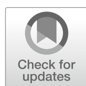

Chapter 13

An Overview of Mass Spectrometry-Based Methods for Functional Proteomics

J. Robert O'Neill

Abstract

The mechanism underlying many biological phenotypes remains unknown despite the increasing availability of whole genome and transcriptome sequencing. Direct measurement of changes in protein expression is an attractive alternative and has the potential to reveal novel processes. Mass spectrometry has become the standard method for proteomics, allowing both the confident identification and quantification of thousands of proteins from biological samples. In this review, mass spectrometry-based proteomic methods and their applications are described.

Key words Mass spectrometry, Proteomics, Quantitation, Label-free, Selective reaction monitoring, MALDI

1 The Challenge of Measuring the Proteome

The study of the entire protein content of an organism, tissue, or cell was first described as proteomics nearly 20 years ago [1]. Mass spectrometry has become the de facto standard method for proteomics, allowing the confident identification of proteins from complex mixtures [2].

Although the goal of measuring an entire eukaryotic proteome has been achieved [3], the human proteome has yet to be described in toto despite the publication of the complete human genome at the turn of the century [4]. The human proteome project has delivered progressive increments toward this goal [5, 6] yet as of the August 2017 data release of neXtProt, the most comprehensive human protein database available, no direct experimental evidence has been provided for 3031 (15%) of the predicted 20,199 proteins comprising the human proteome [7]. Several reasons can be proposed for this disparity.

The polymerase chain reaction (PCR) allows template nucleotide sequences to be copied with an increase in number of many orders of magnitude and very low error rates [8]. Complementary-

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base pairing also allows cryptic nucleotide sequences to be rapidly deciphered [9]. The combination of these methods and advances in computational assembly of short sequence reads allows nucleotide sequencing to proceed in massively parallel configurations to sequence entire genomes within hours [10].

In contrast, the de novo identification of protein sequences contains greater intrinsic challenges. No method exists to amplify protein or peptide sequences and therefore proteomic methods are always restricted by the input mass. Similarly amino acids do not exhibit complementation and identification relies on mass measurement or, historically, chromatography or electrophoresis [11]. The proteome is also significantly larger than the genome with alternative splicing and alternative transcription start sites contributing to transcriptome and ultimately proteome diversity [12].

A further challenge is posed by greater combinatorial possibilities with up to 21 amino acids used interchangeably to generate peptides. This complexity is further increased by posttranslational modifications (PTMs) including the addition of biochemical groups such as a phosphate (phosphorylation), a carbohydrate (glycosylation), and at least 25 other distinct moieties or modifications [11].

A final compounding difficulty is the dynamic nature of the proteome. The genome sequence of an organism is constant across all cells in that organism and is relatively stable in the face of DNA extraction methods even allowing DNA sequences to be obtained from ancient specimens [13]. In contrast, the proteome varies from cell to cell [14] and is highly context-dependent with the post-translational state of a single protein varying across subcellular locations [15]. Extracting the proteome for quantification is also confounded by the rapid alterations in the PTM state induced by hypoxia and changes in intracellular pH with some phosphorylations reported to be lost within 60 min of tissue biopsy [16]. Many of these challenges have been addressed with technological advances, the most significant of which is the use of high scanning speed, high accuracy mass spectrometry [17].

2 Mass Spectrometry for Proteomics

The fundamental components of a mass spectrometer consist of an ion detector coupled to a mass analyzer that measures both the number and the mass-to-charge ratio (m/z) of ions generated into the gas phase from an ionization source. Variations on the instrumentation abound, however, each with their own strengths and weaknesses [18–22]. Despite this, all combine high sensitivity and high mass accuracy to finally bring the measurement of whole proteomes within reach.

Electrospray ionization (ESI) sources ionize analytes directly into the gas phase from liquid, commonly a polar volatile solvent eluted from a chromatography column [23]. These sources are most commonly used for the analysis of complex mixtures including cell lysates. Alternatives include matrix-assisted laser desorption ionization (MALDI) sources which use a laser to ionize analytes directly into the gas phase out of a solid matrix [24]. These sources are limited in the number of ions that can be generated and have previously been reserved for relatively homogenous analytes.

