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Supplementary information

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

In the format provided by the authors and unedited

Materials and methods anticipated result

Supplementary Method 1: PDAC Organoid Preparation

The collection of data and tissue for the generation of organoids was done in accordance with the guidelines of the European Network of Research Ethics Committees following European, national, and local law. PDAC organoids were established and cultured as described previously (Vaes et al., 2020). In brief, pancreatic tumor tissue was cut into small fragments, washed with culture medium (Advanced Dulbecco's Modified Eagle Medium/Ham's F-12) and digested in two steps using collagenase and trypsin. After digestion, the cells were washed, resuspended in ice-cold Geltrex (Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix, Gibco, Cat. No. 1413202) and plated in a 24-wells plate. The organoids were cultured in specific tumor medium (Adv-DMEM/F12+++ medium supplemented with Rspo1 CM (10% v/v), Noggin CM (10% v/v), 1x B27 (1:50), N-acetyl-L-cysteine (nAc) (1.25 mM), epidermal growth factor (mEGF) (5 ng/mL), Primocin (1:500), Gastrin (10 nM), fibroblast growth factor 10 (FGF10) (100 ng/mL), and Nicotinamide (10 mM)). The organoids were cultured at 37°C and 5% CO_2. The organoid growth medium was refreshed every 2-3 days. Characterization of the organoids was performed between passage number 6 and 15.

Supplementary Method 2: MSI sample preparation and data acquisition

Embedded organoids were cryosectioned in 12 µm thick sections, thaw mounted on Indium-Tin oxide (ITO) coated glass slides (Bruker Daltonik, Bremen, Germany) and stored at -80°C. Prior to MALDI-MSI, samples were thawed and dried in a vacuum desiccator for 30 min at room temperature and norharmane (7 mg/mL in 2:1 chloroform:methanol (v/v)) was applied with a TM-Sprayer (HTX Technologies, Chapel Hill, NC, USA) with the following spray conditions: flow rate= 0.12 mL/min, N_2 pressure=10 p.s.i., N_2 temperature= 30 °C, spray-

head velocity= 1,200 mm/min, track spacing= 3 mm, number of layers= 7, drying time between layers= 30 s, nozzle height= 40 mm, pattern= CC. To accurately overlay the MSI data with the optical images, a fiducial marker was applied next to each tissue.

High mass resolution mass spectra were acquired from *m/z* 75-1000 in positive and negative ion mode on a MALDI-Orbitrap mass spectrometer (QExactive HF, Thermo Fisher Scientific GmbH, Bremen, Germany) and equipped with an elevated-pressure MALDI ion source incorporating a dual-ion funnel interface (Spectroglyph LLC, Kennewick, WA, USA) (Belov et al., 2017). The 349-nm MALDI laser (Spectra Physics, Mountain View, CA, USA) was operated at a repetition rate of 1 kHz and pulse energy of ~1.2 µJ. The sample and the entrance to the high-pressure ion funnel were maintained at a pressure of 8.5 mbar, and the exit of the second, low-pressure ion funnel was maintained at 2.0 mbar. The mass spectrometer was operated with an ion-injection time of 200 ms and automatic gain control turned off. FTMS mass resolution was set to the maximum value of 120,000 (FWHM at *m/z* 200). Spectra were acquired in a 20 x 20 µm or 40 x 40 µm raster.

High spatial resolution MSI was performed on a MALDI-ToF mass spectrometer (rapifleX MALDI Tissuetyper,Bruker Daltonik, Bremen, Germany) operating in reflector mode with 200 laser shots accumulated per pixel in negative ion mode. Spectra were acquired in a 10 x 10 µm beam scan region (6 x 6 µm laser focus) raster within a mass range of *m/z* 300–1000 using FlexControl 4.0 (Bruker Daltonik).

Supplementary Method 3: Molecular identification

MS^2 measurements for lipid identification were performed on spotted organoids in positive and negative ion mode on an Orbitrap Elite (Thermo Fisher Scientific GmbH, Bremen, Germany) coupled to an intermediate pressure MALDI source based on a dual ion funnel design (Spectroglyph LLC, Kennewick, WA, USA). The MALDI-ion source and mass

spectrometer settings were as described above for the QExactive HF mass spectrometer, with the main difference that fragmentation spectra were acquired using a data dependent acquisition (DDA) approach, as recently described (Ellis et al., 2018). In brief, full FTMS scans from the Orbitrap (*m/z* 180-2000) were acquired from a 25 x 25 µm pixel. At the same time, in an adjacent 25 x 25 µm pixel, an MS^2 scan is acquired in the linear quadrupole ion trap during orbitrap data acquisition of the previous pixel. This results in a sampling region of 50 x 50 µm. All Ion trap- MS^2 spectra were acquired in parallel to the 768-ms Orbitrap transient using collision-induced dissociation with a normalized collision energy of 30 and 40 for the positive- and negative-ion modes, respectively.

