

## Comprehensive identification of proteins from MALDI imaging

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**Running title:** Identifying proteins from MALDI imaging studies

## Summary

MALDI imaging mass spectrometry (MALDI IMS) is a powerful tool for the visualization of proteins in tissues and has demonstrated considerable diagnostic and prognostic value. One main challenge is that the molecular identity of such potential biomarkers mostly remains unknown. We introduce a generic method that removes this issue by systematically identifying the proteins embedded in the MALDI matrix using a combination of bottom-up and top-down proteomics. The analyses of ten human tissues lead to the identification of 1,400 abundant and soluble proteins constituting the set of proteins detectable by MALDI IMS including >90% of all IMS biomarkers reported in the literature. Top-down analysis of the matrix proteome identified 124 mostly N- and C-terminally fragmented proteins indicating considerable protein processing activity in tissues. All protein identification data from this study as well as the IMS literature has been deposited into MaTisse, a new publically available database which we anticipate will become a valuable resource for the IMS community.

## Abbreviations

FDR	false discovery rate
FFPE	formalin fixed paraffin embedded
HCD	higher collision energy dissociation
IAA	iodoacetamide
IMS	imaging mass spectrometry
ITO	Indium-Tin-Oxide
LDS	lithium dodecyl sulphate
NP40	nonyl phenoxy polyethoxy ethanol 40

## Introduction

Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS) is an emerging technique that can be described as a multi-color molecular microscope as it allows visualizing the distribution of many molecules as mass to charge ( $m/z$ ) signals in parallel *in situ* (1). Originally described some 15 years ago (2) the method has been successfully adapted to different analyte classes including small molecule drugs (3), metabolites (4), lipids (5), proteins (6) and peptides (7) using e. g. formalin fixed paraffin embedded (FFPE) as well as fresh frozen tissue (8). Because the tissue stays intact in the process, MALDI IMS is compatible with histochemistry (9) as well as immunohistochemistry and thus adds an additional dimension of molecular information to classical microscopy based tissue analysis (10). Imaging of proteins is appealing as it conceptually allows determining the localization and abundance of proteoforms (11) that naturally occur in the tissue under investigation including modifications such as phosphorylation, acetylation or ubiquitination, protease mediated cleavage or truncation (12). Therefore a proteinous  $m/z$  species detected by MALDI IMS can be viewed as an *in situ* molecular probe of a particular biological process. In turn,  $m/z$  abundance patterns that discriminate different physiological or pathological conditions might be used as diagnostic or even prognostic markers (13,14). In recent years, MALDI IMS of proteins has been successfully applied to different cancer types from the brain (15), breast (16,17), kidney (18), prostate (19) and skin (20). Furthermore, the technique has been applied in the context of colon inflammation (21), embryonic development (22), Alzheimer's disease (23) and amyotrophic lateral sclerosis (24). With a few notable exceptions (13, 14, 16–18, 20, 24–30), the identity of the proteins constituting the observed characteristic  $m/z$  patterns has generally remained elusive. This not only precludes the validation of the putative biomarkers by for example immunohistochemistry, but also the elucidation of the biological processes that might underlie the observed phenotype.

Here, we introduce a straightforward extraction and identification method for proteins embedded in the MALDI matrix layer which represent the molecular species amenable to MALDI IMS. Using a bottom-up proteomic approach including tryptic digestion and liquid chromatography tandem mass spectrometry (LC-MS/MS), we first created an inventory list of proteins derived from this layer which we term the MALDI matrix proteome. Although the bottom-up approach breaks the link between the identified proteins and the  $m/z$  species detected in MALDI IMS, the list of identified proteins serves as the pool of proteins from which all potential biomarkers are most likely derived. Indeed we detected >90% of all human MALDI IMS biomarkers reported in the literature by analyzing just ten human tissues. In addition, the results demonstrate that the same inventory can be used as a focused database for direct top-down sequencing and identification of proteins extracted from the MALDI matrix layer. The proposed method is generic and can be applied to any MALDI IMS study, which is why we believe that one of the major challenges in identifying MALDI IMS biomarkers has now been overcome. In addition, we provide a list of all proteins and peptides identified in the MALDI matrices and tissues studied here as well as a comprehensive list of  $m/z$  species identified in the literature dealing with MALDI imaging of humans and rodents. This information has been compiled in MaTisse (<http://129.187.44.58:7171/MALDI/protein>), a new publically available and searchable database which we believe will become a valuable tool for the MALDI imaging community.

## **Experimental Procedures**

### **Human tissue**

Fresh frozen tissue specimen from ten different tissues (esophagus, stomach carcinoma stomach normal tissue, colon mucosa, colon muscle, colon adenoma, colon carcinoma, two different mamma carcinomas and osteosarcoma) were provided by the archive of the Institute of Pathology, Technische Universität München and collected from patients on the basis of written

informed consent. This study was approved by the Ethics committee of the Technische Universität München.

### **MALDI IMS Sample preparation**

Cryosections (10  $\mu\text{m}$ ) of fresh frozen tissue were cut on a cryostat (CM1950, Leica Microsystems, Wetzlar, Germany) and transferred to cooled ( $-20\text{ }^{\circ}\text{C}$ ) conductive Indium-Tin-Oxide (ITO) coated glass slides (Bruker Daltonik, Bremen, Germany) and treated with 1:1 poly-lysine: 0.1% NP40 (nonyl phenoxy polyethoxy ethanol 40). The sections were washed in 70% ethanol and 100% ethanol for one minute each, air-dried, and subsequently coated with MALDI matrix ( $10\text{ g l}^{-1}$  sinapinic acid, Sigma Aldrich, in 60% acetonitrile, 0.2% trifluoroacetic acid, TFA) using the ImagePrep spray device (Bruker Daltonik). The detailed method can be found in the Supplemental Material.

