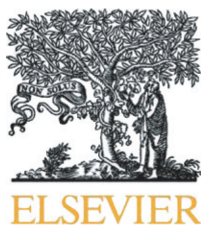
JOURNAL OF PROTEOMICS 79 (2013) 200–218



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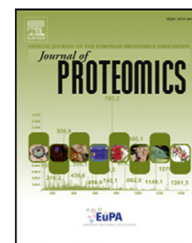

Development of liquid microjunction extraction strategy for improving protein identification from tissue sections

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ARTICLE INFO

Article history:
Received 21 August 2012
Accepted 30 November 2012
Available online 2 January 2013

Keywords:
MALDI MSI
Liquid extraction surface analysis (LESA)
Protein identification
Shotgun proteomics
Micro-extraction
Liquid microjunction

ABSTRACT

MALDI Mass Spectrometry Imaging has shown important potential for molecular classification and pathology marker discovery. Protein markers identification is therefore of prime importance. Direct structural analysis from tissue sections has shown limitations for protein identification because of the high degree of complexity of tissues. Only proteins of major abundance are identified this way. On the contrary, conventional proteomics approaches clearly allow for reliable identification of complex protein extracts but do not provide fine correlation with protein location in their original context. Here is presented an approach to obtain identification of proteins of various abundances while keeping their localization within the section. On-tissue trypsin digestion followed by micro-extraction using a liquid micro-junction interface is an efficient strategy to extract tryptic peptides and further identify the associated proteins off tissues. It was shown that conventional Reverse Phase Liquid Chromatography separation on the extracted material followed by MS/MS analysis on a HR FTMS instrument enabled the identification of 1500 proteins on average with high confidence from an area of about 650 μm in diameter, which corresponds to an estimated number of 1900 cells in average. The approach can be easily integrated in the MALDI MSI workflow and should provide interesting insights for clinical applications.

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Abbreviations: MSI, Mass Spectrometry Imaging; RPLC, Reverse Phase Liquid Chromatography; LCM, Laser Capture Microdissection; ROI, Region of Interest; PCA, Principal Component Analysis; HC, Hierarchical Clustering; FFPE, Formalin Fixed and Paraffin Embedded; TOF, Time-of-Flight; ISD, In Source Decay; ETD, Electron Transfer Dissociation; DESI, Desorption Electrospray Ionization; LESA, Liquid Extraction Surface Analysis; DBS, Dried Blot Spot; LTQ, Linear Ion Trap; MeOH, Methanol; EtOH, Ethanol; HCCA, α-Cyano-4-hydroxycinnamic acid; SA, Sinapinic Acid; ANI, Aniline; HES, Hematoxylin Eosin Safran; ITO, Indium Tin Oxide; Nd:YAG, Neodymium-doped Yttrium Aluminum Garnet; BSA, Bovine Serum Albumin; CID, Collision Induced Dissociation; FWHM, Full Width at Half Maximum; amu., Atomic Mass Unit; FDR, False Discovery Rate; S/N, Signal to Noise; ID, Identification; ppm, Part Per Million; LMJ-SSP, Liquid Microjunction-Surface Sampling Probe; SSSP, Sealing Surface Sampling Probe; CE, Capillary Electrophoresis.

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http://dx.doi.org/10.1016/j.jprot.2012.11.025

1. Introduction

Identification of molecular components involved in cellular mechanisms and their fine regulation is a key point for understanding physiological processes occurring in living organisms. Cell dysfunction processes greatly impact signaling pathways by modifications of molecular players. In particular, proteins are largely affected by cellular changes. These changes have an impact on their regulation (activation/repression of protein synthesis), primary structure (protein cleavage by enzymes, post-translational modifications), spatial arrangement and ability to complex with other partners (directly related to changes in protein concentrations and primary structure or due to changes in the environment such as ionic strength or pH). Over the past 25 years, many efforts were given to gain protein structural information and quantification. To date, proteomics offers a wide range of high performance strategies, methodologies and instrumentation to reach this goal. However, if conventional proteomics offers access to a large number of identified proteins, there is increasing evidence suggesting that such strategies lack correlation of the identified proteins to their location in their original context (i.e. tissues). For tissues, Laser Capture Microdissection (LCM) systems have been developed to collect cells of specific phenotypes based on morphological criteria recognition [1–4]. Currently, for cell collection from tissues, LCM is the better suited method. However, LCM remains technically somehow difficult to setup whatever the used technology. For proteomics, sample collection is time-consuming because of the large number of cells needed to be collected to permit detection given the instrument available. Indeed, few thousands up to several dozen thousands might have to be captured for proteomics analyses. Studies show a clear correlation of collected cell numbers with the number of subsequently identified proteins. Previous studies show identification of about 100–200 proteins for 500 cells, 500–700 proteins for 5000 cells and 1500–2500 proteins for 50,000–60,000 cells [5–9]. However, automatic cell collection, through use of morphological recognition dedicated tools, is not always very reliable and manual collection is very time consuming. Additionally, it must also be noted that cell collection for LCM is based on morphological criteria and thus does not guarantee that all cells are involved in the same physiological processes due to the cells' local microenvironment and cross-talk.

