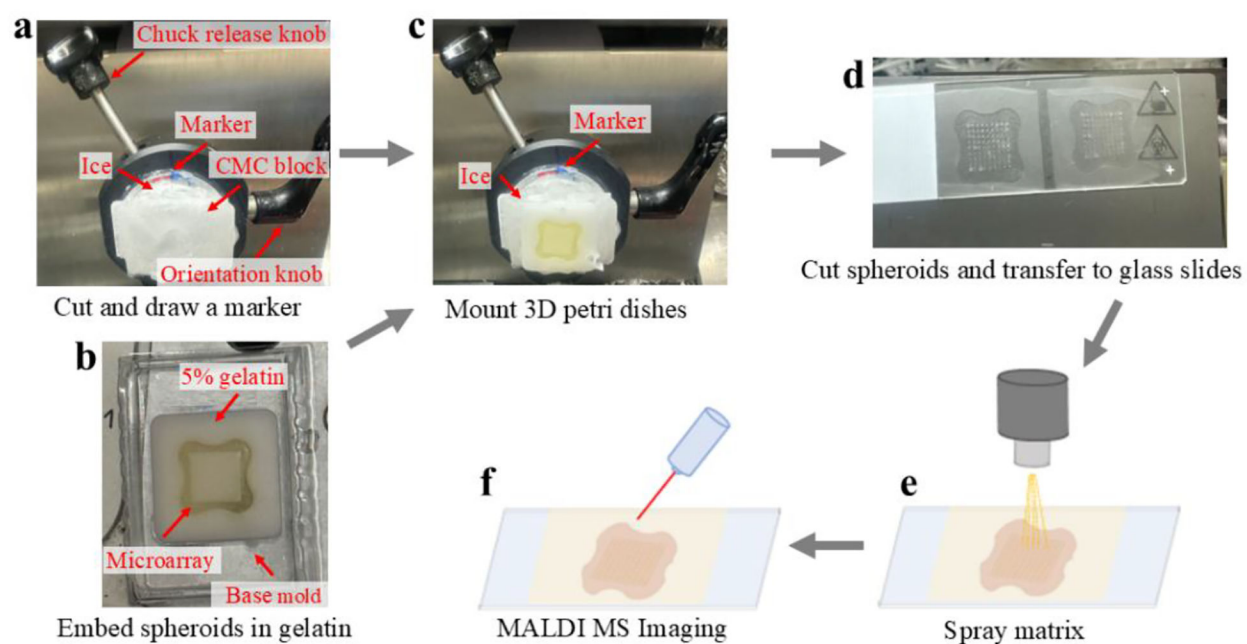


Fig. 1. Workflow of the developed method for high-throughput MSI analysis of MCTS: (a) CMC block was cut, and position marked; (b) MCTSs with agarose-based dish embedded in gelatin solution; (c) MCTSs were transferred on CMC block; (d) MCTSs were sectioned and transferred to glass slides; (e) Matrix application; (f) MALDI MSI analysis.