### **Protein extraction from matrix coated cryosections**

Proteins were extracted from IMS samples in three steps which are illustrated in Supplemental Figure 1 A. In the first step, the matrix coated area of the cryosection (approx. surface area  $5\text{ cm}^2$ ) was covered with  $150\text{ }\mu\text{l}$  of 7.5% acetonitrile in 0.2% TFA, incubated for one minute and the liquid containing the protein extract was recovered using a pipette. This step was repeated using a further  $300\text{ }\mu\text{l}$  of solvent and both extracts were combined. In the second step, the same area was covered with a total of  $150\text{ }\mu\text{l}$  of 60 % acetonitrile 0.2% TFA and the liquid was immediately collected again. Note that the first extraction step (low organic content) allowed extracting the matrix without liquid spreading beyond the covered area. The second extraction step (high organic content) dissolved the matrix completely to recover all of the embedded protein in a separate tube. In a third step, the solvent extracted remaining tissue was removed from the slide by means of a scalpel and collected in a tube as a third sample. The two solvent extracts and the excised tissue section were dried using a vacuum concentrator.

## **SDS gel electrophoresis and in gel digestion**

Seventy-five  $\mu\text{l}$  of 2x LDS sample buffer (lithium dodecyl sulfate, Invitrogen, Carlsbad, CA) containing 100  $\mu\text{M}$  dithiothreitol (DTT) were added to each of the dried samples. After incubation for one hour at 90°C on a shaker, 17.5  $\mu\text{l}$  of a 550 mM iodoacetamide (IAA) was added, and the sample was incubated for 30 min in the dark to alkylate cysteines. After 10 min of centrifugation at 11,340 x g, samples were heated to 90 °C for 10 minutes and 25  $\mu\text{l}$  of the sample were loaded onto a denaturing gradient gel (4–12% NuPAGE, Invitrogen). The run time for acetonitrile extracts was 5 minutes (just to run the samples into the gel) and 45 minutes for the total tissue extracts (to separate the proteins) at a constant voltage of 200 V. Gel lanes derived from total tissue extracts were cut into 12 equally sized slices. Samples derived from the matrix extracts were cut as a single piece. In-gel tryptic digestion of the proteins was performed according to (31) using sequencing grade trypsin (Promega, Mannheim, Germany)

## **LC-MS/MS analysis for bottom-up protein identification**

Nanoflow LC-MS/MS was performed by coupling a nanoLC-Ultra 1D (Eksigent, Dublin, CA, USA) to an LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany). Tryptic peptides were dissolved in 20  $\mu\text{l}$  of 0.1% formic acid, and 10  $\mu\text{l}$  were injected for each analysis. Peptides were delivered to a trap column (100  $\mu\text{m}$  inner diameter 2 cm length, packed with 5  $\mu\text{m}$  C18 resin, Reprosil PUR AQ; Dr. Maisch, Ammerbuch, Germany) at a flow rate of 5  $\mu\text{l}/\text{min}$  in 100% buffer A (0.1% FA in HPLC grade water). After 10 minutes of loading and washing, peptides were transferred to an analytical column (75  $\mu\text{m}$  x 40 cm C18 Reprosil PUR AQ, 3 $\mu\text{m}$ ; Dr. Maisch). Peptides from acetonitrile extracts of the tissues were separated using a 210-min gradient from 7 to 35% of buffer B (0.1% FA in acetonitrile) at 300 nl/min flow rate. Peptides from total lysates of tissue sections were separated by a 90 minute gradient from 2 to 35% of buffer B at 300 nl/min. The LTQ-Orbitrap was operated in data-dependent mode, automatically switching

between MS (in Orbitrap) and MS2 (in ion trap). Full scan MS spectra were acquired in the Orbitrap at 60,000 resolution and an AGC (automatic gain control) target value of 1e6. Internal lock mass calibration was performed using the ion signal  $[(\text{Si}(\text{CH}_3)_2\text{O})_6\text{H}]^+$  at  $m/z$  445.120025 present in ambient laboratory air. Tandem mass spectra were generated by collision induced dissociation (CID) for up to 15 peptide precursors in the linear ion trap at a target value of 5,000 and a normalized collision energy of 35. Precursors selected for fragmentation were dynamically excluded for 30s.

### **MS data processing and protein identification**

Mascot Distiller version 2.2.1 (Matrix Science, London, UK) was used for peak picking, charge deconvolution and de-isotoping. The resulting peak list files were searched against the IPI human database (version 3.68, 87,061 sequences) using the Mascot search engine version 2.2.04 (Matrix Science). Search parameters included a precursor mass tolerance of 10 ppm and fragment tolerance of 0.6 Da. Enzyme specificity was set to trypsin with a maximal number of two missed cleavages. Carbamidomethylation of cysteine residues was set as a fixed modification and deamidation of asparagine and glutamine, oxidation of methionine and acetylation at the peptide N-terminus were set as variable modifications. Mascot search results were loaded into Scaffold (version 3.00.08, Proteome Software, Portland, OR, USA) and filters in Scaffold (32) were applied to result in false discovery rates (FDR) of 0.8% on protein level and 0.4% on peptide level.

### **Sample preparation for top-down protein identification**

Protein containing tissue extracts were prepared as described for the bottom-up approach. The 7.5% and 60% acetonitrile extracts were combined and dried in a vacuum centrifuge. To remove particles that might interfere with LC-MS analysis, the dried samples were reconstituted in 50 % acetonitrile, 0.2% TFA and passed through STAGE tips (33) packed with C18

Empore™ extraction disks (3M). The flow-through fraction containing the proteins was dried and reconstituted in buffer A (0.1% formic acid).

