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Protein Identification in Imaging Mass Spectrometry through Spatially Targeted Liquid Micro-Extractions

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Abstract

Rationale—Liquid extraction surface analysis (LESA) can be used to generate spatially-directed protein identifications in an imaging mass spectrometry (IMS) workflow. This approach involves the use of robotic micro-extractions coupled to online liquid chromatography (LC). We have characterized the extraction efficiency of this method as well as its ability to identify proteins from a matrix assisted laser/desorption ionization (MALDI) IMS experiment.

Methods—Proteins and peptides were extracted from transverse sections of a rat brain and sagittal sections of a mouse pup using liquid surface extractions. Extracts were either analyzed by online LC coupled to a high mass resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometer or collected offline and analyzed by traditional LC-MS methods. Identifications were made using both top-down and bottom-up methodologies. MALDI images were acquired on a 15T FTICR MS at 125 μ m spatial resolution.

Results—Robotic liquid surface extractions are reproducible across various tissue types, providing significantly improved spatial resolution, with respect to extractions, while still allowing for a robust number of protein identifications. A single 2 μ L extract can identify over 14,000 peptides with little sample preparation, increasing throughput for spatially targeted workflows. Surface extractions from tissue were coupled directly to LC to gather spatially relevant proteomics data.

Conclusions—Robotic liquid surface extractions can be used to interrogate discrete regions of tissue to provide protein identifications with high throughput, accuracy, and robustness. The direct coupling of tissue surface extractions and liquid chromatography, offers a new and effective approach to provide spatial proteomics data in an imaging experiment.

Keywords

Liquid surface extractions; proteomics; MALDI IMS; top-down mass spectrometry; bottom-up mass spectrometry

Introduction

Since the emergence of matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS)¹, the field has seen tremendous growth in a diverse range of research areas including the proteomic study of diseased tissue²⁻³, drug development⁴⁻⁵, and biomarker detection.⁶⁻⁷ IMS analysis relies on the systematic interrogation of a sample, generally a thin tissue section, to generate individual mass spectra at discrete x,y- positions. This process gives rise to the spatial mapping of thousands of proteins, lipids, and small molecules within a biological sample.⁸

Molecular identification in an IMS experiment is crucial in determining the physiological role of the detected analytes in the system being studied. Typically, protein IMS experiments have been carried out using time-of-flight (TOF) instruments due to the platform's high sensitivity over a broad mass range, high data acquisition speeds, and relatively low cost. However, identifying proteins during MALDI-TOF IMS analyses can be challenging. Ideally, protein ions would be analyzed and sequenced by tandem mass spectrometry (MS/MS or MSⁿ) directly from tissue. However, generating useful fragmentation data becomes difficult at the higher m/z values ($> \sim 3000$ Da). A second approach for identification during an imaging experiment is the use of high resolution accurate mass measurements to unambiguously identify the empirical chemical formula for the ion of interest. However, collecting accurate mass measurements using MALDI-TOF platforms is difficult because of their limited mass accuracy (typically >20 ppm) and lower mass resolving power, particularly for high m/z ions (i.e., $> m/z 3000$).⁹ Recently, high resolving power, high mass accuracy Fourier transform ion cyclotron resonance mass spectrometers (FTICR MS) have been used to help link identifications through accurate mass measurements to LC-MS/MS proteomics data and TOF-based imaging experiments.¹⁰⁻¹³ For example, in recent studies, we were able to detect intact proteins up to 20 kDa, with resolving powers of $\sim 75,000$ at $m/z 5,000$ and mass accuracies < 5 ppm.¹¹ Although progress has been made, the identification of proteins from tissue still remains a difficult task.

Due to the practical issues associated with fragmenting ions directly from tissue, protein identifications for MALDI IMS experiments are often achieved by performing secondary LC-based experiments to complement the IMS data.¹⁴ By identifying analytes in a secondary experiment, identifications can be correlated back to the imaging data through the matching of intact molecular weights.¹⁵⁻¹⁷ These complimentary experiments typically fall into 3 categories: 1) bulk tissue homogenization and protein extraction, 2) enzymatic digestion performed directly on tissue followed by MS/MS of the resulting peptides, and 3) spatially-targeted surface extractions of peptides or proteins. In the bulk homogenization experiment, the entire tissue or tissue section is homogenized, proteins are extracted and further subjected to bottom-up LC-MS/MS or top-down approaches.¹⁸ Although the more

