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NanoLC-MS coupling of liquid microjunction microextraction for on-tissue proteomic analysis☆

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ABSTRACT

Mass spectrometry (MS)-based microproteomics on localized regions of tissue sections was achieved by direct coupling of liquid microjunction microextraction with a nanoscale liquid chromatography-tandem MS, resulting in the identification of >500 protein groups from a region as small as 250 μm in diameter representing only a few hundred of cells. The method was applied on the examination of benign and tumor regions initially defined by imaging mass spectrometry (IMS) analysis of a consecutive high grade serous ovarian tumor tissue section. Results identified the higher abundance of eukaryotic translation initiation factors eIF4A, its isoform eIF4A2, and eIF5A and its isoform eIF5A2, and lower abundance of actin-binding proteins OBSCN, TAGLN and CNN3 on tumor regions, concomitant with previous findings. This demonstrates the use of the method for downstream characterization of distinct regions identified by IMS. This article is part of a Special Issue entitled: MALDI Imaging, edited by Dr. Corinna Henkel and Prof. Peter Hoffmann.

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1. Background

On-tissue microproteomics provides a direct means to examine proteomic fluctuations at the cellular level in response to changes in the tissue microenvironment. Its importance is evident in cancer, where proteomic analysis of cultured cell lines was observed to not correlate with results from microdissected cells of the same patient [1].

There is an ongoing effort to develop microscale technologies that can achieve reliable identification and quantification of proteins within an area of the most limited size, and correlate these expression changes with alterations in cell phenotypes and/or biological state. Various direct on-tissue mass spectrometry (MS)-based methods, such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) in profiling [2] and imaging modes [3], or desorption electrospray ionization (DESI [4]), have been shown to generate qualitative and quantitative information without *a priori* knowledge of the components present. But many of these techniques can only provide molecular information on low molecular weight compounds such as metabolites or lipids (DESI for example) and only MALDI gives access to information on the proteins. Despite the ability of MALDI to detect proteins that can be specifically associated to a physiological context, currently its major limitation remains the further identification of these proteins. Indeed, because the quality of direct tissue MS^2 spectra generated is greatly affected by the complexity of the tissue matrix, these methods suffer from limited identification. At best, these methods can provide hundreds of protein IDs, a tiny amount compared to tens or possibly hundreds of thousands of proteins expressed in cells. Methods involving the microdissection of cells, on the other hand, provide homogeneous cell populations for proteomics analysis. However, the limited number of cells in a particular region of the tissue entails microdissection in other regions to meet the requirements of extraction, thus compromising the importance of performing localized microenvironment analysis [5].

Abbreviations: ROI, region of interest; DHB, 2,5 dihydrobenzoic acid; RMS, root mean square; HC, hierarchical clustering; EIC, extracted ion chromatogram; CID, collission-induced dissociation; FWHM, full width at half maximum; FDR, false discovery rate; PANTHER, proteomic analysis through evolutionary relationships; LMJ SSP, liquid microjunction surface sampling probe; TCEP, tris(2-carboxyethyl)phosphine; EMT, epithelial-mesenchymal transition.

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More recently, to meet the possible identification and quantification of small exogenous molecules (namely drugs) from specific tissue areas, another strategy involving microextraction on the tissue surface, was developed [6]. Initially introduced for sampling thin layer chromatography (TLC) plates [7,8], this methodology was further extended for sampling biological tissues. This allowed, for example, the direct absolute quantification of propranolol and its metabolites from mouse whole body sections by direct interface to the electrospray (ESI) source of MS instrument [9] or indirectly through high performance liquid chromatography (HPLC) under LC-MS conditions [10]. Several variants of the same concept have been designed [8] provided they are capable of dispensing and aspirating microliter quantities of extracting solvent in a controlled manner. We further applied this micro-extraction strategy for on-tissue microproteomics and developed a workflow that allows correlation of trypsin-digested protein MS images with protein IDs obtained from shotgun MS analysis of extracts taken from the same regions on adjacent tissue sections [11]. This was accomplished by performing microextraction off-line using a commercially available instrument from a micro-digested tissue area by enzyme micro-spotting. This allowed us to identify >1000 proteins from a tissue area of about 650 μm in diameter, corresponding to <2500 analyzed cells. This strategy was shown to be highly efficient and robust but relatively long due to sample preparation steps performed post-micro-extraction such as solvent removal, desalting and reconstitution in a solvent more suitable for injection to the LC-MS instrument.

The present work is focused on direct injection of pre-digested protein extracts from tissues into an LC-MS instrument avoiding the time-consuming post-microextraction steps and concomitant sample losses associated with the sample handling involved. We demonstrate that this approach can be applied to the downstream processing of regions of interest (ROIs) generated from matrix-assisted laser desorption/ionization imaging mass spectrometry (IMS) data to provide identification and relative quantification of hundreds to thousands of proteins from micrometer-sized areas.