3 Protein Identification Using MS: Bottom-Up Approach

The de novo identification of proteins from a complex mixture can be achieved by several means. The most common method, termed "shotgun" or "bottom-up" proteomics relies on the identification of peptides generated by proteolytic digestion of the protein mixture. The presence of a protein in the original mixture is then inferred by interrogation of a protein sequence database with the identified peptide sequences. Matching a peptide sequence unique to a particular protein provides evidence of the protein in the original mixture [2]. An example overview of the workflow is illustrated in Fig. 1.

Shotgun proteomics relies on tandem mass spectrometry (MS/MS) where peptides are ionized to generate precursor ions, analyzed and separated according to their mass-to-charge ratio (m/z) in the primary mass spectrometry run (MS1) . Precursor ions are then fragmented, usually by collision ion dissociation, and the fragment ions are separated and analyzed in the second MS run (MS2) [25]. Multiple fragment species are generated from the same peptide and, with high-quality spectra and sufficient fragment ions, species differing by each individual amino acid in the peptide will be discernible as discrete ion peaks separated by a measured mass difference. As amino acids all have a fixed, defined mass, the measured difference can be used to identify the amino acid [26]. Thus the sequence of the peptide can be determined directly, defined as de novo peptide sequencing [27]. In practice, with complex peptide mixtures it is rarely possible to sequence all peptides directly and this labor-intensive approach is reserved for organisms with limited genome sequence information and therefore limited or absent potential protein databases.

More commonly, database searching is performed to generate peptide-spectrum matches. Several algorithms have been described but they generally follow the same principle; the measured precursor mass is used to filter a database of peptides generated by in silico digestion of a list of potentially identifiable proteins. Theoretical fragment-ion mass differences are generated for all the candidate peptides with a matching precursor mass [28]. These are compared

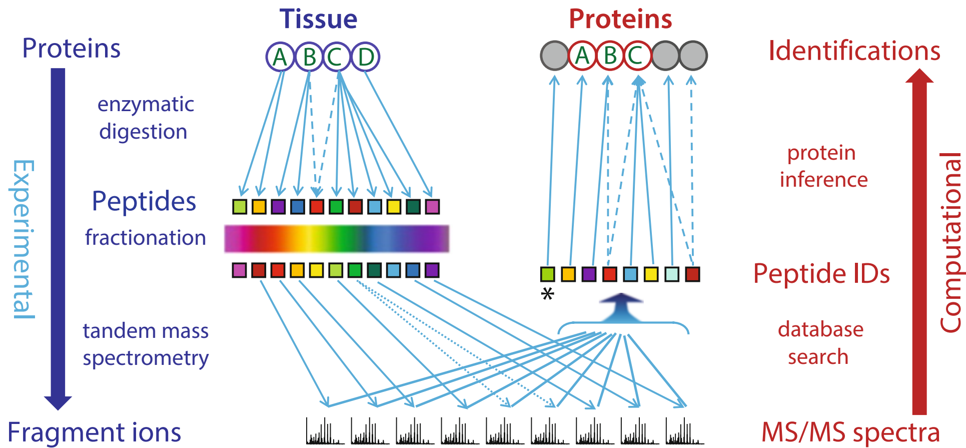

Fig. 1 An overview of protein identification by shotgun proteomics. A complex protein mixture, in this example a tissue sample containing proteins A–D, is proteolytically digested to yield peptides. Each peptide is illustrated as a colored box. To reduce the mixture complexity, peptides are fractionated by a common property such as isoelectric point. Peptide fractions are subjected to tandem mass spectrometry to yield fragment ion spectra. Peptide-spectrum matches (IDs) are made using a protein database, the peptide (precursor) ion masses, and a database search tool [91]. Not all fragment ion spectra result in a peptide match and some peptide matches are of low confidence (e.g., light green peptide; *). Using further statistical tools [28], proteins are identified with unique peptide matches confirming the presence of a protein in the original mixture. Each shotgun experiment only identifies a subset of the proteome from complex mixtures such as tissue lysate, so in this example protein "D" has not been identified

with the identified fragment-ion spectra and candidates ranked using a scoring algorithm, specific to the database search method [29].

