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Imaging Mass Spectrometry and Its Methodological Application to Murine Tissue

J.O.R. Gustafsson[1], S. R. McColl[2], P. Hoffmann[1]

[1]Adelaide Proteomics Centre, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005.
[2]Chemokine Laboratory, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005.

Corresponding author: Peter Hoffmann, Adelaide Proteomics Centre, Gate 8 Victoria Drive, Molecular Life Sciences Building Rm 1.49, University of Adelaide, Adelaide, South Australia 5005. Ph: (08) 8303 7521.

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Abstract

Imaging mass spectrometry (IMS) is an emerging technology, pioneered by Prof. Richard Caprioli's group starting more than a decade ago. In this study we have demonstrated the simplicity of initial technological set up for IMS experiments with commercially available automated matrix deposition, MALDI-TOF mass spectrometry instrumentation and data handling software for image generation. We have applied two different concepts of automated matrix deposition on Murine brain sections and discussed their different features and capabilities in IMS.

Key words: MALDI; mass spectrometry; imaging; tissue; methodology

Abbreviations: IMS, Imaging Mass Spectrometry

Introduction

Proteomics has seen the development of evermore complex and labour intensive methodologies and technologies, with increased qualitative and quantitative analytical power (Schmidt, Kellermann et al. 2005; Vanrobaeys, Van Coster et al. 2005; Mitulovic and Mechtler 2006). As the workhorses of proteomics, two-dimensional gels and high performance liquid chromatography (HPLC) allow exquisite protein/peptide separation and thus in depth proteome analysis in high throughput formats (Vanrobaeys, Van Coster et al. 2005). Such classical proteomics, however, require protein/peptide sample solubilisation and treatment (E.g. precipitation, purification, labelling) prior to separation and downstream analysis by mass spectrometry [MS] (Schmidt, Kellermann et al. 2005; Vanrobaeys, Van Coster et al. 2005). Furthermore, studies seeking diagnostic markers typically use bodily fluids as the basis for investigation, raising the possibility of multiple high abundance species complicating analysis. Spatial information is also lost when solubilisation and protein separation are applied to tissue samples. In this light, it is not surprising that almost a decade ago direct MS analysis on tissue sections was pioneered by Caprioli et al (Caprioli, Farmer etal. 1997; Chaurand, Norris et al. 2006). MS is now regularly being applied to *in situ* tissue analysis (Schwartz, Reyzer et al. 2003; Seeley, Oppenheimer et al. 2008; Stauber, Lemaire etal. 2008). Typically, tissue sections are washed in alcohol, dried and either a homogeneous layer or droplet array of matrix is applied (Schwartz, Reyzer et al. 2003). Direct MS desorption from the matrix co-crystallised tissue surface is then performed: predominantly with Matrix Assisted Laser Desorption/Ionisation (MALDI) sources (Seeley, Oppenheimer etal. 2008; Stauber, Lemaire et al. 2008). Prior knowledge of tissue composition is not necessary, hundreds if not thousands of masses can be measured rapidly and spatial distribution information is main-

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tained (Chaurand, Norris et al. 2006). To date, IMS has been applied in several biological systems including rodent (Schwartz, Reyzer et al. 2003; Baluya, Garrett et al. 2007; Groseclose, Andersson et al. 2007) and crustacean brain (Dekeyser, Kutz-Naber et al. 2007), rodent spinal cord (Monroe, Annangudi et al. 2008), liver (Seeley, Oppenheimer et al. 2008), testes, kidney (Herring, Oppenheimer et al. 2007) and skin (Bunch, Clench et al. 2004). Due to its relative infancy, IMS still requires optimisation of many of its component protocols, including those for section washing, effective and reproducible matrix deposition and processing the large volumes of data generated. Thus, although great progress has been made by several groups no standard IMS methodology has been defined.

