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Preparing ductal epithelial organoids for high-spatial-resolution molecular profiling using mass spectrometry imaging

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Organoid culture systems are self-renewing, three-dimensional (3D) models derived from pluripotent stem cells, adult derived stem cells or cancer cells that recapitulate key molecular and structural characteristics of their tissue of origin. They generally form into hollow structures with apical–basolateral polarization. Mass spectrometry imaging (MSI) is a powerful analytical method for detecting a wide variety of molecules in a single experiment while retaining their spatiotemporal distribution. Here we describe a protocol for preparing organoids for MSI that (1) preserves the 3D morphological structure of hollow organoids, (2) retains the spatiotemporal distribution of a vast array of molecules (3) and enables accurate molecular identification based on tandem mass spectrometry. The protocol specifically focuses on the collection and embedding of the organoids in gelatin, and gives recommendations for MSI-specific sample preparation, data acquisition and molecular identification by tandem mass spectrometry. This method is applicable to a wide range of organoids from different origins, and takes 1 d from organoid collection to MSI data acquisition.

Introduction

Since the discovery by the Clevers lab that Lgr5-positive adult stem cells have self-organizing capacity in vitro[1], organoid models derived from adult stem cells of various organs have been established, including organoids from colon[2], pancreas[3,4], airway[5] and kidney[6] tissue. These models have been shown to mimic the in vivo situation more accurately compared with commonly used monolayer cultures, presumably because they feature a complex 3D environment provided by basement membrane proteins that promotes the development of multiple differentiated cell types[7,8]. Organoids can be established with high efficiency using patient-derived cells and show high concordance with the patient sample they are derived from in terms of morphology and molecular composition[9]. As such, they are valuable model systems that are increasingly used to study biological processes in vitro[5,6]. Many of these organoid models form hollow structures of 20–600 μm with an organ-specific epithelial lining. Retaining the molecular composition and morphology of organoids in experimental procedures for imaging purposes is challenging and requires dedicated sample handling and preparation protocols.

Mass spectrometry imaging (MSI) is a powerful method to detect a wide variety of molecules in a single experiment while retaining their spatiotemporal distribution. Recent developments within the MSI field have resulted in a better spatial resolution that enables single-cell analysis[10]. Together with its high sensitivity, label-free detection methodology and high-throughput analysis compatibility, this makes MSI a useful analytical tool to study biological processes in organoids. Here, we describe a protocol that combines the precision medicine potential of patient-derived organoids[11] with the analytical strengths of MSI. This combination opens up new opportunities for assessing molecular alterations in cancer that can be used to identify novel biomarkers and for developing novel therapeutic strategies.

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MSI has been applied before by our group and others[12–15] to study solid 3D cell culture systems, such as spheroids, and detailed protocols are described elsewhere[16]. These solid 3D models are distinctly different from organoids, because they are larger (>1 mm versus <600 µm), visible by eye, and solid instead of hollow. The protocol that we describe here specifically focuses on the sample preparation and molecular identification of hollow, 3D in vitro models smaller than 600 µm. These include organoid models of salivary gland, taste buds, stomach, colon, esophagus, airways, pancreas, liver, breast, fallopian tube, endometrium, ovary, prostate and kidney. Imaging these organoids is more challenging compared with spheroids because they are not visible to the naked eye and are very fragile. Their visibility is an issue throughout the sample preparation process but also while selecting the region of interest for analysis. Recently, Johnson et al. described an elegant way to organize organoids in an array prior to MSI to assist in selection of the imaging area[17]. In this method, organoids are centrifuged into a gelatin microarray with holes of 200 µm or 800 µm, resulting in a standard alignment of the organoids. The disadvantage of this approach, however, is that smaller organoids may cluster and appear as one organoid, which compromises spatial information. Our method results in a random distribution of the organoids and has the advantage that they are isolated and retain their shape and hollow morphology. This aids in proper histological evaluation of intact organoids and enables accurate co-localization of morphological features with molecular information.

For biological interpretation of MSI data, the identity of the detected masses needs to be determined using tandem mass spectrometry (MS^2) experiments. This is often performed manually through the use of a target list during tissue surface profiling or by analysis of tissue extracts with electrospray-based MS. These methods have the disadvantage that they are laborious, need molecule-specific extraction protocols or require that the sample is visible while positioned inside the mass spectrometer. Recently, Ellis et al. published a method to perform automated matrix-assisted laser desorption/ionization (MALDI)-MS^2 on every detected nominal mass to charge (m/z) value in imaging mode[18]. This method does not require a dedicated extraction protocol and is fully automated. It does, however, require that the spectra from consecutive pixels contain similar spectra. Isolated organoids are too small to achieve this. Therefore, we suggest to prepare a dedicated MS^2 sample containing a dense distribution. Additionally, to ensure that the molecular variation of different biological conditions is represented in this sample, we suggest to combine organoids from all experimental conditions. Steps describing how to generate an organoid sample that is suitable for data-dependent acquisition (DDA) image acquisition, in parallel to the imaging experiment for identification at the single-cell level, are included in the Procedure.

Overview of the protocol

The workflow of the protocol has six major parts: (1) organoid collection, (2) embedding, (3) MSI sample preparation and data acquisition, (4) MS^2 sample generation, (5) (automated) molecular identification and (6) histological staining. These parts result in three products: an m/z distribution image, a morphological image and a list of m/z identities, as outlined in Fig. 1. These data can be merged, and through dedicated data analysis, novel biomedical knowledge can be acquired. The novelty of this protocol lies in the embedding of the organoids and the generation of the MS^2 sample for molecular identification; hence, these steps are described in detail. The protocol describes these parts such that they can be applied to all the above-mentioned organoid models. For each step, a specific explanation for pancreatic cancer organoids is added as an example of a specific application. Generation and cultivation of the organoids as well as the MSI sample preparation and data acquisition are assumed to be of expertise and therefore only mentioned briefly.

Applications and limitations

Instrumentation

Recent technological and methodological advancements have improved the spatial resolution, mass accuracy, detection limit, analysis speed and data management of MSI. Nevertheless, no single MSI instrument performs best on all these parameters, so a compromise has to be made. The example dataset in this paper demonstrates this by using different instruments for high-mass-accuracy data acquisition and high-spatial-resolution imaging. The exact limits of these parameters are instrument dependent, and a comprehensive overview of all possibilities goes beyond the scope of this paper. For more detailed information on this matter, the reader may consult the excellent reviews by Chughtai and Heeren, Liu and Hummon, and Porta Siegel et al.[19–21]. We highlight the capabilities and