These methods can identify peptides without the requirement for prior mass spectrometry. Organism-specific spectral libraries generated using stringent identification thresholds and evaluation of millions of published experimentally derived peptide spectra are now available [30, 31]. An alternative, or complementary, approach is to search identified spectra against these libraries, incorporating other spectral features such as relative ion intensity. This has been reported to enhance the number of peptide identifications compared to standard database search strategies [32, 33].

The sequences of identified peptides are then used to identify proteins using the original search database. A variety of statistical approaches are included in commonly used software packages to deal with protein inference problems such as repeated peptide sequencing events, peptides shared between multiple proteins and estimating the false-discovery rate [28].

3.1 Fractionation

A significant limitation of mass spectrometry is the throughput of ions that can be analyzed. Although this has improved with the current generation of instruments, the number of analytes that can be studied simultaneously is still often the limiting factor. Tissue

lysates contain highly diverse mixtures of proteins. This diversity is further compounded by proteolytic digestion, presenting significant challenges for peptide spectrum matching. Samples are often fractionated to reduce this complexity. Approaches include strong cation exchange [34], subcellular fractionation [35], isoelectric focusing electrophoresis [36], high pH (basic) reversed phase [37], and other chromatography methods [38]. By delivering fractions with reduced numbers of unique peptides into the mass spectrometer, homogenous *m/z* fractions can be produced during the MS1 phase which can be accurately sequenced during the MS2 phase [39, 40].

4 Data-Dependent and -Independent Shotgun Proteomics

A key feature of the shotgun proteomic method as described previously is the selection of precursor ions for fragmentation in the MS2 phase. This is usually performed on the basis of precursor ion intensity and is referred to as the data-dependent approach [41]. This method has the limitation that a precursor ion must be detected to allow peptide sequencing and places an intrinsic bias toward abundant precursor species. An alternative strategy is to systematically fragment all precursor ions within windows of a defined *m/z* range regardless of whether a precursor ion was detected or not [21, 42]. In one iteration of this method, the precursor mass used for peptide spectrum matching is assigned as the center of the MS1 *m/z* window. When this method is applied, fragment ions yielding high-confidence peptide spectrum matches can be detected in up to 10% of cases in the absence of a precursor ion [42, 43] and this approach can enhance the dynamic range of detection by identifying low-abundance peptides. A disadvantage is the long data acquisition times required to obtain spectra across all *m/z* windows although faster instruments and optimized chromatography have reduced this [44].

5 Protein Identification Using MS: Top-Down Approach

An exciting recent development has been the ability to identify intact proteins by mass spectrometry, a so-called "top-down" approach [45]. Proof of concept studies have demonstrated the capacity to identify several thousand distinct protein isoforms (proteoforms) using cultured mammalian cells and extensive orthogonal fractionation in the liquid phase [46–48]. An advantage of this method is the direct identification of proteins, rather than inference from peptide identifications using the shotgun approach. This provides the potential to characterize the entire population of proteoforms generated from a single gene and identify dynamic changes in

protein-processing, alternative-splicing or posttranslational modification often not possible from peptide-level data alone. Although not currently capable of proteome-scale analysis, with further developments in automated fractionation, instrumentation and data analysis methods, this may become feasible in the future [49]. Measuring dynamic changes in cellular states, the goal of most biological proteomic experiments, however, requires quantitation in addition to protein identification. Methods to undertake this using a hypothesis-free top-down approach are in early development and still lack the robustness of shotgun approaches [50].

6 Selective Reaction Monitoring

Mass spectrometry provides an ideal method to allow the hypothesis-free discovery of expressed proteins in biological samples using either the "bottom-up" or "top-down" approaches described. The development of high-quality comprehensive spectral libraries and the availability of synthetic peptides have allowed the development of robust mass spectrometry "assays" covering the entire human proteome and that of several model organisms [30, 51, 52]. These databases can be used for hypothesis-driven studies to quantify protein expression across samples. The commonest application of this method is selective reaction monitoring.