On the other hand, MALDI Mass Spectrometry Imaging (MSI) is a molecular imaging tool that can be used to study the spatial distribution of endogenous and exogenous compounds including, drugs [10–17], metabolites [18–24], lipids [25–31], peptides [32–34] and proteins [35,36] from both vegetal [37] and animal models. MALDI MSI was found to be a powerful technology for many fields of research such as pharmaceuticals [38], biomarker discovery and tracking for various pathologies e.g. brain disorders [32,39,40] or oncology [41–49]. To highlight the regulation of molecules with important functions related to a specific biological process by a simple MALDI MSI acquisition, these molecules have to be subjected to further characterization. For several years, many groups have been developing and improving on tissue protein identification strategies. In particular, on tissue bottom–up approaches through in situ enzymatic digestion have been demonstrated to allow direct identification of proteins from both frozen [50] and formalin fixed paraffin embedded (FFPE) [51] tissues. In order to maintain localization of peptides throughout the digestion process, methodologies were developed to perform enzymatic digestion from discrete locations on the tissues using a micro-spotter [50–52] or from whole tissue sections with micro-sprayer devices [53,54]. In this bottom–up approach, tryptic peptides are subsequently analyzed by MS^2 and identified upon databank interrogation. However, even if MS spectra on each pixel show hundreds of peptides, only few proteins are identified [55]. One of the ways to increase the number of protein identifications is the improvement of tryptic peptide detection. This has been done by incorporation of tissue treatments in the strategy, such as washing to minimize ion suppression effect [52]. Moreover, peptides with a very close m/z that enter and get fragmented together at the collision cell preventing the identification were separated by the inclusion of a gas phase separation using ion mobility [28,56–58]. In addition, improvement of protein fragmentation to aid in identification was also examined by N-terminal derivatization. This orientates the fragmentation of tryptic peptides towards a specific ion series, increasing the protein identification score [59]. All these efforts have led to better protein identification, however, the less abundant proteins which present significant biological activities, in particular pathologies, remain difficult to be detected and identified. More recently, on-tissue top–down strategy [60,61] was introduced by means of In Source Decay (ISD) [62,63]. This method of fragmentation is generally well-suited for purified proteins and leads to large sequence determination. However, in situ ISD has allowed the detection and identification only of the most abundant protein from the pixel analyzed [60] due to the high complexity of the sample.

Another strategy consists of combining MALDI-MSI and LC–MS/MS in a single workflow, allowing the improvement of protein identification. Basically, proteins are in situ digested, fractionated and then extracted prior to nanoLC separation, followed by MS/MS analysis for databank interrogation [40]. This approach was shown to improve protein identification, but since it was performed on a whole or half of a tissue section, information about protein localization is lost. This can be regained if back correlation to imaging data of tryptic peptides is done, as previously demonstrated for FFPE tissue samples [40,51]. More recently, intact proteins were extracted from tissue prior to fractionation by ultracentrifugation, and, in combination with high resolution and accurate mass determination, have allowed the detection of about a hundred proteins [55]. Although in this approach, the localization of proteins is lost, it can be regained by correlation with the tryptic peptide signals from the image of an adjacent section. The number of identified and localized proteins improved, but the approach continues to be limited by ion suppression effect and decreased sensitivity due to dominant signals from the most abundant proteins. Localized intact proteins provided by MALDI-MSI can also be identified after their extraction from a consecutive section prior to analysis by nanoLC-MS/

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