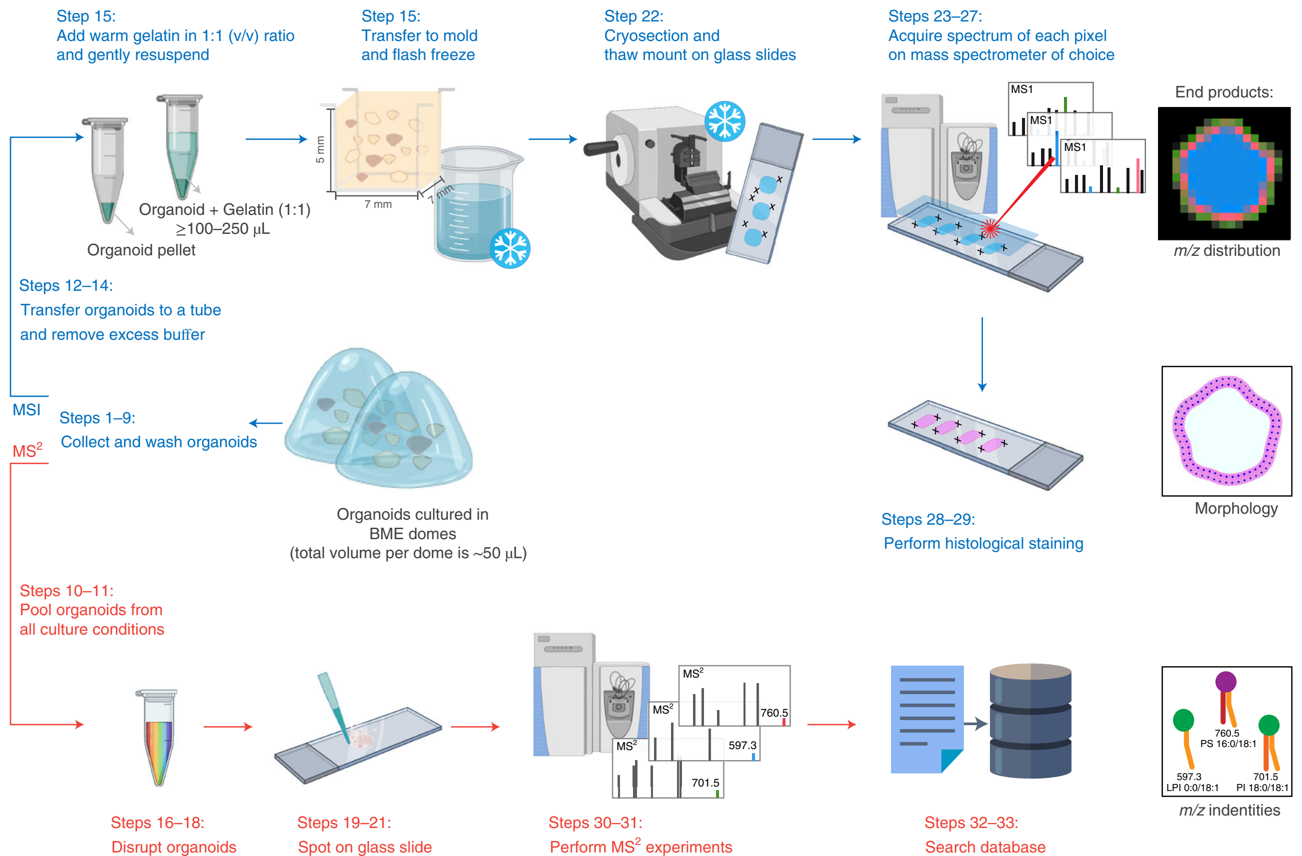

Fig. 1 | Organoid collection and preparation for MSI and, in parallel, molecular identification (MS^2). The protocol has six major parts: (1) organoid collection (Steps 1–11), (2) embedding (Steps 12–15), (3) MSI sample preparation and data acquisition (Steps 22–27), (4) MS^2 sample generation (Steps 16–21), (5) molecular identification (Steps 30–33) and (6) histological staining (Steps 28–29). These parts result in three products: a *m/z* distribution map, a morphological image and a list of *m/z* identities.

limitations of the currently most commonly used types of MSI instruments for biomedical research to give an impression of the possibilities of MSI analysis on organoids.

Mass spectrometers consist of three essential parts: an ionization source, a mass analyzer and an ion detector. Since the spatial resolution is mainly determined by the ionization source, and the most commonly used technique is MALDI, we designed this protocol using MALDI. However, this protocol is not limited to MALDI-MSI experiments. Because of our experience with other common ionization methods, including desorption electrospray ionization, laser ablation electrospray ionization and secondary ion mass spectrometry on sections of similar samples, such as gelatin-embedded spheroids, we are confident that this protocol can also be used to prepare organoids for these ionization methods. Commercial MALDI sources can achieve spatial resolutions from 200 µm to 5 µm, and customized instruments have demonstrated imaging resolutions as fine as 1 µm (ref. [22]). This is one order of magnitude lower than microscopy-based approaches, such as (immuno)histochemistry, which have, under optimal conditions, a resolving power of 200 nm. In the 'Anticipated results', we illustrate the implication of spatial resolution for the morphological features that can be distinguished within a section of embedded organoids.

The ionization method also influences the size and type of molecules that can be detected. Small molecules with a mass <2,000 Da, such as drugs, metabolites, lipids and peptides, ionize well using MALDI. This protocol was designed to detect lipids with MALDI-MSI, but can easily be tailored to detect other small molecules by changing the MALDI matrix. To detect large molecules with MSI, such as glycans and proteins, dedicated sample preparation protocols have already been developed[23,24].

In an MSI experiment, the *m/z* distribution image shows the location and relative intensities of different ions. Furthermore, it enables the first level of molecular identification based on the molecular mass of an analyte, calculated from the *m/z* ratio of the corresponding ion. The accuracy of

the m/z and the ability to distinguish two peaks of slightly different m/z's, defined as the mass resolving power, are mainly determined by the type of mass analyzer employed[25]. The highest available mass resolving power and mass accuracy is acquired from Fourier transform ion cyclotron resonance and Orbitrap mass spectrometry; these instruments can achieve mass accuracies of better than 1 ppm[26]. Another commonly used mass analyzer is the time-of-flight (ToF) mass analyzer[26]. Commercial ToF mass analyzers have a mass accuracy of 5–50 ppm and a resolving power that is about one order of magnitude lower than that of an Orbitrap[27]. The benefit of having a high resolving power is that ions with the same nominal mass, such as m/z 782.3829 and m/z 782.4093, can be clearly distinguished and accurately annotated (see 'Anticipated results'), while in a ToF-based instrument these would have appeared as one peak and identification would not be possible on the m/z alone. Therefore, a second level of molecular identification is necessary. Ion fragmentation experiments (often called 'tandem MS', MS^2 or 'MS/MS' experiments) provide information about the molecular structure of an analyte and, as a second level of molecular identification, confirm the molecular identity when the m/z alone is insufficient. Even though the addition of MS^2 improves the number of m/z values that can be assigned to distinct molecular species, identifying all detected m/z's is currently a limitation of MSI. In the 'Anticipated results', we show that, by combining mass accuracy with MS^2, 150 of the 268 m/z's that were detected could be identified.

Basement membrane extract

Organoids are typically cultured in basement membrane extract (BME), a gel-like substance isolated from mouse Engelbreth–Holm–Swarm sarcomas. BME consists of extracellular matrix proteins such as collagen IV, laminin and fibronectin. While cell isolation protocols and culture conditions vary between organoids from different tissue origins, establishing the organoids is similar. As an example, for pancreatic (cancer) organoids, pancreatic cells are isolated from human pancreas tissue through enzymatic digestion and suspended in BME. Droplets of up to 50 µL are deposited on a cell culture plate, left to solidify at 24–37 °C and overlaid with organoid-specific culture medium. BME is vital for the establishment and subsequent expansion of organoid models as it orchestrates tissue morphogenesis and influences gene expression and cell differentiation[7]. However, the properties of BME prohibit proper sample preparation for MSI, and therefore organoids need to be extracted from BME. Nonetheless, a critical step in preserving organoid morphology is to keep a thin layer of BME around the organoids as this provides a scaffold, and thereby functions as an exogenous exoskeleton. It has been suggested that BME signal interferes with the organoid signal in MSI experiments, though in the 'Anticipated results', we clearly show that the signal from BME can easily be distinguished from the organoid-specific signal after co-registering the MSI with the hematoxylin and eosin (H&E) image. When interested in the molecular composition of the outer surface of the organoid, this thin layer of BME can be removed using cell recovery solution, although, in our experience, this makes the organoids more fragile and may disrupt their morphology. Alternatively, the signal from the BME can be subtracted from the data, revealing tissue-specific molecules.

Materials

Organoids

We decribe a general protocol applicable to organoids cultured in BME with a hollow lumen and thin epithelial outer layer, with additional specific explanation and tips for pancreatic ductal adenocarcinoma (PDAC) organoids. PDAC organoids were generated, cultured and characterized as described previously by our group[4,28].

The sample preparation steps in the Procedure are optimized for the molecular identification of hollow, 3D in vitro models smaller than 600 µm. These include organoid models of salivary gland, taste buds, stomach, colon, esophagus, airways, pancreas, liver, breast, fallopian tube, endometrium, ovary, prostate and kidney. All of these models are cultured in BME and have a hollow lumen and a thin epithelial outer layer. We have shown example results for PDAC organoids, and the precise details for their preparation can be found in Supplementary Method 1.

This protocol can also be used for other 3D in vitro models of similar size that are not cultured in BME. For processing such models, start the protocol at Step 3 **! CAUTION** Consult ethics review board regulations and obtain informed consent from all subjects before collecting and processing human tissues.

Reagents

▲ **CRITICAL** Precool 1× Dulbecco's phosphate-buffered saline (DPBS), cell recovery solution (optional) and ammonium bicarbonate (ABC) to 4 °C using ice. They need to be ice-cold in every wash step.

- 1× DPBS without $CaCl_2$ and $MgCl_2$ (Life Technologies, cat. no. 14190-144)
- 50 mM ABC (NH_4CO_3, Sigma-Aldrich, cat. no. A6141)
- Liquid isopentane (Merck, cat. no. 1060561000)
- Gelatin from porcine skin (Sigma-Aldrich, cat. no. G1890)
- Fetal bovine serum (FBS; Greiner Bio One, cat. no. 758093)
- Klinipath Marker Plus (Klinipath, VWR, cat. no. KLIN98307-R) ▲ **CRITICAL** This marker is used as fiducial marker to assist in making an accurate overlay of the MSI and histological image. This specific marker is preferred over others because it can be detected in positive and negative ion mode, and is ethanol and xylene resistant.
- H&E or other dyes for post-MSI staining

Optional

- Cell recovery solution (Corning, cat. no. 354253)
- Aqueous dye (e.g., cresyl violet)
- MALDI matrix

Equipment

▲ **CRITICAL** To prevent organoids from sticking to plastics, use low-binding plastics and coat all utilities that come in contact with organoids with 50% (vol/vol) FBS/PBS (adapted from ref. [29]).