With this is mind it was the objective to develop an IMS platform in our laboratory, focused on the imaging aspects of an IMS experiment prior to profiling applications for statistical model generation and potential diagnostic applications (Lemaire, Menguellet et al. 2007; Stauber, Lemaire et al. 2008). One of the most challenging aspects of IMS is the reproducible application of matrix on tissue sections. Three different commercial systems are currently available for automated matrix deposition, including the ImagePrep station (Bruker Daltonics), the CHIP-1000 printer (Shimadzu Biotechnology) and the Portrait 630 printer (Labcyte). The ImagePrep station uses vibrational vaporization to generate a matrix aerosol, which gently settles onto tissue samples. The CHIP-1000 and Portrait 630 are both picolitre range droplet printers capable of generating discrete matrix arrays on tissue sections. However, while the CHIP-1000 uses piezoelectric technology to forcefully eject droplets from a printer head, the Portrait 630 uses acoustic ejection of droplets from a liquid surface. We were able to successfully apply IMS to murine brain sections using both the CHIP-1000 and the ImagePrep in conjunction with a MALDI-TOF-MS instrument and image generation software. Under these conditions a comparison between the two instruments using similar sagittal murine brain sections is provided in this communication. Continued optimisation of key methodological steps is still needed, including maintenance of tissue integrity, section washes, matrix solvent composition and matrix deposition strategy. However, we have shown that the technology can be implemented with commercially available instrumentation in a relatively short timeframe. The potential of this technology for future application in biomarker discovery and diagnostics looks extremely promising.

Experimental Methods

Experimental Tissues and Solvents

Murine brain tissue was scavenged from on going experiments in the Chemokine laboratory at the University of Adelaide. All source experiments had full ethics approval from the University of Adelaide Ethics Committee. Solvents, acetonitrile (ACN) and methanol (MeOH) were HPLC grade (Merck, Damstadt, Germany). Ethanol (EtOH) and isopropanol (IPA) were analytical grade or higher (Merck). Trifluoroacetic acid (TFA) was purchased from Applied Biosystems (Foster City, CA). Sinapinic acid (SA) and peptide/protein standards (ClinProT standards, Bruker Daltonics) used to calibrate the Ultra Flex III MALDI-TOF/TOF mass spectrometer were purchased from Bruker Daltonics (Bremen, Germany).

Sample Preparation

Mice were sacrificed by carbon dioxide asphyxiation and tissues of interest were excised within ten minutes of sacrifice. Tissues were slow frozen in foil floated on liquid nitrogen (N2(l)) and transferred on dry ice to a -80°C freezer. Tissues were equilibrated to -20°C, followed by sectioning at -20°C (brain) or -18°C (other organs). Tissue was sectioned at a thickness of 10 ìm with 35 x 80 mm blades in a Shandon cryotome (Thermo Electron, Pittsburgh, PA). Sections were mounted onto pre-chilled Indium Tin Oxide (ITO) slides (Bruker Daltonics) using heat from the operators hand applied underneath the slide. Mounted sections were desiccated for 45 minutes prior to washes for 60 seconds in 100ml of 70% EtOH or IPA followed by 60 seconds submerged in 100ml of 100% EtOH or IPA. Following washes sections were desiccated for 15 minutes. Use of IPA in washes was an alteration to protocols due to published data (Seeley, Oppenheimer et al. 2008).

Matrix Deposition – CHIP-1000

The CHIP-1000 piezo electric printer (Shimadzu Biotechnology, Kyoto, Japan) was operated as per manufacturer's instructions to deposit 250 ìm centre to centre arrays of SA matrix directly onto tissue sections. Dwell voltage and dwell time were modulated as necessary to maintain the quality of droplets for deposition. Prior to printing the piezo printer head was flushed with several volumes of 50% IPA. Following printing the piezo printer head was flushed with 200 ìl of 50% IPA and the outside rinsed with 50% IPA (3x), 100% MeOH (3x) and 100% ultra pure water (Resistivity 18.2 MW-cm, TOC < 1ppb) (3x). SA matrix at 10 mg/ml concentration in 50% ACN, 44.8% ultra pure water, 5% IPA and 0.2% TFA was used. Number of droplets and layers was variable.

Matrix Deposition – ImagePrep

The ImagePrep station (Bruker Daltonics) was operated as per manufacturer's instructions to deposit homogeneous matrix layers onto tissue sections. SA at concentrations of 6, 8 and 10 mg/ml in 50% ACN, 5% IPA and 0.2% TFA were deposited using the default SA ImagePrep method (Bruker Daltonics). Microscopic slide images were captured using a Nikon pathology microscope connected to a digital camera.

