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REVIEW

Microproteomic sample preparation

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Abstract

Multiple applications of proteomics in life and health science, pathology and pharmacology, require handling size-limited cell and tissue samples. During proteomic sample preparation, analyte loss in these samples arises when standard procedures are used. Thus, specific considerations have to be taken into account for processing, that are summarised under the term microproteomics (μPs). Microproteomic workflows include: sampling (e.g., flow cytometry, laser capture microdissection), sample preparation (possible disruption of cells or tissue pieces via lysis, protein extraction, digestion in bottom-up approaches, and sample clean-up) and analysis (chromatographic or electrophoretic separation, mass spectrometric measurements and statistical/bioinformatic evaluation). All these steps must be optimised to reach wide protein dynamic ranges and high numbers of identifications. Under optimal conditions, sampling is adapted to the studied sample types and nature, sample preparation isolates and enriches the whole protein content, clean-up removes salts and other interferences such as detergents or chaotropes, and analysis identifies as many analytes as the instrumental throughput and sensitivity allow. In the suggested review, we present and discuss the current state in μP applications for processing of small number of cells (cell μPs) and microscopic tissue regions (tissue μPs).

KEYWORDS
bottom-up approach, cell microproteomics, mass spectrometry, microproteomics, protein analysis, sample preparation, tissue microproteomics, top-down approach

1 | INTRODUCTION

Given the central importance of proteins as biological effectors in physio-pathological processes, mass spectrometry-based proteomic approaches (MS proteomics) have been incorporated in the toolbox of life and health scientists [1, 2], pathologists [3–5] and pharmacologists [6]. Proteomic approaches can be classified at different levels. For instance, (i.) bottom-up proteomics consists of the analysis of proteolytic peptides that are produced by a controlled enzymatic digestion while a top-down approach analyses proteins in their intact state [7]; (ii.) labelling proteomic approaches are based on the analysis of isotopically labelled peptides while label-free quantification (LFQ), is based on the normalization of non-labelled peptides by extracted ion currents (XIC) [8]; (iii.) manual proteomic workflows are performed by a laboratory personnel while automation is accomplished by a robot [9, 10]; (iv.) standard (or macroscale) proteomics involves sample preparation of large sample amounts while evolution of MS instrumentation in terms of velocity and sensitivity, together with the development of adapted sample preparation approaches now enables to analyse proteomes in limited-size samples [11].

In our review, sample processing of cell isolates or cultured cells and methodologies based on direct handling of dissected tissue microregions, termed as microproteomic approaches, will be described and discussed. First of all, we will briefly discuss limitations brought by the standard proteomic workflows (macroproteomics), that have led to miniaturisation of sample preparation via developing microproteomic workflows. The goal of this overview article is not to provide a fully exhaustive list of already existing studies, but rather a description

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of principal systems for miniaturised proteomic sample preparation. To our knowledge, no abbreviation has been reported for microproteomics; thus, we used μPs (adjective μP).

2 | TOWARD MICROPROTEOMICS

In proteomics, sufficient sample amount is required to reach high numbers of protein identifications [12]. The lack of substantial biological (clinical) sample amount is often a problem [13]. Thus, adapted sample collection ensures the required quantity of starting biological material. Once sampling is done, sample preparation and clean-up can be performed. However, each step of sample preparation workflow contributes to the certain loss of the protein content. Eventually, downstream analysis must be sensitive-enough and analyte concentration should be above the limit of protein detection. The standard (macro)proteomic approach can tolerate sample loss as micrograms to milligrams of proteins are commonly processed. However, standard procedures may not be applicable to limited-size sample, i.e. when hundreds to several thousands of cells are available. In this context, the signal from low-abundant proteins is prone to be lower or even absent, resulting in a reduced number of identified protein groups. Potential biomarkers of a disease can be present at relatively low abundances. Furthermore, biomarkers can be easily masked by major compounds during analyses [12]. Issues related to limited-size samples were addressed by developing μP approaches where micrograms or even sub-micrograms of proteins can be treated while retrieving valuable proteome coverage [14]. The early processes of disease (e.g., cancer) usually take place at a restricted scale and the sample amount available for analysis is thus small. Additionally, when the disease progresses, the analysis of cell or tissue bulks via standard proteomics may not inform about molecular heterogeneities within one sample. The μPs addresses effectively such issues [15–17]. To the note, Bensaddek et al. defined as μPs the analysis of up to 5000 cells [18]. However, such approaches were already used for the analysis of samples containing 7500 cells [19], 10,000 cells [20] or even up to 17,000 cells [21].

Regarding μP pipelines, these are derived from macroscale approaches. Generally, μP procedures involve: (i.) sampling—flow cytometry (FC) is a gold standard for the separation and purification of cells from heterogeneous cell populations while laser capture microdissection (LCM) is mostly used to cut and collect precise region-of-interest (ROI) from tissue sections [5, 22, 23]; (ii.) possible cell/tissue lysis—proteins are present in cells and extracellular matrix; thus, these should be denatured/extracted/purified before analysis. Cell/tissue disruption can be performed using mechanical forces and/or solubilisation via detergents, chaotropes or solvents; (iii.) extraction—proteins undergo extraction to be isolated from interferences; (iv.) possible digestion—in bottom-up approaches, proteins are enzymatically digested to peptides; (v.) clean-up—protein/peptide mixture is cleansed to get a highly pure digest via a solid phase extraction; (vi.) fractionation—redistribution of proteins/peptides via, for example, pH-dependent fractioning to reduce sample complexity [24]; (vii.) separation and analysis—peptides/proteins are separated on chromatographic columns (reverse-phase, ion-exchange or size-exclusion) [25, 26], coupled off-line/on-line to MS analyser (e.g. nano-LC-MS/MS). State-of-the-art mass spectrometers that have been used are Orbitrap instruments enabling analysis down to attomolar protein concentrations. Besides nLC-MS/MS-based μPs, MS-imaging (MSI) which can be used to guide μP workflows, allows to gather specific proteins localisations and/or distribution of molecular signatures in tissues. Here, MALDI-MSI can provide MS images of intact protein, or in-situ digested peptides [27]. The main advantage of MSI is the possibility to analyse proteins directly in their histological context and can be utilised prior to LCM to guide nLC-MS/MS-based μP procedures. The acquired results from both approaches can be correlated for parallel protein analysis [28, 29]. Thus, in this review, we describe this methodology, mostly as an auxiliary technique to guide μPs; (viii.) data assessment—acquired mass spectra with different intensities/areas are matched to those present in mass spectral library and normalised. The ultimate steps involve evaluation of differences of protein concentration or relative abundance between tested groups, followed by a correlation with gene ontologies and/or pathway analysis [29–31]. In the following sections, we will describe μP approaches adapted to cell and tissue samples.

3 | CELL MICROPROTEOMICS

Two types of μP analyses can be distinguished based on the nature of sample such as cells and tissues. Compared to tissue specimens, processing of suspended cells is more straightforward, flexible and better controlled. The dilution and overall handling of freshly isolated or cultured cells is easier and their collection can be done by a wider panel of sampling approaches (FC, LCM). Solution mixtures with suspended cells are commonly handled in collection tubes in which all treatment steps can be performed with no extra transfer or handling. Also, suspended cells can be easily lysed because of the absence of extracellular matrix. Thus, sample loss can be reduced.

In the following sections, we will describe μP approaches when cells suspended in the sample solution are processed. Approaches are classified based on the type of digestion, i.e. in-gel, in-solution and related digestion approaches.

3.1 | Bottom-up approaches

3.1.1 | In-gel digestion

Digestion of proteins to peptides (identified via MS in course of proteomic analysis), is done in the gel material (e.g., polyacrylamide). In-gel digestion was used for LCM-guided μPs to study cellular protrusion subtypes such as filopodia and growth cones, in a Cath.a differentiated cells (CAD) of the mouse B6/D2 F1 hybrid [32]. At first, the cells were fixed with dithiobispropionimidate. Afterwards, LCM of protrusions and whole cell content was performed. The area around the ROI served

as a negative control. Then, collected structures were denatured and run on a Mini-Protean TGX gels to form limited gels for separation via their size. The gels were cut to 1×1 mm^2 regions and proteins were subjected to reduction, alkylation and in-gel digestion. Finally, peptides were extracted, dried, reconstituted and analysed by nLC-MS/MS. Here, the distinct protrusions were analysed on a small scale level. Only 5 or 3 μg of protein from cellular protrusions or whole cells, respectively, were used to eventually reach an acceptable proteome coverage, i.e. up to 3052 proteins identified.

3.1.2 | In-solution digestion

Digestion of proteins is performed with proteases in homogenous sample solutions. In-solution processing is the most effective for both protein solubilization and digestion, since the interaction of the sample compounds with the reagents takes place in a homogenous reaction environment. Incubation time can, however, be long and overnight tryptic digestion is a common practice.

In an initial microproteomic study, ~5000 MCF-7 cells were processed using a trifluoroethanol (TFE)-based extraction [33]. Sample loss was reduced compared to detergent-based sample preparation. TFE facilitated protein solubilisation and clean-up was omitted as TFE was removed simply by evaporation. TFE processing enabled to identify up to 133 proteins in ~500 ng of total protein content.

A high proteome coverage (~5000 expressed proteins) was achieved when processing from 50 to 5000 cells of breast adenocarcinoma (MCF-7) [34]. Proteome profiling and protein assignment to the gene transcripts were performed. First, ultrasonication-assisted cell lysis was performed, followed by in-solution digestion with endoproteinase Lys-C. Afterwards, analysis via nLC-MS/MS was performed. In samples containing 50 cells, around 1000 expressed proteins were identified in three individual MS analyses. When performing overlap evaluation between proteins and their transcripts, ~4000 proteins (from overall 5000 expressed) resulted to be likely present in the sample (80%) while the rest expressed false positivity.

In the work of Brioschi et al. [14], μPs was performed on only 1000 human monocyte-derived macrophages. The cells were sampled from human venous blood and cultured. Thereafter, these were deposited onto membrane slides, air-fixed and selected by LCM. A total of 136 proteins was identified to define spindle- and round-shaped macrophages that were present within a single cell culture. A similar comparative profiling of 6000 macrophages that were captured via LCM from blood-derived monocytes, was done by the same research group [35]. Air-fixed cells were processed by shotgun μPs and different macrophage phenotypes were characterised in spindle and round cells based on their intracellular differences—cytoskeleton or according to responses to stress.