In this method, a peptide unique to the protein of interest and consistently identifiable by mass spectrometry is selected (proteotypic peptide) [53]. The spectral features of this peptide are then used to isolate precursor ions using defined mass windows. This significantly reduces the complexity of the ion mixture for subsequent fragmentation and peptide identification. This also significantly reduces the analysis time so that higher numbers of samples can be analyzed.

Many dozens of proteins can be assayed simultaneously by multiplexing this strategy (multiple reaction monitoring; MRM). By spiking-in isotopically labeled synthetic proteotypic peptides at a defined concentration, the absolute concentration of peptide, and by inference the protein, of interest can be determined with high accuracy. The higher throughput of MRM approaches means that they are commonly employed in the validation phase of biomarker development studies when shortlisted biomarker candidates determined in a discovery proteomic experiment in a small number of samples are assessed in several hundred further samples [54].

Improvements in instrument scanning speeds and the application of data analysis approaches from selective reaction monitoring have been employed in a further hybrid proteomic method. This technique, termed sequential window acquisition of all theoretical fragment-ion spectra or "SWATH MS" [21], has high technical reproducibility and quantitative accuracy [19, 55, 56]. In this

method, data are acquired using a data-independent shotgun proteomic method, and peptide identifications are made on a candidate basis using the SRM approach. Proponents of this technique claim that a "digital" representation of the protein state of a biological sample is created and this can be assessed retrospectively as hypotheses are subsequently developed without the need for further mass spectrometry. Although compelling as a concept, complete proteome coverage is still not routinely achieved using current instruments and identifying biologically significant changes in protein modifications such as phosphorylation still requires careful experimental control and modification-specific sample preparation methods.

7 Quantitative Proteomics

A striking common finding of the increasing number of large-scale proteomic studies is that few proteins exhibit tissue-specific expression [57, 58]. In almost all cases, therefore, diverse phenotypes are manifest through changes in protein expression level, subcellular localization, or posttranslational modifications rather than the presence or absence of protein expression. If the experimental objective is to understand the mechanism governing an observed phenotype, then quantifying protein expression is of central importance.

7.1 Gel-Based Methods for Quantitative Proteomics

A typical proteomic experimental design is to compare a biological sample under two or more conditions and attempt to identify differentially expressed proteins. Historically, 2D gel electrophoresis would be used to separate the lysates from each condition according to protein mass and isoelectric point [59]. Gels could then be stained using a silver-based or other similar methods and differentially expressed proteins could be identified as spots of differing intensities [60]. A variation of this method minimized the gel-to-gel variability by labeling all the proteins in each sample with a different fluorophore and running all the samples together on the same gel [61]. By quantifying the relative emission from each fluorophore across the spots, the relative expression could be determined.

In both examples, differentially expressed spots containing proteins of unknown identify are excised, digested to peptides using proteolytic enzymes, and subjected to mass spectrometry for peptide and subsequent protein identification using similar strategies to shotgun proteomics. This method has the advantage of limiting the protein identifications to a small number of differentially expressed proteins, and providing a relatively homogenous sample for mass spectrometry. Unfortunately, despite advances in the automation of spot detection and quantification, these methods were only semiquantitative, labor-intensive, the data quality was highly user dependent, and protein identifications were limited to a few dozen per experiment.

7.2 Quantitative Shotgun Proteomics

Advances in sample processing and instrumentation have enabled the development of quantitative shotgun proteomic methods. These rely on lysis, digestion, and usually fractionation of samples prior to liquid chromatography (LC) and MS/MS. A labeling phase can be incorporated into the sample preparation stages prior to MS/MS or peptides can be quantified directly using label-free strategies [62].

