Research Article

Spatially-resolved protein surface microsampling from tissue sections using liquid extraction surface analysis

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Tissue microenvironment characterization presents a challenge for a better understanding of the full complexity of a pathology. Unfortunately, making a precise "picture" of the disease needs an efficient microsampling method coupled to an accurate localization for performing region-dependent proteomics. Here, we present a method that enables rapid and reproducible extraction of proteins from a tissue section to analyze a specific region at a millimeter scale. The method used a liquid-microjunction extraction with conventional detergent solution for proteomics analysis. We successfully performed immunoblotting experiments and showed the possibility to retrieve and identify more than 1400 proteins from a 1-mm diameter spot size on tissue sections with a high degree of reproducibility both qualitatively and quantitatively. Moreover, the small size of the extracted region achieved by this sampling method allows the possibility to perform multiple extractions on different tissue section points. Ten points on a sagittal rat brain tissue section were analyzed and the measured proteins clearly distinguished the different parts of the brain, thus permitting precise functional mapping. We thus demonstrate that with this technology, it is possible to map the tissue microenvironment and gain an understanding of the molecular mechanisms at millimeter resolution.

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1 Introduction

Multiscale heterogeneity is inherent in most pathologies, particularly in cancers where the tissue microenvironement has an important role in tumor growth and metastasis. A global study of these pathologies without taking account of this heterogeneity could thus lead to inaccurate conclusions. Nowadays, interest in personalized medicine is growing particularly using molecular information like using the reverse-phase protein arrays [1]. These techniques need to achieve microsampling of intact proteins from low amount of biological samples to overcome this problem of tissue microenvironement. Until now, the most effective technique used to realize the isolation of cell sub-populations within a tissue is laser capture microdissection (LCM) [2]. This technique allows the excision of specific cell types within a thin tissue section. More recently, methods employing mass spectrometry have emerged for the analysis of tissue sections like mass spectrometry imaging (MSI) or liquid-microjunction surface sampling. Of the latter, the most commonly used is the liquid extraction surface analysis technique (LESA) [3]. To perform the analysis in LESA, a droplet of solvent produced on a

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Abbreviations: FASP, filter aided sample preparation; **LCM**, laser capture microdissection; **LESA**, liquid extraction surface analysis; **LFQ**, label free quantification; **MBP**, myelin basic protein; **MSI**, mass spectrometry imaging

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Significance of the study

The study analyzed the quality of proteins extract by a liquid micro-junction extraction procedure using solution of detergents. This method demonstrated the possibility to obtain intact proteins at a millimeter scale allowing the study of isoforms or tracking exogenous high molecular weight compounds. Regionalized proteomics analysis could also be performed with a high degree of reproducibility and quantitative accuracy.

pipette tip is made into contact with the sampling surface to create a liquid-microjunction. This junction is maintained for a few seconds and this step could be repeated to improve the efficiency of extraction. This method is most commonly used for small molecule extraction on tissue sections [4, 5] or in different types of surfaces like food [6], TLC plates [7] or contact lenses [8]. In our previous studies based on direct on-tissue trypsin digestion followed by liquid-microjunction extraction and shotgun proteomics analysis, we showed for the first time the possibility to retrieve from a spot size of 650 μm in diameter (corresponding to an estimated number of 1900 cells) 1500 proteins with a variability of 1.6 ± 0.8% per experiments on both frozen or formalin fixed and paraffin embedded tissue section [9, 10]. Nevertheless, such strategy although highly interesting does not allow to identify isoforms, truncated proteins or PTM of proteins which are important issues to understand the physiopathology of disease progression correlated to the tissue localization. The possibility to use LESA for extraction of intact proteins was also demonstrated from a surface like DBS [11–13], bacteria [14] or liver tissue sections [15]. In these studies, the LESA experiments were realized with an extracting solvent compatible to direct Bottom-up or Top-Down identification which are performed in subsequent analyses (for example, solution of NH4HCO3 or methanol [15]). Nevertheless, the efficacy of extraction is limited to soluble proteins and the number of protein identifications is less than 500 proteins in best cases. We demonstrated here the possibility to used classical detergents to improve protein identification from tissue, perform quantification-based on tissue regional analysis and the possibility to use such technology with classical in gel proteomics to obtain information of proteins isoforms.

2 Materials and methods

2.1 Tissue preparation

Male Wistar rats were used and treated in accordance to the European Communities Council Directive (2010/63/EU) regarding the use of animals in Research, French Law for Animal Protection R214-87 to R214-137 and were approved by the Institutional Animal Care and Use Committee (IACUC) of University Lille 1. Twelve micrometer frozen rat brain tissue sections were cut using a cryostat CM1510S (Leica Microsystems, Nanterre, France) and applied onto superfrost glass slides (Thermo Scientific, France). The tissue sections were vacuum-dried during 10 min then soaked subsequently in 70% EtOH, 95% EtOH and $CHCl_3$ for 30 s each with concomitant drying under vacuum for 5 min between washings. To study the effect of the washing steps in some experiments described in the result part, tissue sections were just dried without any washing steps.