Automation is an option to overcome sample loss for μP applications. Two automation strategies based on microchannel network can be distinguished, such as (i.) microfluidics chips and (ii.) micro LC-injection valve. A highly reproducible microfluidic-based cell sorting system was used to treat THP-1 cells [30]. The microfluidic chip was able to separate from 100 to 1000 cells while keeping these undamaged. Using nLC-MS/MS, up to 911 proteins could be identified. The main adjustment of chip was the horizontal direction of the sheath flow controlled by air pressure. Once the cells were collected in reservoirs, these were transferred to a microtube reactor (coated with a 1% BSA), suspended in a highly salted solution and further treated by a single pot procedure. Apart from treatment of limited-size cell samples, in another work [36] microfluidics was also used to manipulate minute volumes of solvents and buffers. In this study, digital microfluidics was used based on coated electrodes immersed in a carrier liquid to propel driving potential which moves sample and solvents. Briefly, 100–500 mammalian cells (Jurkat T cells) were processed with cell lysis, extraction and single-pot solid-phase-enhanced sample preparation (SP3). In SP3 [37], the sample containing proteins that were initially extracted and solubilised in solutions of detergents, is filled with coated paramagnetic beads to capture proteins by hydrophilic affinity. The sample is then rinsed with organic solutions to remove interferences from proteins that are thereafter eluted and digested. Around 1250 and 2500 proteins were identified with a semi-automated procedure, when analysing 100 and 500 T cells, respectively [36]. The latter microchannel system (micro LC-injection valve) was used for ultrasensitive proteome profiling in only 100 living cells, utilising a partially automated approach termed as integrated proteome analysis device (i-PAD-100) [38]. The Duket's type C colorectal adenocarcinoma cell line (DLD-1), was used as a model sample. Briefly, after culturing cells and performing manual in-solution digestion, proteolytic peptides were online injected in the i-PAD device. Briefly, the system was composed of a 10-post valve that can be switched between two positions for i) cell suspension loading, ii) cell digestion and peptide trapping before nLC-MS/MS analysis. A total of 813 proteins was identified with a 200 zmol limit of protein detection and the system can possibly be used for single cell analysis (SCA). However, issues may appear when using flow automation approach with capillaries of smaller diameters (hundreds of nm). Adsorption of analytes on capillaries humpers the analysis. The problem was addressed by a single tube sample preparation method where a stationary testing tube/reactor was used to process protein sample at the small scale level [39]. A range of 1–100 cells human oocytes was processed. Here, scaling analyses down to single cell proteomics (SCPs) was achieved using SP3 protocol. SCPs enabled to identify ~450 proteins in ~100 ng of protein per cell. Recently, an improved dropled-based microfluidic system was coupled with shotgun proteomics in a nL-scale oil-air-droplet (OAD) microfluidic chip [40]. The system enabled SCPs of a HeLa cell (51 proteins identified) and a mouse oocyte (355 proteins identified). Briefly, 1 μL of cell suspension was aspired in a capillary tip and 100 nL droplets containing one cell, were dispensed into the droplet layer of the OAD chip, followed by oil encapsulating (in the oil layer of the OAD chip) to prevent sample evaporation and ensure a hermetic reaction environment. Afterwards, reagents were dispensed with the capillary probe for sample preparation—cell lysis, reduction, alkylation, in-solution digestion. In this setup, highly precise single cell sample preparation and analysis were performed (<99% sample injection efficiency was achieved).

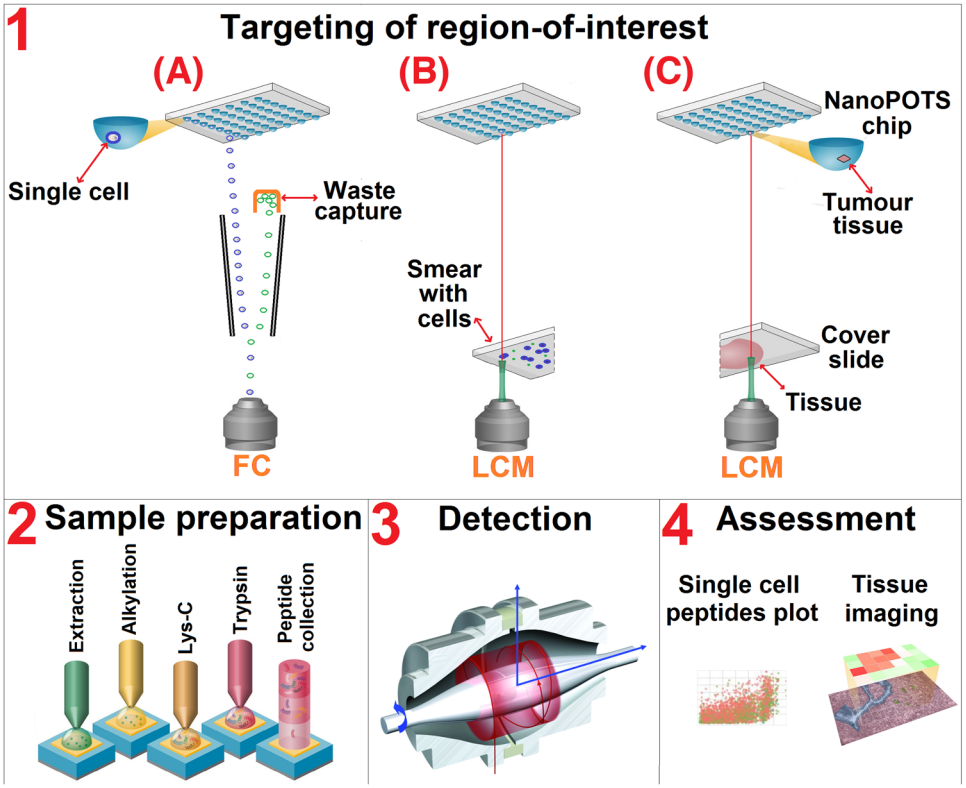

FIGURE 1 nanoPOTS approach for microproteomic sample preparation of cells/tissues [42, 43]. 1. Cells are sorted using FC and deposited into the nanowell droplets on the nanoPOTS chip (A), LCM is used to microdissect cells from smears (B) or tissue sections (C) toward the nanoPOTS; 2. Protein sample preparation is performed in a single nanoPOTS droplet; 3. Analysis is performed via mass spectrometry; 4. Bioinformatics data processing is performed for protein identification/quantification

Another automation strategy based on coupling FC with 96-well plate was suggested by Slavov's research group. The system was called Minimal ProteOmic sample Preparation (mPOP) and used with SCPs of mammalian cells (HEK-293 and U-937) [41]. To lyse these cell types, no detergent/chaotrope was necessary; thus, clean-up was avoided. This improved sample throughput and reduced sample loss. Instead, a focused acoustic sonication-based cell lysis was performed in water. To increase robustness of the lysis and deliver peptides to nLC-MS/MS, a freeze-heat cycles approach was tested and found to be effective before in-solution digestion.

A recently developed state-of-the-art proteomic system which also allowed for automation was called nanodroplet processing in one pot for trace analysis (nanoPOTS). The method has been applied to manipulate and analyse size-limited cell populations (from tens to hundreds of cells) and was suitable to treat cells suspensions as well as tissues (Figure 1) [42–44]. The system is composed of a glass-made chip with a sealed membrane and spacer placed underneath. On the hydrophobic glass slide of the chip, tens of nano-dimensional droplets (nanowells or nanovessels) are created (several nL). Cells in suspension can be collected via FC and cell smears or tissues by LCM. These are further driven to nanoPOTS, for nano-scale sample preparation—extraction, alkylation, two-step in-solution digestion and proteolytic peptides collection. The membrane-coated slide prevented evaporation of the solvent nanowells while processing. Glass composing the chip facilitates sample visualisation via a microscope [44]. The nanoPOTS can be used either for manual sample loading for LC-MS/MS analysis or an autosampler can be applied for online detection [44, 45]. Eventually, nanoPOTS were used for SCPs [42]. It permitted the proteome profiling of 1–5 circulating tumour cells from the whole blood. Using blood spiked with LNCaP cells, 607 protein groups were identified from five cells. In a later work [43], nanoPOTS were also used for TMT-labelling of mammalian cells (epithelial, immune and endothelial) for multiplex analysis of proteins at the single-cell concentration level. From 72 single cells, 2300 proteins were identified in two days. Overall, the method was fairly miniaturised, sensitive and provided comprehensive protein coverage. Recently, authors adapted the principle of nanoPOTS to a benchtop-compatible sample processing workflow and were able to identify ~1800 proteins only from ~25 HeLa cells [46]. Ultimately, authors very recently used nanoPOTS workflow for HeLa cell SCPs, identifying >1000 protein groups using high-field asymmetry ion mobility spectrometry [47].

3.1.3 | Alternative digestion approaches

Protein digestion can be performed with aid of solid surfaces (e.g., on-beads). Here, enzymes are immobilised on active sites of beads and

the sample is pushed across the digestion chamber where proteins are cleaved to peptides.

Spatially targeted optical microproteomics (STOMP) is an approach based on confocal microscopic imaging and tagging of subcellular structures in protein-specific size-limited regions and downsteam digestion of the tagged proteins [48]. The system was used to analyse human skin fibroblasts or mouse bone marrow-derived dendritic cells infected with *Toxoplasma Gondii*. The system was fully automated (autoSTOMP) and consisted of protein tagging, digestion aided by streptavidin magnetic beads, clean-up and protein identification via MS. Here, automation ensured no operator intervention while regional microproteomes could be analysed in small sample areas (1-2 μm size). Overall, 960 human and 736 parasite proteins were identified based on size, shape and location of structures of interest.

3.2 | Top-down approaches

Top-down proteomics refers to the approach performed without digestion, i.e. proteins measured in their intact form. Top-down proteomics has been used to analyse protein isoforms, PTMs or small proteins. In the case of small proteins, the digestion step in shotgun proteomics can result in a lack of proteolytic peptides for eventual identification. Conversely, top-down approach do not rely on the identification of a sufficient number of proteolytic peptides [49].

Labas et al. performed SCP analyses on intact oocyte cells from ovarian follicles, cumulus cells and granulosa cells [50]. Three operational modes were used such as (i.) top-down direct infusion μPs based on introduction of micropurified product (after sample processing and clean-up) into a metalized nanoelectrospray needle and MS analysis; (ii.) direct-injection to nLC-MS/MS and (iii.) combination of manual prefractionations with reverse-phase/gel filtration chromatography prior nLC-MS/MS. The system enabled to identify 386 proteforms encoded by 194 genes. Sixteen markers corresponding to oocyte maturation were found on the single cell level.

In another work [51], top-down proteomics based on nanoPOTS was used for the sensitive analysis in HeLa cells. In the procedure, a combination of dodecyl-β-D-maltopyranoside detergent with urea was used to get a high protein recovery from small sample size, i.e. ~170-620 proteoforms identified in only ~70-770 cells from a total protein amount of <120 ng. Moreover, several PTMs were identified such as acetylation, myrostoylation or iron binding, in only ~800 HeLa cells.

4 | TISSUE MICROPROTEOMICS

Compared to cell suspensions, tissues are solid biological constructions composed of diverse cell populations and extracellular matrix, and necessitate dedicated considerations for μP manipulation. Methods for sampling of tissue ROI are usually grafted to approaches for classical histological evaluation such as microscopy. This is exemplified with LCM, where microscopic evaluation is directly coupled to laser-based dissection of pieces of tissue sections.