common approach, bottom-up identifications are often difficult to correlate back to IMS data of intact proteins because the intact mass information, and post translational information is often lost during the bottom-up experiment. Also, all spatial information is lost during the homogenization process. Another approach is to perform on-tissue enzymatic digestions. An enzyme is applied to the sample in a way that minimizes analyte delocalization on the tissue surface, and proteins are sequenced directly using MALDI MS/MS.¹⁹ These experiments suffer from low throughput as they require additional sample manipulation, relative to intact protein analysis, and each peptide is usually sequenced individually. These bottom-up workflows are also susceptible to missing post translational modifications (PTM) information due to mass mismatches with the IMS data. Finally, spatially-directed approaches, such as enzyme-loaded hydrogels and liquid extraction surface analyses (LESA), allow for the interrogation of discrete regions of the tissue surface, retaining the spatial integrity of an IMS analysis. Hydrogels, small gel polymers that are loaded with trypsin and placed on a tissue for digestion and extraction of peptides, have been shown to be an efficient method of generating protein identifications but are difficult to manipulate due to their small size.^{20–22} LESA workflows involve the dispensing of small volumes of solvent onto the tissue surface, allowing for the diffusion of analytes into the liquid which can be followed by traditional proteomic workflows providing for a sensitive, high-throughput approach to performing spatially targeted proteomics experiments.²³

Liquid micro-extractions between a tissue surface and solvent droplet have been shown to produce robust LC-based proteomics results.²⁴ Schey et al. manually pipetted 1 μ L volumes of solvent onto a tissue surface and detected upwards of 100 intact protein signals by simply aspirating and re-dispensing the solvent on the tissue surface multiple times and then subjecting the extraction to top-down LC-MS/MS.²⁵ This approach is useful when attempting to study a tissue sample in a spatially targeted manner as the sample is kept intact while targeting discrete regions for analyses. A semi-automated approach uses a robotic pumping and positioning system to sample the surface of a substrate with greater accuracy and precision.^{26–31} It's ease of use, reproducibility, automated functionality, and ability to interrogate discrete regions of a sample has been demonstrated for analyzing several classes of biomolecules from a range of surfaces, including tissue, dried blood spots, food surfaces, and latent fingerprints.^{32–35} Previous studies have shown the capabilities and application of LESA, which utilized disposable capillary tips and enabled offline proteomics analysis of tissue extracts.^{26–29, 32–35} Recently, this liquid extraction technology has been modified to replace the disposable capillary with a glass capillary (LESAPLUS) allowing for increased droplet resolution on the sample surface. The capillary outlet is fed into a 6-port valve where the sample can be injected onto a column for online LC-MS analysis.³⁶

Herein, we report the coupling of robotic surface micro-extractions from thin tissue sections directly to LC-MS and LC-MS/MS using a glass capillary setup. Extractions are completed and taken offline for bottom-up MS analysis to assess the extraction process and determine its efficiency and robustness. This methodology provides improved reproducibility and droplet resolution when compared to previous methods using hand pipetting, and has been incorporated directly into online LC-based workflows. The enhanced micro-extraction process allows for the use of both bottom-up and top-down approaches, with a higher throughput than previous IMS protein identification workflows.

Experimental

Chemicals

Acetonitrile (ACN), acetic acid, formic acid (FA), trifluoroacetic acid (TFA), ethanol, ammonium bicarbonate, and chloroform were purchased from Fisher Scientific (Pittsburgh, PA, USA). 2,5-dihydroxyacetophenone (DHA), hematoxylin stain, aluminum potassium sulphate, glycerol, and mass spectrometry sequence-grade trypsin from porcine pancreas were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Tissue Preparation

One week old C57BL/6 control mice that had been stored at -80°C were shaved over dry ice to remove as much hair as possible to avoid contamination during sample preparations and IMS. Frozen rat brain was purchased from Pel-Freeze Biologicals (Rogers, AR, USA), and was stored at -80°C until sectioning. In all cases, tissue was sectioned ($12\text{ }\mu\text{m}$ thickness) at -15°C using a CryoStarTM NX70 Cryostat (Thermo Fisher Scientific, San Jose, CA, USA), thaw mounted onto conductive indium-tin-oxide coated slides (Delta Technologies), and dried in a vacuum desiccator for at least 20 minutes prior to preparation for analysis. To maximize sensitivity for proteins and peptides, tissue sections underwent a washing protocol to remove lipids and salts. The wash steps included 70% ethanol (30s), 100% ethanol (30s), Carnoy's Wash (6:3:1 ethanol:chloroform:acetic acid), 100% ethanol (30s), water (30s), and 100% ethanol (30s) as described previously.³⁷ Animal husbandry and experimental procedures were conducted in agreement with Public Health Service policy and approved by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

