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Gel-Free Proteome Analysis Isotopic Labelling Vs. Label-Free Approaches for Quantitative Proteomics

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1. Introduction

For more than three decades, proteomics have been a crucial tool for deciphering the intricate molecular systems governing biology. O'Farrel was the first to utilise 2 dimensional gel electrophoresis (2DE) to perform actual complex proteomic analyses (O'Farrell, 1975). 2DE has very quickly emerged at the forefront of this rapidly growing field of research and has allowed for thousands of studies in widely varied domains. The development of 2D Fluorescence Gel Electrophoresis (2D DIGE) has provided more accurate and reliable proteins quantification due to the simultaneous migration on a same gel of samples to be compared, avoiding gel-to-gel variation. More recently, technological improvements in liquid chromatography and mass spectrometry have made it possible to develop so called "gel-free proteomics" in which, after total proteome enzymatic digestion, the produced peptides are separated with a high resolution chromatographic system and identified using tandem mass spectrometry. A gel-free approach presents a number of advantages over 2DE, such as a higher sensitivity, an easier automation of procedures to provide a better reproducibility and a reduced influence of intrinsic protein characteristics (pI, molecular weight, etc.). Nevertheless, a high complementarity between 2DE and gel-free approaches has been extensively reported (Finamore et al., 2010; Charro et al., 2011; Matallana-Surget et al., submitted), which suggests that both methods will continue to be considered together for a long time. Furthermore, 2DE also presents some advantages over a gel-free workflow approach in particular contexts. Indeed, 2DE presents the important benefit of allowing for the detection of protein isoforms, which is still complicated using gel-free approaches.

Another example is an immunoproteomic workflow in which 2DE is followed by immunodetection, which allows for the targeted analysis and detection of antigen candidates.

An additional important difference between gel-free approaches and gel-based workflow is found in the quality of the quantitative data obtained. Indeed, 2DE quantification relies on spot volume measurement, which implies each protein is quantified based on a single data point. In contrast, multiple peptides from the same protein can be used for quantification using gel-free approaches. This major difference clearly indicates that gel-free workflow-derived quantitative data are more statistically robust. However, before gel-free workflow approaches can be used in differential proteomics, the intrinsic limitation of mass spectrometry-based peptide analyses, the ion suppression effect, must first be addressed.

2. Ion suppression effect

The ion suppression effect can be defined as a negative influence of the chemical environment of a compound upon its ionisation. In other words, in addition to the chemical characteristics of a compound, the molecules present around it during the ionisation process will influence its ionisation. Even if this phenomenon is also observed under MALDI conditions, this chapter will focus on electrospray ionisation (ESI)-based LC MS/MS workflow, and thus, only ESI ion suppression effects will be discussed here.

Electrospray ionisation results from a complex process that is not fully understood today but most likely relies on ion ejection from a droplet due to electric field strength and on solvent evaporation leading to charge acquisition and gas phase transfer. More details on the ionisation principle are available in a recent review by Wilm (Wilm, 2011). The importance of ion suppression effects in ESI have been mainly investigated in toxicology analyses in the context of LC MS/MS detections and the quantification of target compounds in biological matrices. A mechanistic investigation in 2000 (King et al., 2000) concluded that the gas phase reaction of charge transfer was likely less important than the solution phase processes into ion suppression effect under electrospray conditions. The results of this study point out that the modification of small droplet formation due to non-volatile compounds is the main cause of ionisation suppression, given that other mechanisms can also play minor roles in analyte ionisation. Using LC MS/MS-based toxicological analyses of target compounds in biological matrices, Muller *et al.* (Muller et al., 2002) also confirmed that the majority of the observable ion suppression effect was limited to the early period of reverse phase (RP) chromatography when unretained polar compounds were present in the electrospray solution. In RP-LC MS/MS-based proteomic analyses, the ion suppression effect due to these unretained polar compounds should not be a major concern because peptides will generally be significantly slowed on a C18 reversed phase column and thus, not co-elute with such compounds. Nevertheless, the ion suppression effect also originates from in-solution competition between the co-eluting analytes for charge acquisition and gas phase ejection during ESI. This decreased ionisation efficiency is sometimes referred to as the matrix effect and can be understood using the equilibrium partitioning model (Enke, 1997). The equilibrium partitioning model describes that ESI nano-droplets consist of two phases: an electrically neutral phase containing solvent molecules at its centre and an excess charge containing surface layer. An analyte is distributed between these two phases based on factors such as its hydrophobicity, its charge density or its basicity. Only analytes present at the surface layer will be amenable to ionisation and consequently, a competition can exist

between analytes for distribution in the charged surface. In other words, co-eluting compounds enter into a competition for this distribution and therefore, for ionisation. This model helps explain why the increased non-polar character of peptides, which leads to an increased affinity for the surface phase, results in a more successful competition for surface localisation and thus, ionisation (Cech and Enke, 2000). Ion suppression effects and in particular, the observed competition of co-eluting peptides for ionisation (the matrix effect) have made difficult the quantitative use of gel-free proteomic techniques. The first strategy to address this issue has been to analyse samples that have to be compared simultaneously after mixing them and thus, ensuring that the ionisation of the quantified peptides has been performed with exactly the same matrix effect. In order to achieve this goal, proteins or peptides from the samples that are to be compared are labelled using an isotopic coded tag that will not influence their behaviour during LC MS/MS but rather introduce a mass shift between samples that will allow the discrimination of the origin of the peptide.

The second strategy for quantitative gel-free proteomics relies on an early demonstration (Voyksner and Lee, 1999) that peptide peak intensity correlates with its concentration in a sample and could thus be used to compare one run to another. Nevertheless, to analyse a complex peptide mixture, one must take into account the matrix effect. Indeed, in such a mixture, to be able to compare the run-to-run peak intensities of a peptide, one needs to be able to exactly reproduce the same chromatographic separation for all the samples that are being compared so that all the peptides are always ionised with the same co-eluting peptides. If this prerequisite is not satisfied, the competition between the peptides for ionisation will not be conserved, and a difference in ionisation efficiency will introduce biases into the quantitative data. This manner of interpreting data has led to the development of gel-free quantitative proteomics, which rely on a highly reproducible chromatographic separation and have developed very quickly since the recent apparition of ultra pressure chromatography.