4.1 | Bottom-up approaches

4.1.1 | In-gel digestion

Analysis of breast tumour tissue sample was done using a short-range sodium dodecyl sulphate gel electrophoresis (short-range SDS-PAGE) [20]. In this study, 16% Tricine gel fractionator was used followed by in-gel digestion and nLC-MS/MS. The main modification consisted of shortening the separation distance (2.5 cm) for gel fractionation compared to commercial SDS-PAGE kit (10 cm). As a result, more efficient protein analysis was achieved as reduced gel perimeter ensured high analyte preconcentration. Four-fold more proteins were identified (1100 proteins with two or more unique peptides) in only 10,000 cells. Likewise, the approach was also used for proteome profiling in human prostate cancer tissue pieces [52]. More than 2000 proteins were identified (false discovery rate lower than 1%) and the technical and sample (biological) replicates showed excellent reproducibility (coefficient of variation lower than 15%).

4.1.2 | In-solution digestion

Small mouse brain voxels (~0.75 mm^3 per single brain voxel) were subjected to a 3-dimensional protein profiling via TFE-based proteomic procedure [33]. TFE facilitated protein solubilisation and denaturation, and diminished sample loss as clean-up step was avoided. In another work [53], brainstem nuclei from songbirds samples were processed by μPs to study the vocal learning based on proteomic changes. Shortly, LCM was followed by protein extraction, in-solution digestion and analysis via nLC-MS/MS. Only 1000 tissue cells were necessary to identify up to 300 proteins. A high care was given for each processing step (mainly extraction and digestion) to avoid loss of analytes. The achieved limit of protein detection was <0.75 μg which was close to the initial protein content of 1–2 μg present in the studied sample. The LCM-μPs-nLC-MS/MS workflow with in-solution digestion was also recently used to study protein abundances in various regions and layers of hippocampus dentate gyrus of an electrically stimulated rodent (suffering from emulated hippocampal sclerosis) [54]. At first, the tissue regions/layers were homogenised and centrifuged. Then supernatant proteins were processed by reduction, alkylation, in-solution digestion and clean-up. Differences in protein profiles, i.e. ventral versus dorsal regions and granular versus molecular layers of hippocampus, were found. A comparative proteomic profiling was also performed in human glomerulus [13]. Cells were sieved from a patient with renal cell carcinoma using only 1 μg of protein digest. In this study, 332 distinct proteins were identified (false discovery rate ≤1%) with five different MS analysers.

As presented in our very recent review [10], automation of proteomics has brought benefits over manual procedures. De Graaf et al.

reported partially-robotised μPs workflow based on the SP3 procedure. In SP3, coated magnetic beads are used for protein capture and sample clean-up within a single tube [55]. Small regions from cortex and medulla (5 mm^2 cortex and 1 mm^2 medulla) of mouse kidney tissues were analysed. Deep proteome coverage was achieved—3440 protein groups and 5002 protein groups were identified in cortex and medulla, respectively. Recently, Müller et al. fully automated the SP3 workflow (auto-SP3), to quantify 500–1000 proteins in 100–1000 cells [56]. The approach was used on 5 μM-thick sections of 5 × 5 × 5 mm cubes of formalin-fixed and paraffin-embedded (FFPE) non-small cell lung cancer tissues. Overall, SP3 holds good promise for future sample input reduction and possible adaptation for tissue μPs. In another work [57], SP3 was also used on mouse brain regions with high-grade glioma. At first, high resolution 15T MALDI-FTICR-MSI was compared with MALDI-TOF-MSI to measure intact proteins directly in tissue sections. The first visualisation provided datasets where various protein charges, different proteoforms or isotopic distributions of distinct protein ions, could be distinguished using MALDI-FTICR-MSI. Further, μPs-nLC-MS/MS was performed from only 0.8 mm^2 tissue regions. Here, protein identification could be assigned thanks to the correlation of MALDI-MSI with LC-MS/MS data. However, a limitation was the need to use consecutive tissue sections, on conductive glass slides for MSI and on polyethylenenapthalate (PEN) membrane slides for LCM. The issue was addressed in a later study [58], where atmospheric pressure MALDI-MSI and μPs-nLC-MS/MS where performed from the very same section. In this work, PEN slides were used both for MSI and LCM. Further, LCM-SP3-based μPs-nLC-MS/MS was used for the differential analysis of corpus callosum, motor cortex and sciatic nerves of Twitcher mice (a model for Krabbe disease) and wild type mice, as depicted in Figure 2 [59]. After SP3, 10-plex tandem mass tagging (TMT) of peptides was done. Between Twitcher and wild-type mice, more than 400 expressed protein groups differed and were associated with pathways linked with studied Krabbe disease. The comprehensive proteome coverage represented >3300 protein groups identified for each dataset.

As mentioned upon, nanoPOTS systems enabled analyses from a few tens of cells proteomic analysis to SCA. Apart from cells samples, the system has proved also to be suitable for tissue samples (Figure 1) [60]. Only ≤200 cells were necessary to achieve in-depth proteome coverage (1870 protein groups were identified) in spatially resolved analysis. A total of 422 proteins was identified only within ~0.04 mm^2 tissue region containing only ~8-15 cells (tomato parenchyma). The same research group used nanoPOTS for in-depth proteome profiling [44]. More than 3000 proteins were identified in only 10 cells while ~2400 proteins were quantified in a single human pancreatic tissue section (islet thin areas). Very recently, using spatially resolved nanoPOTS, 2000 proteins could be visualised in mouse uterine tissues with 100-μm spatial resolution [61]. In this study, after tissue cut by LCM, robotic sample preparation (extraction, reduction, double in-solution digestion), was done on a 96-well platform; thus, exhibiting a high reproducibility and throughput.

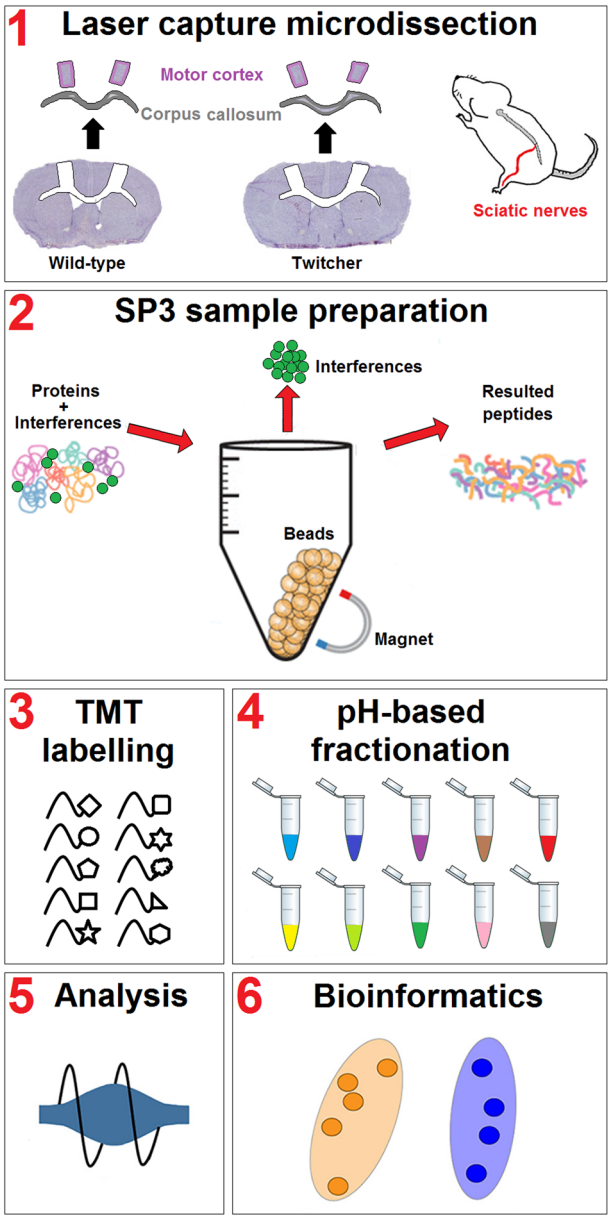

FIGURE 2 SP3-based microproteomic workflow performed on a model mouse [59]. 1. LCM of microregions of different tissue sections are performed; 2. SP3 sample preparation is performed to remove interferences from extracted proteins and elute/digest proteins; 3. Labelling of peptides is performed using tandem-mass-tags for further MS-based relative quantification; 4. pH-based fractionation of peptides takes place; 5. Sensitive analysis is done via mass spectrometry; 6. Identification of protein groups and assignment to molecular pathways are performed

A comparative work to evaluate a single organism analysis μPs (single nematode) versus standard assay, was done on the *Caenorhabditis elegans* worms (Figure 3) [18]. Here, only one worm body was necessary to achieve acceptable protein recovery, that is, ~3000 identifications using a μP workflow versus 5000 identifications from 40,000 worms pool using a macroproteomic approach. The workflow involved cell disruption in a lysis buffer to release proteins, protein reduction,

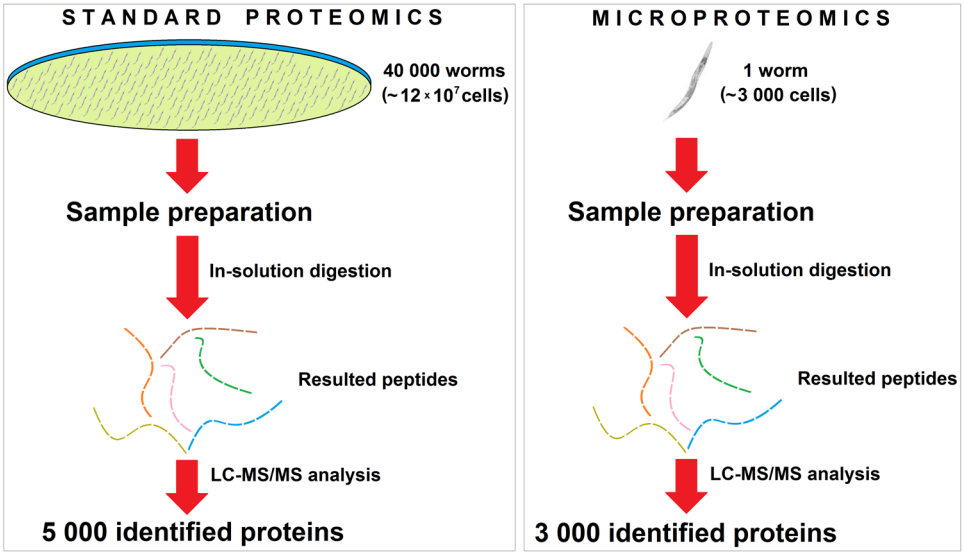

FIGURE 3 Comparison of the microproteomic (1 worm) versus standard proteomic workflow (40,000 worms) performed on *C. elegans*, used as model biological sample [18]

alkylation, double in-solution digestion, sample clean-up and nLC-MS/MS analysis. Using single reaction tube/chamber for lysis, derivatisation and digestion contributed to a higher simplicity of performance. As only a single exchange of lysis buffer could be performed (compared to standard workflow where multiple buffer exchanges are common), sample loss was diminished. The analysis was sensitive (mid-high abundance proteins were identified) and the dynamic working range was 6-orders of magnitude wider than in standard procedure.