MALDI IMS

For imaging experiments, MALDI matrix (DHA) was applied to the sample using a robotic sprayer (TM Sprayer, HTX Technologies, Carrboro, NC, US) at a concentration of 15 mg/mL in 9:1, ACN:H₂O. The sprayer nozzle was set to spray at 80°C using a carrier solvent of 9:1 ACN:H₂O at a flow rate of 0.1 mL/min and a drying sheath gas of dry nitrogen set to 10 psi. Four passes of matrix were applied using alternating offsets (1 mm) and directional rotations (90 degrees) with a 2 mm track spacing. The spray velocity was set to 1100 mm/min with a 2 s dry time between passes and 40 mm nozzle height. The matrix layer on the sample was recrystallized prior to MALDI analysis as previously described using 1.0 mL of 1:1, TFA:H₂O at 37°C for 3 minutes.³⁸ The image was acquired in positive ion mode at $125\text{ }\mu\text{m}$ spatial resolution on a Bruker Solarix 15T FTICR MS (Bruker Daltonics, Billerica, MA, USA). The instrument employs a Smartbeam II 2 kHz frequency tripled Nd:YAG (355 nm) laser, as well as an Apollo II dual MALDI/ESI ion source. Each pixel was the sum of 2000 laser shots, using the smallest laser focus ($\sim 50\text{ }\mu\text{m}$), while random-walking the target within the $125\text{ }\mu\text{m}$ pixel. The mass spectrometer was externally calibrated prior to analysis using a protein mixture (insulin, cytochrome C, trypsinogen, and apomyoglobin). Data were collected from m/z 1,385 - 20,000 with a time-domain file size of 512K (FID length: 1.6078 s), yielding a resolving power of $\sim 42,000$ at m/z 5000. In order to generate an image with a higher mass range, the ion optics were tuned as follows: accumulation hexapole (1.4 MHz, 1700 Vpp), time-of-flight delay (2.1 ms), funnel RF

amplitude (200 Vpp), transfer optics (2 MHz, 380 Vpp), and ICR cell (sweep excitation power: 40%).

On-Tissue Tryptic Digestion

Trypsin was dissolved into 333 μL of ammonium bicarbonate (100 mM, pH~8.50), 67 μL of acetic acid (100 mM), and 40 μL of acetonitrile to a final concentration of 0.045 $\mu\text{g}/\mu\text{L}$. The sample was sprayed at a temperature of 30 $^{\circ}\text{C}$ using the TM Sprayer for 8 total passes using alternating directional rotations and offsets. The sprayer used a flow rate of 0.0075 mL/min, with a 2 mm track spacing, a nozzle height of 40 mm, and a velocity of 700 mm/min using a syringe pump.¹⁶ Immediately following trypsin application, samples were left to digest overnight at 37 $^{\circ}\text{C}$ in a covered dish that contained 3 mL of 100 mM ammonium bicarbonate.

Tissue Extractions

Tissue extractions were completed using the TriVersa NanoMate (Advion, Inc., Ithaca, NY, USA) modified to include a glass capillary (LESAPlus) for improved spatial resolution and online integration with LC-based experiments.³⁶ Scanned images of thaw-mounted samples were uploaded to the ChipSoft Software (Advion, Inc., Ithaca, NY, USA) to allow histological regions of interest to be selected for analysis. For online LC-MS analysis of intact proteins, extractions were completed by aspirating 3 μL of extraction solvent (1:1 ACN:H₂O with 0.5% FA) and dispensing 2 μL on tissue. The dispensed extraction solvent was left on tissue for 15 s prior to re-aspiration back into the capillary. This process was repeated 5 times, after which the capillary was moved back to the solvent well to draw enough solvent to load the sample into the sample loop (~6 μL volume) on a 6-port HPLC valve. All extractions were completed by dispensing the volume from the capillary at a height of 0.5 mm above the surface of the sample. For top-down experiments not directly coupled to the LC, extractions were completed in the same manner but deposited into a single well of a 96-well plate that contained 15 μL of H₂O (0.5% FA). The contents of that well were then injected onto the column immediately for LC-MS analysis (see below for details).