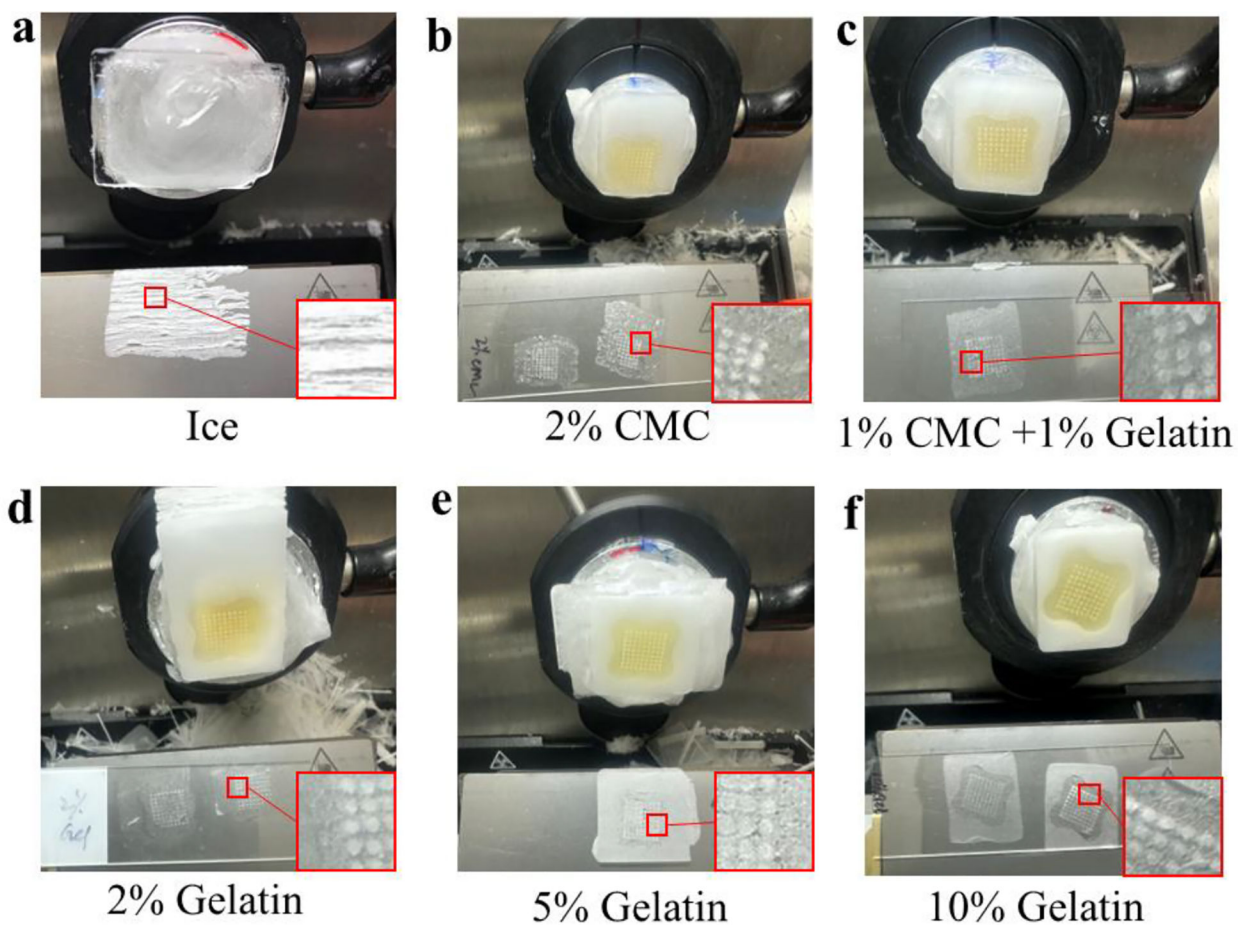