4.1.3 | Related digestion approaches

A STOMP approach based on fluorescent-aggregation was used to identify pathological soluble deposits in target tissue regions [15]. Formalin-fixed tissue regions (amyloid plaques in Alzheimert's disease mouse model) were stained by a fluorescent dye and immersed in a solution containing phototags. A confocal microscope was used for affinity photolabelling of proteins to create a dye-protein-phototag conjugate. Once conjugates were formed within the tissue structures, the sample was solubilised and proteins were extracted via nickel-based affinity beads, on-beads trypsin digestion, clean-up and nLC-MS/MS analysis. A total of 146 proteins were identified within a single tissue microregion (0.38 μm^3), with probability >50% of being associated with amyloid deposits.

On-tissue digestion consists of depositing a proteolytic enzyme directly onto a tissue microregion (localised trypsinization) placed on a glass slide [62]. In another hybrid-based approach between on-surface and in-solution digestion, proteins were directly digested on the tissue surface via a highly concentrated trypsin solution, in which the tissue piece is immersed [16, 63]. On-tissue digestion was used for the comparison between benign and malignant ovarian cells [64–66]. In the work of Wisztorski et al. [64], ROIs were targeted from FFPE tissue sections. A piezoelectric microspotter was used to automatically deposit trypsin on FFPE tissue sections for further direct droplet-based on-tissue digestion of proteins. Resulted peptides were extracted by a liquid-microjunction solvent extraction (LMJ) using a solvent mixture (MeOH and ACN). Isolated analytes were transferred to the collection tubes and analysed by nLC-MS/MS. Only ~2500 cells were used to perform comprehensive proteomics with overall 1109 identified proteins. With FFPE samples, analyte loss was minimised when compared to standard shotgun procedure. In a later study [19], Wisztorski et al. tested the compatibility of detergents for enhanced LMJ of proteins from the rat brain ROI, in comparison with solvent mixtures. Briefly, tissue sections were subjected to liquid extraction surface analysis from areas of ~1.28 mm diameter. The detergent efficiency was evaluated via gel electrophoresis of intact proteins. Downstream filter aided sample preparation (FASP) combined with nLC-MS/MS, allowed the identification of >1400 proteins. Also, ten clusters (spots) from different brain parts were analysed to arrange a protein distribution map across a sagittal section. In a later study [66], authors studied fresh frozen ovarian tumours surrounding tissue regions. Here, proteins were analysed via MALDI-MSI for further segmentation-based definition of ROIs. Thereafter, nanodroplets of trypsin were directly deposited on ROIs from serial sections for further 1-hour digestion and LMJ-based extraction of proteolytic peptides. Digestion was possible in a 250 μm-diameter spot which was ~62% smaller than spots analysed in previous studies of the same group (formerly 650 μm) [67]. In another work from the same group [68], MALDI-MSI was performed (Figure 4A) before on-tissue digestion and nLC-MS/MS analysis (Figure 4B), in glioma grade III samples. More than 2500 proteins were identified, from which 22 were AltProts (i.e., alternative proteins referring to the hidden proteome). A functional analysis was performed and allowed to distinguish three clusters/groups of distinct glioma tissue samples where the sub-networks corresponded to neoplasia (cluster one), inflammation and metastasis (cluster two) and nerve cell differentiation and neurite outgrowth (cluster three). Very

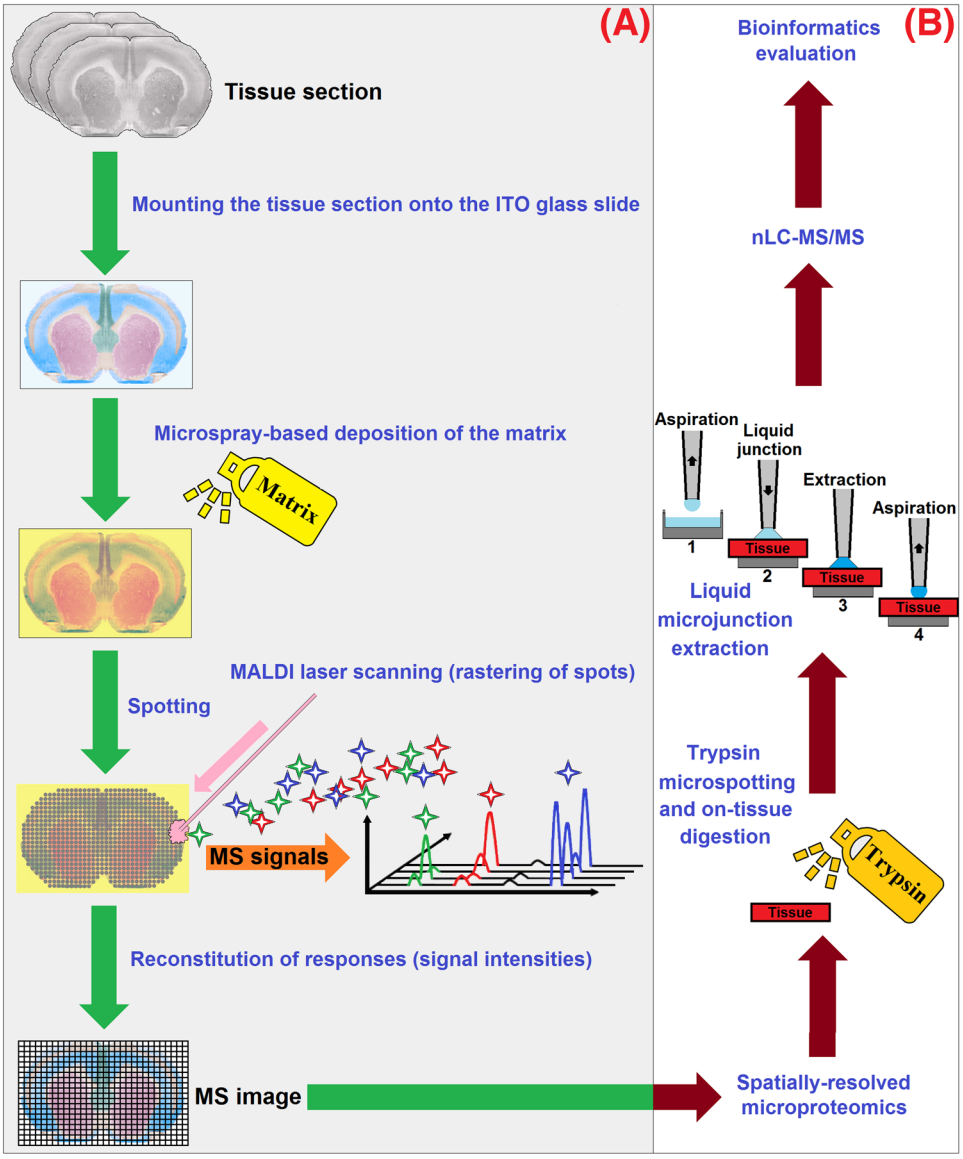

FIGURE 4 MALDI-MSI (A) combined with spatially-resolved microproteomics based on on-tissue digestion and LMJ (B), for protein analysis in human glioma tissues [68]

recently, authors performed spatio-temporal μPs to map a protein profile in microregions of rat brain from a model of traumatic brain injury [69]. A controlled cortical injury was made at different time-points (1st, 3rd, 7th, and 10th day). The acute, subacute and subchronic stages were described via molecular pathways. From only a 1 mm^2 area, proteins were extracted by LMJ and 1950 proteins were identified across all studied regions, from which 306 proteins were quantified. Thus, molecular predispositions for Parkinson disease associated with post-traumatic brain injury could be approached.

As already mentioned, a hybrid on-tissue/in-solution digestion approach can be used by performing the digestion directly from a LCM tissue piece placed in the bottom of a tube, using highly concentrated trypsin solutions and without previous protein extraction. Using this approach, Longuespée et al. analysed FFPE tissue pieces for biomarker discovery between ductal and lobular invasive triple negative breast cancer samples [16, 63]. Once the FFPE sections were stained (hematoxylin-eosin), LCM was performed from serial sections and samples were transferred to the bottom of the tubes (pre-processing) and visualised by microscope to check their presence (Figure 5). Afterwards, citric acid-based antigen retrieval was performed to disrupt methylene bridges between proteins. Reduction, alkylation and double digestion with highly concentrated trypsin solutions were performed. Once the digestion was accomplished, sample was cleansed (desalted) and injected for nLC-MS/MS. Using this approach, >1400 unique proteins were identified in only ~2700 cells, that corresponded to a 375,000 μm^2 region. The approach was also proved to be suitable for other neoplastic tissues [16]. Later, the method was used to analyse FFPE samples of cystic echinococcosis in human liver by comparing *Echinococcus granulosus* tapeworm (sample 1), cystic wall (sample 2) and adjacent liver areas (sample 3) [70].

