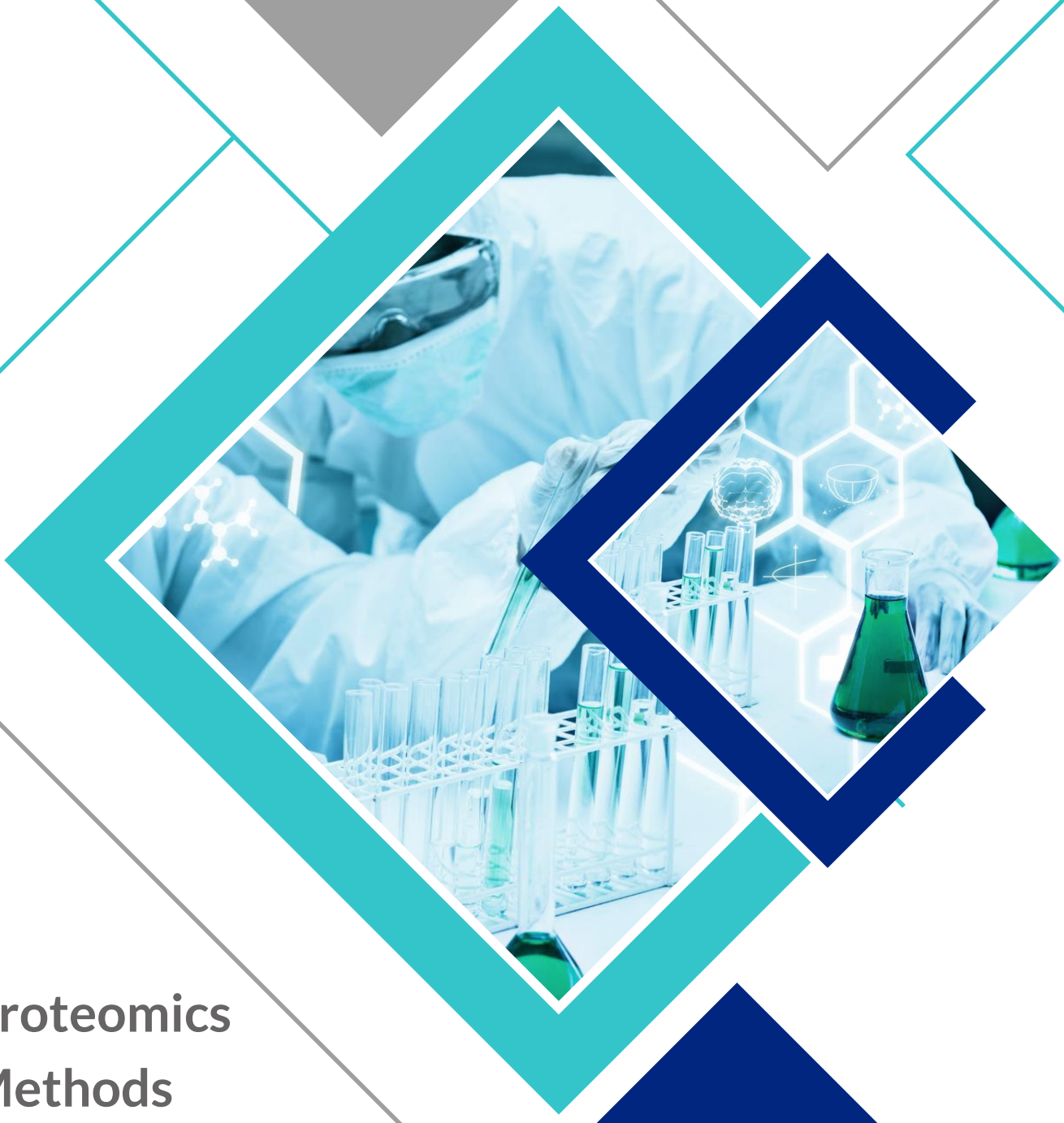
Creative Proteomics



Proteomics Methods for Analysis of Post-Translational Modifications

The importance of post-translational modification research

Most work of early proteome research focused on changes in protein expression levels at different times of cell growth or in response to disease or mitogen stimulation. However, many vital life processes are controlled not only by the relative abundance of proteins, but also by reversible PTMs of spatio-temporal distributions. Therefore, revealing the rules of PTM is a prerequisite for understanding the complex and diverse biological functions of proteins.