2.2 Automatic-LESA for protein extraction

Micro-extraction experiments were performed using the TriVersa Nanomate platform (Advion BioSciences Inc., Ithaca, NY, USA), with the LESA feature. To perform automatic microextraction of proteins using the LESA device several modifications were made on the system. Specific automatic methods for protein extraction were generated using the Advanced User Interface of the Chipsoft software (version 8.3.1.1018 build 101018). A description of the different methods is detailed in supporting information. Different extraction solutions were tested: AcN solution: AcN/H2O (8:2, v/v), MeOH solution: MeOH/H2O (7:3, v/v), NH4HCO3 solution (50 mM), SDS solution (Tris HCl pH8 0.1M / SDS 1% / DTT (50 mM) and CHAPS solution (Tris HCl pH10 0.1M / CHAPS 4% / DTT 50 mM).

2.3 In gel analysis

SDS-PAGE and immunoblotting experiment were done using classical procedures. The complete protocols are detailed in supporting information

2.4 Shot-gun proteomics and MS data acquisition

Shotgun proteomics was performed using the FASP method according the protocol previously published [16]. Samples were then separated by online reversed-phase chromatographic system (Thermo Scientific Proxeon Easy-nLC II system) equipped with a Proxeon trap column (100 μm id × 2 cm, Thermo Scientific) and a C18 packed-tip column (EASY-column, 75 μm id × 10 cm, Thermo Scientific). Peptides were separated using a 120-min gradient with mobile phase solutions of formic acid 0.1% aq (buffer A) and AcN/formic acid (99.9:0.1, vol/vol) (buffer B). The gradient of AcN was

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established from 5 to 35% of B over 100 min at a flow rate of 300 nL/min. Data were acquired on a Thermo Scientific Q-Exactive mass spectrometer set to acquire top 10 MSMS in data-dependent mode. The survey scans were done at a resolving power of 70 000 FWHM (*m/z* 400), in positive mode and using an AGC target of 3e6. Default charge state was set at 2, unassigned and +1 charge states were rejected and dynamic exclusion was enabled for 25 s. The scan range was set to 300–1600 *m/z*. For ddMS^2, the scan range was between 200 and 2000 *m/z*, 1 microscan was acquired at 17 500 FWHM and an isolation window of 4.0 *m/z* was used.

2.5 Protein identification and bioinformatic analysis

All the MS/MS data were processed with MaxQuant [17] (version 1.5.1.2) using Andromeda search engine [18]. Information concerning the search conditions and data analysis was detailed in supporting information. The datasets used for analysis were deposited at the ProteomeXchange Consortium [19] (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [20] with the dataset identifier PXD001925 and PXD002514. Only proteins presenting at least two peptides with one unique peptide were considered and analysis of the proteins identified was done using Perseus software (http://www.perseus-framework.org/, version 1.5.1.6). For quantification-based MSI, LFQ values of proteins present in all positions were analyzed using TIGR Multiexperiment viewer (MEV v4.9) [21] after RMS normalization and clustering by self-organizing trees (SOTA) algorithm [22] using Euclidean distance.

3 Results and discussion

3.1 Solvent extraction parameters

First experiments were done to test the compatibility of different solutions with an automatic system for liquid surface extraction. In this part, no optimizations were made concerning the size of the liquid junction to maximize the amount of proteins extracted. Various solutions were tested and extraction efficiency was evaluated by visualizing the protein yields of the extracts by SDS-PAGE. An optical image of the spot after extraction (obj 2.5x) is presented in Fig. 1A, showing that no lateral diffusion of solvent was observed. Using the same parameters for the positioning of the tip above the tissue section and same dispensing volume, the size of the spot was different depending on the solution used for extraction. Solutions containing MeOH or AcN attained a spot diameter of around 2.5 mm, SDS and CHAPS spots were around 2 mm and NH4HCO3 was approximately 1.7 mm. These results are in accordance with previously published experiments by Kertesz and Van Berkel [23] showing a diameter of the sampling area with high concentration of methanol or acetonitrile between 2 and 2.6 mm. They also demonstrated