2. Materials and methods

2.1. Tissue section preparation

Procedures involving the preparation of animal tissue samples were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of University of Lille 1. Fresh brain tissues were dissected from adult male Wistar rats, flash frozen in liquid nitrogen, and stored in −80 °C until use. 10-μm sections were obtained using a Cryostat (Leica Microsystems, Nanterre, France). These were mounted on polylysine glass slides by finger-thawing, and dried under vacuum for 15 min. The slides were then washed sequentially using 70% and 95% EtOH, and $CHCl_3$ for 30 s each time. Optical images of the sections were acquired using a Super Coolscan 9000 scanner (Nikon, Tokyo, Japan).

Fresh frozen ovarian tumors as well as adjacent benign zone biopsies were obtained from the Centre Hospitalier de Recherche at Oscar Lambret. All patients signed a written consent form prior to biopsy and were informed on the use of the samples. Throughout the experiments, the anonymity of the donors was maintained. The biopsies were stored in −80 °C until use. Prior to sectioning, the biopsies were warmed to −20 °C in a cryostat for at least 30 min. Sections were obtained as described in the previous paragraph, with the first section mounted on an indium-tin oxide coated slide for IMS experiments, and the subsequent sections for extraction mounted on polylysine-coated slides. Sections used for staining were cut at 8 μm thickness. The sections were dried under vacuum prior to both IMS and extraction experiments.

2.2. Hematoxylin-phloxine-saffron (HPS) staining

Sections were soaked for 1 min in hematoxylin, then rinsed 3× in running water. These were then soaked in 0.1% phloxine for 10 s, followed by rinsing 2× in running water. The sections were then soaked in 50% EtOH once and twice in 100% EtOH, then once in saffran for 5 s. Finally the sections were soaked twice in 100% EtOH and in xylene.

2.3. MALDI imaging

To dried ovarian tumor sections, 2,5-dihydrobenzoic acid (DHB) prepared at a concentration of 15 mg/mL in 70:30 methanol/0.1% trifluoroacetic acid in water was deposited by spraying with a nebulizer (an ion trap electrospray source modified in-house) for 30 min. The nebulizer is connected to a compressed air source operating at <1 bar pressure; a 500-μL syringe is also connected and delivers the matrix at a flow rate of 300 μL/h.

The images were acquired using a MALDI-TOF mass spectrometer (AutoFlex III, Bruker Daltonics, Bremen, Germany) equipped with a Smart Beam laser (Nd:YAG, 355 nm) set at a repetition rate of 2 MHz. The instrument was set to acquire in positive reflector mode, at a mass range of 300–1200 m/z, with the obtained spectra being an average of 500 shots per pixel. This results in a minimum of approximately 5000 spectra for the smallest tissue section. The raster size was set at 50 μm.

The generated images were exported into SCiLS Lab software version 2015b. Baseline removal was performed using the top-hat method and the data were normalized using the root mean square (RMS) method. The maximum peak interval was set at ±0.3 Da and peaks were detected using orthogonal matching pursuit applied on individual spectra. The m/z intervals corresponding to the peaks detected were manually validated to remove those corresponding to isotopic peaks as well as those whose widths do not correspond to the set bin width. Peak alignment was then performed by comparing with a randomly selected mean spectrum from one of the images. Automatic spatial segmentation was then performed using Hierarchical Clustering (HC). This leads to the creation of a dendrogram, with each segment pseudocolored and the colors corresponding to the pixels in the segmentation map. Clusters generated from HC were co-registered with optical images obtained from the HPS staining of the adjacent section to verify if the ROIs generated by the clusters correspond to the tumor regions defined by the pathologist.

2.4. On-tissue digestion

Picoliter quantities of 20 μg/mL sequencing grade trypsin (Promega, Charbonnieres, France) dissolved 50 mM NH_4HCO_3 were deposited on tissue sections using a CHIP 1000 instrument (Shimadzu, Japan). Depending on the experiment, parameters such as dwell voltage, dwell time, quantity of a drop, and iterative printing interval and waiting time were adjusted. The quantity of a drop, iterative printing interval and waiting time determine the size of the droplet that is produced. For example, printing using 50 pL/drop at an interval of 5 drops and waiting time of 5000 ms resulted in a droplet size of 250 μm, while a 200 pL droplet at an interval of 15 drops and waiting time of 10,000 ms resulted in a droplet size of 600 μm. Digestion was effected for 1 h, ensuring that the droplet does not dry out by replenishing with a fresh one after each waiting time, except for ovarian samples where it was performed for 2 h.

2.5. On-tissue microextraction

Extraction of the digested components was performed using the modified FAMOS autosampler set-up. 1 mL and 12 μL of 0.1% FA was placed on reservoirs A and C of the autosampler. The digested regions on the tissue section were marked and the slide was placed on the

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