MALDI-TOF Imaging Mass Spectrometry and Data Analysis

Slides for automated MALDI-TOF analysis were scanned at 1200 dpi using a Powerbook III PrePress Digital colour scanner (Amersham Biosciences, Soeborg, Denmark) controlled by Magic Scan software (version 4.6, UMAX). Slides were fitted into a Slide Adapter II MALDI target (Bruker Daltonics). An Ultra Flex III MALDITOF/ TOF mass spectrometer (Bruker Daltonics) operating in linear mode was used for IMS acquisition. Scanned slide images were loaded into Flex Imaging software (version 2.0, Bruker Daltonics), which was used to generate an auto execute sequence and set teach points for each individual IMS experiment. Homogeneous matrix layers generated by the ImagePrep were analysed at a raster suited to the quality of the preparation. Droplet arrays generated by the CHIP-1000 were analysed at a raster of the same size as the centre to centre droplet distance (250 ìm). Auto execute parameters were set by Flex Control software (version 3.0, Bruker Daltonics) and a fixed laser power was selected by the operator. Results from IMS acquisition were observed in Flex Imaging and Flex Analysis software (version 3.0, Bruker Daltonics).

Results and Discussion

Stability of Imaging Mass Spectrometry Instrumentation Allows Successful Application

The IMS methodology is rather straightforward in that it involves use of tissue sections mounted on a conductive surface, which are washed in alcohol to fix protein and wash away contaminants, coated in matrix and analysed by MALDI-TOF MS. Established tissue washes were used for our applications, including simple washes in 70% EtOH or IPA and 100% EtOH or IPA (Schwartz, Reyzer et al. 2003; Seeley, Oppenheimer et al. 2008). The difference in

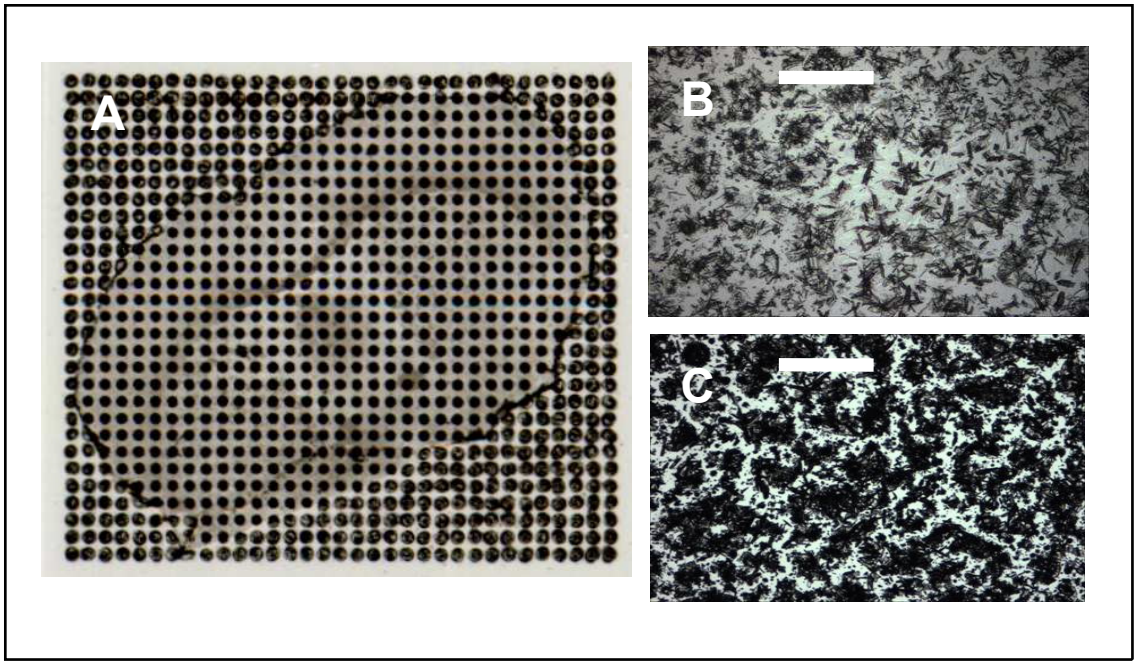

Figure 1: Matrix deposition strategies and resultant crystal formations The two dominant methods of matrix eposition are droplet deposition in a fixed array (A) and spray deposition to generate a homogeneous crystal coating (B and C). The matrix array shown in A was generated by a CHIP-1000 piezo electric printer (Shimadzu) with a centre to centre distance of 250 ìm using 10 mg/ml sinapinic acid (SA) in 50% acetonitrile (ACN), 44.8% ultra pure water, 5% isopropanol (IPA) and 0.2% trifluoroacetic acid (TFA). One droplet was deposited at each position for each of 25 layers. Droplet arrayed tissue section was washed in 70% and 100% IPA prior to matrix deposition. The homogeneous matrix layers generated in B and C were deposited by an ImagePrep station (Bruker Daltonics) using 6 mg/ml (B) or 10 mg/ml (C) SA with identical solvent compositions to those used for the CHIP-1000. The scale bars in B and C are 30 ìm. ImagePrep tissues were washed in 70% and 100% ethanol prior to matrix deposition.