- Container with dry ice
- Container with ice
- Fume hood
- Scissors
- Calibrated pipettes
- Microscope with camera
- Precooled centrifuge (4 °C)
- Heating plate or vacuum desiccator
- Cryotome
- Mass spectrometer equipped with MSI source
- Magnetic hot plate stirrer
- 1 mL pipette filter tips (Corning, cat. no. 4809)
- 15 mL Falcon tubes (Cellstar, cat. no. 188271)
- 5 mL protein low-binding tubes (Eppendorf, cat. no. 0030122356)
- 1.5 mL protein low-binding tubes (Eppendorf, cat. no. 0030120086)
- 200 μL pipette tips (Greiner Bio-One, cat. no. 739291)
- Aluminum foil
- Glass beaker
- Forceps
- Tin cups (Brand, cat. no. 747720)
- Plastic embedding molds (7 × 7 × 5 mm) (Thermo Scientific Shandon, cat. no. 12503557) (alternative mold: F-bottom 96-well plate, Ø ~6.4 μm per well, surface area 32 mm^2, exact dimensions may vary per vendor)
- MSI-compatible glass slides, e.g., indium-tin-oxide-coated glass slides (Bruker, cat. no. 237001)

Optional

- MALDI-matrix application device

Reagent setup

- Prepare 15% gelatin in MilliQ by continuous stirring and heating (70–80 °C). Keep at 37 °C for direct use, store at 4 °C for 1 week or store at −20 °C for up to 6 months.
- Cut the tips of 200 μL pipette tips using scissors; one per sample, plus a few spares. See Fig. 2 for the approximate location of the cut.
- Prepare 50% (vol/vol) FBS/PBS by diluting one part FBS with one part DPBS (store at 4 °C for up to 4 weeks)

- In a fume hood, place a glass beaker with liquid isopentane on dry ice and cover with aluminum foil (Fig. 3) **! CAUTION** Dry ice can cause skin burns; wear protective garments. **! CAUTION** Exposure to isopentane can irritate the eyes, nose and throat and can cause headache, nausea, weakness, dizziness, sleepiness, loss of coordination and loss of consciousness; repeated or prolonged contact with isopentane can cause drying and cracking of the skin; isopentane is a highly flammable liquid and a dangerous fire hazard.

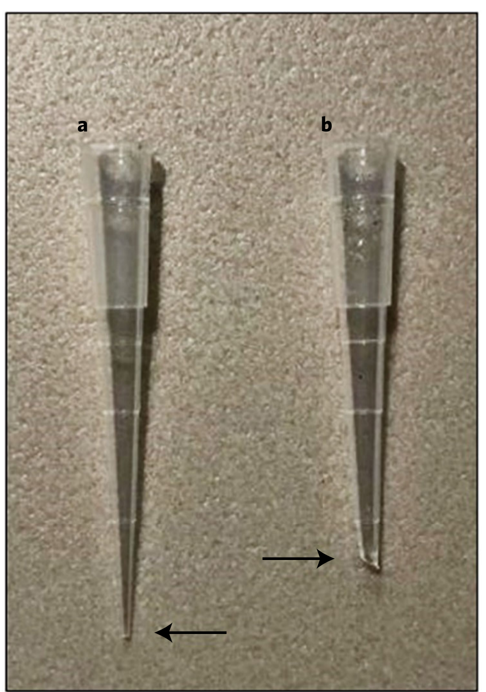

Fig. 2 | Cutoff pipette tips. **a**, Normal 200 μL pipette tip with a narrow opening at the end of the tip. **b**, Cutoff 200 μL pipette tip (established by cutting the end of the tip) with a wider opening. These cutoff tips are used for pipetting the organoid-gelatin suspension and limit shearing off the organoids and ensure that their morphology is maintained.

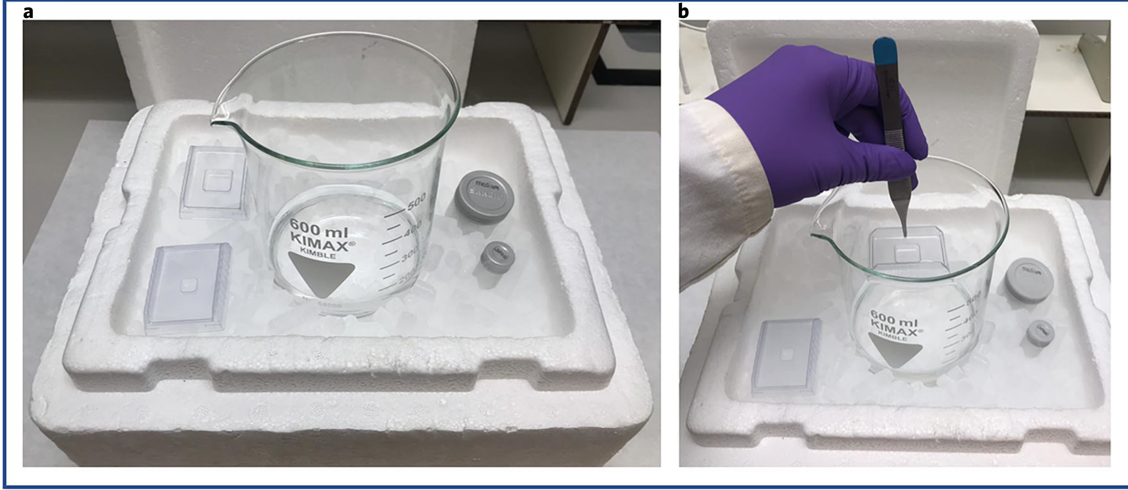

Fig. 3 | Freezing of gelatin-embedded organoids. **a**, Setup for rapidly freezing gelatin-embedded organoids. **b**, After embedding the organoids in warm gelatin, the mold with the organoid--gelatin suspension is rapidly frozen by submerging it into cold isopentane. When completely frozen, evident by a change in color to solid white, the organoid-gelatin block can be taken out of the mold and stored in aluminum foil or tin cups.

Procedure

Organoid collection and embedding ● Timing 90 min to 3 h

▲CRITICAL For acquiring sections that contain isolated, intact organoids with a maximized ratio of on-tissue and off-tissue pixels, it is critical that organoids are embedded at a fixed 1:1 ratio of organoids to gelatin (vol/vol). This ratio was empirically determined and was selected because it allows for gentle mixing with minimal shearing of the organoids. To get a well-shaped block of embedded organoids, the mold (Thermo Scientific Shandon) should be filled with at least 100 µL of organoid-gelatin mix. This means that the **minimum necessary pelleted volume of organoids is 50 µL**. How many organoids this volume contains depends on their size. In the case of PDAC organoids, we use 100–150 µL of confluent BME domes to make one sample. This is equivalent to 2 confluent wells of organoids in a 24-well plate. If the volume of washed organoids is <50 µL, use a smaller mold, such as a well of an F-bottom 384-well plate.

▲CRITICAL For Steps 1–13, the organoid suspension has to be kept cold at all times. Place the samples on ice during waiting steps.

Collect the organoids

1 Aspirate the organoid culture medium, and add 1 mL cold (4 °C) 1× DPBS (1 mL per well of a 24-well plate).
2 To collect the organoids, first disrupt the BME domes by pipetting up and down followed by transferring the suspension to a 5 mL precoated Eppendorf tube.
▲CRITICAL STEP Do not scrape on the bottom of the well as this may damage the organoids.
3 To ensure no organoids are left in the culture dish, wash the well a second time with 1 mL 1× DPBS.
4 (Optional) Wash the organoids in cell recovery solution according to the manufacturer's instructions. This may be useful when research focuses on the molecular composition of the basal side of the organoid and an excess of BME is expected to interfere with either the MSI or morphological image. Please be aware that the composition of cell recovery solution is unknown and may affect the cell membrane and adhesive molecules. Additionally, washing with cell recovery solution makes the organoids more fragile, increasing the chance of disrupting their morphology during sample handling.
5 Finally, fill the Eppendorf tube with 1× DPBS to a final volume of 4 mL.
6 To remove excess BME, incubate on ice for 20–30 min (invert every 5 min) and centrifuge 5 min at 100*g* at 4 °C.
? TROUBLESHOOTING
7 Aspirate excess 1× DPBS and BME, add 4 mL cold 1× DPBS and carefully resuspend the organoids by inverting the tube.
! CAUTION Be careful not to aspirate the organoids.
▲CRITICAL STEP Do not use a pipette to resuspend as this may result in a reduced organoid yield.
8 Centrifuge 5 min at 100*g* at 4 °C.
? TROUBLESHOOTING
9 Repeat Steps 3 and 4 twice (a total of three wash steps).
? TROUBLESHOOTING

Allot organoids for the MS^2 sample

▲CRITICAL This sample will be used to prepare the MS^2 sample in Steps 16–21.