3. Quantification strategies

Quantitative proteomic can be classified in two major approaches: the stable isotope labelling and the label-free techniques (Figure 1).

3.1 Isotope-coded labelling

As suggested above, isotope-coded labelling allows for mass shift introduction between the proteins/peptides of the samples to be compared, which makes it possible to mix them before an LC MS/MS analysis. As the peptides contained within the samples to be compared have been ionised under exactly the same conditions, their intensities can be compared in order to achieve a relative quantification. The first developed method based on this principle was ICAT (isotope-coded affinity tag;(Gygi et al., 1999)), which relies on cysteine tagging followed by the affinity-based enrichment of tagged peptides. Initially, the ICAT tag consisted of a biotin moiety used for affinity enrichment and a thiol-specific reactive group for cysteine labelling. These two groups were separated from each other by a linker group, which contained 8 hydrogens in the light tag and 8 deuteriums in the heavy tag (Gygi et al., 1999). Thus, this tag introduces a mass shift of 8 Da, which will allow the peptides in the samples to be distinguished from one another and compared based on their mass spectrum; this tag also makes it possible to measure the relative abundances of the corresponding proteins in the two samples. This strategy has been referred to as non-
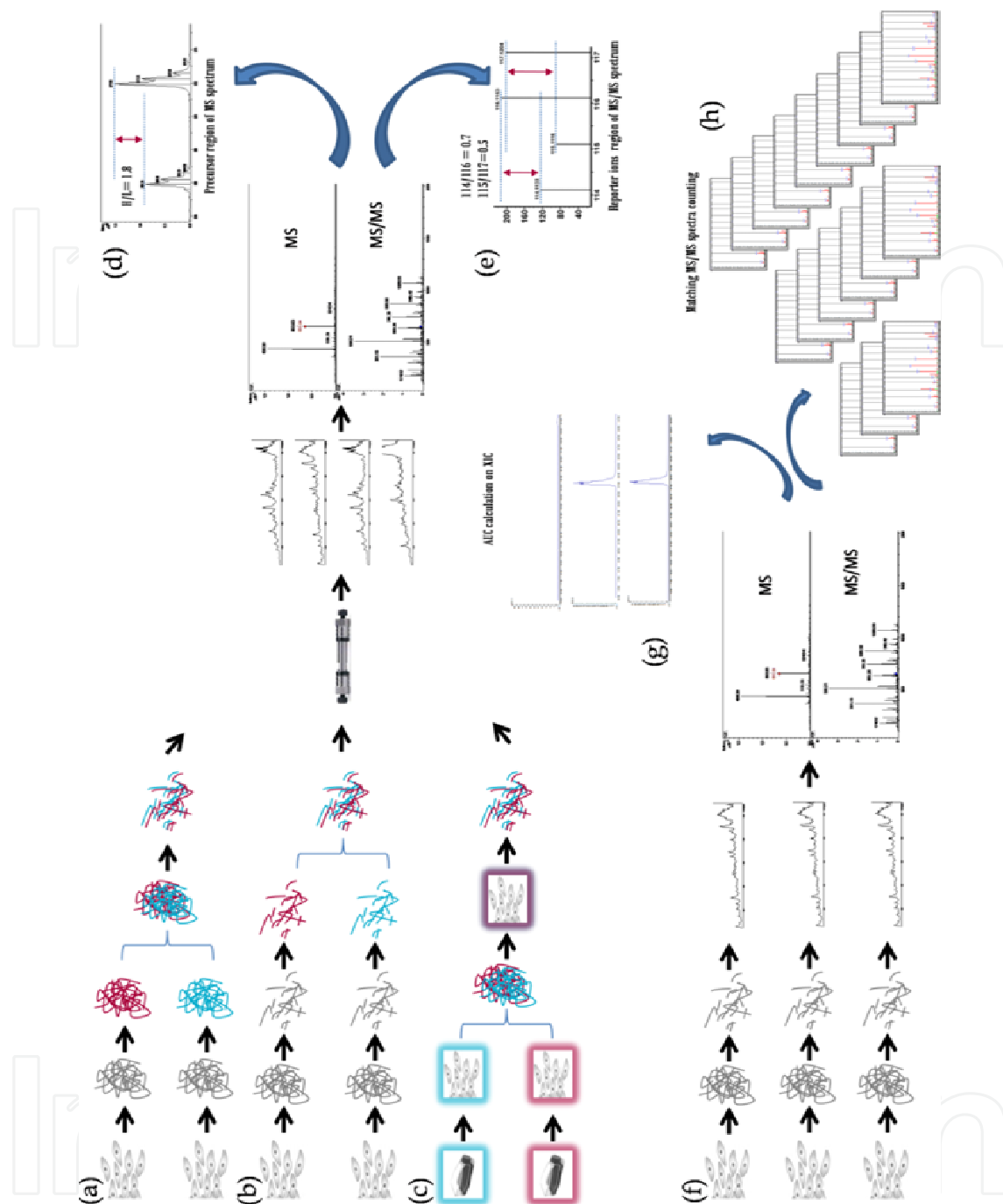
Fig. 1. Schematic representation of the main quantification workflow. (a-e) Isotopic labelling relies on the introduction of a discriminative mass shift, which allows sample mixing before analyses with 2D-LC MS/MS. Quantitative data are obtained in the MS spectrum in the case of non-isobaric labelling (d) or in MS/MS mode due to the release of a reporter group upon fragmentation during isobaric labelling (e). Isotopic labelling can be performed at the protein (a), peptide (b) or cell culture level (c). (f-h) In a label-free workflow, samples are prepared and analysed separately by LC MS/MS (f). Quantitative data can be obtained either from the Area under the curve (AUC) calculated from an extracted ion chromatogram for the representative peptides of a protein (g) or from a number of matching MS/MS associated with a protein (h).

isobaric labelling. ICAT has been continuously improved by first replacing deuterium coding with the C^{13} isotope in order to minimise the chromatographic resolution of the isotope-coded peptides (Zhang and Regnier, 2002) and then introducing a disulphide bond in the linker so affinity-trapped labelled peptides can be more efficiently eluted from the avidin affinity matrix by reductive cleavage of the linker (Hansen et al., 2003). Several non-isobaric tags have also been developed that similarly rely on the quantification of the peptides in the MS due to the isotopically introduced mass shift.