terms of EtOH and IPA is marginal, but as demonstrated previously, IPA generates slightly better spectral data (Seeley, Oppenheimer et al. 2008). IMS requires automated matrix deposition: stability at this stage is critical to ensure reproducibility and prevent protein/peptide delocalisation due to matrix pooling on the section. The two automated systems, the Shimadzu CHIP-1000 Piezoelectric printer and the Bruker Daltonics ImagePrep station, were chosen for their availability and demonstration of applicability, both in the literature (Groseclose, Andersson et al. 2007) and in practical terms. The CHIP-1000 has been utilised for experiments on Murine brain, as shown in figure 1. The SA matrix crystals in figure 1A were arranged in a 250 ìm centre to centre array coating the coronal Murine brain section and these array foci appeared as discrete, dark spots on the section surface. Printer stability was paramount, as instability leads to dispensing of droplets in multiple directions, interrupting the grid pattern, but more importantly, potentially causing random delocalisation of proteins and peptides. However, with fine modulation of piezoelectric voltage during printing, large droplet arrays, as shown in figure 1A, were consistently deposited. Proper printer function was also found to be dependent on the stability of pressure lines feeding into the piezo unit and the condition/cleanliness of the external portions of the print head. These operations were also considered during fine tuning of the printing process and add to the complexity of the instrument. Furthermore, the matrix itself can generate problems in that SA, for example, can cause significant blockages of the piezo printing orifice at concentrations above 10 mg/ml. The ImagePrep station, which generates homogeneous matrix layers, maintains stability dependent on instrument specific methods, the condition of the piezo controlled nebulizer membrane as well as matrix concentration. In a trial of the instrument it was found that while 6 mg/ml SA can generate a homogeneous field of discrete matrix crystals, 10 mg/ml SA in an identical solvent mixture generates larger matrix foci, decreasing the possible resolution of the homogeneous spray preparation (Figure 1B and 1C respectively). Matrix blockages caused by crystallisation of SA on the nebulizer membrane do not have dramatic effects on spray quality until a significant portion (>50%) of the membrane surface is covered. Resolution, while limited in these examples to arrays of 250 ìm or more using a CHIP-1000 printer (Figure 1A, 2A-D), was reduced to 70 ìm with the ImagePrep automated spray system (Figure 2E). Pending further experiments, however, it is unclear how comparable the two systems are in terms of reproducibility.

Imaging the Murine Brain at the Protein Level with the CHIP-1000 and ImagePrep

Rodent and in particular the Murine brain, was a logical start point for IMS experiments due to its well characterized symmetrical structure and ease of handling once frozen. From a single acquisition point in an IMS experiment anywhere from 100-400 masses can be observed with typical array spectra showing high signal to noise (S/N) values for numerous masses in the range of 5-30 kDa (Chaurand, Norris et al. 2006). And as shown in figures 2AD, masses analysed correlate to internal section structure. Similar results are generated for homogeneous matrix layers, but these are generally more difficult to reproduce because control over droplet deposition is lost (data not shown). With proper modulation of instrument settings for both the CHIP-1000 and ImagePrep station, ion intensity maps resulting from multiple successful MALDI-TOF tissue acquisitions were generated as shown in figures 2F and 2G respectively. These results are key proofs of principle: currently these experiments show potential biological significance, which can be verified with future experimental determination of mass/protein identity by LC-MS/MS.

Importantly, it is worth considering that the CHIP-1000 and ImagePrep, as a result of generating different crystal structures, become well suited to different modes of IMS. Droplet arrays are suited to low resolution protein profiling: where different tissue areas are rapidly scanned for identification of a tissue signature based upon molecular ion determinants (Yanagisawa, Shyr et al. 2003). Homogeneous matrix layers on the other hand are suited to pure imaging experiments where high resolution tracking of a defined ion or initial tissue overview is needed. Figure 2F shows the ion maps corresponding to three different m/z-s and their spatial distribution across the tissue following acquisition from a printed droplet array. Compared to the ImagePrep data in figure 2G, the low resolution seems to be a drawback of the CHIP-1000 matrix array. While high resolution is obtainable with the ImagePrep with a hands free automated system, the CHIP-1000 allows rapid deposition of multiple droplets in an array or single point format. Combined with histology, the printer systems are extremely well suited to rapid tissue profiling to provide molecular identification which complements classical pathology. Furthermore, the MS acquisition time for droplet arrays is far less than the high resolution spray preparations. A typical array coating a coronal Murine brain section at a raster of 250 ìm (Figure 1A) takes approximately one hour to analyse by MALDI-TOFMS, where as a similar experiment using a 70 ìm raster or lower can take in excess of four hours. This limits high resolution IMS to the research laboratory until such a time when MALDI laser frequencies have increased by at least two fold.