10 Pool a small volume of organoids from every experimental condition in an FBS/PBS coated 1.5 mL Eppendorf tube. The minimal volume of pelleted organoids needed for this MS^2 sample is 50–75 µL; aim for 150 µL to make multiple samples for MS^2.
11 Leave the MS^2 sample on ice while performing Steps 12–15, and continue with this sample directly after organoid embedding as described under 'MS^2 sample preparation for molecular identification' (Steps 16–21).

Prepare organoids for embedding

12 To remove 1× DPBS, wash the organoids in 5 mL cold ABC and centrifuge for 5 min at 100*g* at 4 °C.
? TROUBLESHOOTING
13 Aspirate excess ABC, and directly continue with embedding.
▲CRITICAL STEP Be careful not to aspirate the organoids.

14 Determine the volume of organoids by adding water to a clean 5 mL tube to visually the same level as the organoid sample. Weigh the tube or measure the volume with a pipette.
▲ CRITICAL STEP To get a well-shaped block of embedded organoids, the mold should be filled with at least 100 µL and a maximum of 250 µL. This means that the minimum necessary volume of organoids is 50 µL. If <50 µL, use an F-bottom 384-well plate as mold instead of the suggested mold.
? TROUBLESHOOTING

15 Gently resuspend the organoids in warm (37 °C) 15% gelatin, at a 1:1 ratio (vol/vol), using a cutoff 200 µL pipette tip. Directly transfer the organoid–gelatin suspension to the plastic mold, and submerge it in ice-cold isopentane until the whole block is frozen (Fig. 3). While freezing, the block changes from transparent to solid white.
▲ CRITICAL STEP Avoid introducing air bubbles in the gelatin.
■ PAUSE POINT When completely frozen, the organoid–gelatin block can be taken out of the mold, wrapped in aluminum foil or stored in a tin cup. Do not forget to label the sample.

16 Temporarily (<4 h) store on dry ice or for several months at −80 °C.

MS^2 sample preparation for molecular identification ● Timing 20 min

▲ CRITICAL The identity of the detected masses needs to be determined using MS^2 experiments for biological interpretation of the MSI data. Therefore, we suggest to prepare a dedicated MS^2 sample containing a dense distribution of organoids from all experimental conditions. To ensure the acquisition of multiple MS^2 spectra per nominal mass, a minimal surface area is required. We empirically tested that a spot of 40–50 µL of disrupted organoids results in a confluent sheet of ~1 cm^2. At a pixel size of 50 μm^2, this should result in ~40,000 MS^2 spectra. We suggest making at least three slides for MS^2, one for positive ion mode, one for negative ion mode and one spare. This means that at least 120–150 µL of disrupted organoids are needed. This sample can also be used for manual profiling using a target list. To ensure the acquisition of multiple MS^2 spectra per nominal mass, a minimal surface area is required. We empirically tested that a spot of 40–50 µL of disrupted organoids results in a confluent sheet of ~1 cm^2. At a pixel size of 50 μm^2, this should result in ~40,000 MS^2 spectra. We suggest making at least three slides for MS^2, one for positive ion mode, one for negative ion mode and one spare. This means that at least 120–150 µL of disrupted organoids are needed. This sample can also be used for manual profiling using a target list.

17 Continue from Step 11 under 'Organoid collection and embedding', where you pooled organoids of all experimental conditions to a total pelleted volume of at least 50–75 µL.

18 Centrifuge for 1 min at 300*g*, aspirate excess of DPBS and resuspend the pellet at a 1:2 (vol/vol) ratio of organoid pellet to ABC, so add 100–150 µL ABC.

19 Disrupt the organoids by firmly pipetting up and down five to ten times with a 200 µL pipette tip.

20 Spot 40–50 µL drops on MSI-compatible glass slides.

21 Check the sample density with a microscope; it needs to form a confluent sheet (see 'Anticipated results'). If one drop is not sufficient, let the drop dry before adding an additional drop of disrupted organoids on top.
? TROUBLESHOOTING

22 Leave to dry for 1–5 min on a 37 °C heating plate or in a vacuum desiccator at room temperature (18–22 °C).
■ PAUSE POINT Store overnight in a vacuum desiccator or for several weeks at −80 °C.

MSI sample preparation and data acquisition ● Timing 2–8 h

▲ CRITICAL The details for the MSI sample preparation are dependent on the molecular class targeted and the ionization method used for imaging. Therefore, we only briefly describe each step and give tips that will assist in imaging and analyzing organoids. Detailed parameters used to the generate the images shown in the 'Anticipated results' are described in Supplementary Method 2.

23 Follow your preferred method of preparing cryosections for MSI. Below are some suggestions:
- It is generally easiest to cut 15% gelatin between −20 °C and −18 °C
- Trim excess gelatin by sectioning 10- to 12-µm-thick sections using a cryotome
- Verify the presence of organoids in the section by thaw-mounting a section on a glass slide, dry for 10 min at room temperature, stain with an aqueous dye (e.g., cresyl violet) for 30 s, rinse briefly with dH_2O and check under the microscope. Repeat until you find organoids in the section
- Collect sections of various experimental conditions on one glass slide, in a random order by using a random sequence generator, to minimize variation in further sample preparation and data acquisition. An example of such an arrangement is illustrated in Supplementary Fig. 1.

■ PAUSE POINT Store overnight in a vacuum desiccator or for several weeks at −80 °C.

24 Follow your preferred protocol to prepare the sample for MSI, e.g., apply MALDI matrix or do an on-tissue enzymatic digestion.

25 Apply a fiducial marker to the slide in close proximity to the sample. To make a fine line drawing, first extract the ink from the marker and use a small rod or 1–2 μL pipetting tip to draw crosses at two corners of the section (Supplementary Fig. 1)

▲ **CRITICAL STEP** This marker is used to accurately overlay the *m*/*z* distribution image with the morphological image. The marker, therefore, needs to be detectable with MSI and should be visible in the post-MSI histological staining, for instance, the Klinipath Marker Plus.

26 Let the marker dry for 1–2 min at room temperature

27 Set up the imaging experiment, and make sure to include the fiducial marker in the acquisition area (Supplementary Fig. 1).

28 Acquire mass spectra of each predefined pixel on your mass spectrometer of choice.

■ **PAUSE POINT** Store the slides overnight in a vacuum desiccator or for several weeks at −80 °C.

29 To find out whether a molecular profile belongs to a particular morphological feature, perform a histological staining (e.g., H&E staining) on the same sample used for MSI acquisition. The method we used to generate the figures shown in the 'Anticipated results' is described in Supplementary Method 4.

30 Create a digitized image of the histologically stained sample.

■ **PAUSE POINT** All imaging data that have been acquired can be analyzed at a later timepoint.

Molecular identification ● Timing 1–4 h

▲ **CRITICAL** MS^2 experiments provide information about the molecular structure of an analyte and can assist in confirming the molecular identity when the mass accuracy alone is insufficient. To ensure ionization of the same molecules as in the MSI experiment, the MSI specific sample preparation should be similar. There are various options for acquiring MS^2 spectra in imaging mode. For profiling studies that aim to detect and identify a large set of molecules, we suggest using a method that automatically selects precursor ions such as described by Ellis et al.[18] or a similar method[30,31]. For targeted experiments that, for instance, target a specific drug and its metabolites, manual profiling of the sample and selecting precursors from a target list works as well. The exact parameters used for the images shown in the 'Anticipated results' are described in Supplementary Method 3.

31 Prepare the MS^2 sample using the same protocol as for the MSI samples; e.g., apply MALDI matrix or do an on-tissue enzymatic digestion.

32 Acquire MS^2 spectra on your mass spectrometer of choice.

33 Search databases for MS^2 spectra interpretation, such as LipidMAPS, ALEX123, METASPACE and the Human Metabolome Database.

34 Assign the molecular identities to the *m*/*z* values.

Data analysis ● Timing variable

▲ **CRITICAL** In the data analysis phase, the data from the *m*/*z* distribution image, the morphological image and the list of *m*/*z* identities are combined. There are multiple open-source, vendor-specific and vendor-neutral software packages available to analyze MSI data. We suggest using a software package that allows for precise co-registration, such as LipostarMSI (Molecular Horizon Srl), SCiLS Lab (Bruker Daltonics), MSireader[32] and the MSI data analysis tool for the Galaxy framework[33]. In our studies, we use Lipostar and have included a detailed description of our steps in Supplementary Method 5.