Following the development of non-isobaric labelling, isobaric tags such as iTRAQ (isobaric tag for relative and absolute quantification) were introduced (Ross et al., 2004). Isobaric tags are composed of an amine-specific reactive group, which allows for the tagging of proteins on lysines and peptides on their N-termini and a reporter group that has a different isotopic composition when comparing the different versions of the tag and thus, different masses. A balance group, which has an isotopic composition complementary to the reporter so that the global mass of reporter + balance group is constant between the different versions of the tag, is placed between the reactive and the reporter group. Thus, the tagged peptides are not discriminated in the MS. Upon peptide fragmentation by MS/MS, the reporter group is released and appears in the low mass range of the MS/MS spectrum. Separated from their balance group, the reporter ions are distinguishable from each other because their different isotopic composition introduces a 1- Da mass shift between them. The relative abundance of the peptides/proteins in the samples to be compared is deduced from the relative abundance of the corresponding reporter ions.

3.1.1 Non-isobaric labelling

ICAT was the first commercially available isotopic-labelling reagent, with a thiol-specific tag to target low abundance amino acids and due to enrichment, enabling a significant decrease of sample complexity (Figure 1a). However, a significant proportion of proteins could not be quantified with ICAT because of a lack of cysteines and hence, a high number of proteins were only quantified based on single peptides. This main limitation, observed with ICAT technique, encouraged researchers to develop alternative tags. ICPL (isotope-coded protein labelling) is one of these tags and was mainly developed to solve the low sequence coverage drawback of ICAT (Schmidt et al., 2005). ICPL, an amine reactive tag targeting lysines on intact proteins, was supposed to address this issue and reduce the proportion of unquantifiable proteins by increasing the number of quantified peptides per protein (Schmidt et al., 2005). Using ICPL, we and other groups have demonstrated that an important proportion of identified peptides in trypsin digested samples still lack a lysine and were not tagged and not quantifiable (Mastroleo et al., 2009b; Paradela et al., 2009). In the protein-labelling conformation, ICPL only allows for the quantification of approximately 70% of the identified proteins. Using the amine reactivity of ICPL, we have developed and optimised a peptide level labelling strategy called post-digest ICPL (Figure 1b), which allows for the tagging of the N-termini of all peptides, making them amenable to quantification (Leroy et al., 2010). This strategy is still currently used in our lab and has allowed for a significant number of successful analyses, some of which are presented below. While increasing the global amount of obtained quantitative data, labelling at the peptide level also implies that a highly cautious sample preparation technique must be employed to avoid bias introduction because samples will be mixed very late in the workflow process. It also impairs the possibility of protein-based sample fractionation, and a high resolution

peptide chromatographic separation (using 2D-LC) or high throughput data acquisition system will be required. It can be assumed that the earlier the sample labelling and mixing the lower the chance of bias introduction. Therefore, the best solution is to mix the samples even before protein extraction so that the chance of bias introduction is extremely decreased, and all protein fractionation methods can be easily applied to the sample. Such a procedure exists and is based on the introduction of a mass shift between the samples to be compared through the metabolic incorporation of isotope-coded amino acids during cell culture (Figure 1c; (Ong et al., 2002)). The two most common metabolic labelling are the ^{15}N labelling, usually used for microorganisms (Li et al., 2007; Ting et al., 2009) whereas the stable isotope labelling in cell culture (SILAC) is mostly used for mammalian cells (Ong et al., 2002; Mann et al., 2006). This method SILAC allows all tryptic peptides to be labelled and quantified if lysine and arginine are used as the isotope-coded amino acids. During such a workflow, harvested cells from treated samples versus control can be mixed immediately and extracted together, which ensures a perfectly unbiased sample treatment. Obviously, all methods suffer from some limitations. This method is only practical for auxotrophically cultivable organisms. Therefore, most bacteria as well as tissue samples are excluded from this workflow (Bantscheff et al., 2007). Recently, metabolically-labelled mice have been introduced to the market, which makes it possible to perform some tissue analyses using this type of workflow (Wu et al., 2004).

As an alternative to chemical (ICAT, ICPL, etc.) and metabolic (SILAC, ^{15}N, etc.) non-isobaric labelling, the enzymatic introduction of isotopic differences between samples has also been developed. In this case, the hydrolysis of the peptide bond during enzymatic digestion is realised in presence of regular water for one sample but with ^{18}O-containing water for the second sample, which results in the exchange of 2 ^{16}O for 2 ^{18}O at the C-terminus of the produced peptide in the latter case (Ye et al., 2009). A 4-Da mass shift will be introduced and used to discriminate between peptides originating from samples that are to be compared. This method is very straightforward, but differences in the rate of oxygen exchange between different peptides are sometimes problematic.

Most non-isobaric labelling strategies were developed as duplex strategies in which two samples can be compared. In order to increase analytical throughput, multiplexing is being introduced, notably with an ICPL tag (SERVA) for which a triplex and a quadruplex version were recently released. In this new version, the introduced mass shift is only 2 Da, and triply charged peptides will only be separated by 0.66 m/z. Under low resolution, such as using an ion trap mass spectrometer, the isotopic pair will become difficult to discriminate, and multiplexing sample analyses using non-isobaric labelling definitely require a high resolution mass spectrometer. On the other hand, a multiplexing capability is clearly an advantage of isobaric labelling strategies in which it can be more easily implemented.