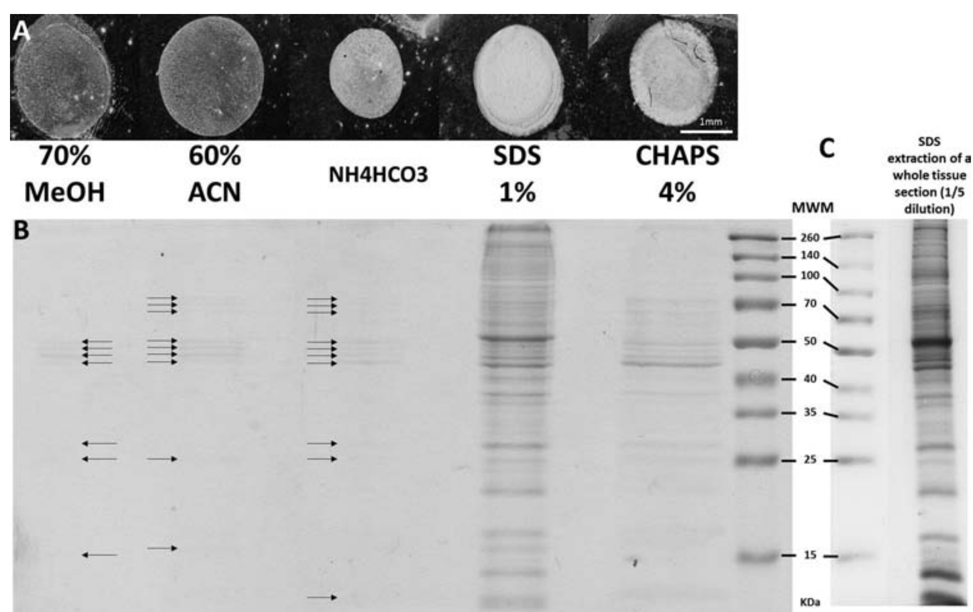

Figure 1. Test of solvent for extraction of proteins in gel-based proteomics. Comparison of various solvents known to be used in proteomics sample preparation (i.e. solutions of 70% methanol, 60% AcN, NH4HCO3, 1% SDS or 4% CHAPS). (A) Microscopic image (obj x2.5) of the extracted region using these different solutions (scale bar: 1 mm). (B) 1D gel electrophoresis of proteins extracted using each solution and with molecular weight marker (MWM) in the last well to the right (in gray-scale for a better contrast). Arrows were used to indicate some bands of low intensity. (C) 1D gel electrophoresis obtained using a solution containing 1% SDS of a whole rat brain tissue section, diluted five times (MWM in the first well to the left).

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that by decreasing the amount of organic solvent a smaller diameter could be achieved like we observed for the solution of NH4HCO3.

Another observation was the partial or total removal of the tissue after extraction depending on the solution used (Fig. 1A and Supporting Information Fig. S2). For MeOH, AcN or NH4HCO3, the tissue remained intact. Examination of the global histological structure showed no disorganization and the tissue became more transparent within the sampling area. By keeping the integrity of the cell structure, only aqueous soluble molecules could be extracted. Using CHAPS, cells were less visible and a loss of integrity was observable at the inner part of the sampling area. For SDS, all the tissue was removed. In this case, all molecules contained in the tissue could be extracted. To confirm these hypotheses, SDS-PAGE was performed using all the extracts (Fig. 1B) and compared to a total extraction of an entire tissue section using a solution of 1% SDS (Fig. 1C). For the extraction using MeOH, ACN and NH4HCO3, only few bands corresponding to proteins were observable. The most intense bands were detected for molecular weight around 50 kDa and corresponded to the most intense bands in the extraction of the whole tissue section. As the gel staining solution allowed the detection of protein bands containing as low as 5ng proteins per band, it was confirmed that a low amount of proteins could be extracted using non-detergent solutions. For extracts obtained using SDS and CHAPS, more bands were detected and both lanes showed similar band profiles but the intensities of the bands varied. As expected from visual inspection of the sampling area after extraction, the SDS solution that removed all the tissue, produced bands with higher intensities than the CHAPS solution. In this case, using detergent as solution for extraction will perform "liquid microdissection" comparable to LCM in the fact that all the tissue was removed. Resolution achieved by this technique is not comparable to the one obtained by LCM that could be at a single cell resolution but handling of the sample in our method is easier. In fact, using liquid-microjunction allows to reduce the number of steps for preparation of the sample by performing dissection and protein extraction in one step.

3.2 Improvement of liquid-microjunction

A previously published experiment showed the possibility to decrease the diameter of the sampling area by decreasing the dispense volume of the solution and the probe-to-surface distance [23]. From our experiments, for solutions containing detergents like SDS or CHAPS, the optimal distance was found to be 0.3 mm with a dispense volume of 0.8 μL. By using these parameters, a diameter of 1.28 ± 0.09 mm was measured (mean of eight extraction points) (Fig. 2A). Using the optimal parameters to obtain a sampling area diameter of approximately 1 mm, extraction using one, three (Fig. 2B)

