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Review

Label-free quantification in clinical proteomics

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ABSTRACT

Nowadays, proteomic studies no longer focus only on identifying as many proteins as possible in a given sample, but aiming for an accurate quantification of them. Especially in clinical proteomics, the investigation of variable protein expression profiles can yield useful information on pathological pathways or biomarkers and drug targets related to a particular disease. Over the time, many quantitative proteomic approaches have been established allowing researchers in the field of proteomics to refer to a comprehensive toolbox of different methodologies. In this review we will give an overview of different methods of quantitative proteomics with focus on label-free proteomics and its use in clinical proteomics.

1. Introduction

Accompanied by rapid technical developments in the field of mass spectrometry, proteomics has evolved into a very powerful bioanalytical platform for answering multidisciplinary scientific questions from medicine, biology, and biochemistry. This widespread applicability of proteomics necessarily implies the need of customized techniques and workflows depending on the scientific question itself, the kind of proteome under investigation (e.g. soluble or membrane proteins, post-translationally modified proteins, protein isoforms) as well as the sample types to be analyzed (e.g. tissue, cultured cells, body fluids, plants, bacteria). To meet all of these demands, a comprehensive repertoire of experimental techniques for isolation, separation, digestion, enrichment, depletion, identification as well as absolute and relative quantification of proteins has been developed over the years and further enhancements are still part of ongoing research. In particular, label-free proteomics has emerged as a high-throughput method for quantitative clinical proteomics studies. In this review we will give an overview about label-free proteomics and its use in the investigation of scientific questions with clinical relevance and a translational intent, widely referred to as clinical proteomics. We will discuss different approaches of label-free proteomics (except MALDI-MS-based strategies like MALDI imaging or quantitative LC-MALDI-MS/MS) in comparison to each other and labeling-based methods in order to shed light on the advantages, disadvantages and limitations of the different techniques. Furthermore, several experimental aspects ranging from sample preparation to data acquisition will be reviewed. Apart from these, software solutions for data analyses of label-free proteomics experiments and further data interpretation will be presented and selected examples from recent clinical proteomics studies will be discussed.

2. Quantitative proteomics

2.1. 2D gel electrophoresis

Since its development almost 40 years ago, the two-dimensional gel electrophoresis is still one of the methods of choice for protein separation and quantification. Using an isoelectric focussing in the first dimension and a separation via SDS-PAGE in the second dimension, thousands of protein spots can be separated, visualized and quantified in a single 2D gel [1,2]. The isolated protein spots of interest are then digested, extracted from the gel and identified via mass spectrometry. Even if the quantification is very accurate and sensitive in this gel-based approach, the relative high amount of protein sample necessary for protein identification as well as multiple experimental steps are the major disadvantages of this technique. Due to these drawbacks and as a consequence of the technical improvements in the fields of chromatography and mass spectrometry, novel mass-spectrometry-based quantification strategies have been developed that allow high-throughput proteome analyses and are complementary to gel-based approaches leading to a higher proteome coverage.

2.2. Labeling-based quantification

Over the years, several mass-spectrometry-based quantitative proteomic strategies utilizing different labeling strategies have been published. Most of these techniques rely on the labeling of samples from different conditions with stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O) and a

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following quantitative analysis in a mass spectrometer. The introduction of the isotopic label can be performed by metabolic, chemical or enzymatic labeling. Approaches utilizing stable-isotope labeling are: stable isotope labeling by amino acids in cell culture (SILAC) [3], stable isotope labeling of mammals (SILAM) [4], isotope-coded affinity tags (ICAT) [5], isotope-coded protein labeling (ICPL) [6], isobaric tags for relative and absolute quantification (iTRAQ) [7], tandem mass tags (TMT) [8,9], isobaric peptide termini labeling (IPTL) [10,11], dimethyl labeling [12] as well as several variants of these techniques. Apart from SILAC and SILAM which are introduced by metabolic labeling, the above-mentioned quantification strategies are directly applicable for proteomic studies of clinical samples. However, to overcome the limitation of SILAC to cell culture models a Super-SILAC approach has been developed. Here, quantitative changes of the proteome in different clinical samples (e.g. tumor tissue samples) can be determined by the comparison to an internal standard consisting of an isotopically-labeled pool of cancer cell lines [13]. Aside from the labeling with stable isotopes, a labeling strategy based on the attachment of a metal complex to peptides or proteins has been reported on. This approach is known as metal-coded affinity tag labeling (MeCAT) and enables absolute quantification with high sensitivity and a wide linear dynamic range via inductively coupled plasma mass spectrometry (ICP-MS) [14,15]. However, its potential applicability for clinical samples has not been tested yet.

2.3. Label-free quantification

Approaches of label-free quantitative proteomics can be divided into two different quantification strategies that are briefly described in the following. A schematic representation of both approaches is shown in Fig. 1.