3.1.2 Isobaric labelling

ITRAQ has been described by Ross and co-workers (Ross et al., 2004). Here, low mass reporter ions produced after peptide fragmentation in a mass spectrometer are used for quantifications (Figure 1). In this low mass range, the 1-Da mass shift between the singly charged reporter ions is easily discriminated and can be used for quantification, even with a low resolution instrument. This facilitates multiplexing analysis and, hence, ITRAQ exists in a 8-plex version and TMT (tandem mass tag; (Dayon et al., 2008) exists in a 6-plex version. This multiplexing capability undoubtedly represents a major advantage of isobaric labelling

because as many as 4 pairs of control/case samples can be simultaneously analysed. Alternatively, multiplexing can be used to perform technical replicates at the same time to increase the statistical power of the dataset. To date, ITRAQ represents the most commonly used quantification strategy with more than 500 entries found in a Pubmed bibliographic search engine using the keyword "ITRAQ" versus less than 350 for "SILAC". As in the post-digest ICPL, ITRAQ and TMT rely on peptides labelled at their N-termini due to an amine reactive group and also allows for the quantification of all identified peptides. Nevertheless, ITRAQ also has its own limitations, mainly due to the necessity of analysing a low mass range of the MS/MS spectra, which is generally not performed using a quadrupole /ion trap mass spectrometer.

Non-isobaric labelling also presents another advantage over isobaric tagging, *i.e.*, the ability to include differences in relative abundances for an isotopic pair in the precursor selection criteria to determine which ion will be selected for fragmentation. In other words, the mass spectrometer could preferentially select peptides for which a differential abundance has been detected and can virtually decrease the sample complexity and focus on the differentially abundant proteins. This is obviously not possible with isobaric tags because quantitative data are only available after precursor selection and fragmentation have occurred.

3.1.3 Examples of isotopic labelling applications

The major benefit of using isotopic labelling workflow in differential proteomics is the high accuracy of the obtained quantitative data. As discussed below, isotopic labelling definitely surpasses label-free approaches in this aspect. In this section, we will emphasise case studies in which high precision data were obtained and validated using alternative methods.

In our lab, we are currently involved in the analysis of naive T cell activation through anti-CD3/CD28 in the presence or absence of co-activating interleukins, notably IL-6. This project aims to better understand the mechanisms that underlie T cell differentiation, particularly T follicular helpers (Eddahri et al., 2009). In this study using post-digest ICPL and 2D-LC MS/MS, some obvious markers of Th2 polarisation of IL6-activated T cells were detected, as was expected (unpublished data). In addition, slight differences were also observed for proteins related to cellular trafficking. As T cell cellular trafficking is already known to be important for T cell differentiation (Tanaka et al., 2007), the validation of these observations is essential. We are focusing our efforts on a microtubule (Mi) polymerisation factor for which only a slight increase of abundance could be observed in IL6-activated T cells. The fold change observed in two biological replicates were only 1.33 and 1.48 with 2 and 5 peptides being used for quantification, respectively (Figure 2). This protein was selected because its means fold change (calculated on the 2 biological replicates) was statistically different from 1 based on t-student analysis (t <0.05). Western blot analysis was used to quantify the relative abundance of this protein on a third biological replicate using image based quantification (Figure 2). A fold change of 1.4, obtained by western blotting, confirmed the accuracy as well as the reproducibility of the observation made using post-digest ICPL.

A second example comes from the analysis of the fear-conditioning influence on neuronal plasticity. In this context, fear-conditioned rat cerebral tissue was compared to unconditioned controls using post-digest ICPL and 2D-LC MS/MS. In this analysis, the abundance of very few proteins was altered between the samples and control and only very
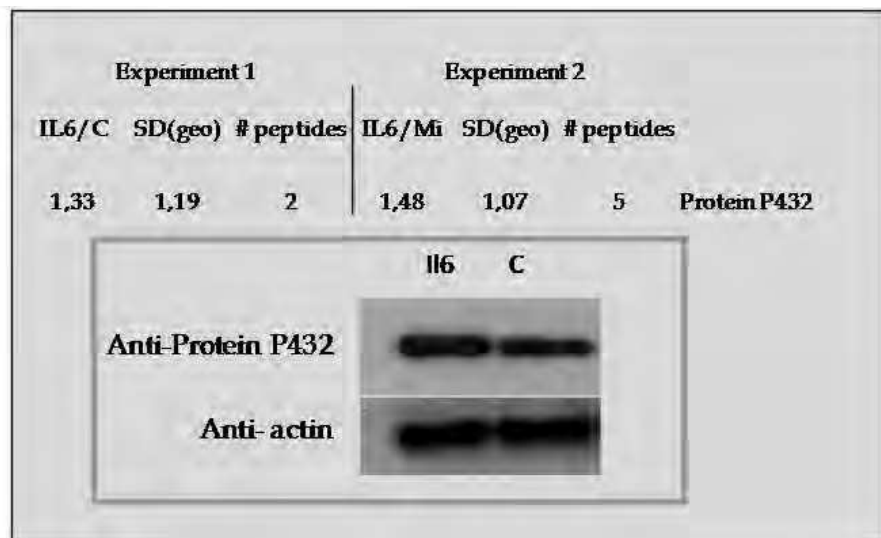
Fig. 2. Western blot analysis was used to validate the data obtained by post-digest ICPL. C : control; Mi, Microtubules; SD(geo), geometric standard deviation.
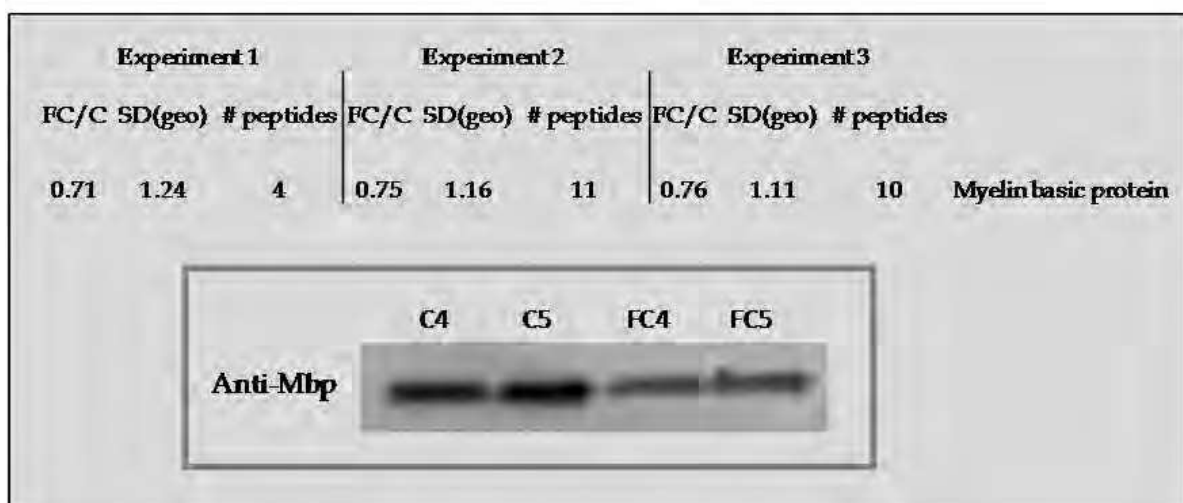