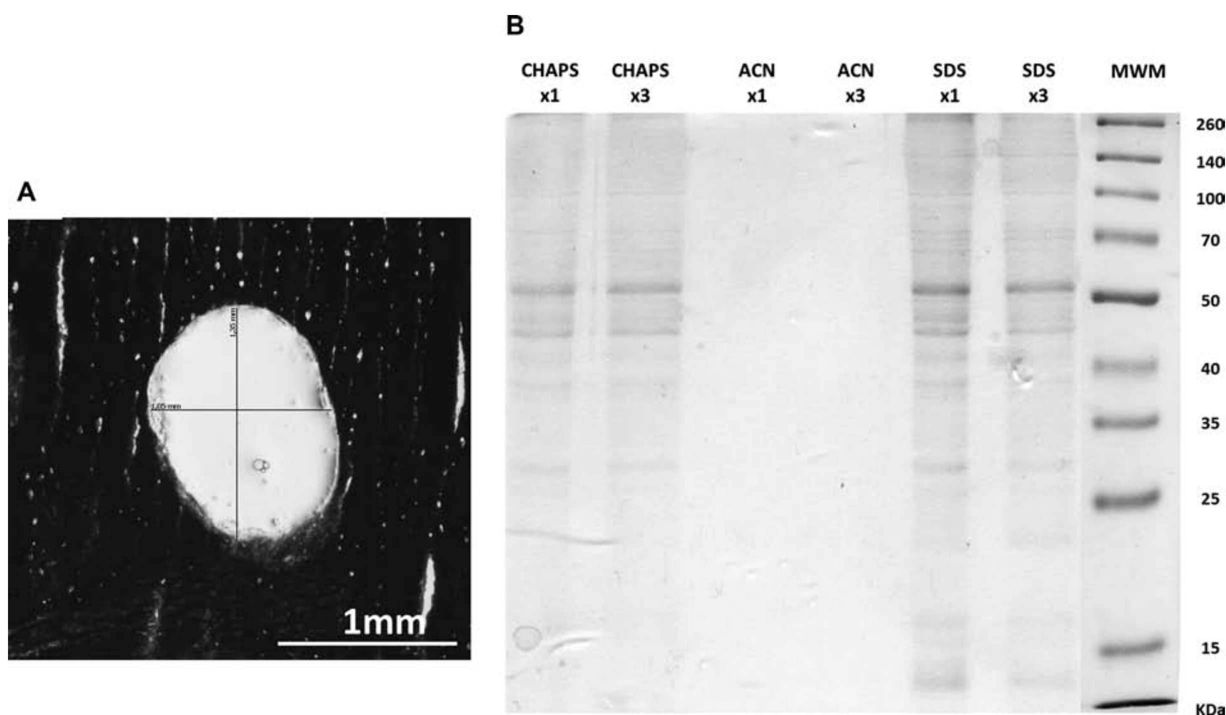

Figure 2. Optimization of the liquid-microjunction and influence of the number of extraction cycle. (A) Optical observation of the extracted region after optimization of liquid junction for three cycles of extraction. Scale bar: 1 mm. (B) 1D gel electrophoresis obtained with proteins extracted with different numbers of extraction cycles (1 or 3) using 4% CHAPS, AcN or 1% SDS. Molecular weight marker (MWM) in the last well to the right (in gray-scale for a better contrast).

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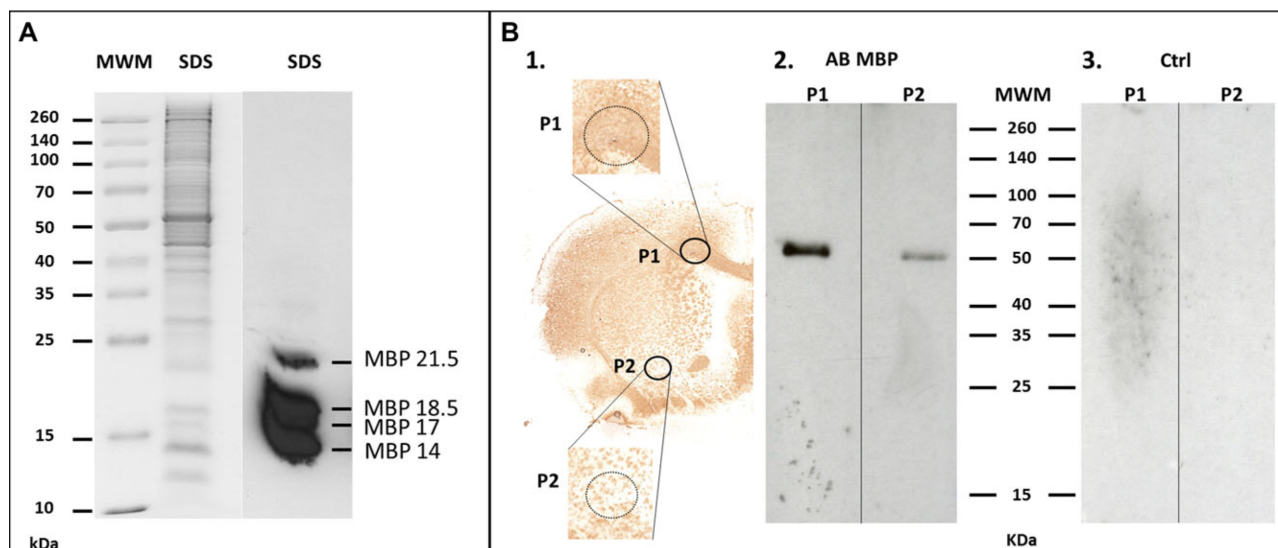

Figure 3. Immunoblotting of proteins extracted by liquid-microjunction. (A) The left part shows the liquid-microjunction protein extraction using a solution of 1% SDS of a region of a rat brain tissue section (corpus callosum) resolved by SDS-PAGE (MWM: Molecular weight marker) and subjected to western blot analysis (right part) showing the expression of Myelin Basic Protein (MBP). (B) 1. Optical observation of a rat brain tissue section after immunohistochemistry directed against MBP. Circles point out the region extracted to perform immunoblotting experiments (P1: corpus callosum P2: caudate putamen). 2. Western blot analysis with detection of the primary antibody against MBP, extracted using 1% SDS from the two regions pointed out in the part B-1. 3. Western blot analysis after extraction of a serial control tissue section without incubation of the anti-MBP antibody on the tissue section.

or five (Supporting Information Fig. S3) cycles of extraction on the same position were compared. The decreased size of the sampling region led to a concomitant decrease of the intensity of the protein bands in the gel. For AcN, decreasing the size of extraction resulted to a disappearance of the bands for one cycle of extraction. After three cycles, the amount of extracted proteins was still below the LOD. The same results were obtained with solutions of MeOH or NH4HCO3 (data not shown). After three cycles of extraction, no tissue remained for both SDS and CHAPS (Fig. 2A and Supporting Information Fig. S2 for coloured optical image) and the gel showed more intense bands correlated to the increase of the number of extractions (Fig. 2B). Repeated extraction cycles up to three times on the same position did not give any improvement (Supporting Information Fig. S3) and only increased the diameter of the sampling area due to spreading of the solvent. Our modified sample support allowed the use of different solutions in order to perform extraction in the same region, as shown in gel wells SDS-CHAPS or CHAPS-SDS (Supporting Information Fig. S3).