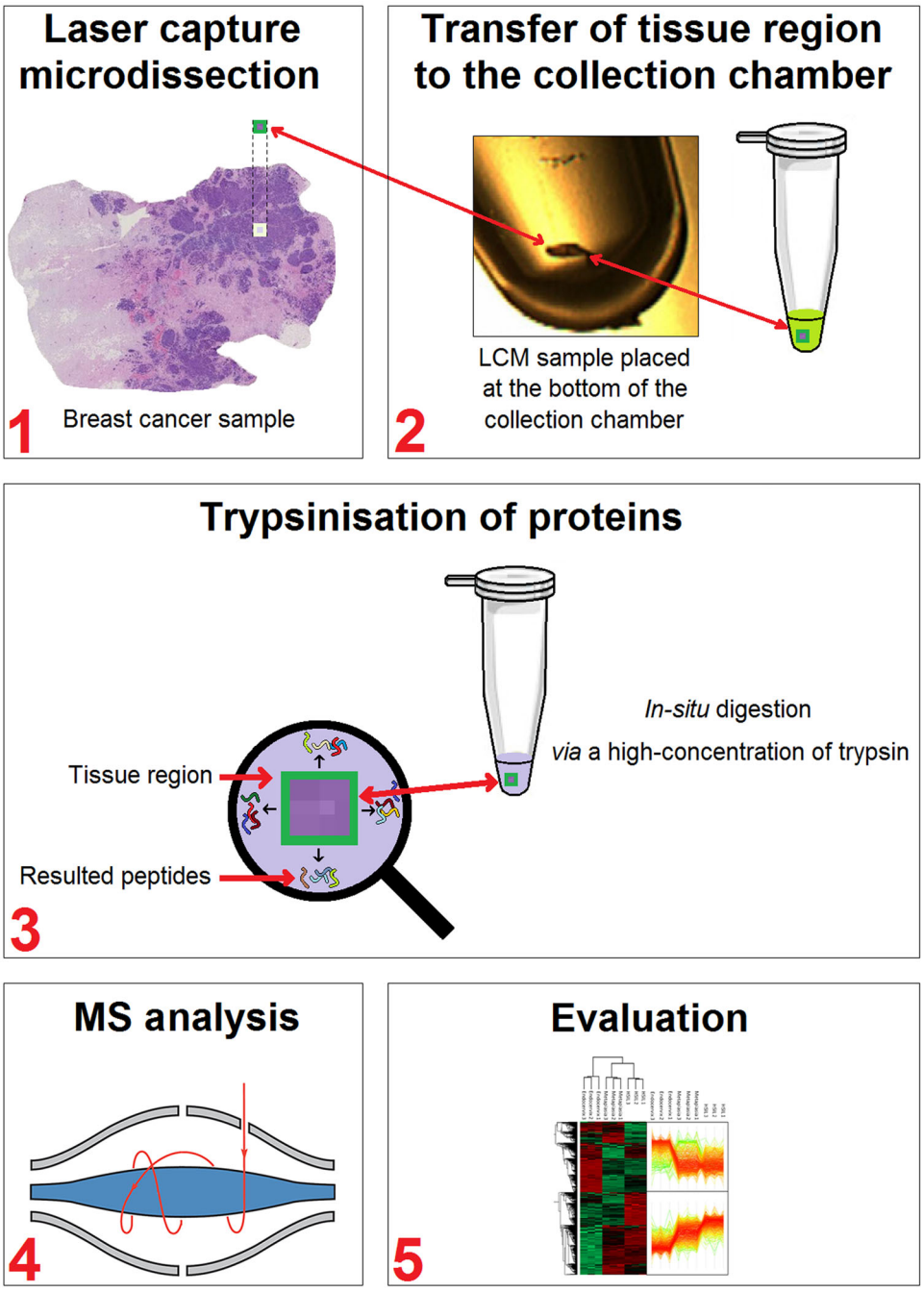

FIGURE 5 A LCM-guided microproteomic workflow with hybrid in-solution/on-tissue digestion of proteins performed on different biological tissues—human/animal [63]. 1. LCM of tissue region-of-interest is performed; 2. Transfer of the target sample to the bottom of the collection tube is performed; 3. Sample processing consists of reduction, alkylation, reduction and direct in-solution/on tissue digestion with a high concentration of enzyme; 4. Analysis via orbitrap-mass spectrometry; 5. Data processing

The tapewormt's nesting was studied based on proteomics and 85, 184, and 927 specific proteins were detected in sample 1, 2, and 3, respectively. This hybrid on-surface/in-solution method could also be used for investigations in anal canal carcinoma in order to discriminate proteomic profiles between dualistic and clinically relevant classes, originating from the squamous zone and from the transitional zone of the anal canal [71]. In another study [31], the authors used FFPE tissues and LCM-μPs for the profiling of high-grade squamous intraepithelial lesion of the cervix (HSIL). The proteome of HSIL was compared to normal samples taken from ectocervix and endocervix (all sampled from five individuals). In total, 3072 proteins were identified via nLC-MS/MS. After statistical analysis and IHC validation, several biomarker candidates (e.g., MCMs or ASF1B) of cervical dysplastic lesions were highlighted. The biomarker discovery data from this study were further correlated with MALDI images in order to assign with more precision protein identification to peaks of interest [72]. Here, it was

TABLE 1 An overview of recent bottom-up and top-down microproteomic applications

Sample	Approach	Number of cells	Digestion	Quantification	Comment	Ref.
Human breast cancer tissue	Bottom-up	10,000	In-gel	Label-free	1123 proteins were identified[a]	[20]
Human prostate cancer tissue	Bottom-up	N.i.	In-gel	Label-free	>2000 proteins were overall identified	[52]
Mouse CAD cells[b]	Bottom-up	N.i.	In-gel	Label-free	≤3052 proteins were overall identified	[32]
Human macrophages[b]	Bottom-up	1000	In-solution	Label-free	136 proteins were overall identified	[14]
THP-1 cells[b]	Bottom-up	100	In-solution	Label-free	Up to 549 proteins were overall identified	[30]
THP-1 cells[b]	Bottom-up	1000	In-solution	Label-free	Up to 911 proteins were overall identified	[30]
MCF-7 cells[b]	Bottom-up	50-5000	In-solution	Label-free	~5000 proteins were overall identified	[34]
MCF-7 cells[b]	Bottom-up	~5000	In-solution	Label-free	83-133 proteins were identified in four samples	[33]
Jurkat T cells[b]	Bottom-up	100, 500	In-solution	Label-free	1851 proteins were identified[a]	[36]
HeLa cells[b]	Bottom-up	~25	In-solution	Label-free	~1800 proteins were overall identified	[46]
HeLa cells[b]	Bottom-up	2-3[c]	In-solution	Label-free	1475 protein groups were overall identified	[47]
HeLa cells[b]	Bottom-up	1[c]	In-solution	Label-free	51 proteins were overall identified	[40]
HeLa cells[b]	Bottom-up	10-100	In-solution	Label-free	192-1360 proteins were overall identified	[40]
Mouse oocyte[b]	Bottom-up	1[c]	In-solution	Label-free	355 proteins were overall identified	[40]
Human oocyte[b]	Bottom-up	1[c]	In-solution	Label-free	~450 proteins were overall identified	[39]
Human oocyte[b]	Bottom-up	100	In-solution	Label-free	~2154 proteins were overall identified	[39]
Circulating tumour cells from human whole blood[b]	Bottom-up	1-10	In-solution	Label-free	up to 607 protein groups were identified	[42]
DLD-1 cells[b]	Bottom-up	100	In-solution	Label-free	813 proteins were overall identified	[38]
Tomato tissue with parenchymal cells	Bottom-up	≤200	In-solution	Label-free	1870 protein groups were overall identified	[60]
Human pancreatic tissue	Bottom-up	~10	In-solution	Label-free	>3000 proteins were overall identified	[44]
Human kidney tissue	Bottom-up	N.i.	In-solution	Label-free	332 human glomerular proteins were overall identified	[13]
Songbird brainstem motor tissue	Bottom-up	1000	In-solution	Label-free	Up to 300 proteins were overall identified	[53]
C. elegans	Bottom-up	~3000	In-solution	Label-free	>3000 proteins were overall identified in the single worm	[18]
Rat hippocampus tissue	Bottom-up	N.i.	In-solution	Label-free	Up to 959 proteins were overall identified	[54]
Mouse tissue *voxels*	Bottom-up	N.i.	In-solution	Label-free	587-855 proteins were identified in four different voxels	[33]
Mouse uterine tissue	Bottom-up	N.i.	In-solution	Label-free	>3000 proteins were overall identified with 100 μm spatial resolution	[61]
Mouse liver tissue	Bottom-up	N.i.	In-solution	Label-free	~1200 proteins were overall identified	[46]
GBM mouse brain tissue	Bottom-up	N.i.	In-solution	Label-free	N.i.	[57]
Mouse brain glioma tissue	Bottom-up	N.i.	In-solution	Label-free	3440 protein groups were overall identified in 0.5 mm^2 area	[58]
Mouse brain glioma tissue	Bottom-up	N.i.	In-solution	Label-free	5002 protein groups were overall identified in 1 mm^2 area	[58]
Mouse kidney tissue	Bottom-up	N.i.	In-solution	Label-free	N.i.	[55]
HEK-293 cells[b], U-937 cells[b]	Bottom-up	1[c]	In-solution	Labelling	N.i.	[41]
U-937 cells[b], Jurkat cells[b]	Bottom-up	10000	In-solution	Labelling	2438 proteins were overall identified	[41]

(Continues)

TABLE 1 (Continued)

Sample	Approach	Number of cells	Digestion	Quantification	Comment	Ref.
Murine cells[b]	Bottom-up	72[c]	In-solution	Labelling	2300 proteins were overall identified	[43]
Mouse kidney	Bottom-up	N.i.	In-solution	Labelling	6052 protein groups were overall identified	[55]
Mouse brain tissue	Bottom-up	N.i.	In-solution	Labelling	>3300 protein groups were overall identified	[59]
Human skin fibroblasts[b]	Bottom-up	N.i.	On-beads	Labelling	960 proteins were overall identified	[48]
Mouse bone marrow-derived dendritic cells with *Tg*[b]	Bottom-up	N.i.	On-beads	Labelling	736 proteins were overall identified	[48]
Alzheimert's disease mouse brain tissue	Bottom-up	N.i.	On-beads	Labelling	146 proteins were overall identified	[15]
Rat brain tissue	Bottom-up	7300	On tissue extraction-On-filter digestion	Label-free	>1400 proteins were overall identified in 1 mm diameter spot size	[19]
Rat brain tissue	Bottom-up	N.i.	On-tissue	Label-free	1950 proteins were overall identified	[69]
Rat brain tissue	Bottom-up	1900	On-tissue	Label-free	1500 proteins were overall identified in ~650 μm diameter spot size	[67]
Human ovarian cancer tissue	Bottom-up	~2500	On-tissue	Label-free	792[d], 983[e] nonredundant protein groups were overall identified	[64]
Human ovarian cancer tissue	Bottom-up	275	On-tissue	Label-free	>500 protein groups were overall identified in 250 μm diameter spot size	[66]
Human glioma tissue	Bottom-up	N.i.	On-tissue	Label-free	>2500 proteins were overall identified	[68]
Human breast cancer tissue	Bottom-up	~2000	On-tissue	Label-free	>1000 proteins were overall identified	[29]
Human breast cancer tissue	Bottom-up	2700	Hybrid on-tissue/in solution	Label-free	>1400 proteins were overall identified from a single biopsy	[16]
Human cyst liquid with *Eg*, cystic wall and adjacent liver tissue areas	Bottom-up	N.i.	Hybrid on-tissue/in solution	Label-free	927 proteins were overall identified	[70]
Invasive ductal breast cancer tissue	Bottom-up	~3500	Hybrid on-tissue/in solution	Label-free	3538 protein were overall identified	[28]
Anal canal carcinoma	Bottom-up	~5000	Hybrid on-tissue/in solution	Label-free	4465 proteins were overall identified	[71]
HSIL, endoC and ectoC tissues	Bottom-up	3500	Hybrid on-tissue/in solution	Label-free	Up to 3072 proteins were overall identified	[31]
Mouse dental cementum tissue	Bottom-up	N.i.	On-tissue	Label-free	519 proteins were overall identified	[74]
Mouse dental cementum, alveolar bone, dentin tissues	Bottom-up	N.i.	On-tissue	Label-free	243 proteins were overall identified	[75]
Bovine oocyte[b]	Top-down	1[c]	-	Label-free	386 proteoforms were identified	[50]
HeLa cells[b]	Top-down	~70-770	-	Label-free	~170-620 proteoforms were identified	[51]
Rat brain tissue	Top-down	N.i.	-	Label-free	123 proteins were overall identified	[76]
Human ovarian cancer tissue	Top-down	N.i.	-	Label-free	237 proteins were overall identified	[17]

CAD, Cath.A differentiated cells derived from a catecholaminergic neural tumour; DLD-1 cells, Duket's type C colorectal adenocarcinoma; ectoC, normal tissue ectocervix; *Eg*, *Echinococcus granulosus*; endoC, normal tissue endocervix; GBM, Glioblastoma multiforme; HeLa, cervical cancer cells; HSIL, High grade squamous intraepithelial lesion of the cervix; MCF-7 cells, breast adenocarcinoma; N.i., not indicated in the manuscript; *Tg*, *Toxoplasma Gondii*; THP-1, human leukemia monocytic cells.