For peptide extractions, on-tissue tryptic digestion was performed as described above. For all bottom-up offline experiments, a combination of 1:1 ACN:H₂O (0.5% FA) was used as the extraction solvent. Extractions were all completed using an initial aspirated volume of 3.0 μL while varying the total amount dispensed on tissue. Instead of being pulled into the 6-port valve for the dispensing/aspirating, the aliquots were deposited into a single well of a 96-well plate that contained 100 μL of H₂O (0.5% FA). Samples were dried down using a desktop vacuum centrifuge (Thermo Fisher Scientific, San Jose, CA, USA) and stored at -80 $^{\circ}\text{C}$ until analysis. For the droplet resolution experiments, hematoxylin stain was used for the extraction solvent as the liquid can penetrate the cell nucleus, allowing for visualization of solvent diffusion and measurement of extraction spot size on rat brain tissue. Extraction solvents were able to interact with the surface for 30 seconds and repeated twice to ensure the stain had enough time to penetrate the cells. Additionally, extraction spot size measurements were made by dispensing varying volumes of solvent onto water sensitive paper (Rittenhouse, St. Catherines, Ontario, CA). Following spot size experiments, the tissue

sections or water sensitive paper were left to dry under vacuum and spot size measurements were made using an optical microscope (Olympus, Center Valley, PA, USA).

Proteomic Workflows

All of the offline top-down and bottom-up LC-MS/MS data were collected using Orbitrap platforms (Thermo Scientific, San Jose, CA, USA) and were completed using standard approaches as described in supplemental information. For the online LC-MS experiments, proteins were eluted using an analytical column which was packed with 20 cm of C4 reverse phase material (Halo Protein C4, 3.4 μm , 400Å) with a laser-pulled emitter tip. Proteins were loaded on the capillary reverse phase analytical column (360 μm O.D. $\times$ 150 μm I.D.) using a Waters nanoACQUITY UPLC (Waters Corporations, Milford, MA, USA) where mobile phase A consisted of 0.1% formic acid, 99.99% water, and mobile phase B consisted of 0.1% formic acid, 99.99% acetonitrile, eluting at 0.600 $\mu\text{L}/\text{min}$. Ions were generated using a Bruker Captive Spray nanoelectrospray source (Bruker Daltonics, Billerica, MA, USA) and directed into a Bruker Solarix 15T FTICR MS (Bruker Daltonics, Billerica, MA, USA). The mass spectrometer was set to scan from m/z 230-2,000, with a file size of 1M yielding a resolving power of 150,000 at m/z 400 (FID length: 0.5243 s). Ion optics were tuned as follows: accumulation hexapole (2 MHz, 1200 Vpp), time-of-flight delay (0.8 ms), funnel RF amplitude (280 Vpp), transfer optics (4 MHz, 290 Vpp), and ICR cell (sweep excitation power: 18%).

Results/Discussion

Surface Extraction Performance

In order to visualize the effect of solvent composition on droplet resolution, various combinations of solvents were tested using an increasing volume of extraction solvent while dispensing onto water sensitive paper using the LESApplus platform (Figure 1 and Supplemental Table 1). Three replicates were measured to assess the reproducibility of the droplet diameters. For 100% H_2O , the average extraction volume diameter on water sensitive paper is below 600 μm for the smallest extraction volume (0.5 μL), ~70% smaller than previous efforts using manual pipetting.²⁵ We also see an improvement in droplet resolution compared to the older, air-infused mandrel for the LESA experiments. The prior setup yielded an average spot size of 2.2 ± 0.04 mm dispensing 1:1 ACN: H_2O (~50% decrease in resolution). However, the spot size increases significantly with increasing percentages of organic solvents and extraction volume. LESA droplet sizes were also assessed on tissue using hematoxylin stain (Supplemental Table 1). The values measured from the on-tissue experiment are slightly larger than their water sensitive paper counterparts. This is mainly due to the water-sensitive paper being more hydrophobic than the tissue, allowing the surface tension of the liquid to maintain a smaller droplet profile. Even though the dispensing/aspiration step was held for twice as long as the water sensitive paper tests in order to allow for the stain to penetrate the cells, no change in diameter was observed using the increased dispensing time for the on-tissue measurements. The spot size measurements from tissue were 1.23 mm (5.6% RSD), 1.78 mm (4.5% RSD), 2.29 mm (2.2% RSD), and 2.55 mm (3.2% RSD) for 0.5, 1.0, 1.5, and 2.0 μL extraction volumes,

respectively. These results demonstrate the reproducibility of a LESA experiment, and improvements compared to similar techniques, with regards to droplet resolution.

