MS Imaging-Guided Microproteomics for Spatial Omics on a Single Instrument

Frédéric Dewez, Janina Oejten, Corinna Henkel, Romano Hebeler, Heiko Neuweger, Edwin De Pauw, Ron M. A. Heeren,* and Benjamin Balluff

Mass spectrometry imaging (MSI) is an omics technique that offers spatially resolved molecular analyses of tissues with high-throughput and high spatial resolution down to the single-cell level.\(^1\) The fact that it does not need labeling of the molecules has facilitated the annotation of tissues beyond histology.\(^2\) This unique capability is challenged by the limited analytical depth of MSI due to the absence of chromatographic separation which leads to a limited molecular characterization caused by local ion suppression.\(^3\)

To obtain deeper insights into the local biochemical processes, MSI needs to be complemented with a more sensitive liquid-based approach such as liquid chromatography-mass spectrometry (LC-MS). Recently, matrix-assisted laser desorption/ionization (MALDI)-MSI has been used to guide a laser microdissection system (LMD) to dissect regions highlighted by MSI for their subsequent proteomic analysis by LC-MS.\(^4\)–\(^8\) The coupling of these two technologies necessitates optimal instrumentation capabilities for every component of the pipeline. State-of-the-art MSI instrumentation needs to provide fast acquisition rates at high mass and high spatial resolution, whereas the LC-MS system needs to provide high sensitivity from minute amounts of material since the microdissected regions can be very small (in the sub-mm\(^2\) scale). This has so far required specialized and therefore separate instrumentation.

Recent advances in mass spectrometry (MS) instrumentation, however, promise to perform both state-of-the-art MALDI-MSI and LC-MS experiments on a single instrument. In this study, we use a hybrid-source system (timsTOF fleX, Bruker Daltonik, Bremen, Germany), which is equipped with a 10 kHz MALDI laser for rapid MSI and an orthogonal electrospray source. The latter combines ion mobility separation (trapped ion mobility spectrometry, TIMS), which separates and accumulates ions for high sensitivity, with the parallel accumulation serial fragmentation (PASEF) technology which synchronizes the quadrupole with the elution time of the TIMS for high-speed MS/MS acquisitions (up to 100 kHz).\(^9\)

The aim of this study is to bring together the spatial molecular information provided by MALDI-MSI with the microproteomic characterization by LC-MS on the exact same tissue section via...
LMD, which has never been performed on a single instrument before. This workflow is depicted in Figure 1.

First, MALDI-MSI was performed on a 12 µm thick section from a breast cancer sample, which was collected by the Tissue Biobank of the University of Liège. The consent form was obtained from the involved patient and the study was approved by the Ethics Committee of the University Hospital Center of Liège. Importantly, the section was mounted on a polyethylene naphtalate (PEN) membrane slide, which is compatible with the employed MSI system and required for the later LMD.

The section was dried in a desiccator for 30 min at room temperature before being coated with norharmane (7 mg mL\(^{-1}\) in 2:1 chloroform:methanol) for lipid MSI using the TM-sprayer (HTX Technologies, Chapel Hill, NC, USA) with the following parameters: 12 layers, flow rate = 0.120 mL min\(^{-1}\), velocity = 1200 mm min\(^{-1}\), and nozzle temperature = 30 °C. The MSI measurement was performed in positive ion mode at 50 µm spatial resolution within a mass range of m/z 300–1600 at 25 pixels per second. After the MSI experiment, the sample was rinsed in 70% ethanol to remove the matrix and stained with only hematoxylin. After scanning of the slide with a high-resolution scanner (M8 scanner, Freising, Germany), the tumor area was delineated by a trained pathologist.

The MSI data were loaded into SCilS Lab 2020a (Bruker Daltonik) for coregistration to the annotated histological image. Peak picking was performed on the exported average spectrum in mMass (v.5.5.0) with a minimum signal-to-noise threshold of three, a minimum relative base peak intensity of 0.02%, and a picking height of 80. The annotated area was segmented by k-means clustering using all 994 peaks into three subpopulations that are not distinguishable on a histological level (Figure 2A,B).

As the segmentation provides fine structures that are not easily processable by the LMD, the segmentation image needs to undergo image processing in Matlab R2018a (MathWorks, Natick, MA, USA) as previously reported by us (Figure 2B). First, a smoothing with a neutral-density filter (imfilter) and a square filter of size 2 × 2 was accomplished providing smooth cutting lines to the LMD (Figure 2C). Although the LMD is able to dissect single cells, the vast number of small objects encountered (here 92 with less than 30 pixels) makes a dissection infeasible. Small objects were therefore removed and assigned to the surrounding cluster (filling holes; Figure 2D,E). After up-scaling of the detected boundaries to the resolution of the optical image, the coordinates were transferred to the LMD software via an XML file (Figure 2F–H). The coregistration was performed using fiducial markers placed onto the slide prior to the MSI experiment and visible in the LMD system (Leica LMD7000, Leica Microsystems, Wetzlar, Germany).

Three subregions (≈2000 cells) from every segment were randomly selected and microdissected for further analysis (details in the Supporting Information), making a total of nine samples. These samples were individually prepared for LC-MS as previously described (details in the Supporting Information).[10]

The samples were run in random order on a nanoElute HPLC system (Bruker Daltonik) using a one-column setup equipped with an Aurora 25 cm × 75 µm × 1.7 µm C18 column (IonOpticks, Parkville, Australia). 0.1% formic acid, 2% acetonitrile in water was used as eluent A and 0.1% formic acid in acetonitrile as eluent B. Chromatographic separation of the peptides was carried
out over a 100 min nonlinear gradient up to 37% eluent B at a flow rate of 400 nL min$^{-1}$ and an oven temperature of 50°C.