Fig. 3. Western blot analysis was used to validate data obtained by post-digest ICPL. Mbp: Myelin basic protein

slight changes were observed (unpublished data). Among the three biological replicates analysed, a protein was always modified with the same fold change of around 0.75 (Figure 3, t <0.05), which meant a slightly lowered abundance in the fear-conditioned animals. As this protein is known to be related to neuronal plasticity, it was mandatory to be able to confirm the 2D-LC MS/MS-obtained data. Here, again using a western blot (Figure 3), this protein has been selected for validation, which made it possible to confirm, after image-based quantification, a 30% decrease in the abundance of this protein.

The third example has been recently published by a Finnish group and is also related to the T cell differentiation mechanism but in presence of an alternative interleukin, namely IL4 (Moulder et al., 2010). In this study, the nuclear fraction was analysed 6 and 24 hrs after IL4 supplementation or control anti-CD3/CD28 activation of naive T cells. As observed in our study, the differences between the IL4-activated cells and control activation were very scarce

and of a low amplitude. Moulder and co-workers considered 3 biological replicates, and all were analysed three times using an ITRAQ 4-plex kit. In this study, a random effect meta-analysis model was used to estimate the representative expression ratios for each protein. Thanks to this elaborate study design they were able to apply a fold change cut-off of 1.2 (a 20% abundance variation) to their dataset and highlight abundance modifications for important proteins. Moreover, their observations can also be confirmed by fluorescence-based western blot analysis.

Finally, there is another example of a very well-designed study in which ITRAQ was proven to be highly reproducible. Uwin and co-workers (Unwin et al., 2006) analysed the differences in proteomes of two lineages of stem cells LSK+ (Lin+, Sca+, Kit+) and LSK- (Lin-, Sca+, Kit-) and also applied a cut-off of 1.2 to their obtained dataset, even though only 2 biological replicates were analysed. The use of such a low fold change threshold was justified by filtering their dataset based on intra-condition variability limits. Indeed, a 4-plex ITRAQ kit was used to label and analyse the two LSK+ biological replicates together and the two LSK- biological replicates. For a protein to be considered of a different abundance, the LSK+1 vs. LSK+2 as well as the LSK-1 vs. LSK-2 ratios of that protein had to first be between 1.10 and 0.92 (minimal intra-condition variability), and in addition, both the LSK+1 vs. LSK-1 and LSK-2 and LSK+2 vs. LSK-1 and LSK-2 ratios had to be higher than 1.2 with a $p<0.05$ in a pairwise Student's t-test analysis. This analysis clearly indicates the very high value of the multiplexing capability of isobaric labelling workflow and the extreme accuracy of the quantitative data that can be obtained using isotopic labelling strategies.

3.2 Label-free approaches

Label-free approaches fundamentally demonstrate that a MS signal observed for a peptide correlates very well with its abundance in the sample (Chelius and Bondarenko, 2002). A difficulty arises from the effect of the matrix, which may differ between the two separate LC MS/MS runs, and thus impair a fair comparison of the data sets acquired consecutively. Bondarenko and co-workers (Bondarenko et al., 2002) were the first to demonstrate that such a comparison of MS signals between individually acquired datasets was possible even with complex protein mixtures like serums. Thus, it appears that if a chromatographic separation is sufficiently controlled, the matrix effect is not that different between successive runs, and MS data can be used to quantify MS/MS- identified peptides (Figure 1g).

In addition, relying on the assumption that the matrix effect can be controlled, other approaches for label-free protein abundance comparisons have been described that rely on MS/MS data. Indeed, Liu *et al.* (Liu et al., 2004) demonstrated that the number of MS/MS spectra acquired by LC MS/MS for a defined protein correlates over 2 orders of magnitude with its abundance in the sample (Figure 1h). This very simple measurement relies on the principle that the more we see a protein the more abundant it should be in the sample. This type of strategy is termed spectral counting and can be opposed to "MS spectral intensity measurements" as "MS/MS features analyses".

3.2.1 MS-based label-free analysis

Since the first demonstration of the linearity of the MS signal and protein abundance relationship by Chelius and Bondarenko (Chelius and Bondarenko, 2002) as well as Wang