In order to determine the reproducibility of the proteomic extraction from the tissue using the glass capillary mandrel, peptide extractions were performed from rat brain cerebellum following on-tissue proteolytic digestion using trypsin. A single, 2 μ L volume was dispensed/aspirated 5 times (75 seconds total) onto the tissue surface, and subjected to offline, bottom-up LC-MS/MS where an average of 1620 proteins were identified across the 5 replicates. These results showed that the extraction was extremely reproducible with a relative standard deviation of 2.7% (1st Replicate: 1560 unique proteins, 2nd Replicate: 1661 unique proteins, 3rd Replicate: 1615 unique proteins, 4th Replicate: 1603 unique proteins, 5th Replicate: 1662 unique proteins) between the 5 samples. It is also important to maintain sensitivity at decreasing spot size in order to allow for the interrogation of smaller foci. The overall sensitivity and reproducibility of the LESAplus platform was assessed by measuring the total number of proteins identified and peptides observed from tissue as a function of extraction volume. Supplemental Figure 1 highlights the results from triplicate surface extractions that were completed using 2.0, 1.5, 1.0, and 0.5 μ L volumes of 1:1 ACN: H₂O (0.5% FA) from the cerebellum of a rat brain that had been digested overnight using trypsin. This solvent composition was selected for these experiments based on previous tissue extraction experiments in our lab. The total number of peptides observed across the interrogated volumes, from largest to smallest, were 8475 ± 331 (2.0 μ L, RSD 3.9%), 8361 ± 361 (1.5 μ L, RSD 4.3%), 7791 ± 568 (1.0 μ L, RSD 7.3%), and 4837 ± 933 (0.5 μ L, RSD 19.3%) peptides. The total number of protein identifications were found to be 1165 ± 55 (RSD 4.7%), 1175 ± 43 (RSD 3.7% RSD), 1119 ± 48 (RSD 4.3%), and 860 ± 104 (RSD 12.1%) as LESA volumes are varied from 2.0-0.5 μ L. The relative standard deviations across both the peptide and protein replicates were found to be quite low for most volumes (RSD < 5%), with even the smallest droplet size experiments (0.5 μ L) having an RSD of just over 12%.

One of the advantages of utilizing an automated liquid surface extraction platform is the ability to increase throughput, minimizing sample preparation time and allowing for multiple extraction experiments to be completed in a single experiment. Comparing the LESA experiment to a similar, spatially-targeted technology such as hydrogels highlights the increase in throughput achieved. Whereas the typical hydrogel experiment takes ~2-2.5 days to complete, the LESA workflow takes ~1 day for bottom-up and ~2 hours for top-down analysis. A hydrogel experiment requires the casting of the gel (~16 hours), reswelling and gel placement (~1-2 hours), digestion (~14 hours), and extraction and LC-MS/MS analysis (~1-2 hours). The bottom-up LESA experiment can be completed in ~16 hours total. This includes the overnight digestion and LC-MS/MS runs. The number of identifications are comparable to other spatially targeted approaches such as hydrogels. A hydrogel fabricated with a diameter of 1.66 mm has been shown to be capable of identifying 1052 proteins, while the LESA experiment at 1.0 μ L (~1.71 mm diameter measured previously) identified 1119 proteins.²⁰ The top-down experiments can be completed in as little as 1-2 hours, where the majority of the time is devoted to LC-MS/MS analysis, as the tissue washes and extractions only take ~10 minutes to complete. For both bottom-up LESA methodologies, the analyte extraction steps require minimal time (~10 minutes) relative to the digestion

(overnight) and LC-MS/MS (~1-2 hour) steps. This improved throughput allows multiple LESA experiments to be performed rapidly, enabling the generation of multiple extractions and analyses from a single tissue section.