The analytical strategies for post-translational modification of proteins

PTM of proteins is a complex process. Currently, the most common modification types found are glycosylation, ubiquitination and phosphorylation (Figure 1).

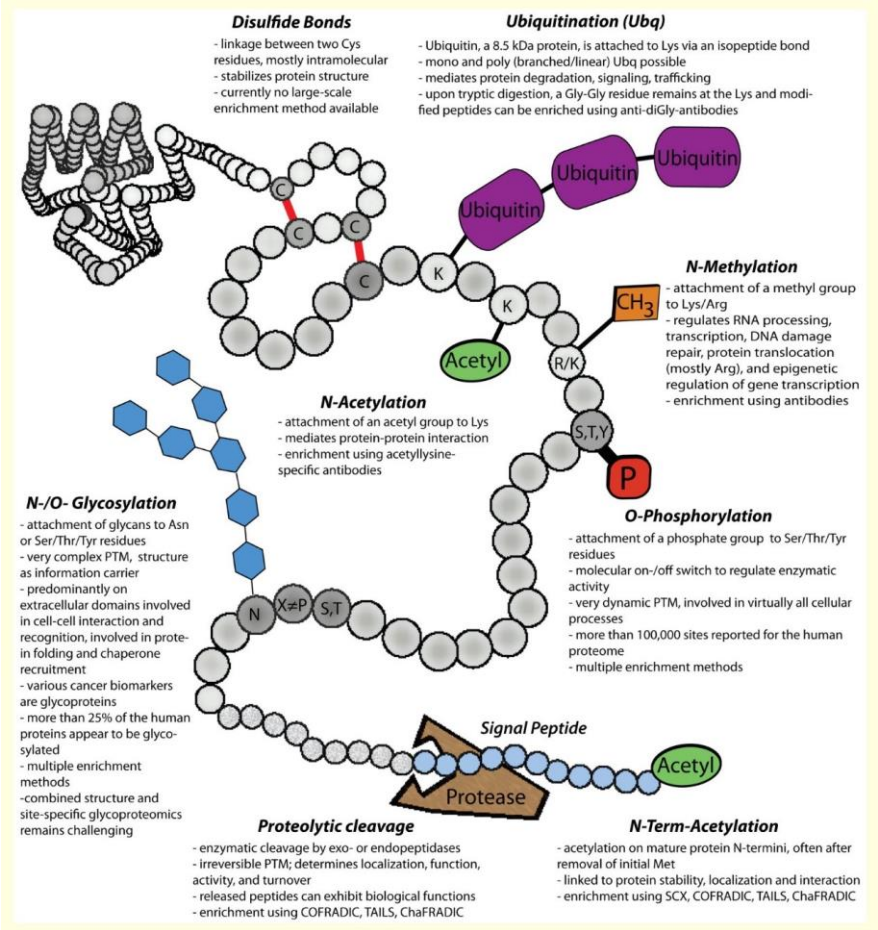


Figure 1. Diversity of post-translational modifications (Pagel *et al*, 2015).

The low content and wide dynamic range of post-translational modified proteins in samples have brought great challenges to relevant researches. Based on the characteristics of heterogeneity and low relative abundance, post-translational modified proteins are mainly studied by using gels, biomass spectra and bioinformatics tools. The mass spectrometry method used for PTM analysis is similar to that used for "routine" protein identification, including bottom-up, middle-down, and top-down proteomics analysis. A typical flow based on these three analytical approaches is shown in Figure 2.