Supplementary Method 4: Post-MSI histology

A standard H&E staining was performed to visualize organoids with a light microscope. In brief, norharmane was removed with 70% ethanol for 1 min, followed by a 30 s rinse in dH_2O. Nuclei were stained with Haematoxylin for 3 min, and after a 10 min rinse in running tap water, the remaining tissues were counterstained with Eosin for 30 s. Excess dye was removed with dH_2O (10 s) and samples were dehydrated with ethanol (2x 70% ethanol for 10s and 2x 100% for 1 min), followed by 2x xylene for 5 minutes and mounting. Optical images were generated using the M8 scanner (Precipoint, München, Germany).

Supplementary Method 5: MSI data processing

All data were processed and visualized using LipostarMSI (Molecular Horizon Srl, Bettona, Italy). Thermo Fisher raw files were first converted into the mzML format using msconvert (ProteoWizard) (Chambers et al., 2012) followed by merging with the MALDI-MSI position file and conversion to imzML using the in-built converter of LipostarMSI. Bruker raw files were exported to imzML using Fleximaging (Bruker Daltonik, Bremen, Germany) prior to

import in LipostarMSI. LipostarMSI parameters setting for the pre-processing of the raw data files can be found in supplemental table S1.

Supplementary Method 6: Lipid identification

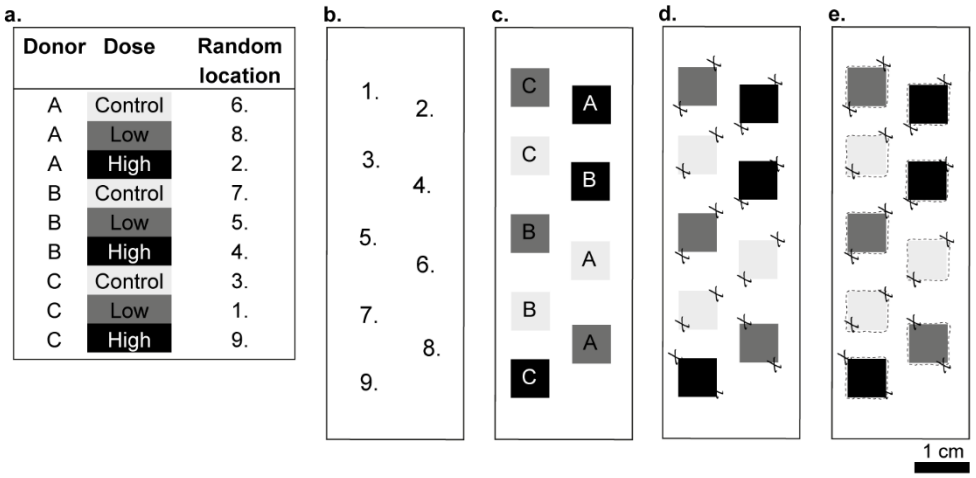
High mass resolution data from the MALDI-Orbitrap imaging experiment were used for lipid identifications and lipids were assigned based on accurate mass with a mass error of ± 2 ppm using LipostarMSI. The LIPIDMAPS and HMDB databases were used as reference for identification. Import parameters of the MS^2 data and identification settings can be found in supplemental table S1.

The database matches were manually approved with the following criteria:

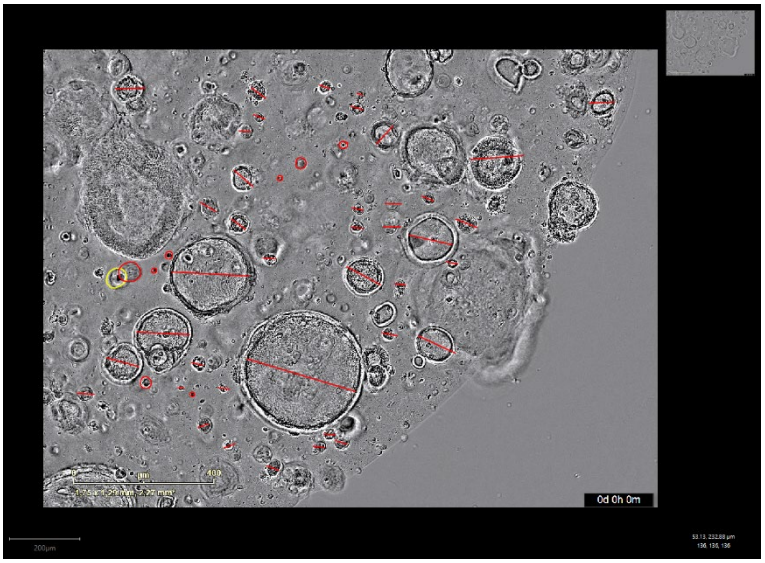
1. Mass error ~2 ppm + 1 database match
2. Mass error ~2 ppm + multiple database matches + MS^2
3. Mass error < 10 ppm + MS^2

The database matches were <u>not</u> approved when they were matched to a phospholipid with a fatty acyl chain with an odd number of carbon atoms.