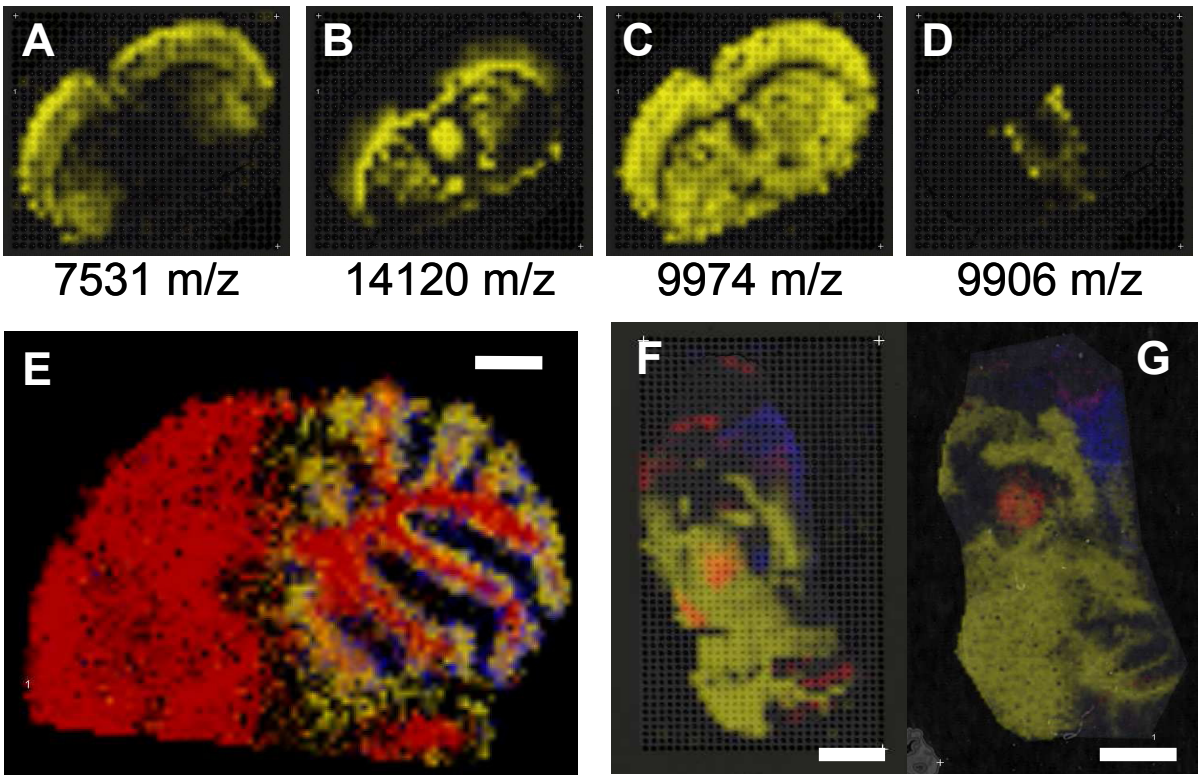

Figure 2: Imaging the Murine brain utilising automated matrix deposition Figures **A-D** show single ion intenity map examples from an imaging mass spectrometry experiment where 10 mg/ml sinapinic acid (SA) in 50% acetonitrile ACN), 44.8% ultra pure water, 5% isopropanol (IPA) and 0.2% trifluoroacetic acid (TFA) was deposited in 1 droplet iterations per layer in 25 layers onto a 10 ìm thick section of coronal Murine brain tissue using the CHIP-1000 (all experiments below used identical matrix solvent compositions). Array was 250 ìm centre to centre and can be seen overlaid onto figures **A-D**. Tissues in A-D were washed in 70% and 100% IPA prior to matrix deposition. Figure **E** shows a combination of three separate ion intensity maps where intensity of the individual colour correlates to relative ion intensity at that location. A 10 ìm thick sagittal Murine brain section was coated with 10 mg/ml SA using an ImagePrep station. The instrument was operated with default SA settings and manual determination of spray power. Scale bare in **E** is 1 mm. Figure **F** shows three combined ion intensity maps from an experiment where 10 mg/ml SA was deposited in 24 droplet layers onto a 10 ìm thick sagittal Murine brain section. Figure **G** shows a combination of the same three separate ion intensity maps at a resolution of 100 ìm. This separate 10 ìm thick sagittal Murine brain section was coated with 10 mg/ml SA in identical solvents to those above using an ImagePrep station. The instrument was operated with default SA settings and manual determination of spray power. Scale bars for F and G are 2 mm. An Ultra Flex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) was used for acquisition and analysis was performed in Flex Imaging software (version 2.0, Bruker Daltonics).

Conclusion

IMS technology, although in its infancy compared to established proteomic methods, including HPLC and two dimensional gels, has shown and continues to show its extreme potential through numerous applications in biological systems. Our laboratory has set up and begun the process of evaluating the methodology for performing high quality IMS experiments using automated systems such as the CHIP-1000 printer and ImagePrep station for matrix deposition. As a result we have observed that both systems can be confidently applied to Murine brain tissue for the purpose of imaging, at both rapid medium resolution (CHIP-1000) and high resolution (ImagePrep).

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References