Sorting is done based on performed digestion mode.

[a]Number of identified proteins with more than one unique peptide.

[b]Cultured cells and body fluid cells were analysed.

[c]Single cell analysis was done.

[d]Proteins were identified in malignant ovarian cells.

[e]Proteins were identified in benign ovarian cells.

demonstrated that statistical analyses resulting from biomarker discovery acted as filter of the several protein candidates for identification. Very recently, differences in aldo-keto reductase family 1 member C3 protein were found between cases of ulcerative colitis and Crohnt's disease, using this approach [21]. Overall, the proteomic analyses revealed eight proteins more abundant in colitis samples compared to those of Crohnt's disease, highlighting a differential neutrophil activity between these two groups of specimens. Approximately 1400 proteins were identified from which 666 were quantified. Finally, the approach was used to propose the concept of MALDI-MSI-guided μPs [28, 73]. MALDI-MSI and image segmentation were first performed to reveal heterogeneous regions within the tumour. Heterogeneous regions were used to guide microdissection and μP processing was performed to compare these regions on a proteomic level. Approximately 3000 proteins were identified in each breast cancer case. Lateron, further improvement of the method was proposed for the precise LMD-based collection of heterogenous regions defined by MSI segmented data [29]. MATLAB software was used to coregister segmented regions obtained via MSI with the same section further stained by haematoxilin and eosin, based on an affine geometric transformation. Segments were recalculated based on coordinates of fiducial markers reported in an XML file that were compatible with the LCM device. Such combination of MSI with LCM-μPs-nLC-MS/MS is a representative illustration of the complementarily of two methods to study the biological mechanisms associated to intratumour heterogeneity.

Other authors [74, 75], used FFPE-based tissues for μPs of dental cementum, alveolar bone and dentin specimens to assign molecular targets to the pathological or reparation dental processes.

4.2 | Top-down approaches

Delcourt et al. analysed microproteins in rat brain microregions. Microproteins and AltProts were translated from the protein-coding sequences other than the ones from reference proteins. In their procedure, MALDI-MSI of lipids and spatial segmentation of brain tissue were performed. Three different regions were chosen and submitted to LMJ or parafilm-assisted microdissection (PAM). Afterwards, top-down proteomics was performed and back-correlated to MALDI-MSI data [76]. The approach has been also used to detect and characterise AltProts in human ovarian cancer cells [17]. Protein profiles of benign, malignant and necrotic tissue microregions of serous ovarian cancer biopsies were studied. Ovarian cancer progression was revealed by profiling of protein changes related to different cell phenotypes from benign, tumour and necrotic-fibrotic tissues. A total of 237 proteins was detected by combining all acquired proteomic data. Each cluster contained specific proteins: 48 in benign, 61 in malignant and 44 in necrotic regions. Apart from reference proteins, from four to six AltProts were detected across the particular clusters.

Approaches based on μPs are summarized and compared in Table 1.

5 | SUMMARY AND OUTLOOKS

In this review, we surveyed the past and recent development in μPs. Given the specificity of μP processing compared to standard workflows, and the large panel of possibilities in this field, it was necessary to summarize the principal μP worflows in a single document. The methods were distinguished based on the processing of either cell or tissue samples and further sub-classified using either bottom-up (in-gel, in-solution or related digestions—on-beads, on-tissue, hybrid in-solution/on-tissue) or top-down proteomic approaches. Both cell and tissue μPs still have several pros-&-cons. However, the development of new strategies for low-input sample preparation together with the evolution of MS instrumentations in terms of sensitivity and throughput, have paved the way for μPs. It is now possible to obtain deep proteome coverage at the single cell level (SCPs) or with high spatial resolution using tissue sections. Method combinations allow for the deep proteomic exploration of the biological mechanism related to intratumour heterogeneity. Overall, these developments represent new opportunities for histological or cellular-based pathological stratification [3–5], but also for pharmacodynamics at anatomical and cellular sites of action [6].

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Laputková, G., Bencková, M., Alexovič, M., Schwartzová, V., Talian, I., & Sabo, J. (2017). Proteomic and bioinformatics analysis of human saliva for the dental-risk assessment. *Open Life Science*, *12*(1), 248–265.
2. Bober, P., Alexovič, M., Tomková, Z., Kilík, R., & Sabo, J. (2019). RHOA and mDia1 promotes apoptosis of breast cancer cells via a high dose of doxorubicin treatment. *Open Life Science*, *14*(1), 619–627.
3. Longuespée, R., Casadonte, R., Schwamborn, K., & Kriegsmann, M. (2019). Proteomics in pathology: The special issue. *Proteomics. Clinical Applications*, *13*(1), 1800167. https://doi.org/10.1002/prca.201800167
4. Longuespée, R., Casadonte, R., Schwamborn, K., Reuss, D., Kazdal, D., Kriegsmann, K., Von Deimling, A., Weichert, W., Schirmacher, P., Kriegsmann, J., & Kriegsmann, M. (2018). Proteomics in Pathology. *Proteomics*, *18*(2), 1700361. https://doi.org/10.1002/pmic.201700361

5. Longuespée, R., Fléron, M., Pottier, C., Quesada-Calvo, F., Meuwis, M.-A., Baiwir, D., Smargiasso, N., Mazzucchelli, G., De Pauw-Gillet, M.-C., Delvenne, P., & De Pauw, E. (2014). Tissue proteomics for the next decade? Towards a molecular dimension in histology. *Omics*, *18*(9), 539–552. https://doi.org/10.1089/omi.2014.0033
6. Longuespée, R., Theile, D., Fresnais, M., Burhenne, J., Weiss, J., & Haefeli, W. E. (2020). Approaching sites of action of drugs in clinical pharmacology: New analytical options and their challenges. *British Journal of Clinical Pharmacology*. https://doi.org/10.1111/bcp.14543
7. Szabo, Z., & Janaky, T. (2015). Challenges and developments in protein identification using mass spectrometry. *Trends in Analytical Chemistry: TRAC*, *69*, 76–87.
8. Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., & Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular and Cellular Proteomics*, *13*(9), 2513–2526. https://doi.org/10.1074/mcp.M113.031591
9. Quadroni, M., & James, P. (1999). Proteomics and automation. *Electrophoresis*, *20*(4-5), 664–677. https://doi.org/10.1002/(SICI)1522-2683(19990101)20:4/5⟨664::AID-ELPS664⟩3.0.CO;2-A
10. Alexovič, M., Urban, P. L., Tabani, H., & Sabo, J. (2020). Recent advances in robotic protein sample preparation for clinical analysis and other biomedical applications. *Clinica Chimica Acta*, *507*, 104–116. https://doi.org/10.1016/j.cca.2020.04.015
11. Feist, P., & Hummon, A. (2015). Proteomic challenges: sample preparation techniques for microgram-quantity protein analysis from biological samples. *International Journal of Molecular Sciences*, *16*(2), 3537–3563. https://doi.org/10.3390/ijms16023537
12. Gutstein, H. B., Morris, J. S., Annangudi, S. P., & Sweedler, J. V. (2008). Microproteomics: analysis of protein diversity in small samples. *Mass Spectrometry Reviews*, *27*(4), 316–330. https://doi.org/10.1002/mas.20161
13. Zhang, Y., Yoshida, Y., Xu, Bo, Magdeldin, S., Fujinaka, H., Liu, Z., Miyamoto, M., Yaoita, E., & Yamamoto, T. (2011). Comparison of human glomerulus proteomic profiles obtained from low quantities of samples by different mass spectrometry with the comprehensive database. *Proteome Science*, *9*(1), 47. https://doi.org/10.1186/1477-5956-9-47
14. Brioschi, M., Eligini, S., Crisci, M., Fiorelli, S., Tremoli, E., Colli, S., & Banfi, C. (2014). A mass spectrometry-based workflow for the proteomic analysis of in vitro cultured cell subsets isolated by means of laser capture microdissection. *Analytical and Bioanalytical Chemistry*, *406*(12), 2817–2825. https://doi.org/10.1007/s00216-014-7724-9
15. Hadley, K. C., Rakhit, R., Guo, H., Sun, Y., Jonkman, J. E., Mclaurin, J., Hazrati, L.-N., Emili, A., & Chakrabartty, A. (2015). Determining composition of micron-scale protein deposits in neurodegenerative disease by spatially targeted optical microproteomics. *Elife*, *4*, e09579. https://doi.org/10.7554/eLife.09579
16. Longuespée, R., Alberts, D., Pottier, C., Smargiasso, N., Mazzucchelli, G., Baiwir, D., Kriegsmann, M., Herfs, M., Kriegsmann, J., Delvenne, P., & De Pauw, E. (2016). A laser microdissection-based workflow for FFPE tissue microproteomics: Important considerations for small sample processing. *Methods*, *104*, 154–162. https://doi.org/10.1016/j.ymeth.2015.12.008
17. Delcourt, V., Franck, J., Leblanc, E., Narducci, F., Robin, Y.-M., Gimeno, J.-P., Quanico, J., Wisztorski, M., Kobeissy, F., Jacques, J.-F., Roucou, X., Salzet, M., & Fournier, I. (2017). Combined mass spectrometry imaging and top-down microproteomics reveals evidence of a hidden proteome in ovarian cancer. *EBioMedicine*, *21*, 55–64. https://doi.org/10.1016/j.ebiom.2017.06.001
18. Bensaddek, D., Narayan, V., Nicolas, A., Brenes Murillo, A., Gartner, A., Kenyon, C. J., & Lamond, A. I. (2016). Micro-proteomics with iterative data analysis: Proteome analysis in *C. elegans* at the single worm level. *Proteomics*, *16*(3), 381–392. https://doi.org/10.1002/pmic.201500264
19. Wisztorski, M., Desmons, A., Quanico, J., Fatou, B., Gimeno, J.-P., Franck, J., Salzet, M. & Fournier, I. (2016). Spatially-resolved protein surface microsampling from tissue sections using liquid extraction surface analysis. *Proteomics*, *16*(11-12), 1622–1632. https://doi.org/10.1002/pmic.201500508
20. Thakur, D., Rejtar, T., Wang, D., Bones, J., Cha, S., Clodfelder-Miller, B., Richardson, E., Binns, S., Dahiya, S., Sgroi, D., & Karger, B. L. (2011). Microproteomic analysis of 10,000 laser captured microdissected breast tumor cells using short-range sodium dodecyl sulfate-polyacrylamide gel electrophoresis and porous layer open tubular liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*, *1218*(45), 8168–8174. https://doi.org/10.1016/j.chroma.2011.09.022
21. Arafah, K., Kriegsmann, M., Renner, M., Lasitschka, F., Fresnais, M., Kriegsmann, K., Winterfeld, M., Goeppert, B., Kriegsmann, J., Casadonte, R., Kazdal, D., Bulet, P., & Longuespée, R. (2020). Microproteomics and immunohistochemistry reveal differences in aldo-keto reductase family 1 member C3 in tissue specimens of ulcerative colitis and Crohn's disease. *Proteomics. Clinical Applications*, *14*(4), 1900110. https://doi.org/10.1002/prca.201900110
22. Gutstein, H. B., & Morris, J. S. (2007). Laser capture sampling and analytical issues in proteomics. *Expert Review of Proteomics*, *4*(5), 627–637. https://doi.org/10.1586/14789450.4.5.627
23. Eggeling, F., & Hoffmann, F. (2020). Microdissection-an essential prerequisite for spatial cancer omics. *Proteomics*, *20*(17-18), 2000077. https://doi.org/10.1002/pmic.202000077
24. Manadas, B., Mendes, V. M., English, J., & Dunn, M. J. (2010). Peptide fractionation in proteomics approaches. *Expert Review of Proteomics*, *7*(5), 655–663. https://doi.org/10.1586/epr.10.46
25. Xu, P., Duong, D. M., & Peng, J. (2009). Systematical optimization of reverse-phase chromatography for shotgun proteomics. *Journal of Proteome Research*, *8*(8), 3944–3950. https://doi.org/10.1021/pr900251d
26. Lane, R. E., Korbie, D., Trau, M., & Hill, M. M. (2019). Optimizing size exclusion chromatography for extracellular vesicle enrichment and proteomic analysis from clinically relevant samples. *Proteomics*, *19*(8), 1800156. https://doi.org/10.1002/pmic.201800156
27. Longuespée, R., Casadonte, R., Kriegsmann, M., Pottier, C., Picard De Muller, G., Delvenne, P., Kriegsmann, J., & De Pauw, E. (2016). MALDI mass spectrometry imaging: A cutting-edge tool for fundamental and clinical histopathology. *Proteomics. Clinical Applications*, *10*(7), 701–719. https://doi.org/10.1002/prca.201500140
28. Alberts, D., Pottier, C., Smargiasso, N., Baiwir, D., Mazzucchelli, G., Delvenne, P., Kriegsmann, M., Kazdal, D., Warth, A., De Pauw, E., & Longuespée, R. (2018). MALDI imaging-guided microproteomic analyses of heterogeneous breast tumors-a pilot study. *Proteomics. Clinical Applications*, *12*(1), 1700062. https://doi.org/10.1002/prca.201700062
29. Dewez, F., Martin-Lorenzo, M., Herfs, M., Baiwir, D., Mazzucchelli, G., De Pauw, E., Heeren, R. M. A., & Balluff, B. (2019). Precise co-registration of mass spectrometry imaging, histology, and laser microdissection-based omics. *Analytical and Bioanalytical Chemistry*, *411*(22), 5647–5653. https://doi.org/10.1007/s00216-019-01983-z
30. Kasuga, K., Katoh, Y., Nagase, K., & Igarashi, K. (2017). Microproteomics with microfluidic-based cell sorting: Application to 1000 and 100 immune cells. *Proteomics*, *17*(13-14), 1600420. https://doi.org/10.1002/pmic.201600420
31. Pottier, C., Kriegsmann, M., Alberts, D., Smargiasso, N., Baiwir, D., Mazzucchelli, G., Herfs, M., Fresnais, M., Casadonte, R., Delvenne, P., Pauw, E., & Longuespée, R. (2019). Microproteomic profiling of high-grade squamous intraepithelial lesion of the cervix: Insight into biological mechanisms of dysplasia and new potential diagnostic markers. *Proteomics. Clinical Applications*, *13*(1), 1800052. https://doi.org/10.1002/prca.201800052
32. Gousset, K., Gordon, A., Kumar Kannan, S., & Tovar, J. (2019). A novel microproteomic approach using laser capture microdissection to study