LESA also has the advantage of allowing for sequential surface extractions to effectively 'build-up' analyte concentrations enabling both detection and structural identification by MS/MS even for low abundance species. This is particularly important for tissue analysis where the abundance of analytes can vary orders of magnitude across the surface. To determine the total amounts of extractable proteins gathered in sequential liquid surface extractions, peptides were extracted from the cerebral cortex of a trypsin-digested rat brain. A single 2 μ L extraction was gathered from the cerebral cortex and collected for offline LC analysis. Following this, the tissue was allowed to dry and another extraction was then collected from the same spot and analyzed separately. This process was repeated 5 times and the total peptide and protein identification counts were compared across each subsequent extraction (Figure 2). It is evident that after the initial extraction, the losses in both peptides observed and proteins identified are substantial. Between the 1st and 2nd extractions there is a decrease of approximately 30% and 42% in the peptides and proteins identified, respectively. By the 5th extraction a total decrease of 96% (peptides) and 98% (proteins) from the 1st extraction is observed. Between the 1st and last extraction, there are no new species being detected, only a loss in identifications of proteins detected in the prior runs. As there are no new species being detected, the results suggest that after the 2nd extraction from the same spot the results are diminishing, and combining subsequent extractions after this is unnecessary. However, the ability to perform multiple extractions at the same spot is important for improving sensitivity and generating sufficient signal to produce quality MS/MS data from liquid surface extractions.

Correlating IMS with LESApplus Protein Experiments

Coupling LESApplus with LC-based experiments provides a high throughput method for generating spatially-targeted proteomics data for the various proteins and proteoforms observed in imaging MS experiments by minimizing sample preparation and time between experiments. In order to determine the efficacy of coupling LESA directly to LC-MS/MS to analyze intact proteins from tissue, full body tissue sections of mouse pup were prepared. Full body tissue sections serve as an excellent sample to test this spatially-targeted workflow because there are many large heterogeneous substructures in the tissue that can be easily targeted for analysis using the LESApplus system without the risk of collecting data from overlapping regions.

A full body protein image from a mouse pup was acquired on a 15T FTICR MS at 125 μ m spatial resolution. The data showed a number of unique protein distributions, including m/z 4,898.57, m/z 5675.21, m/z 6,628.37, and m/z 7,513.88 (Supplemental Figure 2). MALDI FTICR protein data provide the spectral quality necessary to fully resolve the isotopic envelopes and provide high mass accuracy for the observed ions. Based on these results, the cerebellum, cerebral cortex, and kidney were chosen for interrogation using spatially targeted surface extractions. Adjacent serial tissue sections of the mouse pup were used for LESApplus extractions for both bottom-up and top-down experiments. For the bottom-up

analysis, two separate, 2 μ L surface extractions were combine for each target area. The aliquots were dried down and reconstituted into 10 μ L of H₂O (0.5% FA) prior to analysis. The results are presented in Figure 3 and Table 1 using a Venn diagram to compare the total number of protein identifications and a table highlighting some of the proteins identified that are unique to the area sampled. Both the cerebellum and cerebral cortex extractions yielded a large number of identifications proteins respectively, many of which were identified in both substructures of the brain (1536). Interestingly, the kidney produced far fewer protein ID's than the other two areas sampled.