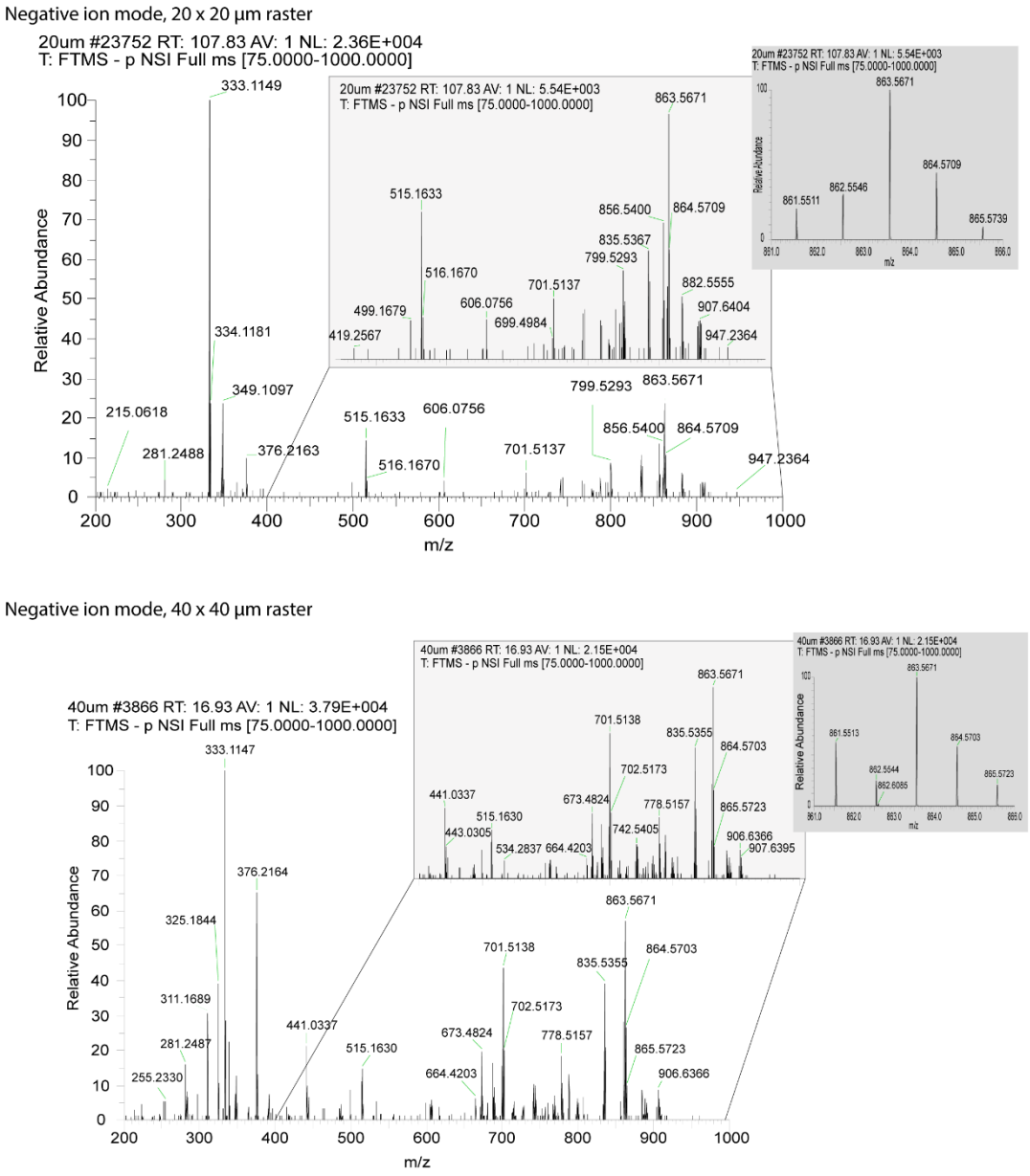
Supporting data



Supplementary figure 1: Cryosection organization and randomization. Randomization of a hypothetical experiment with three biological replicates (A-C) and three experimental conditions (a). Random locations refer to one of the spots in image b. About nine sections of 7 x 7 mm can be thaw mounted onto a standard microscope glass slide of 25 x 75 mm (c). After sample preparation, apply a fiducial marker to each section (d) and include part of the marker into the MSI experiment (e). The segmented line refers to the imaging area (e).