1. Baluya DL, Garrett TJ, etal. (2007) Automated MALDI matrix deposition method with inkjet printing for imaging mass spectrometry. Anal Chem 79: 6862-7.

2. Bunch J, Clench MR, etal. (2004) Determination of pharmaceutical compounds in skin by imaging matrix-assisted laser desorption/ionisation mass spectrometry. Rapid Commun Mass Spectrom 18: 3051-60.

3. Caprioli RM, Farmer TB, etal. (1997) Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. Anal Chem 69: 4751-60.

4. Chaurand P, Norris JL, etal. (2006) New developments in profiling and imaging of proteins from tissue sections by MALDI mass spectrometry. J Proteome Res 5: 2889-900.

5. Dekeyser SS, Kutz NKK, etal. (2007) Imaging mass spectrometry of neuropeptides in decapod crustacean neuronal tissues. J Proteome Res 6: 1782-91.

6. Groseclose MR, Andersson M, etal. (2007) Identification of proteins directly from tissue: in situ tryptic digestions coupled with imaging mass spectrometry. J Mass Spectrom 42: 254-62.

7. Herring KD, Oppenheimer SR, etal. (2007) Direct issue analysis by matrixassisted laser desorption ionization mass spectrometry: application to kidney biology. Semin Nephrol 27: 597-608.

8. Lemaire R, Menguellet SA, etal. (2007) Specific MALDI imaging and profiling for biomarker hunting and validation: fragment of the 11S proteasome activator complex, Reg alpha fragment, is a new potential ovary cancer biomarker. J Proteome Res 6: 4127-34.

9. Mitulovic G, Mechtler K (2006) HPLC techniques for proteomics analysis-a short overview of latest developments. Brief Funct Genomic Proteomic 5: 249-60.

10. Monroe EB, Annangudi SP, etal. (2008) SIMS and MALDI MS imaging of the spinal cord. Proteomics 8: 1-9.

11. Schmidt A, Kellermann J, etal. (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5: 4-15.

12. Schwartz SA, Reyzer ML, etal. (2003) Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: practical aspects of sample preparation. J Mass Spectrom 38: 699-708.

13. Seeley EH, Oppenheimer SR, etal. (2008) Enhancement of Protein Sensitivity for MALDI Imaging Mass Spectrometry After Chemical Treatment of Tissue Sections. J Am Soc Mass Spectrom.

14. Stauber J, Lemaire R, etal. (2008) MALDI Imaging of Formalin-Fixed Paraffin-Embedded Tissues: Application to Model Animals of Parkinson Disease for Biomarker Hunting. J Proteome Res.

15. Vanrobaeys F, Van Coster R, etal. (2005) Profiling of myelin proteins by 2D-gel electrophoresis and multidimensional liquid chromatography coupled to MALDI TOF-TOF mass spectrometry. J Proteome Res 4: 2283-93.

16. Yanagisawa K, Shyr Y, etal. (2003) Proteomic patterns of tumour subsets in nonsmall-cell lung cancer. Lancet 362: 433-9.