Spatially targeted liquid surface extractions were also used to generate top-down data from full body mouse pup tissue sections. Here, two separate 2 μ L surface extractions were combined for each target area prior to offline LC-MS/MS analysis. Top-down results were initially processed using the proteomics search engine Byonic optimized for ETD data. The results from these searches identified 27, 33, and 38 intact proteins from the kidney, cerebral cortex, and cerebellum, respectively, out of a total of 81, 69, and 72 intact protein species detected during LC-MS/MS analysis. Based on these results, selected proteins were then chosen for de novo manual interpretation guided by the database results to improve overall sequence coverage (Figure 4). Proteins detected in the imaging experiment were correlated to identifications from the top-down data by matching the intact accurate masses of the two experiments to within 2 ppm. The ion at m/z 4,936.540 was determined to be N-term Acetylated Thymosin β 10 (0.36 ppm), a protein that has been shown to bind and sequester G-actin (Figure 4D).³⁹ Thymosin β 10 is a known to be abundant in brain tissue and was found localized mainly to cerebral cortex (Figure 4C).⁴⁰ The ion at m/z 5707.118 was determined to be ATP synthase subunit ϵ (-1.08 ppm). ATP Synthase is a general mitochondrial protein responsible for converting ADP to the ATP (Figure 4F) and can be localized to many different tissue types.^{41–42} This protein was found to be localized to all 3 targeted regions (Figure 4E). The ion observed at m/z 7023.974 in the MALDI IMS data was identified as the N-terminally acetylated form of Histone H2A (-0.56 ppm) (Figure 4H). This ion was found to be localized in the cerebellum and kidney (Figure 4G). Histone H2A plays an important role in the folding of DNA in chromatin⁴³ and is known to be elevated in tissue that has undergone trauma or disease.⁴⁴ Here it was found localized to the cerebellum and kidney regions. The dimethylated form of hemoglobin subunit A was determined to be the ion at m/z 7491.884 (-1.41 ppm) and can be seen localized to the kidney (Figures 4I and 4J). Subunit A is one of the 4 major subunits that make up hemoglobin, an important oxygen-transporting metalloprotein found in red blood cells, which are produced in the kidney.^{45–46} The mass range of the proteins identified span from 4,500 to 18,000 Da. From the 4 proteins identified by top-down sequencing, 3 were found to be correlated to the 3 regions subjected for bottom-up mass spectrometry in the prior section. Thymosin beta-10 was not found to be in any of the bottom-up extractions.

A unique advantage of the LESApplus system is the ability to perform online LC-MS experiments with a surface extraction. This feature maximizes throughput and minimizes potential sample loss during preparation steps. In order to test the performance of this methodology, intact proteins were extracted from the same three target areas mentioned above (cerebellum, cerebral cortex, and kidney) within the full body mouse pup tissue section and subjected to online LC-MS in triplicate. A single 2 μ L extract was gathered by

dispensing the extraction volume five times (15 s each) onto the tissue surface and then automatically injecting it onto the sample loop directly. The total number of proteins detected for each run ($S/N > 3$) was determined using the deconvoluted average mass spectra over the entire LC experiment (select chromatograms for each target area are depicted in Supplemental Figure 3). From the cerebellum a total of 60 ± 6 proteins were detected while the kidney and cerebral cortex yielded 57 ± 13 , and 52 ± 15 respectively. Though the relatively slow scan time of the FTICR prevented LC-MS/MS identification, the number of protein species detected are comparable to previous methods using hand pipetting.²⁵ Additionally, although previous studies have demonstrated online LC-based analysis from liquid surface extractions using home-built systems,^{30–31} this is the first example of this capability being demonstrated for proteins using the commercial LESAplus platform. Future studies will examine implementing this online workflow on a MS platform more amenable to top-down LC-MS/MS experiments.

Conclusions

This work has demonstrated the capabilities of robotically-controlled, spatially-targeted liquid surface extractions coupled to online and offline proteomics workflows for identifying proteins observed in imaging mass spectrometry experiments. A number of experimental characteristics were measured to assess the efficiency of the LESAplus extraction including extraction spot size, number of peptides detected, and number of proteins identified across multiple experiments. Utilization of a glass capillary for extractions allows for improved droplet resolutions ($< 600 \mu\text{m}$ on water sensitive paper) and reproducibility (relative standard deviations of $< 5\%$) for most experiments. Comparisons of spatially-targeted proteomic techniques such as hydrogel experiments, laser capture laser microscopy (LCM), and tissue homogenization have been discussed elsewhere.²⁰ In these studies, 1.5 mm LCM, 1.5 mm tissue punch/digestion, and 1.67 mm hydrogel experiments yielded ~800-1000 protein identifications whereas the 1.0 μL LESA experiment (1.71 mm on tissue) described herein yielded ~1120 protein identifications. Although a modest improvement in the number of protein identifications, the LESAplus workflow is a significant improvement in throughput relative to other approaches. Hydrogels, tissue punches, and LCM are all time-consuming experiments, while the automated LESA extraction can generate samples, ready for LC-MS/MS analysis, in ~10 minutes. Additionally, the tissue is preserved during LESA experiments, allowing for sequential extractions that effectively improve the sensitivity of the technique. Finally, direct coupling of LESAplus to HPLC allows for the generation of online LC-MS data directly from tissue with minimal sample preparation. Using only a single 2 μL extraction, we were able to detect upwards of 60 proteins from various regions of tissue. Robotic liquid micro-extractions allow for a more reproducible approach to generating protein identifications to complement an IMS workflow. The combination of robotic extraction and online LC technologies allows for the rapid collection of spatially-targeted protein identifications that can be correlated to imaging MS data using accurate mass-matching. The spatial sampling precision, coupled with robustness and reproducibility, make the LESAplus technique more suitable for high-throughput spatial proteomic experiments than previous manual methodologies and will aid in the challenge of identifying proteins observed by MALDI IMS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) The measured droplet resolution of the LESAplus system on water-sensitive paper decreases as the volume dispensed increases and as the relative organic fraction of the solvent increases. B) The measured droplet resolution of the LESAplus system on rat brain tissue (cerebellum) using hematoxylin stain to visualize the solvent diffusion during extraction. For both experiments, droplet diameter measurements were made using bright field microscopy.