### **LC-MS/MS analysis for top-down protein identification**

Nano-LC-MS measurements of intact proteins were carried out using a nanoLC-Ultra 1D+ (Eksigent) coupled to an LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific). Samples were loaded onto the same columns, using the same solvents as above. Gradient separation was performed as follows: 2-10% B in 1 min; 10-35% B in 34 min; 35-90% B in 2 min; 90% B for 4 min; 90-2% B in 0.5 min; 2% B for 3.5 min. The Orbitrap Elite was operated in data dependent mode automatically switching between full MS and MS/MS mode. Full MS spectra were acquired in the Orbitrap over a mass range from 430 to 1800  $m/z$  collecting four microscans. The three most intense precursor ions with charge states greater than three were selected for fragmentation using higher-energy collisional dissociation (HCD) using a normalized collision energy of 30. Eight, respectively four microscans were acquired for each precursor in separate LC-MS/MS runs to generate tandem mass spectra with sufficient quality for peptide identification. Both MS and MS/MS were acquired at a resolution of 60,000 in the Orbitrap and using a target value of  $1e6$ . Internal calibration was performed as described above. Raw mass spectra were processed using Mascot Distiller version 2.4.2 (Matrix Science). The resulting charge-deconvoluted and de-isotoped spectra were searched against the IPI human database (version 3.68, 87,061 sequences) using the Mascot search engine version 2.3.02 (Matrix Science). The same data was also searched against an in-house generated fasta file (using the export function of Scaffold) containing 5,465 sequences assembled from the bottom-up analysis described in this study (3,738 protein sequences and 1,727 isoform sequences, Supplemental File 1). All searches used 7 ppm precursor tolerance and 0.04 Da fragment ion tolerance. Acetylation of lysine, protein and peptide N-terminus, deamidation of glutamine and asparagine as well as oxidation of methionine were set as variable modifications. No enzyme

was specified for searching. To sequence less abundant precursors, the data dependent acquisition search results of the first measurement were used to compile an exclusion list for further LC-MS/MS iterations. The peak list files of all runs were merged into one file using MASCOT daemon. Top-down searches were run without the decoy option i) because searching against human IPI would have taken excessively long (an estimated 8 days on a 12 CPU server) and ii) because the target-decoy approach is not well suited to estimate FDRs in low complexity samples such as the ones analysed here. Search results were loaded into Scaffold version 3.6.3. For protein identifications to be accepted, the mascot ion score had to be equal or above the mascot homology score of 52. In addition, proteins identified by a single peptide were verified by manual inspection of the tandem mass spectra. Identifications based on tryptic peptides originating from carry over from previous runs were excluded.

### **Bioinformatic data analysis and data availability**

Lists of identified proteins were analyzed for cellular compartment, biological function and canonical pathways using Ingenuity Pathway Analysis, IPA (Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)). For the survey of proteins from published human MALDI IMS studies, the reported protein accession numbers were mapped to IPI accession numbers using the ID mapping tool of Uniprot (<http://www.uniprot.org>). For papers reporting proteins from other organisms, respective sequences were first analysed by BLAST (<http://www.uniprot.org/>) and subsequently mapped to the corresponding human ortholog.

A complete list of identified proteins and peptides along with all supporting evidence such as annotated tandem mass spectra has been assembled into two Scaffold files available from <http://www.proteomexchange.org>, accession PXD000125). Protein identification data as generated in this study as well as that extracted from the literature is available via the newly created MaTisse database under <http://129.187.44.58:7171/MALDI/protein>.

## Results

### Characterization of the matrix proteome

In keeping with the idea that any IMS protein biomarker must be contained in the MALDI matrix layer in order to be detectable by the mass spectrometer, we set out to identify these proteins systematically (Fig. 1). Tissue samples were prepared for MALDI IMS according to standard procedures but instead of executing an imaging experiment, we extracted proteins from the MALDI matrix layer using 7.5% acetonitrile (ACN) followed by 60% ACN and finally recovered the entire remaining material from the glass slide. Analytical SDS-PAGE separation of the three protein pools (Fig. 1B) shows that the 7.5% ACN extract mainly contains small proteins (<35 kDa). Increasing the concentration of the organic solvent to 60% ACN dissolves the MALDI matrix layer completely and extracts proteins up to ~70 kDa in size (the strong band is human albumin). The total tissue protein extract (Fig. 1B) shows the typical range of proteins expressed in human tissues covering the entire mass scale. We processed samples from ten different tissues in this way (Fig. 2A) and subjected all to tryptic digestion and protein identification using LC-MS/MS (the bottom-up proteomic approach). Using 20% of each extract of a single microscopic slide with approximately 5 cm<sup>2</sup> tissue surface, 400, 700 and 2,100 proteins were identified on average from the 7.5% ACN, 60% ACN and total extracts respectively (Fig. 2A). The total number of proteins identified from all matrix extracts was 1,435 and 3,709 from all total extracts. With a few exceptions (37 proteins), all proteins identified from the matrix extracts were also present in the total extracts (the core proteome (34)) showing that the sample preparation used for MALDI IMS studies extracts a subset of the total proteome of a human tissue.