and co-workers (Wang et al., 2003), who performed a large scale demonstration of the applicability of this finding on large numbers of samples, MS-based label-free analyses have continuously been optimised and used more frequently in biological studies and especially in clinical research. Although sample preparation and MS data acquisition must be performed very cautiously as they represent a mandatory step, processing the data from a MS-based label-free approach is far for being trivial. Indeed, for all ions to be quantified, an area under the curve (AUC), based on the m/z and chromatographic retention time of the ion, has to be determined for all samples. Even with the best chromatographic system, this step will first necessitate a realignment of multiple chromatograms to compensate for the long analysis-induced retention time drift. This aspect is critically dependent on the quality of the chromatographic system and in particular, on its stability. The implementation of an ultra-HPLC system presenting excellent stability in terms of retention time now alleviates this step and will probably become mandatory to achieve a high quality MS-based label-free quantitative analysis. Once the chromatograms are suitably aligned, the AUC can be calculated for a particular ion based on its measured m/z. Of course, the accuracy of the data will depend on the ability to calculate the AUC for particular peptides and to avoid contamination by co-eluting peptides with similar m/z values. In that aspect, mass spectrometric resolution is critical and can help narrow the AUC calculation windows and, thus, eliminate most of the contaminating signal (figure 4).
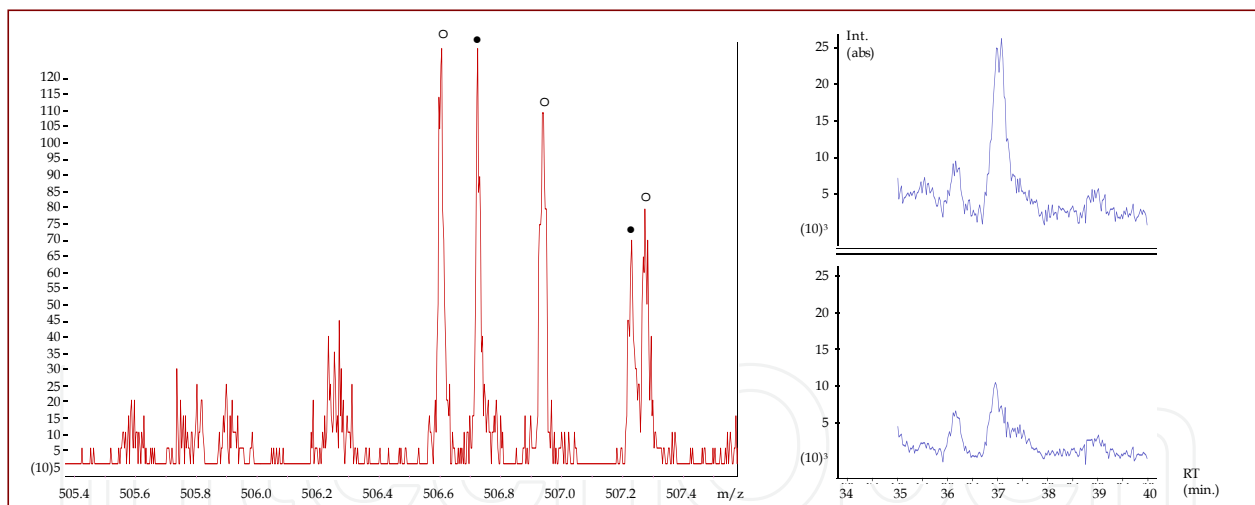

Fig. 4. High resolution mass spectrometry allows AUC calculations based on narrow m/z windows. In the case of co-eluting peptides of similar m/z values (left panel), the calculated AUC can be very different if narrow m/z windows (0.05; right lower panel) are used or if larger m/z windows (0.15; right higher panel) are taken into account due to a lower mass spectrometer resolution. Personal data obtained using the Triple TOF5600 (ABSciex).

A large amount of software has been developed for MS-based label-free quantification, and new tools are frequently released, which indicates a keen interest in these methods. A description of these softwares is beyond the scope of this chapter and has recently been performed by Neilson *et al.* (Neilson et al., 2011). However, it is interesting to note a

dichotomy in MS-based label-free data processing strategies. Indeed, if most of the early implemented data processing tools relied on an identified peptide list for AUC calculation, software now exists that allows for an unbiased total ion quantification independent of positive identification during the database search process. Quantitative data are calculated for all detected m/z notwithstanding an identified ion or even selected for fragmentation. Such an approach allows for the circumvention of a low sampling drawback of data-dependent acquisition in MS, which generally results in missing low level peptides. Here, all detectable ions are quantified and ions for which differential abundances have been observed can be identified by a subsequent targeted analysis. Obviously, as in this case, a quantification step only relies on accurate masses and RT measurements (AMRT or AMT workflow) without prior confirmation by MS/MS, such a workflow is only applicable to high resolution mass spectrometry-based platforms.

Another way to address the sampling bias of data-dependent acquisition has been proposed by Plumb and co-workers (Plumb et al., 2006). These authors developped the first real data-independent acquisition (DIA) workflow called MS^E (E states for elevated energy) and is available as an acquisition mode with WATERS instruments. This strategy is aimed to obtain the fragmentation data for all detectable ions by avoiding precursor selection (as in data-dependent acquisition, DDA) and isolation and rather acquiring alternatively low and high collision energy mass spectra for a full mass range. Using multiple criteria, a tremendous algorithm is then charged to associate a precursor mass deduced from low energy spectra and its fragment ions obtained in the high collision energy spectra. The grouping of fragment ions with their parent ions mainly relies on intensity and the elution profile. This theoretically comprehensive quantification and identification of all detectable ions has triggered significant interest and already been used in numerous publications (Blackburn et al., 2010; Herberth et al., 2011; Mbeunkui and Goshe, 2011). A variant of this workflow has recently been implemented by ABSciex (MS^{All}) on its triple TOF5600, and it can be assumed that all MS vendors will implement a DIA-like workflow on their instruments.

3.2.2 MS/MS-based label-free analysis

MS/MS-based label-free derived data represent the simplest process to quantify information. Indeed, there is no need to align a chromatogram to calculate AUC or to detect isotopic pairs, and everything required is contained in the database search results. MS/MS-based label-free quantification relies on the assumption that in data-dependent acquisition (DDA) analysis the sampling probability of a protein (*i.e.*, the number of MS/MS spectra related to a protein) is a function of the protein's abundance in the sample (Liu et al., 2004), which can be estimated by the so-called "spectral count" of a protein. MS/MS-based label-free quantification has been diversified using different parameters such as peptide counts (the number of unique peptides; (Gao et al., 2003)), sequence coverage and several tentatively normalised indices (NSAF, Normalised spectral abundance factor, etc.;(Ishihama et al., 2005; Florens et al., 2006)).