Figure 2.

Five LESA extractions gathered sequentially from the same spot on trypsin-digested rat brain that show a decrease in unique protein identifications and peptides identified through sequential extractions.

Figure 3.

Proteins identified from 3 regions of a mouse pup tissue section by combining 2 liquid surface extractions from the same spot. In total, 2879 proteins were identified from the cerebral cortex, 2290 proteins were identified from the cerebellum, and 602 proteins were identified from the kidney. The 3 regions tested displayed overlaps in the proteins identified as highlighted in the Venn diagram.

Figure 4.

A & B) Spectral and imaging data from a 125 μm spatial resolution MALDI protein image from a sagittal section of a mouse pup acquired on a 15T FTICR MS. Four ions were chosen to exemplify the many tissue substructures present as highlighted in the image overlay (B). The proteins sequenced below are highlighted by a star in the average mass spectrum of the protein image. C & D) The high resolution, top-down mass spectrum of m/z 4,898.57 allows for its identification as N-acetylated Thymosin β 10. E & F) The high resolution, top-down mass spectrum of m/z 5,675.21 allows for its identification as ATP synthase subunit ϵ . G &

H) The high resolution, top-down mass spectrum of m/z 6,628.37 allows for its identification as N-acetylated histone H2A Type 1. I & J) The high resolution, top-down mass spectrum of m/z 7,513.88 allows for its identification as dimethylated hemoglobin subunit A.

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Table 1

Selected proteins identified from LESApplus extracts that are unique to the cerebral cortex, cerebellum, and kidney in mouse pup full body tissue sections.

	Accession Number	Description
Kidney	Q91Y97	Fructose-bisphosphate aldolase B
	Q8CGP1	Histone H2B type 1-K
	Q8C196	Carbamoyl-phosphate synthase [ammonia]
	Q9JIL4	Na(+)/H(+) exchange regulatory cofactor NHE-RF3
	Q9QXD1	Peroxisomal acyl-coenzyme A oxidase 2
	P51667	Myosin regulatory light chain 2
	Q9QXD6	Fructose-1,6-bisphosphatase 1
	P52825	Carnitine O-palmitoyltransferase 2, mitochondrial
	Q64442	Sorbitol dehydrogenase
	P70694	Estradiol 17 beta-dehydrogenase 5
Cerebellum	Q8VHQ9	Acyl-coenzyme A thioesterase 11
	Q924X2	Carnitine O-palmitoyltransferase 1, muscle isoform
	Q62203	Splicing factor 3A subunit 2
	Q3UZA1	CapZ-interacting protein
	Q3TMP8	Trimeric intracellular cation channel type A
	Q9Z1E4	Glycogen [starch] synthase, muscle
	P84244	Histone H3.3
	Q9CY02	Alpha-hemoglobin-stabilizing protein
	P57722	Poly(rC)-binding protein 3
	Q9D783	Kelch-like protein 40
Cerebral Cortex	O35143	ATPase inhibitor, mitochondrial
	Q5QNQ9	Collagen alpha-1(XXVII) chain
	P68500	Contactin-5
	P62889	60S ribosomal protein L30
	O54901	OX-2 membrane glycoprotein
	Q91VJ2	Protein kinase C delta-binding protein
	O35668	Huntingtin-associated protein 1
	P55937	Golgin subfamily A member 3
	Q9WU84	Copper chaperone for superoxide dismutase
	Q91YE6	Importin-9