The CaptiveSpray nano source was used as inlet into the MS instrument operated with 3.0 L min$^{-1}$ dry gas at 180 °C and 1.6 kV capillary voltage. Data were acquired in the mass range of m/z 100–1700 and the mobility range was set to 0.6–1.6 1/k0. In the dual TIMS cell, the accumulation as well as the ramp time was set to 100 ms. For MS/MS fragment spectra acquisition, one MS scan (100 ms) was followed by ten subsequent PASEF scans (each 100 ms). The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier “PXD020362”.[11] MaxQuant (v.1.6.10.43) was used for protein identification and label-free quantification (LFQ). Database search was performed using the Swiss-Prot human database (downloaded on February 12, 2020, with 42 368 entries including isoforms) with a parent
mass tolerance of 20 ppm, a fragment mass tolerance of 50 mDa, and a FDR ≤ 0.01. Trypsin was chosen as proteolytic enzyme with a maximum of two missed cleavages. Carbamidomethylation of cysteine residues was set as fixed modification, acetylation of protein N termini and methionine oxidation as variable modifications. The identification was first performed on individual replicates providing on average 2000 protein identifications per replicate, giving evidence of the high-sensitivity of the LC-MS setup for the analysis of minute amounts of samples, and then on all three replicates from every segment using the “matching between runs” feature (Figure 3A Table S1, Supporting Information). Based on a control tissue experiment of the same region and involving the same number of technical replicates, we observed no detrimental effect of the lipid MSI experiment on the number of identified proteins (Figure S1, Supporting Information). As expected when analyzing samples from the same tissue section, the overlap in identified proteins between the three segments was high with on average only 21.8% segment-exclusive identifications (Figure 3B).

LFQ calculations were performed on all replicates across all segments using the “matching between runs” feature. Proteins identified as “reverse,” “only identified by site,” and “potential contaminants,” or with one or more zero-LFQ intensities across the replicates were removed from further analysis (Table S1, Supporting Information).

Principal component analysis (PCA) was performed on the z-scored and log2-transformed LFQ intensities in R (v3.6.2) which showed the presence of two molecular distinct segments (Figure 3C). PCA was also performed for the MSI lipid data in Matlab R2018a (Figure 3D) to identify lipid signals that are particular to each segment. A putative annotation of the five loadings with the highest contribution to the PCA was achieved by MetaboScape (v5.0, Bruker Daltonik) using LipidMaps (43 080 entries) with a mass tolerance of 2 mDa (details in the Supporting Information). Putative annotations of m/z 786.60, 734.57, 760.58, 788.62, and 703.58 correspond to phosphatidylcholine PC(36:2), PC(32:0), PC(34:1), PC(36:1), and sphingomyelin SM(34:1), respectively. Among these, other MSI studies have found PC(32:0) and SM(34:1) to be associated with poor survival, which are lipids present at higher abundance in segment 3 (Figure 3D). [13]

We also performed statistical comparisons using t-tests to identify the most discriminating proteins between segments 1 and 2 versus segment 3. After multiple-testing correction (Benjamini–Hochberg method), 164 proteins were found significantly (p ≤ 0.05) altered (Table S2, Supporting Information). We also found several proteins altered that are related to lipid metabolism. For instance, we observed the two proteins...
sphingosine-1-phosphate lyase 1 (SGPL1; $p = 0.086$), which is known to be increased in cancer,\textsuperscript{[14]} and vesicle-associated membrane protein-associated protein B/C (VAPB; $p = 0.038$) to be down-regulated in segment 3. Since these proteins are involved in the breakdown and biosynthesis of sphingolipids, they could be related to the observed higher abundance of certain lipid species in segment 3, such as SM(34:1) (Figure 3D). We also observed the phospholipid transfer protein STA10 (STARD10; $p = 0.056$), which known to be overexpressed in breast cancers,\textsuperscript{[15]} to be up-regulated in segments 1 and 2 compared to segment 3. This could be related to the higher levels of several phospholipids in segments 1 and 2 such as PC(36:2), PC(34:1), and PC(36:1) (Figure 3D). This demonstrates how the presented workflow provides a useful tool to connect different MS-based omics data via the spatial context.

Other approaches in spatial omics pursue the systematic dissection of the tissue section in order to map protein abundances and biochemical processes in tissues, which is very time-consuming.\textsuperscript{[16]} Our approach, in contrast, uses MSI as a screening tool to reveal first obvious molecular spatial differences in tissues to direct further investigations on the same instrument. While there are many non-commercial or commercial systems equipped with hybrid sources, this kind of experiment has never been reported previously. Moreover, the performance in both MALDI and LC-proteomics is comparable to specialized MS instrumentation for either part of the workflow.

In conclusion, the present work combined the molecular spatial dimension provided by MSI with proteomics data obtained from locally microdissected material of the exact same tissue section on one single MS instrument.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

C.H., J.O., H.N., and R.H. are employees of Bruker Daltonik, the manufacturer of the mass spectrometer and the chromatographic system used in this study.

**Keywords**

laser microdissection, mass spectrometry imaging, microproteomics, spatial omics

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