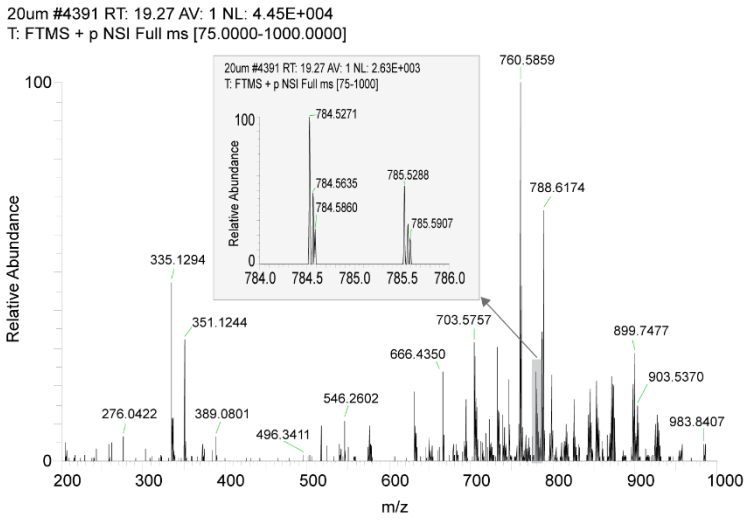


Supplementary figure 2: The size of organoids grown in basement membrane extract was measured manually using QuPath's line and area tools. The diameter is 57.8 µm ± 56.0 µm (average ± sd; range 12.8-320.4 µm)

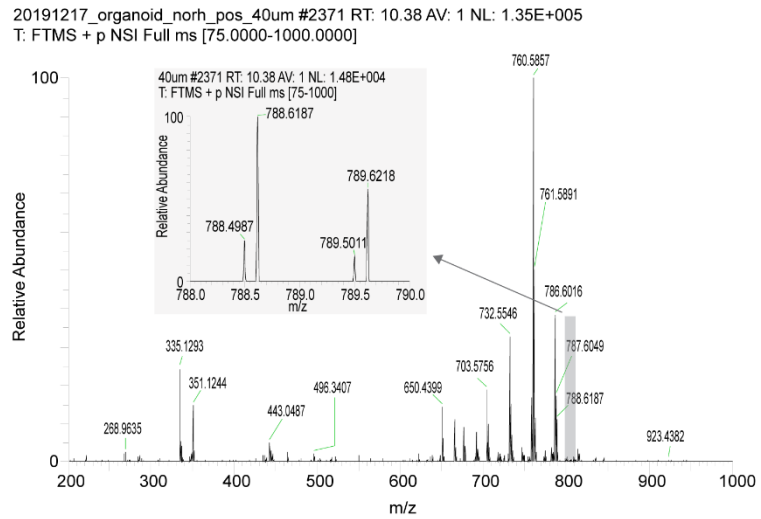


Supplementary figure 3: Single pixel spectra acquired with MALDI-Orbitrap in negative ion mode.

Positive ion mode, 20 x 20 µm raster



Positive ion mode, 40 x 40 µm raster



Supplementary figure 4: Single pixel spectra acquired with MALDI-Orbitrap in positive ion mode.

Supplementary table 1: LipostarMSI parameters setting for the processing of the raw data files

Instrument	Thermo instruments (Orbitrap)	Bruker (Time-of-Flight)
MSI processing		
Peak detection		
Savitzky-Golay	Window size: 7 points, Degree: 2,	Window size: 7 points, Degree: 2,
Baseline correction	0.1 amu	1 amu
Peak picking	Min SNR: 1 Noise window size: 0.1	Min SNR: 1, Noise window size: 0.1
Calibration		
Spectral Calibration	Lock mass: *m/z*: 863.5655 ± 0.002	Mass warping: 0.05 amu +3 ppm,
Dataset		
m/z tolerance	0.005 amu + 3 ppm	0.05 amu 3 ppm
min peak frequency	0.10	1.0
min peak intensity	0.20	0
min spatial chaos	0.00	0
m/z for normalization	0.00	0
Isotope clustering	Pattern abundance deviation: 30%,	Pattern abundance deviation: 30%,
Import MS/MS		n.a
precursor *m/z*	0.25 + 0 amu	
MS/MS *m/z* tolerance	0.25 + 0 amu	
MS/MS *m/z*	0 + 0 amu	
Discard MS/MS with	4 spectra for the same precursor	
Remove peaks lower	0.02 and 2% of the base peak	
Identification method		n.a
MS tolerance	0.005 amu + 3 ppm	
MS/MS tolerance	0.3 amu + 0 ppm	
Minimum chain	12	
Oxidations	Discard oxidized if any other match	