-Figure 1 near here-

Notably, the core tissue proteomes were rather similar between tissues as accumulating all distinct protein identifications across the tissues lead to rapid saturation (Fig. 2B). A similar but

less pronounced effect was also observed for the acetonitrile extracts indicating that the data mainly covers ubiquitously expressed abundant proteins. An analysis of the molecular weight (MW) distribution of the identified proteins (Fig. 2C) shows that many more small proteins (<25kDa) are detected in the matrix layer compared to the total tissue, which is consistent with the data obtained by SDS-PAGE analysis and MALDI IMS data from the literature where most protein features are detected between 2-25 kDs (1) and seldom beyond 40 kDa (35). The bottom-up protein identification data from the matrix layer did, however, also identify many nominally medium sized and large proteins (by MW in Uniprot). This is inconsistent with the SDS-PAGE data shown in Figure 1 and indicates that the larger proteins are either present in quantities below the detection limit of silver staining or that these represent protein fragments arising from protease cleavage (see also below). The majority of all proteins identified from extracts are cytoplasmic proteins (Fig. 2D) and the matrix extracts contain relatively more extracellular proteins but relatively fewer plasma membrane proteins than the tissue extracts. Taken together, the characterization of the matrix proteome shows that it mainly consists of small, soluble, abundant and well annotated human proteins.

-Figure 2 near here-

-Table 1 near here-

### **A near complete list of published MALDI IMS biomarkers**

As noted in the introduction, the molecular identities of  $m/z$  patterns detected in MALDI IMS studies often remain unknown. We conducted a comprehensive literature survey and compiled a list of 254  $m/z$  species that were identified from human and rodent tissue (Supplemental Table 4). Thirteen of the 22 reports analysed human tissue specimen and identified 60 distinct human proteins (Table 1). We then asked if these proteins were also identified in our work and, if so, in which extracts and how often they were found. Interestingly, 90% of all IMS markers reported in

the literature were found in our matrix proteomes despite the fact that the organs used in the literature were different from the ones used here. It is also noteworthy that the same proteins tend to be identified in multiple studies indicating they are rather abundant and not necessarily specific for a particular tissue or disease. Many IMS studies use rodents as disease models and our list of identified human matrix proteins also covers nearly 80% of the rodent orthologs (Supplemental Table 5). To assess protein abundance specifically, we ranked all 3,700 proteins identified in the total tissue extracts by the number of tandem mass spectra with which they were identified (divided by the size of the protein) and highlighted the IMS biomarkers from the literature in orange (Fig. 3A). The expression range of proteins in the total tissue extract spans approximately four orders of magnitude and most of the IMS biomarkers appear to be abundant cellular proteins. When plotting the 1,400 matrix proteins on the same scale (Fig. 3B), most of the reported IMS markers are also abundant in the matrix. However, there are also some proteins with quite high abundance in the matrix but only medium or low abundance in the tissue (e.g. cytochrome c oxidase). This may suggest that MALDI IMS, at least in principle, can access proteins across several orders of magnitude in cellular protein expression. An alternative explanation is that the spray coating process may strongly favor the solubilization of some particular proteins.

-Figure 3 near here-

### **Closing the gap: top-down protein identification**

The above bottom-up proteomic approach created a list of proteins from which essentially all  $m/z$  features detectable by MALDI IMS in human tissue should be derived. Eventually, any particular  $m/z$  feature of interest in a MALDI IMS spectrum (i.e. discriminating between two or more tissue states) must be identified. This is ideally done directly and without the use of a protease because it can otherwise not be ascertained that the detected  $m/z$  feature and the

protein identified following proteolysis are indeed the same. Therefore, the mass spectrometric fragmentation of the intact molecule is required (top-down proteomic approach). Progress in the performance of mass spectrometers is now making this more readily possible (36) and the short-list of MALDI IMS proteins generated in this study is of tremendous help for this purpose. As a proof-of-concept, we prepared MALDI matrix extracts from esophagus and colon tissue sections, mixed the extracts and analyzed these by LC-MS/MS on a high field Orbitrap mass spectrometer (36). Tandem mass spectra were charge deconvoluted and deisotoped prior to searching against a collection of ~5,500 protein sequences assembled from protein identifications made in MALDI IMS studies including the ones presented above (subsetDB, Supplemental File 1 and 2). An example of an acetylated and top-down sequenced 5 kDa peptide including nine internal lysine residues is shown in Figure 4A (full length thymosin beta-4, TMSB4X). When searching either the commonly used human IPI database (37) or the 15x smaller subsetDB, 124 proteins were identified in common and only very few identifications were exclusive for either database (Fig. 4B). However, the much reduced search space enabled the search engine (here Mascot) to assign over four times the number of spectra above homology threshold (~2,500) when using subsetDB compared to human IPI (Supplemental Fig. 5) resulting in more than 3 times the number of identified peptides for the respective proteoforms. The vast majority of the identified peptides are smaller than 4 kDa but it is noteworthy that peptides as large as 12 kDa could also be identified top-down using sample quantities recovered from a single microscopic slide (Fig. 4C). Interestingly, many of the identified proteins are represented as C-Terminal and N-Terminal fragments (Fig. 4D) suggesting significant protein processing within the tissue that gives rise to these species.

-Figure 4 near here-

**MaTisse: a searchable database of protein identifications in MALDI IMS studies**

To facilitate the translation of protein identifications made in different IMS studies, we have created a new database, MaTisse, which contains the complete bottom up matrix and tissue proteomes as well as the top-down identifications made in this study. In addition, we supplemented MaTisse with protein identification data from the published literature. MaTisse enables scientists to search either for proteins or for  $m/z$  values resulting in the rapid identification of potential protein candidates which generally facilitates the association of  $m/z$  values to proteins in any MALDI IMS study. Because of this unique feature, we anticipate that MaTisse will become a valuable source of information for the MALDI IMS community.