35 For MSI processing,
- Perform peak detection and calibrate spectra with settings most suited to the mass analyzer of choice
- (Optional) Remove known background *m*/*z* values from all spectra, e.g., *m*/*z* values that originate from the MALDI matrix

36 Use the fiducial marker to manually overlay the MSI image with the morphological image. Automated morphology-based co-registration methods are also available[10].

37 Distinguish organoid-specific pixels from background.
- Use a multivariate statistical method, such as principal component analysis or cluster analysis, to identify on- and off-tissue areas based on the spectra
- Inspect if the test accurately separates on- and off-tissue areas by comparing the separate regions with the morphological image
- Select organoids by generating regions of interest or by removing background pixels from the dataset

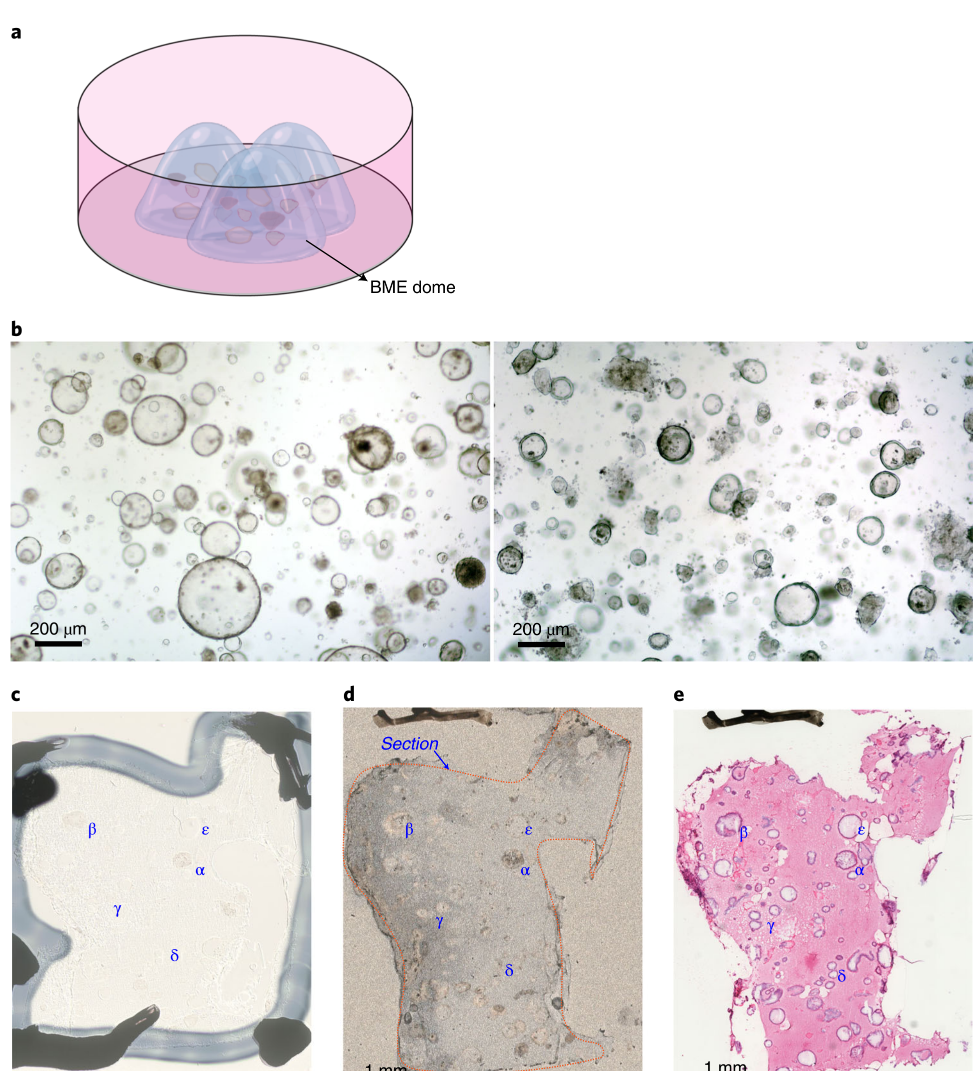

Fig. 4 | Appearance of the organoids at various stages of the protocol. a, Schematic of conventional organoid culture method using BME domes. The domes vary in size depending on the organoid model and laboratory-specific protocols, but are generally ~15–50 μL. **b**, Brightfield photographs of PDAC organoids grown in BME from cells obtained from two different human donors showing the characteristic spherical shape of these ductal organoids. **c**,**d**, After embedding, organoids can barely be distinguished macroscopically during sample preparation before (**c**) and after matrix application (**d**). Only larger organoids with luminal content (α and β) are clearly visible (**c**,**d**). **e**, After histological staining, all organoids are visible.

38 Combine the imaging data with the molecular identities, and manually scan through the data to identify molecules that are specific for a morphological feature.

39 (Optional) Apply univariate and multivariate statistical analysis to identify morphology or disease-specific biomarkers.

Troubleshooting

Steps 6, 8 and 12, centrifugation

This protocol was designed using PDAC organoids with a size between 20 and 300 μm. These organoids consist of an epithelial wall one or two cell layers thick, with a relatively large lumen (see ‘Anticipated results’). As a consequence, these organoids are very fragile. Therefore, they require gentle centrifugation. Whether centrifugation can be used depends on the organoid model, and if morphology is not adequately preserved, it is suggested to remove the centrifugation steps and let the organoids settle by gravity for ±20 min during each wash step.

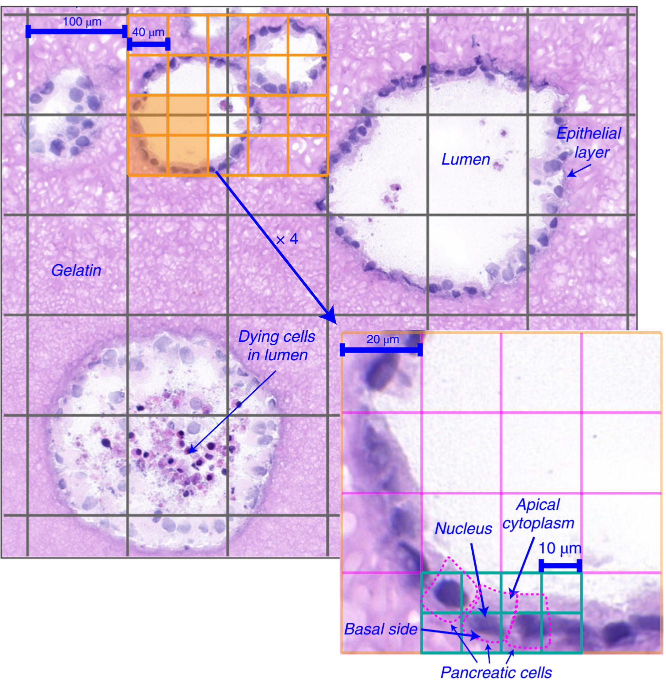

Fig. 5 | PDAC organoid morphology. PDAC organoids were embedded in gelatin, cryosectioned, and stained with H&E. Depending on the raster size, different morphological features can be identified.

Steps 7–9, wash steps

BME liquefies at temperatures below 24 °C and, at this temperature, mixes well with PBS. These properties allow for gentle washing. After washing and pellet formation, a small layer of BME can easily be distinguished above the organoid pellet. Three wash steps are generally sufficient to remove ~95% of BME, though more washing steps may be required. Take into account that adding more centrifugation steps may disrupt the morphology of the organoids.

Steps 19–20, spot formation for automated MS^2 experiments

To perform an automated MS^2 experiment, the spectra from consecutive pixels should be similar. To achieve this, the organoids are disrupted and spotted on a glass slide. The H&E-stained organoid droplet that was used for MS^2 shows that the characteristic tubular morphology is no longer visible and the overall structure is more compact than the sectioned organoids (see 'Anticipated results'). It also shows that there are open areas between the organoid cells, so the spot is not 100% confluent. This means that there are pixels 'lost' for the MS^2, though this is of no concern since there are still sufficient pixels left. For this particular sample, the confluency is estimated at 80%, resulting in ~32,000 pixels for MS^2.

Step 34, removing BME-specific spectra and other background contaminants from the MSI data

A number of methods can be used to remove background signal from MSI data. First, the signal from BME can be removed based on its location; by manually selecting the BME in the H&E stained image and precisely overlaying it with the MSI image, the BME pixels can be removed in the dataset. Alternatively, multivariate statistical methods including principal component analysis and partial least square discriminant analysis are common examples of unsupervised and supervised multivariate methods used to reveal patterns within MSI data[34], including the molecular pattern of BME. These methods can be used to filter the data on the basis of spectral content.