7.3 Quantitative Shotgun Proteomics Using Labeling

7.3.1 Stable Isotope Labeling of Amino Acids in Culture (SILAC)

Chemical labeling can take place at the protein or peptide level. The use of stable carbon, hydrogen, and nitrogen isotopes allows differential labeling of amino acids such as Leucine, Lysine, and Arginine that will remain biochemically identical but through their mass differences are resolvable as discrete spectral peaks. This approach, termed Stable Isotopic Labeling of Amino Acids in Culture (SILAC), allows the proteins in mammalian cells in culture to be isotopically labeled by the use of medium containing only "heavy" amino acids [63]. A typical experiment would comprise one treated, "heavy"-labeled cell line and a control unlabeled, "light", cell line. Cell lysates are mixed in a 1:1 ratio and then subjected to standard LC-MS/MS workflow. Peptides are identified in the usual fashion and the relative expression between cell line conditions identified at the MS1 level by the ratio of heavy to light peptide ion intensities. This approach has been shown to be reproducible across the proteome with a coefficient of variation of ~30% [62]. By using both heavy lysine and heavy arginine combinations, three conditions can be compared simultaneously.

A disadvantage of SILAC approaches is the requirement for complete label uptake by cultured cells, which limits the application to cells which express stable phenotypes of interest across several passages. The requirement for prior labeling in the conventional SILAC method also precludes the study of human tissues samples although fully isotopically labeled organisms have been described which may have application in disease models [64–66].

7.3.2 Super-SILAC

A variation of the SILAC method, termed super-SILAC, has been applied to quantify the proteome of human cancer samples [67]. In this procedure a mixture of cell lines derived from the cancer tissue of interest and approximately covering the expression profile of the tissue of interest are heavy-labeled using the SILAC method. A mixture of lysates from these cell lines with a defined protein mass is spiked-in to each tissue lysate in a 1:1 ratio before digestion, fractionation, and LC-MS/MS using standard procedures. Peptide identification and quantitation then proceeds as for a standard SILAC experiment. The ratio of expression between heavy and light peptides is calculated for each tissue sample. The constant SILAC spike-in mass provides a method of normalizing between experimental runs and also, by calculating the ratio of ratios, allows the relative expression between tissue types to be calculated [68].

An advantage is the spike-in standard can be used in multiple experiments on multiple platforms and still allow normalization between experiments and, once the spike-in standard is generated, there is no further labeling steps or reagent costs. A disadvantage is that the accuracy of SILAC is highest at ratios <2 and therefore a relatively close match to the tissue expression profile is required [62]. SILAC media is not yet available for many primary cells and therefore primary human tissues or cancers with few available cell lines may be difficult to analyze with this technique. Similarly, proteins unique to a tissue sample will not be quantified.

7.3.3 Isotope-Coded Affinity Tags (ICAT)

In this method, the cysteine residues of reduced proteins are labeled with tags comprising a composite of a sulfhydryl reacting group, a deuterated linker, and a biotin affinity tag [69]. Proteins from discrete samples can be differentially labeled as both "light" and "heavy" isotopes of the linker are available. Labeled samples are then pooled and digested together. Cysteine-containing peptides are then enriched by avidin-affinity chromatography. Peptides can then be further fractionated or directly subjected to LC-MS/MS. The different isotopes of the deuterated linker provide discrete mass peaks during MS1 analysis to allow differential expression analysis.

Unfortunately only cysteine-containing proteins can be studied, limiting proteome-wide efforts and the bulky affinity group, biotin, introduces significant background into the MS/MS spectra [70]. Furthermore, deuterated labels are more hydrophobic and therefore are differentially eluted during reverse phase LC, complicating the MS analysis [71]. This technique still has a role, however, as the affinity enrichment step allows the study of low-abundance proteins, not easily accessible by other methods.

7.3.4 ^{18}O Labeling

An advantage of this strategy is that it can be applied to almost any sample. In an approach that predates the SILAC method, samples for comparison are either proteolytically digested in ^{18}O-containing water for the "heavy" sample or standard "light" water [72]. As the protease, in most cases trypsin, cleaves the peptide bonds, the heavy isotope is incorporated, so all tryptic peptides will be labeled. The subsequent data analysis is identical to SILAC methods. A disadvantage of this approach is the relative expense of $H_2{}^{18}O$.