## **Discussion**

The application of MALDI IMS of proteins has grown steadily over the past 15 years. At the same time, there still is a striking discrepancy between the sophistication with which such images can be generated and the ability to identify the underlying proteins. Out of the many hundreds of publications reporting MALDI IMS data and biomarkers, only about 22 (13 human, 9 rodent), and mostly very recent papers, have also revealed the name of the protein. There are many reasons for this general inability to identify a protein using MALDI IMS. First, a mass measurement of a protein or fragment thereof as provided by MALDI IMS is not a good indicator for protein identity because any such mass can be generated from nearly any protein sequence within the mass accuracy of the experiment (1-2 Da). Second, the direct mass spectrometric sequencing of the detected  $m/z$  feature in a MALDI mass spectrum is generally not possible owing to the generally inadequate sensitivity, resolution and mass accuracy and mass range of these instruments in tandem MS mode. Many researchers have therefore decoupled the imaging experiment from the protein identification experiment and resorted to detailed bottom-up proteomic analysis of a separate tissue sample from which the image was created (28). In a more sophisticated approach, on-tissue tryptic digestion followed by MS analysis has been employed for protein identification (7). While these have sometimes been successful, they are

also time consuming and, in fact, there has often been a fair amount of 'educated guessing' in trying to connect molecular weight information from the MALDI IMS experiment to a list of proteins identified from a tissue. The above shortcomings have plagued the field to an extent that there is growing concern, at least in parts of the community, about the general value of  $m/z$  feature information for biomarker discovery in the absence of protein identification information. This is because the identity of any biomarker must be known in order to validate and develop it into a useful clinical tool.

In this study, we have taken an alternative approach to solving the above issues. We reasoned that any protein detectable by MALDI IMS must not only be present in the underlying tissue, but also reside in the matrix layer of the tissue preparation. We therefore developed a simple extraction procedure that recovers the proteins from the matrix, digested them with the protease trypsin and identified the 'matrix proteome' by high performance LC-MS/MS. Exemplified on ten human tissues, this enabled us to generate a shortlist of ~1,400 mostly small, abundant and soluble proteins that contain nearly all MALDI IMS protein biomarkers reported in the literature thus far. Proteins (e.g., E2F3, S100B) that were found only rarely (or never) may have been difficult to distinguish from closely related proteins, possibly misidentified in the original publication, or of too low abundance in the tissues analyzed here (e.g., myelin basic protein, MBP, P02686, which is essentially only expressed in central nervous system tissue, <http://www.proteinatlas.org/ENSG00000197971>). Many of the reported biomarkers were identified in many if not most of the tissues analyzed here. For example, the pro-inflammatory cytokine MIF (macrophage migration inhibitory factor, P14174) was found in all ten tissues as well as each of the acetonitrile extracts and this is also true for many other cases. Ingenuity pathway analysis (IPA) of protein identified here and in the MALDI IMS literature (both human and rodents) show significant overrepresentation of, for example, 'mitochondrial dysfunction' and 'EIF2 signaling' both of which are intimately linked to cancer (Supplemental Table 8). In light of

the above and because IMS biomarkers are generally abundant proteins, our observations strongly suggest that IMS biomarkers are mostly molecular surrogate markers of rather broad specificity rather than proteins involved in the molecular mechanism underlying a specific disease or physiology.

Compared to direct MALDI MS/MS or on-tissue digestion, the bottom-up LC-MS/MS approach has the advantage of superior sensitivity and analytical depth (i.e., the number of proteins that can be identified). Still, it is important to connect a protein identification back to the  $m/z$  feature measured in the IMS experiment. To affect this, we subjected the proteins extracted from the MALDI matrix layer to direct top-down LC-MS/MS and confidently identified 124 proteins in this way from four human tissues. To the best of our knowledge, it is the first time that this has been successfully demonstrated on MALDI IMS samples. The top-down analysis has been greatly aided by the shortlist of proteins identified from the tissues and assembled in the subsetDB and by employing a very high performance mass spectrometer (36). This subsetDB is 4x smaller than Swissprot and 15x smaller than the original human IPI database commonly used in proteomics (37) and thus vastly reduces the number of proteins that have to be considered in a database search and increases the confidence in peptide identification scores. Because no protease can be specified in the search, the very high mass accuracy afforded by the mass spectrometer (single digit ppm) for intact peptides as well as fragment ions together with the small sequence database enables searching the data in a reasonable time and reduces the number of possible false positive identifications (38).

An interesting observation from our literature survey and the top-down analysis is that the majority of the IMS biomarkers reported in the literature were detected as full length, unmodified proteins while the top-down analysis clearly showed that many of the  $m/z$  species in the matrix layer are fragments of much larger proteins (Fig. 4D) or post-translationally modified (Fig. 4A). This has been generally overlooked in the past because these fragments could technically not

be readily identified. It further suggests that many more IMS biomarkers could now be identified without the common 'educated molecular weight guessing' based on the simple (but often invalid) assumption that a protein would be detected as an intact, unmodified molecule. Recent evidence suggests that the fragmented proteins are not the result of sample preparation and degradation artifacts but are genuine and discriminating surrogates of biological processes occurring in the respective tissue (25;27;39).