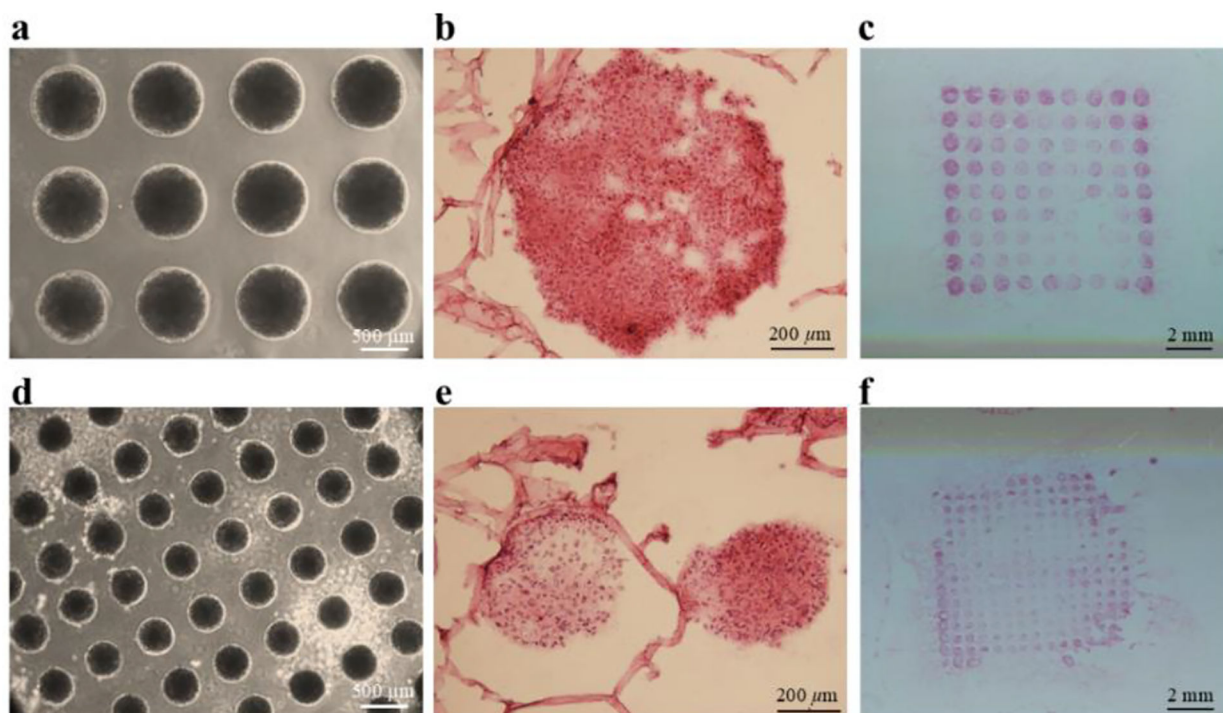