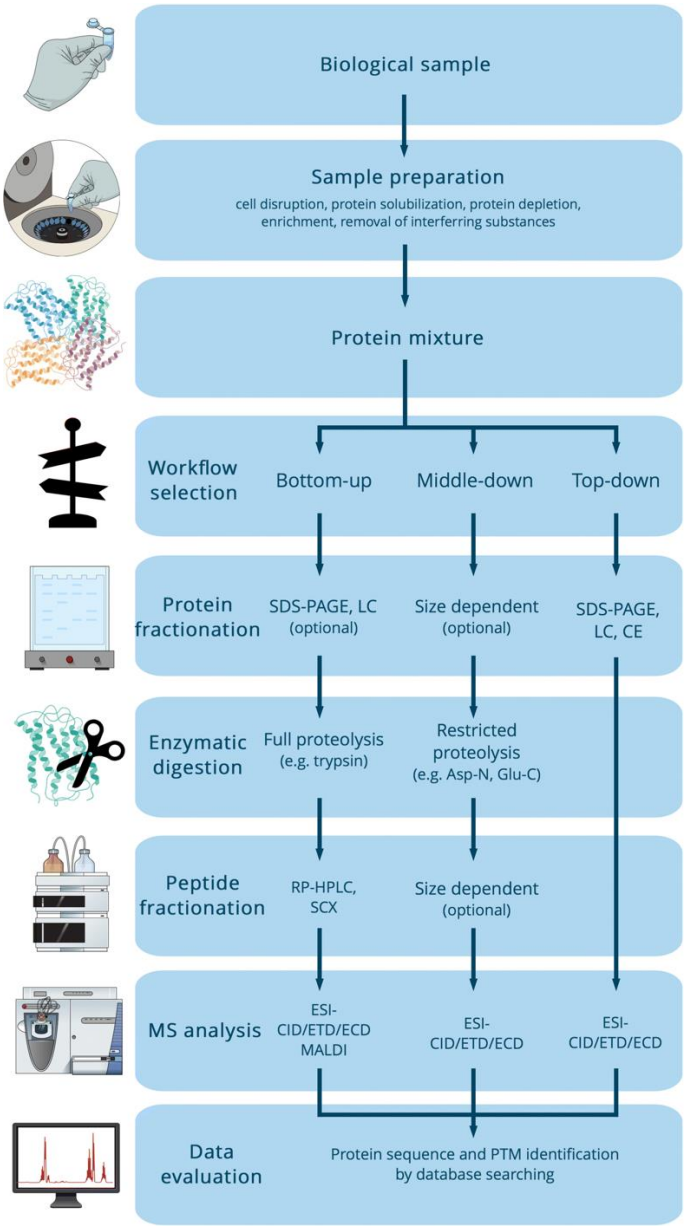


Figure 2. Typical proteomic workflows (Virág *et al*, 2019).

Bottom-up proteomics techniques for PTMs analysis

Bottom-up proteomics is a technique based on peptide analysis. For different types of PTM, there are differences in specific analysis strategies.

Glycosylation

Glycosylation of proteins is characterized by heterogeneity. Different sugar chains can be linked to the same site and different sites can be connected to different sugar chains on the same protein. The heterogeneity of glycation seriously hinders the separation and analysis of glycoprotein. The same protein with different sugar types will appear scattered bands on the electrophoresis, leading to signal dispersion. In addition, poor identification of proteins with low abundance results in poor separation of glycoproteins in the chromatographic spectrum and a cluster of poorly resolved peaks in the mass spectrum without accurate molecular weight.

Currently, the main research strategy for glycosylation of proteins adopts the existing technology system to separate and enrich glycosylation peptides of glycosylation proteins, eliminate the heterogeneity of glycosylation and its influence on mass spectrometry, and mark glycosylation sites. Thus, the identification of high-throughput glycoproteins and glycosylation sites can be realized. The commonly applied glycoprotein isolation and enrichment technologies include: (1) Lectin affinity technology; (2) Hydrazide chemistry enrichment; (3) Hydrophilic interaction chromatography; (4) β-elimination/Michael addition reaction. Mass spectrometry-based glycoprotein identification and glycosylation site determination methods include: (1) PNGase F enzymatic method; (2) Endo H enzymatic method; (3) Trifluoromethanesulfonic acid (TFMS) method.