The accuracy of MS/MS-based label-free quantification has also been extensively investigated and proven unexpectedly high given the extreme simplicity of the measurement. In 2006, Zhang *et al.* (Zhang et al., 2006) compared spectral count, peptide count and sequence coverage in terms of reliability and also investigated the statistical relevance of such measurements. Interestingly, they linked the fold change, which can be

perceived statistically, with the actual number of spectral counts. This analysis showed that below 15 spectral counts only fold change higher than 2 could be detected no matter what statistical test was used. However, if more than 50 spectral counts were obtained, a fold change of 1.5 was detected. More recently, Colaert and co-workers (Colaert et al., 2011) estimated the global standard deviation of three different MS/MS-based label-free techniques and concluded that all of them had global SD of around 0.5. If a simple threshold in the format of mean +/- 2 SD is applied to such a dataset, fold changes higher than 2 were generally measurable.

Spectral counting has also been modified to allow for comparisons of the abundances of different proteins and absolute quantification. emPAI (**e**xponentially **m**odified **p**rotein **a**bundance **i**ndex) normalises the number of identified peptides of a protein by the number of theoretically observable peptides to account for differences in sequence characteristics between different proteins and allows for their quantitative comparison. More recently, APEX (**a**bsolute **p**rotein **ex**pression; (Lu et al., 2007)) profiling was developed to measure the absolute protein concentration per cell from the proportionality between the protein abundance and the number of peptides observed, by using a correction factor that correlates the likelihood of peptides observed to their intrinsic characteristics (length, amino acid composition, etc.).

3.2.3 Examples of label-free analysis

Recently, we had the opportunity to challenge the label-free analytical platform from WATERS using the ion mobility-implemented synapt G2 mass spectrometer (unpublished data) and its MS^E data-independent acquisition features. We analysed three biological replicates of crude protein extracts from *Variovorax* sp. SRS16 cultured in the presence or absence of the phenylurea herbicide linuron. This strain has already been shown to catabolise linuron (Breugelmans et al., 2007), and we have already performed gel-based (Breugelmans et al., 2010) as well as isotopic labelling gel-free proteomic analyses on these samples (Bers et al., *In Press*), indicating us what changes should be expected. All three biological replicates were injected three times, and only proteins identified in at least 2 out of the 3 technical replicates as well as in each biological replicate were considered for quantification. A statistical analysis was performed on the mean linuron/control ratio and, thus, only proteins with a rejected null hypothesis (ratio = 1) and a p-value <0.05 were accepted as modified in abundance. An arbitrary cut-off of 1.5 and 0.66 was additionally applied. Using these stringent criteria (identification in all three biological replicates and in at least 2 out of 3 technical replicates with a p-value <0.05), 83 proteins (33 up, 50 down) out of the 1500 identified were considered to differ in amount between the linuron and control condition. This label-free analysis gives us a tremendous increase in proteome coverage, multiplying the number of detected and quantified proteins by a factor of 3.

A particular feature of label-free analysis is its higher throughput, which facilitates large sample size analysis in clinical research and biomarker discovery. Moon and co-workers (Moon et al., 2011) recently used a MS^E-based label-free strategy efficiently in order to discover biomarker candidates. From the urinary exosome proteome of IgA nephropathy (IgAN), **t**hin **b**asement **m**embrane **n**ephropathy (TBMN) and healthy patients, they were able to identify and quantify more than 1800 proteins, among which 83 differed in amount between IgAN and TBMN. Four IgAN/TBMN-discriminating biomarkers were selected

among this dataset (aminopeptidase N, vasorin precursor ceruloplasmin and alpha-1-antitrypsin). These candidate biomarkers were submitted to western blot analysis on an independent set of samples, which failed to confirm differential expression for one of them but was validated in the other three. ROCs for differentiation between IgAN and TBMN indicated a high potential use for ceruloplasmin in this context because it provided a specificity of 91% for a sensitivity of 100%.

Using spectral counting, Saydam and co-workers (Saydam et al., 2010) recently analysed differences between human meningioma cells and primary arachnoidal cells. Proteins were separated by SDS-PAGE, the gels were cut into ten bands, submitted to in-gel digestion and the peptides were analysed by LC MS/MS. For all identified proteins, the spectral count was determined, and the differences between the samples were evaluated for statistical relevance using beta-binomial test. In this very simple workflow, 2800 proteins were identified (protein prophet probability >99% and at least 2 peptides), and 10% of them were statistically different in amount between the archnoidal cells and meningioma. Proteins belonging to the **m**ini**c**hromosome **m**aintenance (MCM) family were observed in a higher abundance in meningioma cells and were submitted for further validation by qRT-PCR and western blotting.

4. Isotopic labelling or label-free approaches?

4.1 Relative quantification

Most proteomic studies aim to compare different states of a proteome rather than obtaining absolute quantitative data. When designing such a differential proteomic analysis, one has to face, with Cornelian dilemma, the question of which method would be most suitable for obtaining valuable data useful for better characterising a biological system. This is a very difficult and important topic for which many parameters must be considered. In the above section, we have tried to describe and exemplify the main existing methods for relative comparisons of protein abundances. Here, we will try to summarise, based on their pros and cons, which methods best suit which needs.

A first principle could be to use the most straightforward and simple method possible. In regards to this aspect, MS/MS-based label-free analysis clearly comes first. This method only requires that sample preparation and data acquisition are reproducible, which is usually expected. Here, there is no requirement for time-consuming sample labelling or for an analytical platform using ultra-HPLC and high resolution MS. This type of analysis can be applied to a variety of samples, such as very large sample cohort, often required for clinical research, and no limitation exists concerning the number of conditions that can be compared at a time. Finally, assuming a convenient correction factor is used, absolute quantitative data can be obtained, which allows not only for a comparison of the abundance of a protein in different samples, but also ranks the proteins in a defined proteome based on their abundance (Mastroleo et al., 2009a). Of course, as no ideal method exists, a MS/MS-based label-free approach also has a major drawback, data accuracy. As already described above, numerous analyses have been conducted to estimate this accuracy and concluded this technique does not easily detect fold changes lower than 2 (Zhang et al., 2006; Colaert et al., 2011). Nevertheless, it is important to replace this accuracy in the context of a biological question. Indeed, if only major changes are of interest or if the samples to be compared are expected to be highly different, MS/MS-based quantitative data could be sufficient. Equivalently, if one goal is to

discover biomarker candidates able to discriminate diseased from healthy patients, it is not mandatory to be able to detect very slight fold changes. On the contrary, only proteins presenting major differences between controls and clinical cases will ultimately be useful for physicians to help them in their diagnostics or prognostics. It appears that pure biomarker discovery studies can be typically performed using MS/MS-based label-free approaches, and a more elaborate workflow would only be helpful if functional data could also be gained or are needed.