Fig. 2.

Evaluation of different embedding materials for sectioning. (a) Ice; (b) 2% CMC; (c) 1% CMC + 1% gelatin; (d) 2% gelatin; (e) 5% gelatin; (f) 10% gelatin. An enlarged area of the cross-section on the slide is shown by red outlined square for each panel highlighting the changes in the shape of the cross-section due to differences in the embedding method.

**Fig. 3.**

Preparation of MCTS sections with different sizes. (a, d) Microscope images of MCTSs cultured in medium, showing larger MCTSs (600–800 μm diameter) in 9×9 array (a) and smaller MCTSs (300–400 μm diameter) in 16×16 array (d). (b, e) Microscope images of representative spheroid sections after H&E staining, demonstrating preserved tissue morphology for both larger (b) and smaller (e) MCTSs. (c, f) Scans of complete MCTS array sections on glass slides after H&E staining, showing successful high-throughput sectioning achieved for both larger (c) and smaller (f) spheroid arrays. Scale bars: 500 μm (a, d), 200 μm (b, e), and 2 mm (c, f).

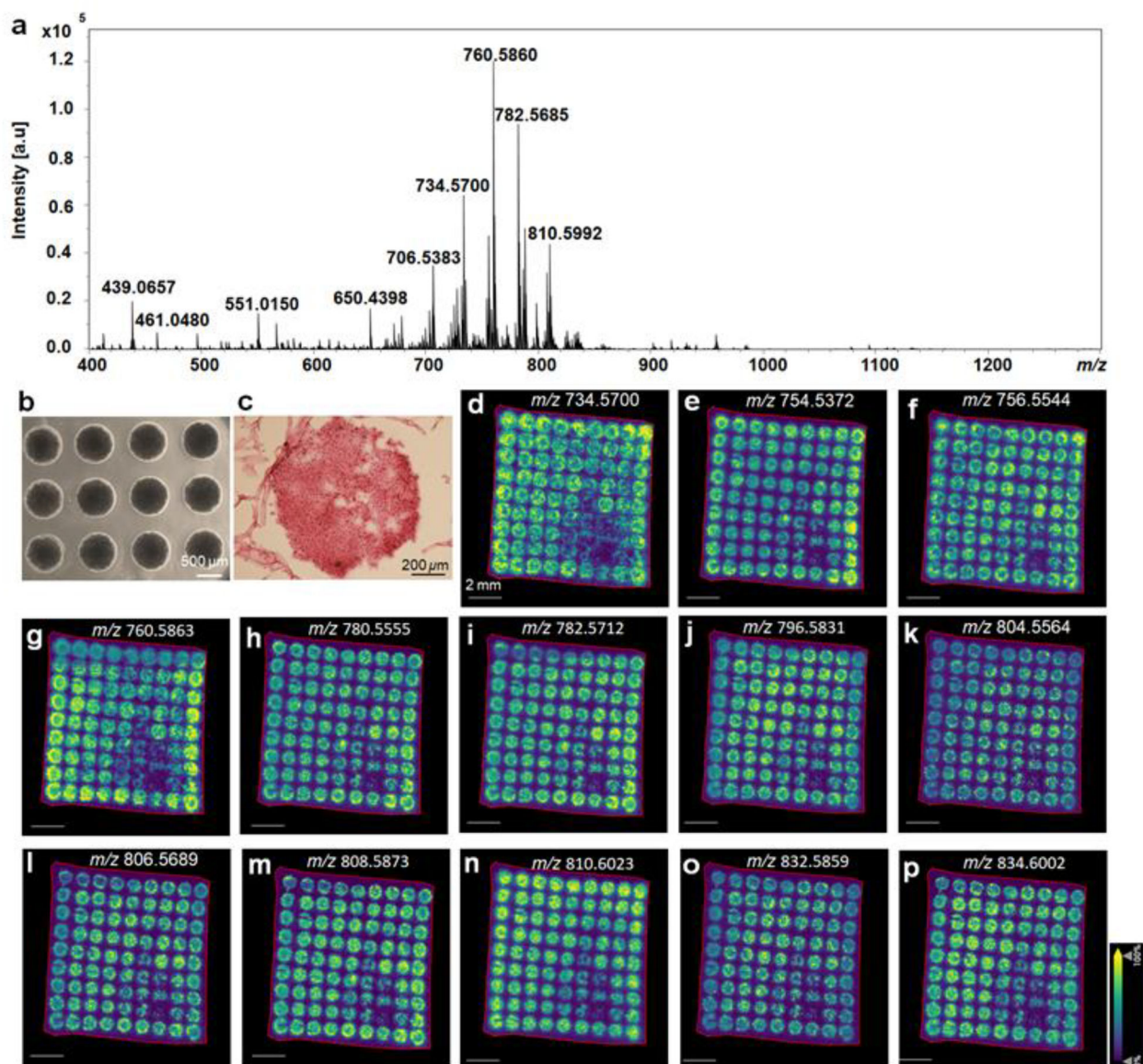


Fig. 4. MS imaging of MCTSs.

(a) Mass spectra acquired from PANC-1 spheroids; (b) optical images of PANC1 spheroids cultured in 3D petri dishes; (c) optical images of PANC-1 spheroid after H&E staining; MS images of representative lipid species detected from PANC-1 spheroids. (d) PC (32:0) ($[M + H]^+$, m/z 734.5694), (e) PC (34:4) ($[M + H]^+$, m/z 754.5372), (f) PC (34:3) ($[M + H]^+$, m/z 756.5544), (g) PC (34:1) ($[M + H]^+$, m/z 760.5863), (h) PC (36:5) ($[M + H]^+$, m/z 780.5555), (i) PC (36:4) ($[M + H]^+$, m/z 782.5712), (j) PC(22:4/15:0) ($[M + H]^+$, m/z 796.5831), (k) PC (38:7) ($[M + H]^+$, m/z 804.5564), (l) PC (38:6) ($[M + H]^+$, m/z 806.5689), (m) PC (38:5) ($[M + H]^+$, m/z 808.5873), (n) PC (38:4) ($[M + H]^+$, m/z 810.6023), (o) PC (40:7) ($[M + H]^+$, m/z 832.5859), (p) PC (40:6) ($[M + H]^+$, m/z 834.6002). All MCTS MSI images were obtained with mass error tolerance of 10 ppm; scale bar, 2 mm.