Ubiquitination

Ubiquitin is a 76 amino acid polypeptide that is highly conserved in eukaryotes and can be covalently linked to the epsilon amino group of a lysine residue of a target protein by an isopeptide bond. Enrichment of ubiquitinated proteins is primarily based on labeling. Ubiquitin is labeled with affinity label (usually 6xHis), and the ubiquitin proteins are extracted by nickel chelation chromatography affinity. Since the C-terminus of ubiquitin is Arg-Gly-Gly structure, after trypsin hydrolysis, Gly-Gly remains on the peptide chain of the modified protein, increasing the mass of the peptide by 114, which serves as a mass marker for the ubiquitin localization site. Furthermore, the ubiquitination sites can be identified by tandem mass spectrometry. Pre-separation of samples using HPLC and enrichment of ubiquitinated peptides using antibodies against specific residue structures can greatly raise the concentration of ubiquitinated proteins.

- **Phosphorylation**

Studies of phosphorylated proteomes have focused on the phosphorylation of serine, threonine and tyrosine prevalent in eukaryotes. Since the content of the phosphorylated protein in the living body is very low, it must be separated and enriched before analysis. Currently, common separation and enrichment techniques for phosphorylated proteins include immobilized metal affinity chromatography (IMAC), immunoprecipitation (IP), strong cation exchange (SCX) chromatography, strong anion exchange (SAX) chromatography, reverse phase chromatography and so on. These techniques are integrated and optimized for phosphorylation proteome analysis in different biological samples.

Middle-down proteomics techniques for PTMs analysis

Middle-down proteomics techniques get involved in the analysis of histone modifications, as is shown in Figure 3. Sample preparation is the same as the widely used bottom-up analysis strategy until purified histones are obtained. After histone extraction, digestion is performed with GluC. Samples are then ideally separated using weak cation exchange/hydrophilic interaction chromatography (WCX-HILIC) coupled online with high resolution MS equipped with electron transfer dissociation (ETD). Identification of spectra can be performed with traditional software, but the result needs to be filtered due to the issues of estimating a proper false discovery rate.

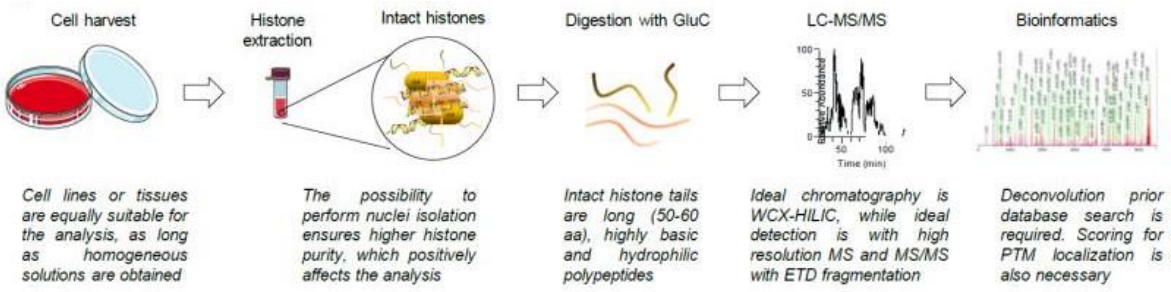


Figure 3. Middle-down MS workflow (Sidoli *et al*, 2017).

Top-down proteomics techniques for PTMs analysis

Top-down techniques can directly introduce intact proteins and fragment them in tandem mass spectrometers without proteolytic digestion. Currently, there are two effective methods for complete protein separation: off-line and on-line. The former is represented by four-dimensional separation method, while the latter by WCX-HILIC.

Early protein fragmentation mass spectrometry techniques used low-energy collision induced dissociation (CID). Although CID can obtain abundant fragmentation information for most peptides, it is not sufficient for intact protein molecules. The electron capture dissociation (ECD) technology developed by McLafferty *et al.* is more suitable for the fragmentation of intact proteins, which overcomes the shortcomings of CID. ECD uses free hot electrons to interact with multi-charged protein ions, resulting in the fracture of protein molecules. As ECD is non-ergodic, it can retain the modified group on the fragment ion. Compared with CID, ECD can accurately determine the modified site.

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