Timing

Culturing organoids: depending on the type of organoids and at which ratio they were passaged, it can take 5–14 d to grow to full confluency. For PDAC organoids, this usually takes ~5–7 d.

Steps 1–11, organoid collection; can be performed per batch (up to 30 samples): 1–2 h, depending on the number of samples

Steps 12–15, organoid embedding; should be performed sequentially per sample: ~10 min per sample
Steps 16–21, MS^2 sample generation: 20 min
Step 22, sectioning of embedded organoids: 20 min per sample (depending on the level of experience and number of sections)
Steps 23–27, MSI sample preparation and data acquisition: highly variable and, depending on the target molecule(s) and ionization method used for MSI, can take up to 24 h in case an on-tissue enzymatic digestion or derivatization is needed
Steps 28–29, post-MSI histological staining and digital image acquisition: 1 h
Step 30, sample preparation for MS^2: highly variable and, depending on the target molecule(s) and ionization method used for MSI, and can take up to 24 h in case an on-tissue enzymatic digestion or derivatization is needed
Step 31, MS^2 spectra acquisition: 1–4 h
Steps 32–38, data analysis: time varies depending on the data size and purpose of the analysis

Anticipated results

Sample processing stages

PDAC organoids cultured in BME form spherical, hollow structures with a diameter of 12.8–320.4 µm (average 57.8 ± 56.0 µm; n = 49) (Supplementary Fig. 2). Gelatin has a similar consistency as BME and the organoids, which makes it a suitable embedding medium; however, this also makes it difficult to distinguish individual organoids in the various stages of sample preparation (Fig. 4). Post-MSI histological staining is therefore indispensable for visualizing all organoids and for

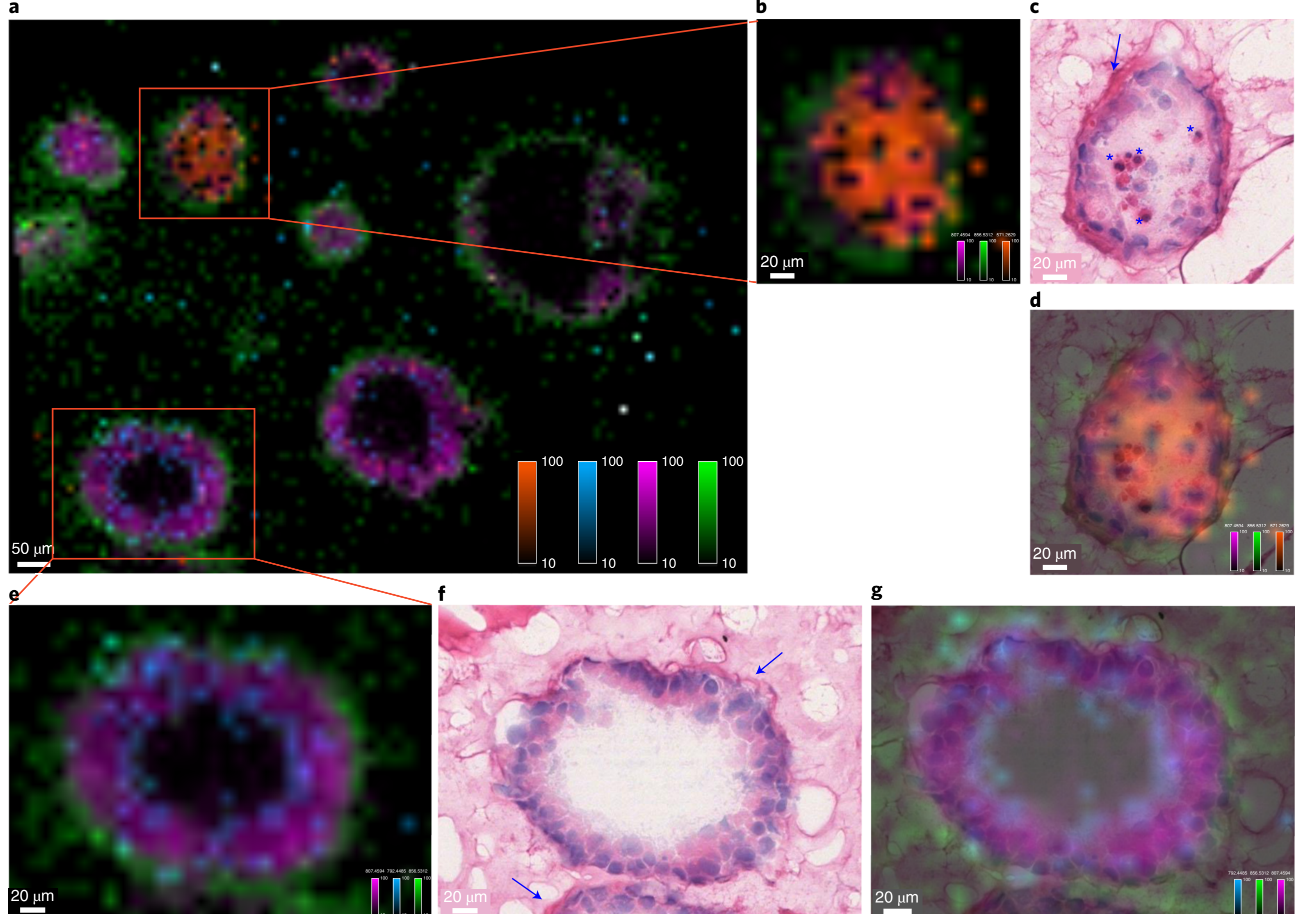

Fig. 6 | High-spatial-resolution MALDI-MSI. a–c,e,f, Mass spectra were acquired in negative ion mode in a 10 × 10 µm raster using MALDI-ToF (**a**,**b**,**e**) and subsequently stained with H&E (**c**,**f**). **d**,**g**, Overlaying the MALDI-ToF and H&E reveals morphology-specific masses. Asterisks indicate dying cells in the organoid lumen, and arrows indicate BME that is attached to the basal side of the epithelial cells. Orange, m/z 571.3; blue, m/z 792.4; purple, m/z 807.4 (PI 32:1); green, m/z 856.5. Ion intensities are normalized to the total ion count and scaled from 10% to 100%.