cellular protrusions. *International Journal of Molecular Sciences*, *20*(5), 1172. https://doi.org/10.3390/ijms20051172

33. Wang, H., Qian, W.-J., Mottaz, H. M., Clauss, T. R. W., Anderson, D. J., Moore, R. J., Camp, D. G., Khan, A. H., Sforza, D. M., Pallavicini, M., Smith, D. J., & Smith, R. D. (2005). Development and evaluation of a micro- and nanoscale proteomic sample preparation method. *Journal of Proteome Research*, *4*(6), 2397–2403. https://doi.org/10.1021/pr050160f
34. Sun, J., Zhang, G., Li, S., Ivanov, A. R., Fenyo, D., Lisacek, F., Murthy, S. K., Karger, B. L., & Brusic, V. (2014). Pathway analysis and transcriptomics improve protein identification by shotgun proteomics from samples comprising small number of cells–a benchmarking study. *Bmc Genomics [Electronic Resource], 15 Suppl 9*, S1. https://doi.org/10.1186/1471-2164-15-S9-S1
35. Eligini, S., Brioschi, M., Fiorelli, S., Tremoli, E., Banfi, C., & Colli, S. (2015). Human monocyte-derived macrophages are heterogenous: Proteomic profile of different phenotypes. *Journal of Proteomics*, *124*, 112–123. https://doi.org/10.1016/j.jprot.2015.03.026
36. Leipert, J., & Tholey, A. (2019). Miniaturized sample preparation on a digital microfluidics device for sensitive bottom-up microproteomics of mammalian cells using magnetic beads and mass spectrometry-compatible surfactants. *Lab on A Chip*, *19*(20), 3490–3498. https://doi.org/10.1039/c9lc00715f
37. Hughes, C. S., Foehr, S., Garfield, D. A., Furlong, E. E., Steinmetz, L. M., & Krijgsveld, J. (2014). Ultrasensitive proteome analysis using paramagnetic bead technology. *Molecular Systems Biology*, *10*, 757. https://doi.org/10.15252/msb.20145625
38. Chen, Qi, Yan, G., Gao, M., & Zhang, X. (2015). Ultrasensitive proteome profiling for 100 living cells by direct cell injection, online digestion and nano-LC-MS/MS analysis. *Analytical Chemistry*, *87*(13), 6674–6680. https://doi.org/10.1021/acs.analchem.5b00808
39. Virant-Klun, I., Leicht, S., Hughes, C., & Krijgsveld, J. (2016). Identification of maturation-specific proteins by single-cell proteomics of human oocytes. *Molecular and Cellular Proteomics*, *15*(8), 2616–2627. https://doi.org/10.1074/mcp.M115.056887
40. Li, Zi-Yi, Huang, M., Wang, X.-K., Zhu, Y., Li, J.-S., Wong, C. C. L., & Fang, Q. (2018). Nanoliter-scale oil-air-droplet chip-based single cell proteomic analysis. *Analytical Chemistry*, *90*(8), 5430–5438. https://doi.org/10.1021/acs.analchem.8b00661
41. Specht, H., Harmange, G., Perlman, D. H., Emmont, E., Niziolek, Z., Budnik, B., & Slavov, N. (2018). Automated sample preparation for high-throughput single-cell proteomics. *medRxiv and bioRxiv*, 399774. https://doi.org/10.1101/399774
42. Zhu, Y., Podolak, J., Zhao, R., Shukla, A. K., Moore, R. J., Thomas, G. V., & Kelly, R. T. (2018). Proteome profiling of 1 to 5 spiked circulating tumor cells isolated from whole blood using immunodensity enrichment, laser capture microdissection, nanodroplet sample processing, and ultrasensitive nanoLC-MS. *Analytical Chemistry*, *90*(20), 11756–11759. https://doi.org/10.1021/acs.analchem.8b03268
43. Dou, M., Clair, G., Tsai, C.-F., Xu, K., Chrisler, W. B., Sontag, R. L., Zhao, R., Moore, R. J., Liu, T., Pasa-Tolic, L., Smith, R. D., Shi, T., Adkins, J. N., Qian, W.-J., Kelly, R. T., Ansong, C., & Zhu, Y. (2019). High-throughput single cell proteomics enabled by multiplex isobaric labeling in a nanodroplet sample preparation platform. *Analytical Chemistry*, *91*(20), 13119–13127. https://doi.org/10.1021/acs.analchem.9b03349
44. Zhu, Y., Piehowski, P. D., Zhao, R., Chen, J., Shen, Y., Moore, R. J., Shukla, A. K., Petyuk, V. A., Campbell-Thompson, M., Mathews, C. E., Smith, R. D., Qian, W.-J., & Kelly, R. T. (2018). Nanodroplet processing platform for deep and quantitative proteome profiling of 10–100 mammalian cells. *Nature communications*, *9*(1), 882. https://doi.org/10.1038/s41467-018-03367-w
45. Williams, S. M., Liyu, A. V., Tsai, C.-F., Moore, R. J., Orton, D. J., Chrisler, W. B., Gaffrey, M. J., Liu, T., Smith, R. D., Kelly, R. T., Pasa-Tolic, L., & Zhu, Y. (2020). Automated coupling of nanodroplet sample preparation with liquid chromatography-mass spectrometry for high-throughput single-cell proteomics. *Analytical Chemistry*, *92*(15), 10588–10596. https://doi.org/10.1021/acs.analchem.0c01551
46. Xu, K., Liang, Y., Piehowski, P. D., Dou, M., Schwarz, K. C., Zhao, R., Sontag, R. L., Moore, R. J., Zhu, Y., & Kelly, R. T. (2019). Benchtop-compatible sample processing workflow for proteome profiling of <100 mammalian cells. *Analytical and Bioanalytical Chemistry*, *411*(19), 4587–4596. https://doi.org/10.1007/s00216-018-1493-9
47. Cong, Y., Motamedchaboki, K., Misal, S. A., Liang, Y., Guise, A. J., Truong, T., Huguet, R., Plowey, E. D., Zhu, Y., Lopez-Ferrerb, D., & Kelly, R. T. (2021). Ultrasensitive single-cell proteomics workflow identifies >1000 protein groups per mammalian cell. *Chemical Science*, *12*(3), 1001–1006. https://doi.org/10.1039/D0SC03636F
48. Yin, B., Mendez, R., Zhao, X.-Y., Rakhit, R., Hsu, K.-L., & Ewald, S. E. (2020). Automated spatially targeted optical microproteomics (autoSTOMP) to determine protein complexity of subcellular structures. *Analytical Chemistry*, *92*(2), 2005–2010. https://doi.org/10.1021/acs.analchem.9b04396
49. Capriotti, A. L., Cavaliere, C., Foglia, P., Samperi, R., & Laganà, A. (2011). Intact protein separation by chromatographic and/or electrophoretic techniques for top-down proteomics. *Journal of Chromatography A*, *1218*(49), 8760–8776. https://doi.org/10.1016/j.chroma.2011.05.094
50. Labas, V., Teixeira-Gomes, A.-P., Bouguereau, L., Gargaros, A., Spina, L., Marestaing, A., & Uzbekova, S. (2018). Intact cell MALDI-TOF mass spectrometry on single bovine oocyte and follicular cells combined with top-down proteomics: A novel approach to characterise markers of oocyte maturation. *Journal of Proteomics*, *175*, 56–74. https://doi.org/10.1016/j.jprot.2017.03.027
51. Zhou, M., Uwugiaren, N., Williams, S. M., Moore, R. J., Zhao, R., Goodlett, D., Dapic, I., Paša-Tolić, L., & Zhu, Y. (2020). Sensitive top-down proteomics analysis of a low number of mammalian cells using a nanodroplet sample processing platform. *Analytical Chemistry*, *92*(10), 7087–7095. https://doi.org/10.1021/acs.analchem.0c00467
52. Staunton, L., Tonry, C., Lis, R., Finn, S., O'Leary, J., Loda, M., Bowden, M., & Pennington, S. R. (2016). Profiling the tumor microenvironment proteome in prostate cancer using laser capture microdissection coupled to LC-MS - A technical report. *EuPA Open Proteomics*, *10*, 19–23.
53. Roulhac, P. L., Ward, J. M., Thompson, J. W., Soderblom, E. J., Silva, M., Moseley, M. A., & Jarvis, E. D. (2011). Microproteomics: quantitative proteomic profiling of small numbers of laser-captured cells. *Cold Spring Harbor Protocols*, *2011*(2), pdb prot5573. https://doi.org/10.1101/pdb.prot5573
54. Do Canto, A. M., Vieira, A. S., H.B. Matos, A., Carvalho, B. S., Henning, B., Norwood, B. A., Bauer, S., Rosenow, F., Gilioli, R., Cendes, F., & Lopes-Cendes, I. (2020). Laser microdissection-based microproteomics of the hippocampus of a rat epilepsy model reveals regional differences in protein abundances. *Scientific Reports*, *10*(1), 4412. https://doi.org/10.1038/s41598-020-61401-8
55. De Graaf, E. L., Pellegrini, D., & Mcdonnell, L. A. (2016). Set of novel automated quantitative microproteomics protocols for small sample amounts and its application to kidney tissue substructures. *Journal of Proteome Research*, *15*(12), 4722–4730. https://doi.org/10.1021/acs.jproteome.6b00889
56. Müller, T., Kalxdorf, M., Longuespée, R., Kazdal, D. N., Stenzinger, A., & Krijgsveld, J. (2020). Automated sample preparation with SP3 for low-input clinical proteomics. *Molecular Systems Biology*, *16*(1), e9111. https://doi.org/10.15252/msb.20199111
57. Dilillo, M., Ait-Belkacem, R., Esteve, C., Pellegrini, D., Nicolardi, S., Costa, M., Vannini, E., Graaf, E. L. De, Caleo, M., & Mcdonnell, L. A. (2017). Ultra-high mass resolution MALDI imaging mass spectrometry of proteins and metabolites in a mouse model of glioblastoma. *Scientific Reports*, *7*(1), 603. https://doi.org/10.1038/s41598-017-00703-w
58. Dilillo, M., Pellegrini, D., Ait-Belkacem, R., De Graaf, E. L., Caleo, M., & Mcdonnell, L. A. (2017). Mass spectrometry imaging, laser capture