3.3 Extraction of protein isoforms

Figure 3A shows, on the left part, a SDS-PAGE generated from the protein extract obtained using optimal parameters and a solution of 1% SDS as extraction solution applied on the *Corpus callosum* region of a rat brain tissue section. The right part is the result of a western blot against Myelin Basic Protein (MBP). The four major isoforms of MBP with a molecular weight of 21.5, 18.5, 17 and 14 kDa [24] can be detected intact directly after liquid-microjunction extraction using SDS. This result is in adequacy with the high level of expression of MBP in the white matter where myelinated axons are most abundant. This result indicates that intact proteins as well isoforms or modified proteins can also be extracted by this method. Based on such development it could be possible to perform experiments using microwestern arrays [25] or reverse phase protein array [26] on a localized region in less than 5 min.

3.4 Extraction of high molecular mass proteins

To see whether the procedure can extract high mass proteins from tissue, an antibody was deposited on the tissue surface and immunohistochemistry was realized. Figure 3B shows the result of this experiment. First, a complete reaction was done to localize the MBP on a coronal rat brain tissue section (Fig. 3B part 1). Then, two different regions were selected, and in this case, partial immunochemical reaction was performed, using only the primary antibody against MBP but without the addition of the secondary antibody and revelation. Extractions of the two regions selected previously were done and the proteins were transferred on a nitrocellulose membrane after separation by SDS-PAGE. The

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membrane was incubated only with an antibody against the primary antibody used on the tissue section for direct detection (Fig. 3B part 2). A band corresponding to the heavy chain of the primary antibody (around 50 kDa) was detected and revealed a difference in intensity of the bands between the *Corpus callosum* and *Cingulum* (P1) and the *Caudate putamen* (P2). The fold change of 4.69 between both regions in the western blot was in correlation with the difference of signal observed in the IHC experiment. The control (Fig. 3B part 3) was the result of extraction of the same regions in a consecutive section after the same experiment but without the use of the primary antibody. In this case, no signal was detected. This experiment shows the possible use of the technique for the extraction and quantification of high mass proteins like antibodies from tissue, and also the possibility of applying the method to track, validate and quantify therapeutic antibodies from tissue, and discover possible modifications or degradation in a quantitative manner.

3.5 Mass spectrometry-based microproteomics of tissue proteins

3.5.1 Washing step

In MALDI MSI experiments, solvent washing has been demonstrated as the most critical step during the preparation of the tissue itself [27]. To obtain optimal protein extraction for tissue-based proteomics, the same parameter has been found to be the most crucial as well. It was observed that when using tissue section not subjected to solvent washing that the signal of the gel bands of the proteins extracted was very low. Moreover, shotgun proteomics analysis of the extracts from unwashed tissue sections only yielded protein identifications of around 100 proteins per spot, whereas extracts obtained from tissue sections subjected to washing steps produced around 1000 proteins per spot (Supporting Information Fig. S4). Observation of the extracted region on the unwashed tissue section showed that a lot of tissue is remaining compared to the same region on a tissue section after washing with a different solution than the one used for extraction. A possible explanation for this observation is the destabilization of the structure of the tissue after chloroform treatment to remove lipids of the plasma membrane, thus facilitating protein extraction.

3.5.2 Influence of extraction solvent

To perform shotgun proteomics, the FASP protocol was used due to its compatibility with detergents [16]. In our case, the amount of proteins was very low. In fact, using our extraction method, with an average diameter for the extracted region of 1.28 ± 0.09 mm, this represents around 7300 cells or 13 nL of tissue (sample volume was calculated as a cylindrical volume using the average surface of extraction and tissue section thickness). Assuming that there is an average of 200 pg of protein/cell (for HeLA cell), a total maximum amount of protein of around 1.5 μg per extracted region was estimated. In our case, the use of SDS as detergent resulted to a dramatic decrease in the number of identified proteins after FASP compared to the use of CHAPS (less than 300 for SDS compared to more than 1500 for CHAPS). The detergents used were of high concentration (5 to 10 fold higher) than their critical micellar concentration (CMC) and formed micelles with a molecular weight of 18 kDa for SDS and 6 kDa for CHAPS. It seems to be less critical when using high amounts of tissue or proteins but in our conditions, 6X dilution using UA buffer decreased the detergent concentration just near to the CMC and remaining micelles could be still present. In this case, remaining micelles of CHAPS could be more easily eluted through the 30 kDa membrane of the filter unit than the remaining SDS micelles and the high amount of SDS micelles could result to a decrease of the enzyme digestion efficiency. As we have shown before a complete remove of the tissue was achieved using both CHAPS or SDS solution and no difference in gel were observed with optimal protocol of extraction. For all these reasons the solution of CHAPS was used for the shotgun analysis experiment.