Despite the very considerable methodological progress represented by this study, there are areas that should be improved. First, the typical productive range of MALDI IMS for the detection of biomarkers is about 25 kDa yet our top-down analysis so far only extends to 12 kDa. This is not a principle limit of the method and we indeed detected larger precursor ions in the primary mass spectrometry data, but the current sensitivity of the LC-MS/MS system precluded their identification by tandem mass spectrometry. Second, by recovering proteins from the MALDI matrix layer, the spatial context, i.e. the x-y coordinates at which the MALDI IMS experiment detected a particular protein within the tissue is lost. It would obviously be highly desirable if this information could be retained. Using laser capture microdissection (LCM) (40) or more elegant local extraction of the matrix (41) should improve this but, again, the sensitivity of current LC-MS/MS systems cannot yet analyze such material.

Overall, we believe that the method proposed in this study is a very significant improvement over current practice in the field as it removes the protein identification issue as perhaps the most disabling factor in MALDI IMS. The bottom-up identification of proteins from the MALDI matrix layer is generic, very easily performed and the database MaTisse in which all the information of our study as well as that of the literature is assembled, constitutes a valuable database for the identification of biomarkers. In fact, we recommend including this step in any MALDI IMS study to define the set of proteins from which a biomarker will eventually be drawn. The top-down approach is technically somewhat more demanding but as more and more high

performance mass spectrometry systems become available, this crucial step will also become more easily taken than previously possible. Using this or similar future approaches, we think that more and more IMS biomarkers will be furnished with a protein name from now on which will be of tremendous value in validating and developing these molecules for use in basic research as well as the clinic.

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## Figure Legends

Figure 1: Extraction of proteins from MALDI IMS slides. A) the sinapinic acid matrix layer of a ready-to-measure MALDI IMS microscopy slide (here prepared from normal stomach tissue is removed by a two-step extraction procedure using 7.5% and 60% acetonitrile in 0.2% tri-fluoro acetic acid (TFA). The remaining tissue is recovered by means of a scalpel (see also Supplemental Figure 1 A) B) silver stained SDS PAGE of the proteins recovered from the microscopy slide showing that the acetonitrile extractions primarily recover low molecular weight proteins (see also Supplemental Figure 1 B).

Figure 2: Bottom-up proteomic characterization of MALDI IMS samples. A) protein identification summary of ten human tissues. B) cumulated number of identified proteins from the ten different tissues and three protein extractions. Samples are ordered such that the highest number of identifications is obtained for the smallest number of samples combined. C) distribution of identified proteins by molecular weight. Bins were chosen according to the marker used in the SDS PAGE shown in Figure 1B. Blue bars represent the distribution of entries in the Uniprot database (human only). D) distribution of proteins by cellular compartment.

Figure 3: Relative abundance of proteins identified in MALDI IMS. A) Proteins from all 10 human tissues were ranked according to the number of assigned spectra divided by the molecular weight of the proteins' database entry. MALDI IMS markers extracted from the literature (see also Table 1 and Supplemental Table 5) are indicated in orange showing that these are mainly abundant cellular proteins. B) Proteins identified from the MALDI matrix layer plotted against the ranked protein abundance used in panel A).

Figure 4: Top-down protein identification. A) charge deconvoluted and deisotoped tandem mass spectrum of intact and acetylated thymosin beta-4. (only b and y ions are shown, for a completely annotated spectrum see Supplemental Fig. 3). B) comparison of top-down protein

identifications made by searching the human IPI database (87,061 entires) or subsetDB (5,455 entires) showing that almost all proteins can be identified in the 15x smaller subsetDB. C) distribution of precursor ion masses leading to a successful top-down identification. D) normalized sequence coverage plot of top-down identified proteins in which each line represents one protein. The darker the grey scale density, the more spectra matched to the respective region of the protein indicating that most proteins identified from MALDI IMS samples are fragments of the original protein sequence.

Table 1: Proteins previously reported in published MALDI IMS studies and their frequency of observation in the present study. The maximum number of observations 'in extracts' is 20 (i.e. 10 human samples, two acetonitrile extractions each) and 10 'in tissue'. The column 'reference' points to the publication in which the respective protein was originally identified.

## Tables

Table 1

Gene name	UniProtKB entry	Frequency of observations		Reference
		in extracts	in tissue	
AGR3	Q8TD06	6	5	26
ATP5J	P18859	3	4	20
B2M	P61769	18	9	20
CALM1	P62158	16	9	20
CFL1	P23528	20	10	20
COX17	Q14061	7	1	20
COX5A	P20674	13	10	20
COX5B	P10606	13	7	18
COX6C	P09669	18	10	18, 20
COX7A2	P14406	5	9	13, 18
COX7C	P15954	4	8	18
COX8A	P10176	0	0	18
CRIP1	P50238	13	6	14, 16
CSTB	P04080	18	10	20
CYCS	P99999	20	10	18, 20
DBI	P07108	15	9	13, 24
DCD	P81605	12	10	20
DEFA1	P59665	18	10	14, 17, 20, 26
DYNLRB1	Q9NP97	13	9	20
E2F3	Q499G5	0	0	20
FXYD3	Q14802	0	0	28
GSTM3	P21266	0	9	28
HBA1	P69905	20	10	20, 26, 30
HBB	P68871	20	10	20, 26, 30
HINT1	P49773	18	10	20
H2BFF	P33778	18	9	24
HIST1H4A	P62805	20	10	20, 17
HSPE1	P61604	20	10	20
LGALS1	P09382	18	10	20
LSM6	P62312	9	9	20
LYZ	P61626	16	9	20, 26
MAP3K2	Q9Y2U5	0	0	25
MBP	P02686	0	0	24
MIF	P14174	20	10	18, 20
MT2A	P02795	8	4	20
MTPN	P58546	10	10	20
NDUFA4	O00483	9	10	18
NEDD8	Q15843	4	9	20
NME1	Q32Q12	17	10	20
PFN1	P07737	20	10	20
PSME1	Q06323	20	10	27
RPL22	P35268	15	10	20
RPLP2	P05387	12	10	20
RPS21	P63220	15	10	20
RPS27A	P62979	20	10	20, 24
RPS28	P62857	18	10	20
S100A1	P23297	1	3	20
S100A10	P60903	11	9	13, 24, 29
S100A11	P31949	20	10	20, 24, 28
S100A6	P06703	19	10	14, 13, 18, 20, 29
S100A8	P05109	16	9	20, 26
S100A9	P06702	15	9	20, 26
S100B	P04271	1	1	20
TAGLN2	P37802	20	10	13
TBCA	O75347	17	9	20
TIMM8A	O60220	5	6	20
TMSB4X	P62328	10	5	24
TXN	P10599	20	10	13, 20, 29
TMSB10	P63313	9	4	18
UQCRH	P07919	10	8	18