The first approach is termed spectral counting and implies a counting and a comparison of the number of fragment-ion spectra (MS/MS) acquired for peptides of a given protein. Due to the empirical observation that the number of tandem mass spectra of a particular peptide increases with an increasing amount of the corresponding protein, a relative quantification of proteins between different samples is possible [16]. However, as in this method the quantification relies on a simple counting of acquired spectra rather than on measuring physical data, the spectral counting method is controversial [17]. Nevertheless, spectral counting is widely used and was further developed over the years. For example, modified approaches of spectral counting have been reported that take into account aspects influencing the number of spectral counts, like physicochemical properties of peptides as well as the lengths of the corresponding proteins. These approaches are known as absolute protein expression (APEX) [18] and normalized spectral abundance factor (NSAF) [19,20]. More recently, normalized spectral index (SI_N) was introduced which combines three MS abundance features, namely peptide count, spectral count and fragment-ion intensity. This approach has shown to eliminate variances between replicate measurements and allows quantitative reproducibility and significant quantification in replicate MS measurements [21]. For more detailed methodological reviews of spectral counting, see [22,23].

The second approach of label-free quantitative proteomics implies the measurement of chromatographic peak areas (also termed mass spectrometric signal intensities) of peptide precursor ions. Depending on the chromatographic method (e.g. reversed-phase liquid chromatography) the peptides are separated according to their particular physical properties (e.g. hydrophobicity, charge), subsequently ionized in an ion source and finally detected in a mass spectrometer. In the acquired mass spectrum each peptide of a particular charge and mass generates one mono-isotopic mass peak. The intensity of this peak as a function of the retention time can be visualized in an extracted ion chromatogram (XIC) and the area under the curve (AUC) can be determined. The areas of chromatographic peaks have been shown to correlate linearly in a wide range with the protein abundance which makes their measurement feasible for quantitative studies [24,25]. At a first glance, this approach looks straightforward and very convenient, but to obtain reliable results several experimental and technical aspects have to be considered (see: Section 3). Furthermore, raw LC–MS data generated in the experiments have to be post-processed (e.g. feature detection, alignment of retention times, normalization of MS intensities, peak picking, noise reduction) in the course of a quantitative analysis (see: Section 4).

2.4. Labeling-based versus label-free quantification

The first question arising prior to a quantitative proteome analysis refers to the quantification method itself. In principle, it is beneficial to use more than one technique for quantification, as the complementarity of various approaches implies a greater proteome coverage if they are used in combination. Apart from this aspect, different approaches have their particular advantages and limitations. For example, a clear advantage of labeling-based strategies over label-free approaches is the possibility of a multiplexed analysis, allowing the simultaneous measurement of differentially labeled samples in a single experiment. In particular, multiplexing capacities of 2-plex, 4-plex and 8-plex can be achieved with commercially available iTRAQ and TMT reagents. However, one should keep in mind that different quantification techniques imply variable requirements to the sample type and amount as well as the mass spectrometer used for the analysis. As mentioned before, metabolic labeling strategies are for example not applicable for proteome analysis of clinical samples and therefore limited with respect to the sample type. Chemical labeling strategies like iTRAQ or TMT on the other hand have special requirements concerning ion trap mass spectrometers. Contrary to label-free approaches, iTRAQ or TMT requires alternative fragmentation methods like HCD (higher-energy collisional dissociation) or ETD (electron transfer dissociation) that should be used instead of CID (collision induced dissociation), which is not compatible with the low mass range reporter ions. For label-free analysis via peptide ion intensities a high-resolution mass spectrometer is recommended, because the mass of the precursor ions needs to be determined very accurately. Contrarily, label-free analysis by spectral counting can also be performed on low-resolution mass spectrometers and was shown to give more accurate quantitative results than the ion-intensity-based approach in such a case [21].

To assess the performance of variable quantification approaches several comparative studies were carried out. A comprehensive study, for example, was performed in 2006 by the Association of Biomolecular Resource Facilities aiming the quantification of eight known proteins in different sample mixtures. The methods used in this study included gel-based approaches as well as MS-based techniques, either label-free or labeling-based. In case of label-free methods, the monitored protein ratios were close to the expected values, especially for the protein with the lowest abundance in the investigated mixture [26]. More recent comparative studies were published by Li et al. as well as Merl et al. [27,28]. In the former study, a comprehensive systematic comparison of label-free quantification based on spectral counting with SILAC, iTRAQ and TMT was performed. The authors were able to show that among these techniques the label-free approach has the largest dynamic range and the highest proteome coverage for identification. However, the quantification accuracy and reproducibility are worse in comparison to the investigated labeling-based strategies [27]. In the latter study, the authors used a combination of label-free quantification based on peptide ion intensities and SILAC for proteomic profiling of primary retinal Müller cells. Here, a significant complementarity concerning quantified and identified proteins was shown. In agreement with earlier studies, the label-free approach was also found to yield a higher proteome coverage. Within the label-free approach itself significant differences were also monitored depending on the software used for the data analysis [28]. In the literature, many more studies can be found that focused