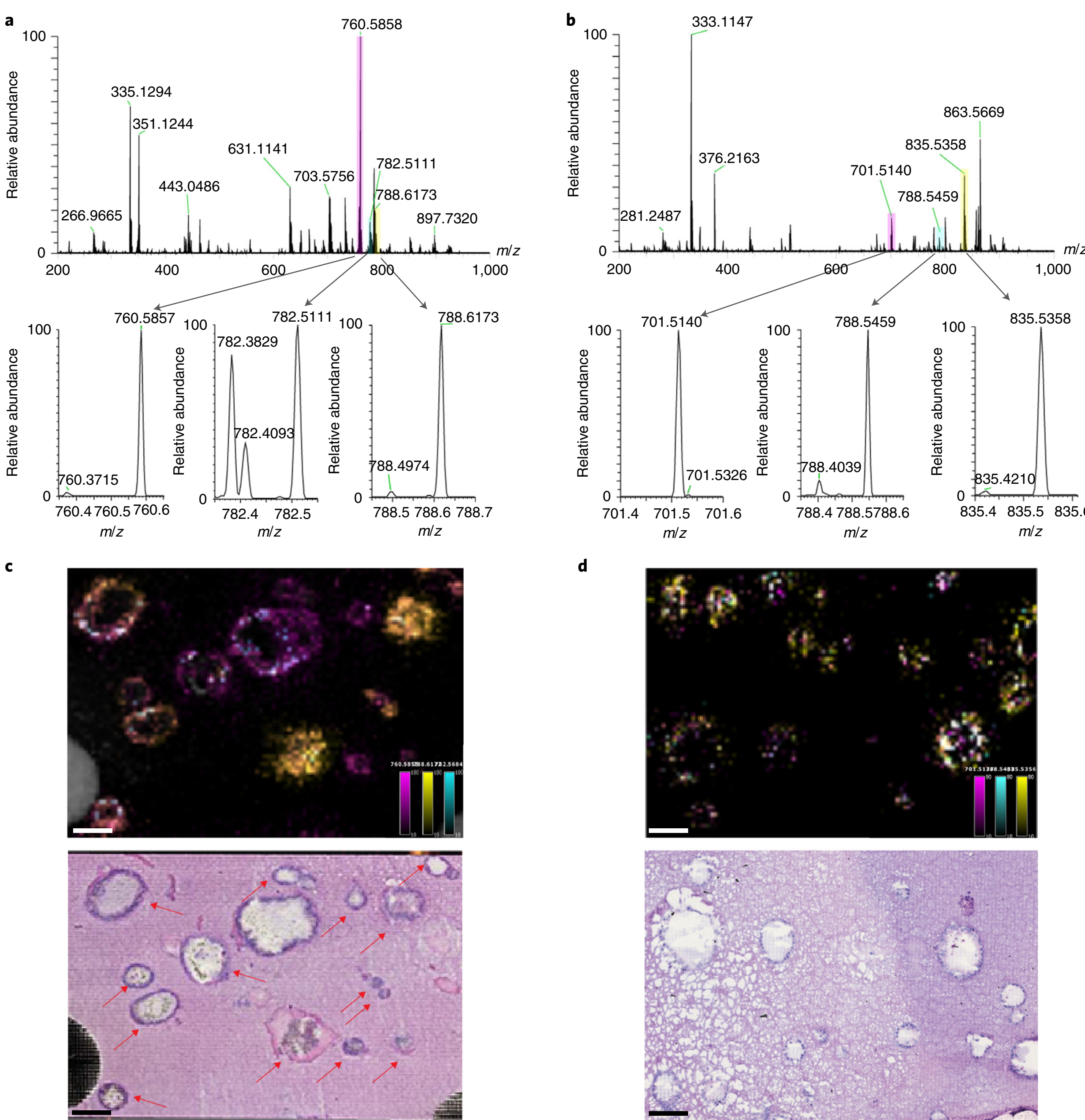

Fig. 7 | High-mass-resolution MALDI-MSI. a–d, The cryosections were analyzed with MALDI-Orbitrap in positive (**a**,**c**) and negative (**b**,**d**) ion mode with a raster of 20 × 20 µm and subsequently stained with H&E. The average spectrum shows a clear lipid signature in both polarities (**a**,**b**). In positive ion mode, various masses, including *m/z* 760.5857, 782.5111 and 788.6173, clearly co-localize with the epithelial layer of the organoids (indicated by arrows). These masses were identified as phosphatidylcholine 34:1, 36:4 and 36:1, respectively (**c**). In negative ion mode, *m/z* 701.5140, 788.5459 and 835.5358 co-localize with the epithelial layer and were identified as phosphatidic acid 36:1, phosphatidylserine 36:1 and phosphatidylinositol 34:1, respectively (**a**). Intensity images are root mean square normalized (the standard method to normalize Orbitrap and Ion Cyclotron Resonance data) and scaled from 10% to 100%. Scale bars, 200 µm.

proper morphological evaluation and co-registration. H&E staining is the most widely used staining to visualize morphological features, such as lumen, epithelial layer, luminal content, individual ductal epithelial cells, cytoplasm and nucleus (Fig. 5).

Organoid morphology

MSI experiments commonly involve trade-offs between lateral resolution, signal intensity and mass resolution. The biological question is the basis for this compromise, and as the strength of MSI is

Table 1 | Molecular identification based on exact mass

Detected *m/z*	Database hits	Adduct	Theoretical *m/z*	Mass error (ppm)
760.5855	PC 34:1 PE-NMe 36:1	$[M + H]^+$	760.5851	0.6
788.6172	PC 36:1 PE-NMe 38:1	$[M + H]^+$	788.6164	1.1
782.5684	PC 36:4 PE-NMe 38:4	$[M + H]^+$	782.5694	2.1
	PC 34:1 PE-NMe 36:1	$[M + Na]^+$	782.5670	1.0
701.5140	PA 36:1	$[M - H]^-$	701.5127	1.7
788.5459	PS 36:1	$[M - H]^-$	788.5447	1.6
	GlcCer 36:3	$[M + Cl]^-$		1.7
835.5358	PI 34:1	$[M - H]^-$	835.5342	1.7

visualizing the spatial localization of molecules, we first investigated which morphological features can be separated at various raster sizes. Within a raster of 100 µm, large PDAC organoids are resolved, making it useful to localize individual organoids. However, this raster size does not reveal organoid-specific features. When the raster size is decreased to 40 µm, also smaller organoids are clearly visible, and the epithelial layer and lumen of larger organoids can be identified. When the raster size is further reduced to 20 µm or 10 µm, individual PDAC cells and the polarity of the cells (apical versus basal) can be distinguished (Fig. 5).

Acquiring spectra every 10 µm in negative ion mode using a high-spatial-resolution MALDI-ToF instrument reveals a distinct distribution of various masses, including *m/z* 571.3, *m/z* 792.4, *m/z* 807.4 and *m/z* 856.5 (Fig. 6). Co-registering the MSI intensity images with the post-MSI H&E reveals that *m/z* 807.4 originates from the epithelial layer and *m/z* 792.4 from the apical side of the cell, and that *m/z* 856.5 surrounds the organoid and seems to come from BME that is attached to the basal side of the epithelial cells. Interestingly, *m/z* 571.3 is of high abundance in the lumen of organoids that contain dying cells (Fig. 6).

Combining molecular distribution and molecular identification

A high lateral resolution may give proper information about the location of various masses. However, to answer biological questions, we generally also need their molecular identity. This can be achieved by using a mass analyzer with a high mass resolving power that provides the elemental composition of the molecules. We analyzed gelatin-embedded organoid samples using a MALDI-Orbitrap in a 20 × 20 µm raster. In both positive and negative ion mode, various masses were clearly localized within the epithelial layer, including *m/z* 760.5855, 788.6172 and 782.5684 in positive mode and *m/z* 701.5140, 788.5459 and 835.5358 in negative mode (Fig. 7). Based on their exact mass, the phospholipid head group and the sum of the acyl chains could be identified (Table 1). In total, 110 masses (54 in positive mode and 56 in negative mode) were identified based on exact mass (mass error <2 ppm; Fig. 8).

For masses that have multiple database hits within a mass error of ~2 ppm, additional MS^2 experiments are required for identification. Additionally, MS^2 experiments give structural information about the molecules, as they provide the length and degree of insaturations of the acyl chains. In MSI, MS^2 experiments are often performed manually using a target list that requires that the tissue is visible by eye while inside the mass spectrometer. As the organoids are very small and are barely or not visible while inside the mass spectrometer, manual MS^2 experiments are not possible. We solved this by making a separate sample dedicated for MS^2. Sheared organoids were deposited onto a glass slide that, after drying, formed a thin, continuous sheet that allowed for automated MS^2 using a DDA approach (Fig. 9). The collection and preparation of this sample can easily be performed in parallel to the samples for MSI, minimizing day-to-day variation. DDA on the spotted organoids resulted in a library of MS^2 spectra of each detected nominal mass. The imaging experiment performed in a 40 × 40 um raster on a MALDI-Orbitrap resulted in 149 and 119 tissue-specific masses in positive and negative ion mode, respectively (Fig. 10). These masses were searched against

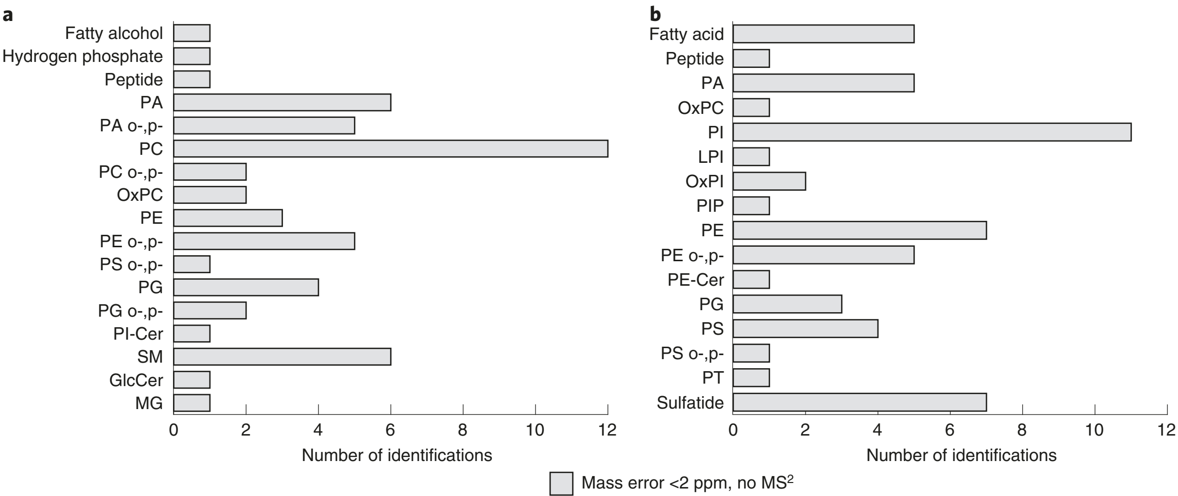

Fig. 8 | Number of *m/z* values that could be assigned to a distinct molecular species based on exact mass (mass error <2 ppm). a, Positive ion mode. **b**, Negative ion mode. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; MG, monoacylglycerol; LPI, lysoPI; PIP, phosphatidylinositol phosphate; Cer, ceramide; GlcCer, glycosphingolipids: o-, p-, alkyl or alkenyl ether; Ox, oxidized.