7.3.5 Dimethyl Isotopic Labeling

A further method uses standard and deuterium isotopes of formaldehyde to label the amino-terminus of peptides or the amino group of Lysine residues [73]. The isotopes are subsequently resolved by their mass differences allowing peptide-level quantitation from the MS1 scan. A further limitation common to SILAC, ^{18}O, and Dimethyl labeling is that maximum of three samples can be compared per mass spectrometry analysis.

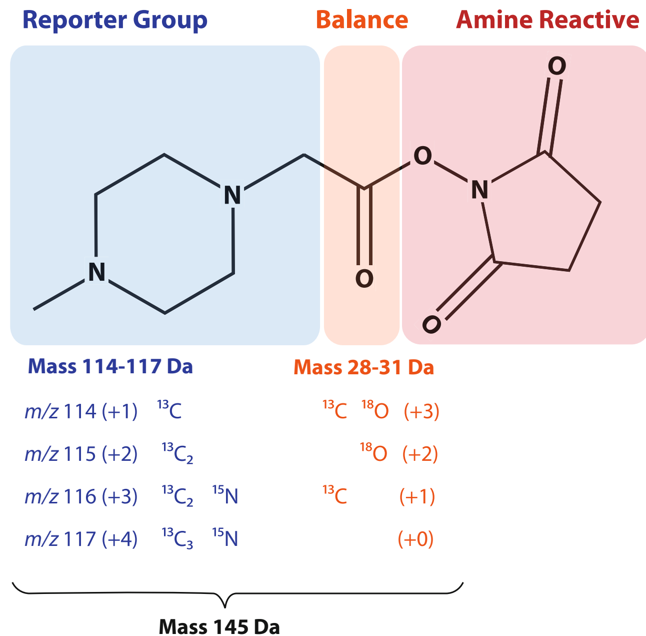

Fig. 2 Schematic of the four-plex iTRAQ peptide label. A reporter group with a defined mass between 114 and 117 Da is connected to a balancing linker. Together the reporter and linkers have a fixed mass of 145 Da and they are connected to an amine-reactive group which binds peptide amino-termini and lysine residues. The label is cleaved at the balancing linker during MS2 fragment ion generation to allow reporter ion detection. Figure adapted from [74]

7.3.6 Isobaric Peptide Labeling

Isobaric peptide labels offer greater multiplexing capabilities with 4-plex or 8-plex (Isobaric Tag for Relative and Absolute Quantification; iTRAQ) [74] or 6-plex, 10-plex or 11-plex (Tandem Mass Tags; TMT) commercial kits available [75]. These kits all rely on the same underlying principle.

Each label consists of an amine-reactive ester, a balancing carbonyl linker, and a reporter ion (Fig. 2). Tryptic peptides form amide linkages with the labels via N-termini or lysine residues. A label with a different reporter is used for each different sample and all the samples are mixed prior to fractionation and LC-MS/MS. Each label has the same total mass and chromatographic properties and therefore the LC retention time and mass/charge (*m/z*) separation of each sample are not differentially affected during the MS1 scan [74].

Precursor ions are then sampled for MS/MS analysis and the ionized-labeled peptides are fragmented with dissociation of the reporter ions from the balancing carbonyl linker. The peptide fragments are detected generating mass spectra in the usual manner. The reporter ions are also detected as peaks at a predefined *m/z*. For a four-plex iTRAQ experiment the reporters are detected at 114.1, 115.1, 116.1, and 117.1 *m/z* [74]. For a six-plex TMT

experiment, the reporters are detected between 126.1 and 131.1 m/z [75].

Assuming complete peptide labeling of each sample, the more abundant peptides within each sample will have accumulated more label. When equal amounts of each sample are mixed and subjected to LC-MS/MS together, those samples with a greater original concentration of a peptide of interest will produce higher reporter ion peak intensities in the MS/MS scan. By comparing the relative reporter ion intensities, the relative peptide and therefore protein abundances in the original samples can be determined [76].

The multiplexing capabilities of isobaric labels are directly offset by the consequent dilution of each sample leading to challenges in identifying low-abundance peptides [44]. Samples also require to be lysed and digested separately which has the potential to introduce error. In contrast, cell populations can be mixed prior to lysis in SILAC experiments. The quantitative accuracy and dynamic range offered by isobaric labels are excellent, however, surpassing SILAC in a direct comparison [62].