3.5.3 Evaluation of method reproducibility for microproteomics analysis

As illustrated in Fig. 4, reproducibility was assessed for our method at the protein level by comparing triplicate extractions on consecutive sections of two regions, i.e. the ventral orbital cortex, a part of the prefrontal cortex (region 1) and the posterior thalamic nuclear group, a part of the thalamus (region 2) (Fig. 4A). Concerning the estimated molecular weight of the proteins (Supporting Information Fig. S6), a complete coverage of the mass range was observed between 10 to 500 kDa with majority of the proteins falling within 10 to 70 kDa. Reproducibility was qualitatively evaluated in terms of overlap of protein identifications (Venn diagrams) and quantitatively expressed as Pearson correlation coefficients (dot plots and r value). For Venn diagrams, the value was worked out based on the number of proteins identified in all replicates independent of their abundance while the scattering plots were calculated based on the quantitative values (LFQ values) obtained for each protein in two different replicates, for a total of three replicate combinations per method. Extraction of proteins by LESA showed high reproducibility, as shown by the high protein identification overlap (approximately 82%) for the three replicates (Fig. 4B) and the average Pearson correlation (0.976, Fig. 4C). A second region was also extracted in triplicate and showed around 80% of overlapping and an average Pearson correlation of 0.976 (Supporting Information Fig. S5). Same observations were obtained at the peptide level with corresponding values of 79.3% (Fig. 4E) and 0.874 for the region 1 (Fig. 4F) and 67.1% and 0.863 for the region 2 (Supporting Information Fig. S5). In terms of

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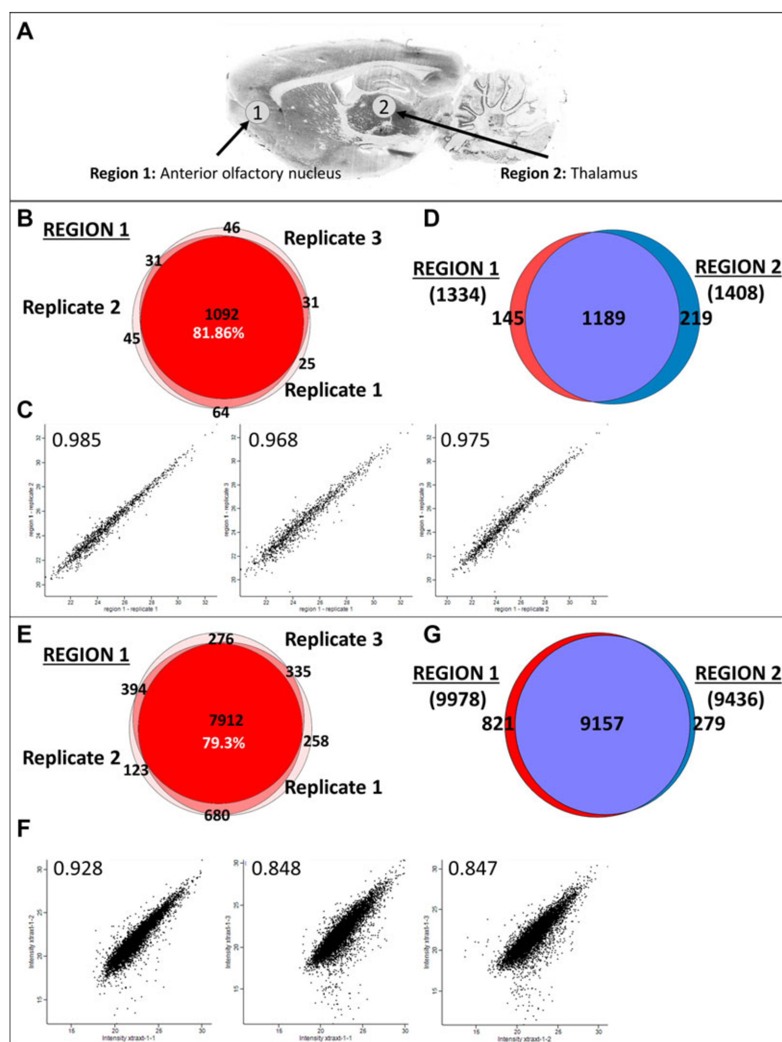

Figure 4. Qualitative and quantitative reproducibility of the protein extraction by LESA methods for shotgun proteomics analysis. (A) Localization of the extracted regions on a sagittal rat brain tissue section. Two regions were extracted i.e. the ventral orbital cortex (region 1) and the posterior thalamic nuclear group (region 2). Venn diagrams show the distribution of identified proteins (B) or peptides (E) among replicates. Scatter plots of correlation for quantification values between all replicate combinations at proteins (C) or peptides (F) level using, respectively, log2 of LFQ value or Log2 of intensity. Pearson correlation coefficients are reported (same data are also available for the region 2 in Supporting Information Fig. S5). Venn diagrams depict distribution of all identified proteins (D) and peptides (G) for the two extracted regions.

number of identifications, extraction of region 1 resulted to the identification of 1334 groups of proteins (9978 peptides) and 1408 groups of proteins (9436 peptides) for the region 2 (Fig. 4D and G). For region 2, a higher variability was observed for the identification especially against replicates 1 and 3, certainly due to an evolution of the histological features. But for the quantification, the values of the Pearson correlation were the same as in region 1, showing that identified proteins gave the same value of quantification between replicates of the same region. For region 1, a gene ontology-based analysis was performed using PANTHER (Supporting Information Fig. S7). In molecular function, majority of the proteins were associated with catalytic activity (35.9%) and binding (29.6%). For the biological process, proteins were involved in metabolic process (29.3%) and cellular process (21.6%). Finally, concerning the cellular component, 41.4% were in the cell part and 29.4% in the organelle. Proteins associated with the membrane were also observable (12.2%) as well as synapse proteins (1.8%). A rapid functional analysis was done using STRING v10 [28] with proteins specific to each region. For Region 1, some proteins where found to be involved in addiction processes. The extraction was done in the Ventral orbital cortex, a part of the orbitofrontal cortex. This region of the brain is known to be implicated in addictive behaviour via dopaminergic activation of rewards circuits. For the region 2, a part of the thalamus, majority of proteins were involved in metabolic processes.