## Figures

Figure 1

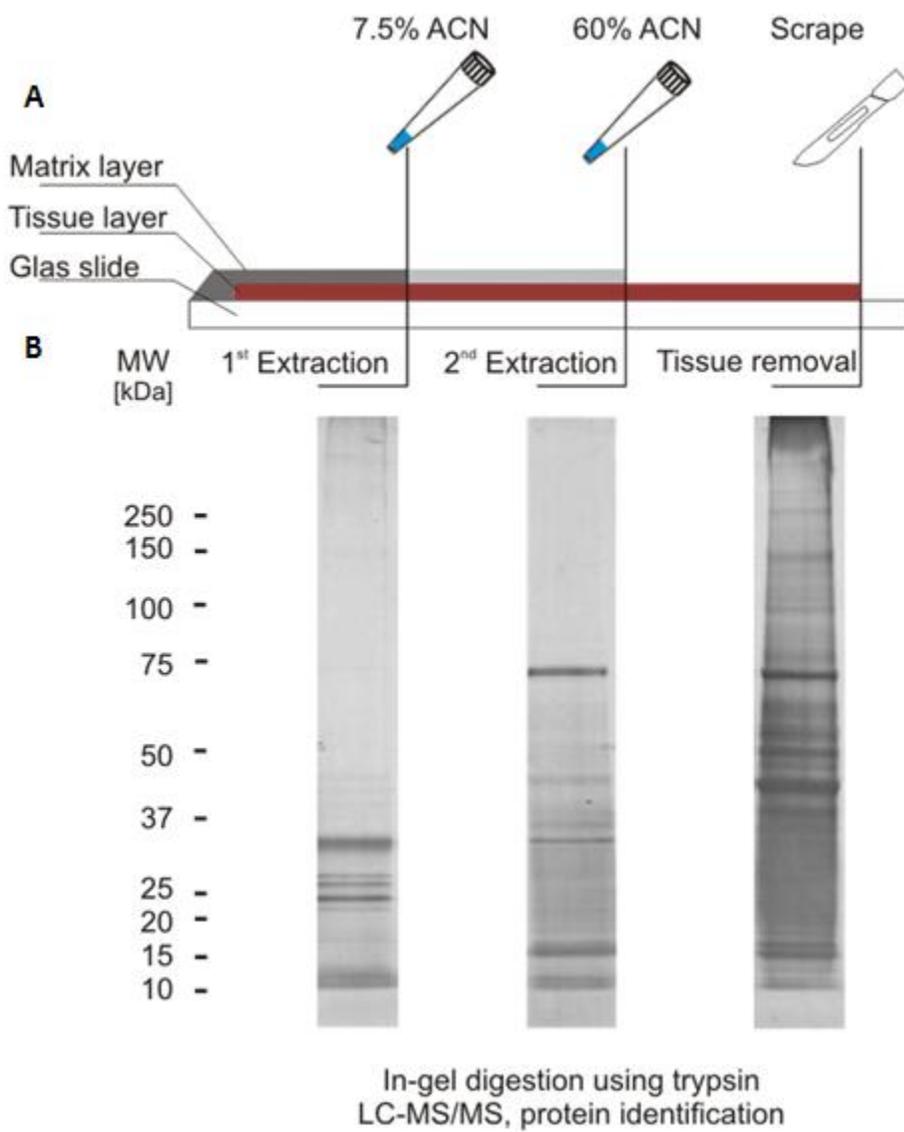
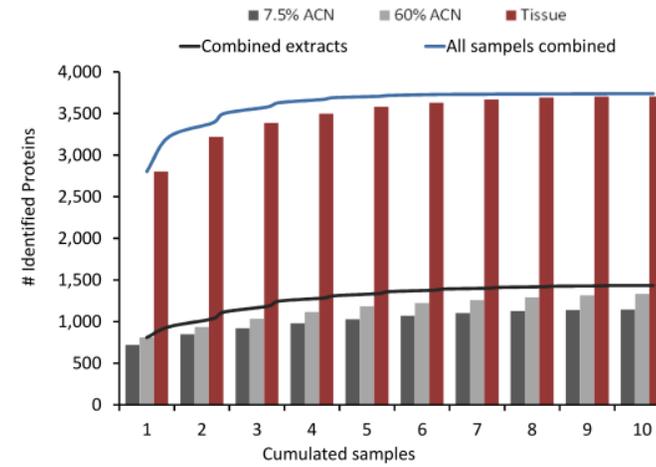