microdissection, and LC-MS/MS of the same tissue section. *Journal of Proteome Research*, *16*(8), 2993–3001. https://doi.org/10.1021/acs.jproteome.7b00284
59. Pellegrini, D., Del Grosso, A., Angella, L., Giordano, N., Dilillo, M., Tonazzini, I., Caleo, M., Cecchini, M., & Mcdonnell, L. A. (2019). Quantitative microproteomics based characterization of the central and peripheral nervous system of a mouse model of Krabbe disease. *Molecular and Cellular Proteomics*, *18*(6), 1227–1241. https://doi.org/10.1074/mcp.RA118.001267
60. Liang, Y., Zhu, Y., Dou, M., Xu, K., Chu, R. K., Chrisler, W. B., Zhao, R., Hixson, K. K., & Kelly, R. T. (2018). Spatially resolved proteome profiling of <200 cells from tomato fruit pericarp by integrating laser-capture microdissection with nanodroplet sample preparation. *Analytical Chemistry*, *90*(18), 11106–11114. https://doi.org/10.1021/acs.analchem.8b03005
61. Piehowski, P. D., Zhu, Y., Bramer, L. M., Stratton, K. G., Zhao, R., Orton, D. J., Moore, R. J., Yuan, J., Mitchell, H. D., Gao, Y., Webb-Robertson, B.-J. M., Dey, S. K., Kelly, R. T., & Burnum-Johnson, K. E. (2020). Automated mass spectrometry imaging of over 2000 proteins from tissue sections at 100-mum spatial resolution. *Nature communications*, *11*(1), 8. https://doi.org/10.1038/s41467-019-13858-z
62. Wisztorski, M., Quanico, J., Franck, J., Fatou, B., Salzet, M., & Fournier, I. (2017). Droplet-based liquid extraction for spatially-resolved microproteomics analysis of tissue sections. *Methods in Molecular Biology*, *1618*, 49–63. https://doi.org/10.1007/978-1-4939-7051-3_6
63. Longuespee, R., Baiwir, D., Mazzucchelli, G., Smargiasso, N., & De Pauw, E. (2018). Laser microdissection-based microproteomics of formalin-fixed and paraffin-embedded (FFPE) tissues. *Methods in Molecular Biology*, *1723*, 19–31. https://doi.org/10.1007/978-1-4939-7558-7_2
64. Wisztorski, M., Fatou, B., Franck, J., Desmons, A., Farré, I., Leblanc, E., Fournier, I., & Salzet, M. (2013). Microproteomics by liquid extraction surface analysis: application to FFPE tissue to study the fimbria region of tubo-ovarian cancer. *Proteomics. Clinical Applications*, *7*(3-4), 234–240. https://doi.org/10.1002/prca.201200070
65. Longuespée, R., Gagnon, H., Boyon, C., Strupat, K., Dauly, C., Kerdraon, O., Ighodaro, A., Desmons, A., Dupuis, J., Wisztorski, M., Vinatier, D., Fournier, I., Day, R., & Salzet, M. (2013). Proteomic analyses of serous and endometrioid epithelial ovarian cancers - cases studies - molecular insights of a possible histological etiology of serous ovarian cancer. *Proteomics. Clinical Applications*, *7*(5-6), 337–354. https://doi.org/10.1002/prca.201200079
66. Quanico, J., Franck, J., Cardon, T., Leblanc, E., Wisztorski, M., Salzet, M., & Fournier, I. (2017). NanoLC-MS coupling of liquid microjunction microextraction for on-tissue proteomic analysis. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1865*(7), 891–900. https://doi.org/10.1016/j.bbapap.2016.11.002
67. Quanico, J., Franck, J., Dauly, C., Strupat, K., Dupuy, J., Day, R., Salzet, M., Fournier, I., & Wisztorski, M. (2013). Development of liquid microjunction extraction strategy for improving protein identification from tissue sections. *Journal of Proteomics*, *79*, 200–218. https://doi.org/10.1016/j.jprot.2012.11.025
68. Le Rhun, E., Duhamel, M., Wisztorski, M., Gimeno, J.-P., Zairi, F., Escande, F., Reyns, N., Kobeissy, F., Maurage, C.-A., Salzet, M., & Fournier, I. (2017). Evaluation of non-supervised MALDI mass spectrometry imaging combined with microproteomics for glioma grade III classification. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1865*(7), 875–890. https://doi.org/10.1016/j.bbapap.2016.11.012
69. Mallah, K., Quanico, J., Raffo-Romero, A., Cardon, T., Aboulouard, S., Devos, D., Kobeissy, F., Zibara, K., Salzet, M., & Fournier, I. (2019). Mapping spatiotemporal microproteomics landscape in experimental model of traumatic brain injury unveils a link to Parkinson's disease. *Molecular and Cellular Proteomics*, *18*(8), 1669–1682. https://doi.org/10.1074/mcp.RA119.001604
70. Longuespee, R., Casadonte, R., Kriegsmann, M., Wandernoth, P., et al. (2017). Proteomic investigation of human cystic echinococcosis in the liver. *Mol Biochem Parasitol*, *211*, 9–14.*
71. Herfs, M., Longuespée, R., Quick, C. M., Roncarati, P., Suarez-Carmona, M., Hubert, P., Lebeau, A., Bruyere, D., Mazzucchelli, G., Smargiasso, N., Baiwir, D., Lai, K., Dunn, A., Obregon, F., Yang, E. J., Pauw, E. D., Crum, C. P., & Delvenne, P. (2017). Proteomic signatures reveal a dualistic and clinically relevant classification of anal canal carcinoma. *Journal of Pathology 241*(4), 522–533. https://doi.org/10.1002/path.4858
72. Longuespée, R., Ly, A., Casadonte, R., Schwamborn, K., Kazdal, D., Zgorzelski, C., Bollwein, C., Kriegsmann, K., Weichert, W., Kriegsmann, J., Schirmacher, P., Fresnais, M., Oliveira, C., & Kriegsmann, M. (2019). Identification of MALDI imaging proteolytic peptides using LC-MS/MS-based biomarker discovery data: A proof of concept. *Proteomics. Clinical Applications*, *13*(1), 1800158. https://doi.org/10.1002/prca.201800158
73. Longuespee, R., Alberts, D., Baiwir, D., Mazzucchelli, G., Smargiasso, N., & De Pauw, E. (2018). MALDI imaging combined with laser microdissection-based microproteomics for protein identification: Application to intratumor heterogeneity studies. *Methods in Molecular Biology*, *1788*, 297–312. https://doi.org/10.1007/7651_2017_114
74. Salmon, C. R., Giorgetti, A. P. O., Paes Leme, A. F., Domingues, R. R., Sallum, E. A., Alves, M. C., Kolli, T. N., Foster, B. L., & Nociti, F. H. (2016). Global proteome profiling of dental cementum under experimentally-induced apposition. *Journal of Proteomics*, *141*, 12–23. https://doi.org/10.1016/j.jprot.2016.03.036
75. Salmon, C. R., Giorgetti, A. P. O., Paes Leme, A. F., Domingues, R. R., Kolli, T. N., Foster, B. L., & Nociti, F. H. (2017). Microproteome of dentoalveolar tissues. *Bone*, *101*, 219–229. https://doi.org/10.1016/j.bone.2017.05.014
76. Delcourt, V., Franck, J., Quanico, J., Gimeno, J.-P., Wisztorski, M., Raffo-Romero, A., Kobeissy, F., Roucou, X., Salzet, M., & Fournier, I., (2018). Spatially-resolved top-down proteomics bridged to MALDI MS imaging reveals the molecular physiome of brain regions. *Molecular and Cellular Proteomics*, *17*(2), 357–372. https://doi.org/10.1074/mcp.M116.065755

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* Correction added on 25 March 2021, after first online publication: Reference 70 was changed.

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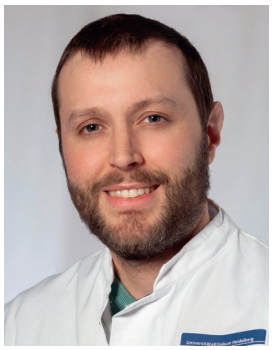

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