3.5.4 Application to quantification-based mass spectrometry profiling

Quantification-based mass spectrometry profiling was performed according to our recent publication [29]. Extractions of 10 consecutive regions on a sagittal rat brain tissue section (Supporting Information Table S1) were performed followed by shotgun analysis of each sample (Fig. 5A). A total number of 1777 proteins were obtained for these ten positions with

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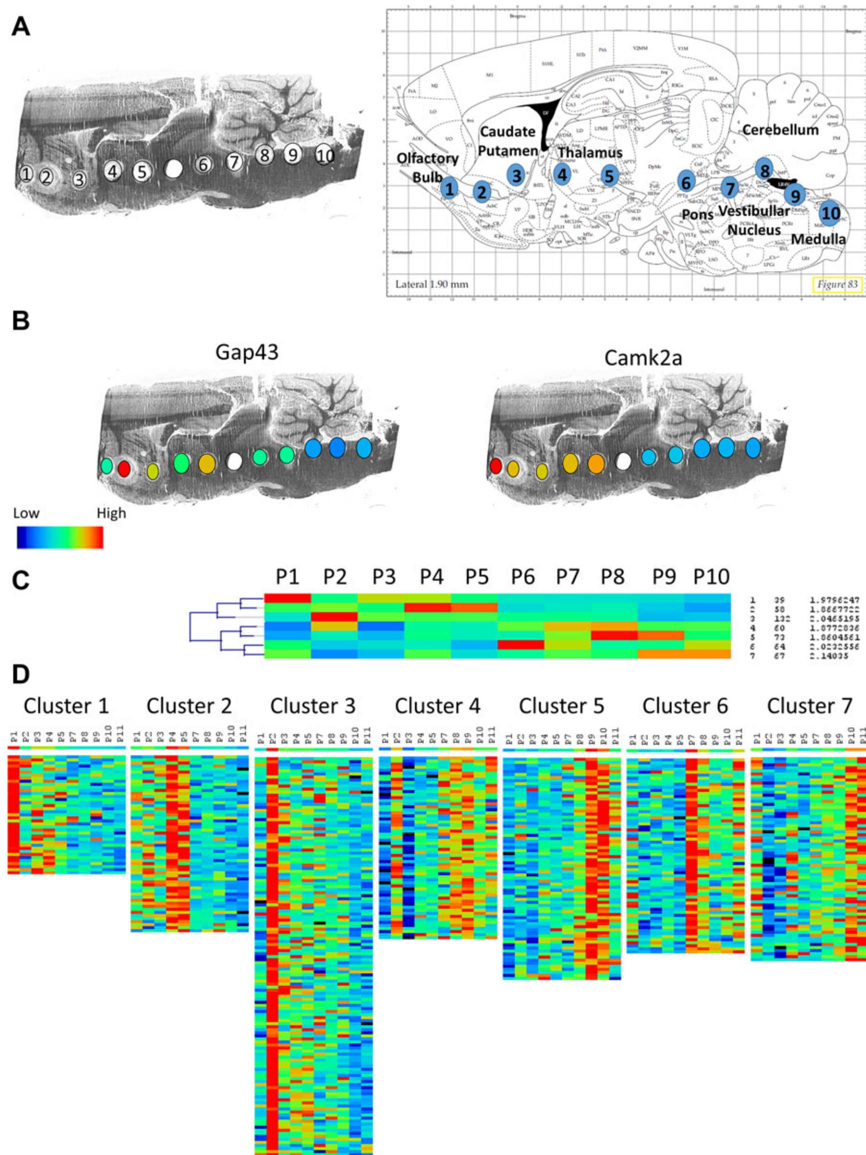

Figure 5. Quantification-based mass spectrometry imaging experiment realized using ten extracted regions distributed on a sagittal rat brain tissue section. (A) Optical image of the rat brain after extraction (position 6 not used) on the left part and localization of the extracted regions on a sagittal diagram from a rat brain atlas (right part). (B) Reconstructed distribution of proteins gap43 and camk2a based on LFQ values provided by nanoLC-MS/MS analysis. (C) SOTA dendogram obtained by clustering of proteins present in all the positions. (D) Cluster trees correspond to each SOTA dendogram line.