7.4 Label-Free Quantitative Shotgun Proteomics

All sample manipulation steps during a proteomic workflow reduce the data yield due to loss of proteins [77] and are additional sources of variation [78]. Eliminating the sample processing steps to incorporate labels for quantitation is clearly an advantage and underlies the rationale to develop label-free methods of quantitation.

The total number of spectra matched to each peptide contributing to a protein identification, termed the spectral count, has been reported to correlate with absolute protein abundance [79]. Various methods have been proposed to refine the spectral count such as normalizing for protein length [80], or combination scores including peptide count and fragment-ion intensity [81]. For complex protein mixtures, spectral counts are still subject to significant between-run variability and are highly dependent on LC conditions and precursor ion selection. As a result, the quantitative reproducibility of spectral counting is inferior to isobaric labeling methods [82].

An alternative relies on the capture of precursor ion intensity as a function of time to produce an ion chromatogram. The area under the ion chromatogram curve is linearly proportional to the peptide concentration [83]. Challenges exist in applying this method across LC-MS/MS runs to allow differential analysis as the same peptide ion must be identified and quantified despite background noise, co-eluting peptides causing signal overlap, technical variations in retention time and total protein loading among other factors [84].

A simultaneous advantage and disadvantage of both label-free approaches is the requirement to analyze one sample per LC-MS/MS run. This prevents sample dilution, provides maximum potential coverage, and prevents the potential failure to identify

dysregulated low-abundance proteins that occurs with multiplexed approaches. By comparing conditions across separate LC-MS/MS runs, however, the inherent changes in LC performance and the stochastic nature of protein identification by shotgun proteomics both contribute to data heterogeneity. Until these concerns are addressed, labeling strategies will still be widely employed.

8 MALDI-Imaging MS (MALDI-IMS)

A major disadvantage of lysing tissue biopsies for downstream mass spectrometry analysis is the loss of microscopic spatial information relating to protein expression. The local microenvironmental context of a cell is critical in determining behavior with cancer tissues being a well-recognized example [85]. Understanding the changes in protein expression that occur within a defined cellular niche may unveil novel insights not apparent from analysis of tissue biopsies in toto [86].

To preserve this heterogeneity, matrix-assisted laser desorption and ionization (MALDI) techniques have been adapted to allow direct ionization and mass spectrometry from tissue sections [87]. By co-registering spectra and histological images, patterns of protein expression can be interpreted within their biological and cellular context. The significant advantages of this imaging mass spectrometry (MALDI-IMS) method are offset by some of the limitations common to all MALDI approaches [88].

MALDI-IMS generates spectral features (m/z) which can be used to differentiate samples but does not provide protein identities. Hybrid approaches with downstream tandem mass spectrometry allow low mass proteins to be identified directly although proteome coverage has not yet reached parity with LC-MS/MS analysis of tissue lysates [89]. A further significant limitation is in the resolution of ionization sources. Current technologies allow a minimum resolvable area of 10 μm but most analyses are practically limited to areas of 100 μm [90]. This allows a granular expression map to be generated but the goal of identifying subcellular expression patterns, for example, at the tumor-stromal interface, remains elusive.

9 Conclusion

Biological phenotypes are governed by protein interactions and the correlation between RNA and protein expression is limited in many circumstances. The large-scale, direct measurement of protein expression is therefore an attractive prospect for the biologist. Mass spectrometry offers the potential to identify expressed proteins from biological samples in a hypothesis-free manner.

Technological advances have allowed the goal of proteome-wide measurement to become a reality in some model systems.

Monitoring dynamic changes in protein abundance has become feasible using both biochemical labeling strategies to provide highly accurate protein quantitation and label-free techniques. The evolution of selective reactive monitoring methods allows robust identification and quantitation across conditions. MALDI-IMS allows the spatial diversity of protein expression in biological tissues to be preserved and novel insights into disease processes such as cancer can be gleaned directly from tissue sections. By applying this array of proteomic techniques, scientists can address fundamental questions and begin to understand biological processes and disease pathophysiology.

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