Figure 2

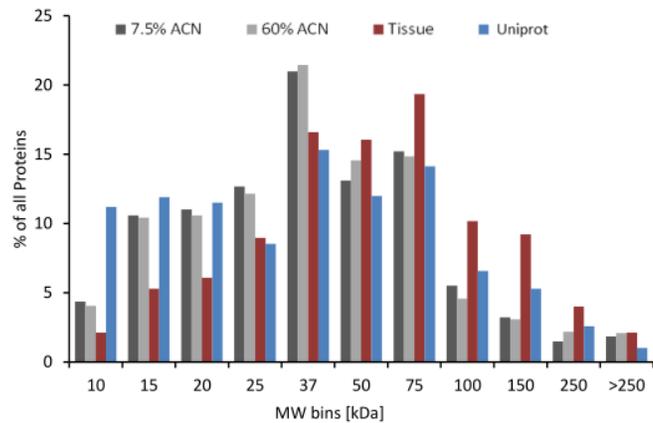
**A**

Identifications per single tissue	7.5% ACN	60.0% ACN	Tissue
Esophagus (carcinoma)	414	319	1333
Stomach (normal tissue)	314	360	1510
Stomach (carcinoma)	427	684	2532
Colon (mucosa)	293	501	2316
Colon (muscle)	283	344	1389
Colon (adenoma)	720	809	2802
Colon (carcinoma)	554	701	2315
Mamma (carcinoma I)	492	396	2474
Mamma (carcinoma II)	441	458	2523
Osteosarcoma	327	501	2178
<b>Combined results</b>			
Average	428	689	2137
Cumulated	1144	1333	3709
Combined Extracts	1435		
Exclusive	37		2268
<b>Total number of IDs</b>			<b>3738</b>

**B**



**C**



**D**

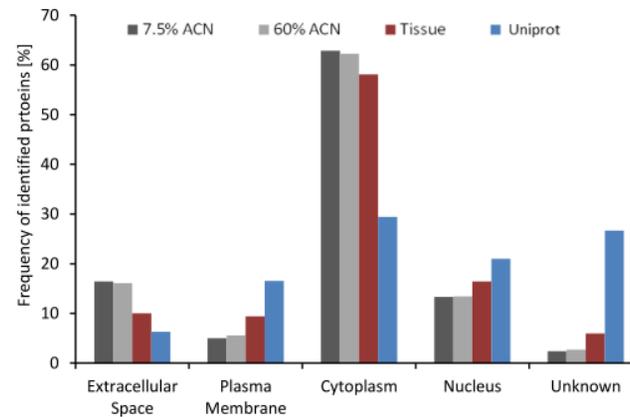


Figure 3

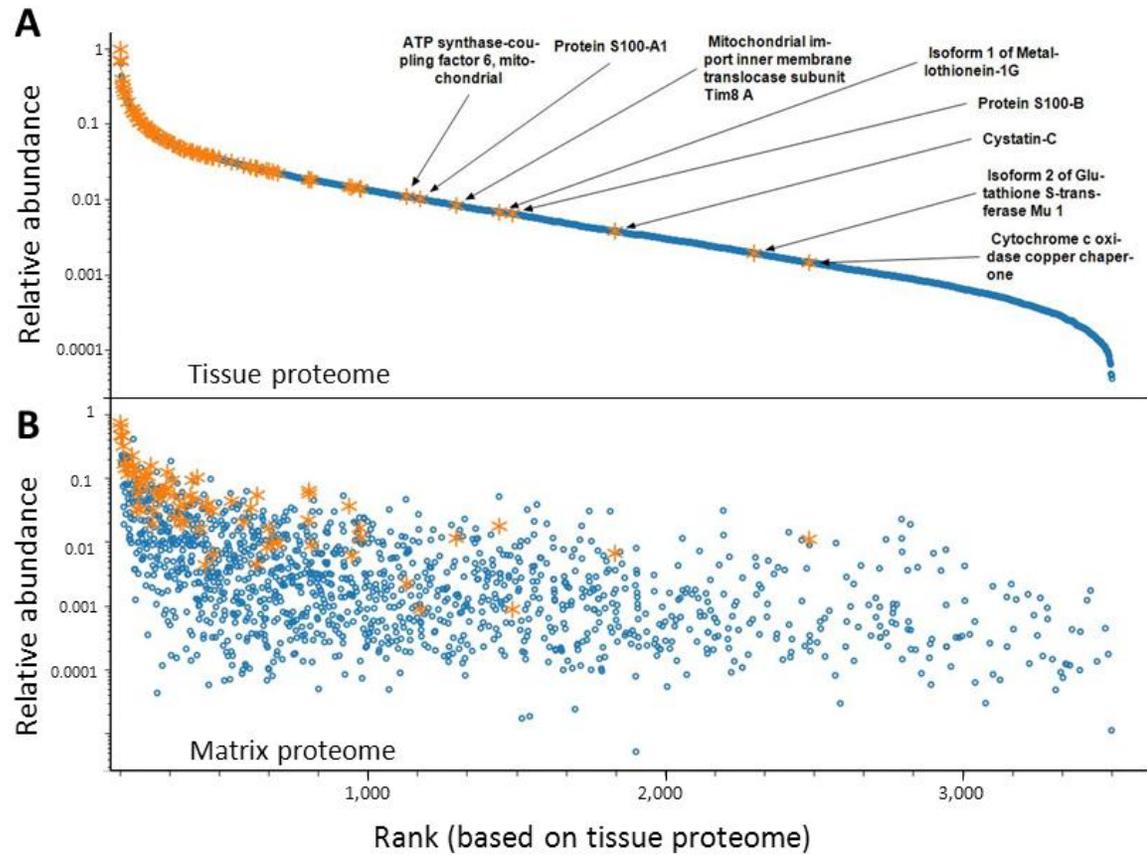


Figure 4