493 proteins common to all positions (supporting information Excel file total_and_only_P1-11.xlsx). Expression of specific proteins in each brain region was then examined. For example, in the striatum (position P2 and P3, respectively, the nucleus accumbens and the *Caudate putamen*), several proteins involved in the neuronal cyclic AMP (cAMP) signaling pathway were found (Table 1). Adenylate cyclase type 5 (Adcy5), Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-7 (Gng7), Aromatic-L-amino-acid decarboxylase (Ddc) and Protein phosphatase inhibitor 2 (Ppp1r2) were localized exclusively in the nucleus accumbens. Two other proteins, calcium/calmodulin-dependent 3′,5′-cyclic nucleotide phosphodiesterase 1B (Pde1b) and Protein phosphatase 1 regulatory subunit 1B (Ppp1r1b) were highly expressed in the nucleus accumbens. These proteins have been shown to maintain cAMP level in the striatum and exclusively expressed in this region of the brain particularly in the nucleus accumbens [30]. Lists of proteins specific to each position are presented in the supporting Excel file only-P1-11.xlsx. For proteins common to all positions, a number of housekeeping genes could be identified (examples in supporting Excel file HKGs.xlsx). All the six members of the 14-3-3 proteins expressed in most mammalian tissues were found as well as 24 of 26 proteins from the class of the citric acid cycle-related proteins. For this class of proteins, the two not identified were integral membrane proteins (SDHC and SDHD) for which a specific extraction solution needs to be used. Some other proteins known to have low expression variation within tissues and used for normalization for Western Blotting were also observed like Actb, Pfkm, Ppia or Rpsa (with a coefficient of variation of 8.63, 1.88, 3.59 and 8.38%, respectively). Finally, many tissue-specific proteins were identified

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Table 1. Proteins found in the striatum part involved in the cAMP signaling pathway. Values correspond to log2(LFQ)

Gene name	Protein name	1	2	3	4	5	6	7	8	9	10
Adcy5	Adenylate cyclase type 5	NaN	21.8129	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
Ddc	Aromatic-L-amino-acid decarboxylase	NaN	22.1816	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
Gng7	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-7	NaN	25.8567	25.5251	NaN	NaN	NaN	NaN	NaN	NaN	NaN
Pde1b	Calcium/calmodulin-dependent 3′,5′-cyclic nucleotide phosphodiesterase 1B	21.5073	26.0811	24.2862	21.465	NaN	NaN	NaN	NaN	NaN	NaN
Ppp1r1b	Protein phosphatase 1 regulatory subunit 1B	22.151	26.995	26.3403	23.8397	23.7232	NaN	NaN	21.9042	NaN	NaN
Ppp1r2	Protein phosphatase inhibitor 2	NaN	22.5997	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN

like Mbp, Ncam1/2 or Sncb. For these proteins, the coefficients of variation were higher, suggesting specific localization in the brain. The protein Neuromodulin (Gap43) was highly present in the position 2, localized in the ventral part of the striatum, the nucleus accumbens. This is a region of high plasticity involved in learning processes. Previous works have shown that Gap43 is considered to be a crucial component of neurite formation, regeneration and mediation of experience-dependent plasticity [31] explaining its specific repartition at a high level in the nucleus accumbens and decreasing in other positions (Fig. 5B). Camk2a is a CAMK protein, a class of enzymes, Ca2+/calmodulin-dependent protein kinases, also known to be involved in synaptic development and plasticity [32]. Previous reports [33, 34] demonstrated a higher level of expression in the forebrain than in the hindbrain (constituted by the Pons and the Medulla). These observations were confirmed in more recent publications especially for the olfactory bulb [35]. This specific localization was also found using our data (Fig. 5B). Clustering of the 493 proteins found in the ten regions was then performed using label free quantification (LFQ) values by applying a SOTA [22] to highlight proteins more specifically present in one position. Fig. 5C presents the SOTA dendogram, where each line corresponds to a cluster and each column to an extracted position (position 1 to 10). In this diagram, the first five positions were grouped in the same node and correspond to the forebrain. Positions P6 to P10 (midbrain and hindbrain) were also grouped together in the second part of this SOTA diagram. Moreover, by selecting a line of this dendogram, it was easily possible to extract the expression cluster corresponding to a specific brain structure (Fig. 5D). Extractions done in the same major region of the brain were grouped in the SOTA dendogram, like Positions 4 and 5 (cluster 2), localized in the thalamus or Positions 8, 9 and 10 in the hindbrain (clusters 5 and 7). For Position 7, the proteins were divided into two different clusters, i.e. cluster 4 and cluster 6, respectively, with a profile closed to one of positions 8 and 6. Indeed, position 7 was in the borderline between the end of the Midbrain and the start of the Pons in the hindbrain which explains the result.

4 Concluding remarks

Taken together, we showed here that based on a spatially-resolved extraction on a tissue surface, it is possible to retrieve highly specific information on protein content.

First, we demonstrated the efficient extraction of proteins on restricted regions at a millimeter resolution. Contrary to the previously published experiment, we used here conventional detergents for proteomics instead of solvent mixtures composed of methanol or ACN. These detergents gave a better extraction efficiency making it possible to use the extracts for in gel separation.

Second, this sampling method allows the retrieval of intact proteins with their PTMs or truncations as well as isoforms. Extraction and quantification of high mass compounds can be achieved.

Third, shotgun proteomics analyses were possible directly in combination with methods like FASP with the identification of more than 1400 proteins with a high degree of reproducibility from a surface of 1 mm^2. A good correlation in the quantitative data between each replicate allows a confident comparison of protein content in different regions from the same tissue section.

Finally, using this sampling method on consecutive positions on a surface, it is possible to obtain a quantitative distribution map of proteins across different regions of a tissue section. These results clearly open the door to the understanding of molecular cross-talk in the tissue micro-environment.

Such a strategy could be used complementary to a MALDI MSI experiment. For example, molecular information obtained by MALDI MSI can be subjected to statistical analysis to generate regions of interest [9, 10, 29]. The ROIs can then serve as regions where liquid microextraction of the proteins can be performed in the manner presented in this work. Such information will allow to link individual cell phenotypes expressed in a particular tissue region with the tissue microenvironment.

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