If not only discriminative but also functional data are to be obtained, acquiring accurate quantitative data is absolutely required. In these cases, both MS-based label-free approaches and isotopic labelling could be suitable. Nevertheless, the pros and cons of both strategies can help in the decision.

First, MS-based label-free techniques are only able to reach the isotopic labelling accuracy of quantitative data (CV>20%) if the analyses are performed on the latest generation mass spectrometers. Although MS vendors are continuing their efforts to allow access to such pieces of equipment to an increasing number of labs, to date, they are not considered as a benchtop device that is easily handled and accessible. On the other hand, isotopic labelling is easily amenable to high accuracy studies using a first generation Q-TOF device, or quadrupole ion trap.

The number of conditions to be analysed needs to be carefully considered. Indeed, isotopic labelling is limited in its multiplexing capacity, since so far only TMT and iTRAQ allow the comparison of multiple (up to 6 and 8 respectively) samples at the same time. For non-isobaric labelling, multiplexing capacities are, to date, limited at 4 samples, and in this case again, high resolution MS would be required.

Analysis throughput is generally considered to be lower when isotopic labelling is used because 2D-LC peptide separation is usually necessary to avoid the co-elution of peptides with similar m/z values, which might introduce errors in quantification. Label-free approaches, which rely on high resolution MS systems and ultra-HPLC, generally more efficiently deal with co-eluting peptides with similar m/z values and can be performed using 1D-LC. Nevertheless, it must be kept in mind that if the analysis of two mixed samples using 2D-LC requires around 12 hours, no gain in machine time will be obtained if the same samples are analysed using a 2-hour gradient in 1D-LC because, to obtain statistical relevance using data from a label-free analysis, a triplicate injection of all samples is generally required.

Another advantage of isotopic labelling is that when tagging occurs at the protein or even at the cell culture level, such as in SILAC, samples can be mixed very early in the workflow, and, thus, potential biases are avoided. During label-free approaches, full sample processing is performed separately, and the risk of a biased treatment is obviously increased.

In some cases, a high accuracy will not be sufficient and ultimate precision will be required. This is the case if very slight modifications are expected, such as in the example of T cell differentiation we have highlighted above. Post-translational modifications of proteins can also dramatically change a protein's function even if the fold changes are extremely small. In regards to this aspect, isotopic labelling still surpass label-free approaches and is the method of choice if fold changes lower than 1.4 must be efficiently characterised, as described above.

4.2 Absolute quantification

It has already been described that MS/MS-based label-free approaches can be used to reach an absolute quantification, but here again the precision is generally low and only orders of magnitude can be determined. Isotopic labelling is more easily amenable to the accurate absolute quantification of targeted proteins in a MS workflow. Absolute quantitative methods aim to measure the absolute protein level using a standard peptide to the corresponding protein. This is achieved by mixing a known amount of the synthesized isotope-coded form of a peptide from the protein to be quantified and using it as an internal standard to calculate the endogenous amount of the protein (method AQUA, Absolute quantification) (Gerber et al., 2003). This principle has been diversified in order to multiplex the proteins being quantified as well as to decrease biases introduced during sample treatment. In a new workflow termed concat, a chimeric protein composed of concatenated isotope-coded peptides to be quantified is introduced in the sample before enzymatic digestion. The common enzymatic digestion of reference peptides and endogenous peptides ensures a higher accuracy and allows for easy multiplexing.

Until recently, MS-based label-free approaches did not support absolute quantification. Nevertheless, Silva and co-workers from WATERS Corporation (Silva et al., 2006) reported that MS^E was the most accurate label-free technique for estimating absolute abundance by using average of the three most abundant tryptic peptides, which was reported to be proportional to protein molarity. This discovery used a unique internal standard to obtain absolute quantitative data for 6 exogenous standard proteins spiked into serum with a relative error below 15%. Moreover, 11 proteins of the serum matrix could also be quantified, and the obtained data correlates very well with the values available in the literature. To date, this absolute quantification feature has, to our knowledge, only been implemented in WATERS software packages.

5. Conclusion

In this chapter, we have described the most widely used strategies for quantitative proteomics studies. All have their pros and cons, which makes the choice of one of them difficult for non-proteomic researchers. Different criteria can be used in order to distinguish which method is best-suited to a given biological question. Among these, the data accuracy level required is probably the most interesting. With numerous proteomic analyses focusing on biomarker discovery, MS/MS-based label-free workflows are, to date, underutilised. When accurate data must be obtained, isotopic labelling methods and label-free approaches work equally well. Isotopic labelling will nevertheless still be of interest when high precision is required. It is expected that, in the future, easier access and development of highly reproducible nano-HPLC separation, high resolution mass spectrometer, and efficient computational tools will greatly improve the reliability and the use of label-free workflows.

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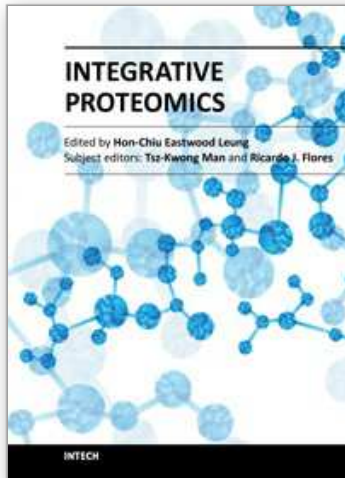
Integrative Proteomics

Edited by Dr. Hon-Chiu Leung

Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceutics development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

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