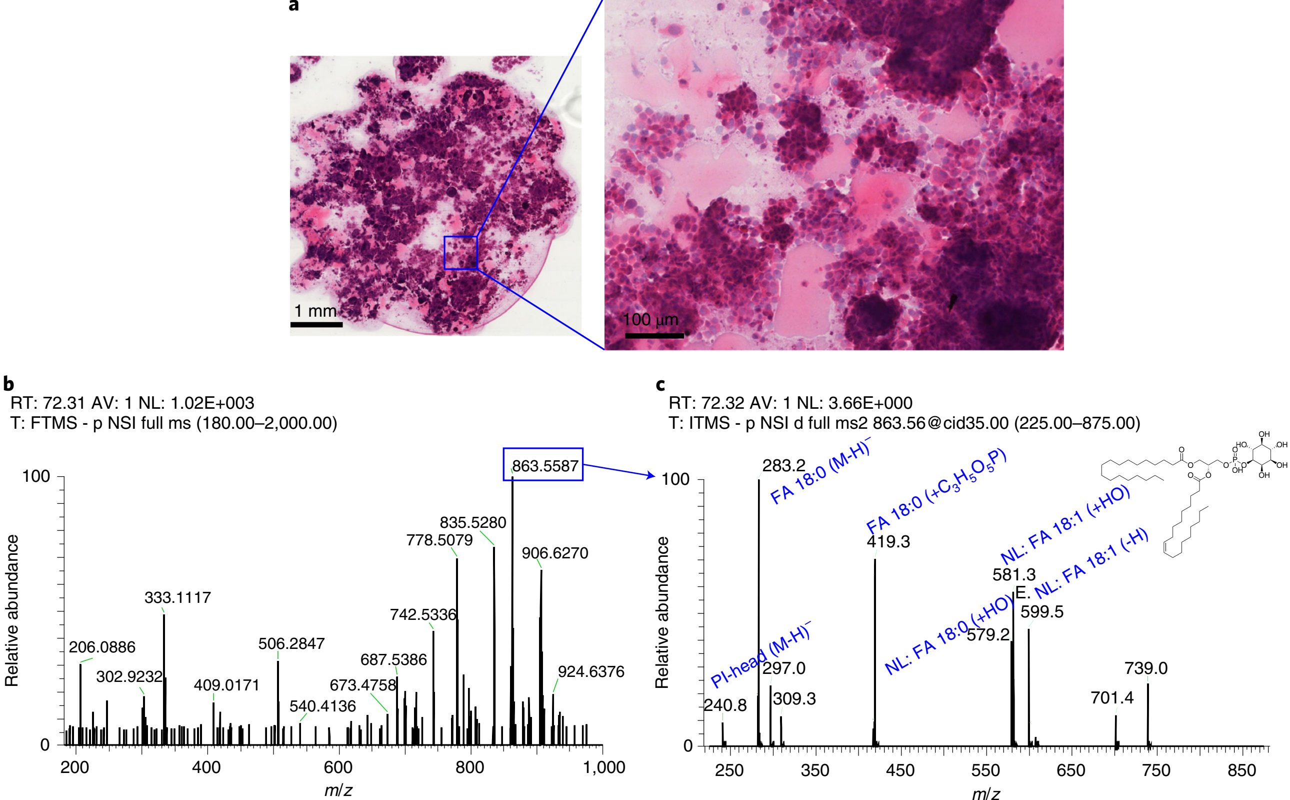

Fig. 9 | MALDI-MS^2 on spotted organoid. a, Organoids from all experimental conditions were pooled, sheared, spotted on a glass slide, analyzed with MALDI-MS^2 and subsequently stained with H&E. **b**,**c**, MALDI-MS^2 was perfomed in DDA mode on a MALDI-Orbitrap by acquiring a MS scan (**b**) on a pixel and a MS^2 scan (**c**) on the subsequent pixel.

lipid-specific databases and could be assigned to a distinct molecular species with the following criteria: (1) mass error ~2 ppm + 1 database match, (2) mass error ~2 ppm + multiple database matches + MS^2, or (3) mass error <10 ppm + MS^2 (Table 2). The majority of the identified molecules are lipids, especially from the lipid subclass glycerophospholipids. Glycerophospholipids are the most

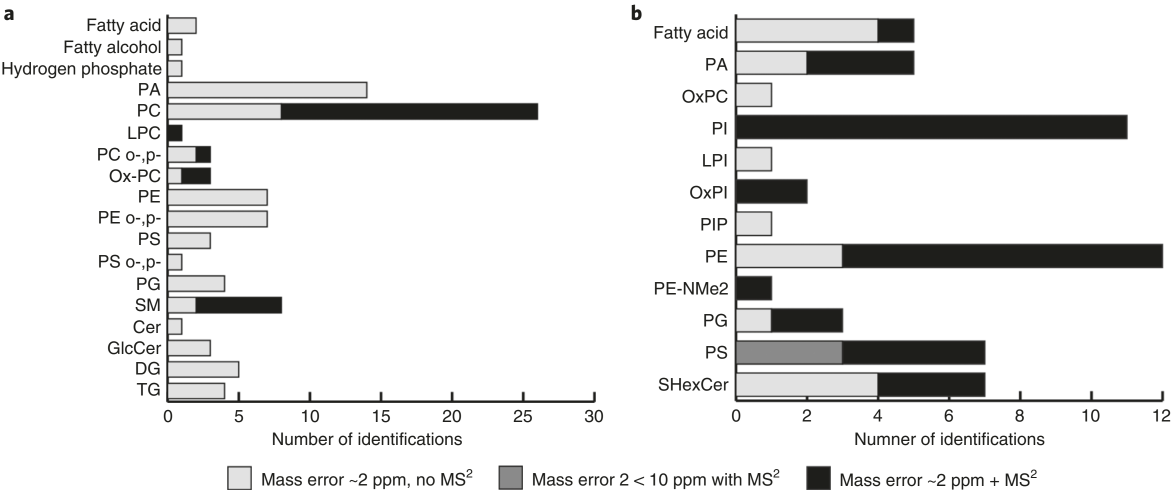

Fig. 10 | Number of *m/z* values that could be assigned to a distinct molecular species on the basis of exact mass and MS^2 data. a, Positive ion mode. **b**, Negative ion mode. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; DG, diacylglycerol; TG, triacylglycerol; LPC, lysoPC; LPI, lysoPI; PIP, phosphatidylinositol phosphate; PE-NMe2, dimethylphosphatidylethanolamine; Cer, ceramide; GlcCer, glycosphingolipids; o-, p-, alkyl or alkenyl ether; Ox, oxidized.

Table 2 | Number of molecular identifications based on exact mass and MS^2

Identification criteria	Positive ion mode	Negative ion mode
Mass error ~2 ppm + 1 database match	61	17
Mass error ~2 ppm + multiple database matches + MS^2	33	39
Mass error <10 ppm + MS^2	0	3

abundant lipid species and are the key building blocks of cellular membranes. They play important roles in energy storage, cellular signaling, cell membranes and cell–cell interactions. The role of a glycerophospholipid is determined by its polar head group and the length of the acyl chains. By performing MS^2 experiments in parallel to the imaging experiments, we were able to confidently identify the head group and length of the individual acyl chains, which may give insight into the biological state of the cells.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The example data are available from the corresponding author upon reasonable request.

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Author contributions

B.B.: writing, protocol development, data acquisition, data processing. R.D.W.V.: writing, organoid culture, protocol development. M.R.A.: writing, organoid culture, protocol development. T.W.: writing, protocol development, organoid culture. T.H.: supervision. S.S.R.: writing, supervision, funding, project conception. S.W.M.O.D.: supervision, funding, project conception. R.M.A.H.: supervision, funding, project conception.

Competing interests

The authors declare no competing interests.

Additional information

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Key references using this protocol

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Ellis, S. R. et al. *Nat. Methods* **15**, 515–518 (2018): https://doi.org/10.1038/s41592-018-0010-6

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Xcalibur 4.3 (Thermo Electon) FlexControl 4.0 (Bruker Daltonik)
Data analysis	Fleximaging (Bruker Daltonik, Bremen, Germany) LipostarMSI (Molecular Horizon Srl, Bettona, Italy) QuPath (v.0.2.0.m8)

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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For the development of this procedure organoids from two different donors was used. For the detection and identification of lipids in the organoids of one donor was used.
Data exclusions	No data were excluded from the analysis.
Replication	The data used for the example result section was not replicated, because the results were in line with previous observations.
Randomization	The experimental design did not include different experimental groups that could be randomized.
Blinding	The experimental design did not include different experimental groups that could be blinded.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging