

Towards a better understanding of nuclear processes based on proteomics

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Abstract The complex structural and functional organisation of the brain warrants the application of high-throughput approaches to study its functional alterations in physiological and pathological conditions. Such approaches have greatly benefited from advances in proteomics and genomics, and from their combination with computational modelling. They have been particularly instrumental for the analysis of processes such as the post-translational modification (PTM) of proteins, a critical biological process in the nervous system that remains not well studied. Protein PTMs are dynamic covalent marks that can be induced by activity and allow the maintenance of a trace of this activity. In the nucleus, they can modulate histone proteins and the components of the transcriptional machinery, and thereby contribute to regulating gene expression. PTMs do however need to be tightly controlled for proper chromatin functions. This review provides a synopsis of methods available to study PTMs and protein expression based on high-throughput mass spectrometry (MS), and covers basic concepts of traditional ‘shot-gun’-based MS. It describes classical and emerging proteomic approaches such as multiple reaction monitoring and electron transfer dissociation, and their

application to the analyses of nuclear processes in the brain.

Keywords Neuroproteomics · Nucleus · Neuroscience · Histones · PTMs · Signalling · Transcription · Quantitation · Mass spectrometry

Abbreviations

CID	Collision-induced dissociation
ECD	Electron capture dissociation
ESI	Electrospray ionisation
ETD	Electron transfer dissociation
FFE	Free-flow electrophoresis
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HMT	Histone methyl transferase
HPLC	High-performance liquid chromatography
iTRAQ	Isobaric tag for relative and absolute quantitation
MALDI	Matrix-assisted laser desorption ionisation
MRM/SRM	Multiple/selected reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
PTM	Post-translational modification
PTP	Proteotypic peptide
RP	Reversed-phase
SCX	Strong cationic exchange
SILAC/SILAM	Stable isotope labelling of amino acids in cell culture/in mammals
SUMO	Small ubiquitin-like modifier
TOF	Time of flight

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Introduction

Eukaryotic cells carry their genome within the nucleus, a dedicated organelle with complex organisation and structure. Although nuclear processes are extremely important for cellular functions, their mechanisms and modes of regulation remain only partially understood. An important goal for the characterisation of nuclear organisation and nuclear events is to identify and quantify the proteins present in the chromatin, and in its different sub-nuclear domains. Mass spectrometry (MS)-based proteomics is a method of choice for such characterisation. It has contributed tremendously to a better understanding of nuclear functions and is expected to continue to advance this understanding in the coming years.

It is well established that post-translational modifications (PTMs) on proteins are key to cellular signalling. Most signalling pathways rely on reversible and site-specific PTMs of proteins in most cellular compartments, in particular the nucleus. When occurring on histone proteins, PTMs can change chromatin structure, and positively or negatively regulate transcriptional activity. PTMs such as phosphorylation, acetylation, methylation and ubiquitination establish a specific ‘mark’ on histones called the ‘histone code’, which is dynamically induced and can persist for long periods of time. The presence of these PTMs alters the chromatin structure and affects the binding of transcription factors, proteins that control gene activity, to the DNA (Boi 2008). In the brain, histone PTMs are critical for basic cellular processes such as activity-dependent gene transcription, required for long-lasting neuronal events, but also for complex brain functions such as learning and memory. They are also emerging as critical components of enduring effects on brain functions and behaviour induced by early life experience, trauma, hormonal exposure or cognitive activation (Franklin and Mansuy 2009; Graff and Mansuy 2008; McCarthy et al. 2009). The ability to interfere with histone PTMs using drugs like for instance, inhibitors of histone deacetylases (HDACs), is a promising approach to block gene activation and transcription in pathologies such as cancer (Insinga et al. 2005). However, a full and comprehensive understanding of regulatory mechanisms that control the histone code in the nucleus is still required. The establishment of appropriate methods to study these mechanisms is therefore, of primary importance for several areas of biology.

Proteomics in the brain and animal/cellular models

Advances in the sensitivity and temporal resolution of MS-based proteomics has permitted quantitative analyses of complex proteomes, and their change in response to cell

growth, activation, and death. Although fairly recent, the application of MS methods to neuroscience has grown significantly over the past years, and has allowed novel investigations of biochemical processes in the nervous system (Bayes and Grant 2009; Liao et al. 2009; Tweedie-Cullen et al. 2007). Even though no single proteomic strategy can lead to the full and routine analysis of the full proteome of a given organism, the combined use of different techniques has allowed the extensive characterisation of sub-proteomes and organelles in several organisms. Multiple studies have been carried out to systematically identify proteins and their PTMs in given samples, and many have provided quantitative measures.

Over the past few years, MS has been extensively applied to cell-based systems and genetically engineered mouse models for the study of biological processes. Quantitative proteomic technologies when used in combination with cellular or animal models with, for instance, controllable alterations in synaptic or nuclear functions (Lee and Silva 2009) have allowed a better understanding of specific proteins in these pathways. While complementary, they have their own advantages and limitations. Cell culture-based systems provide more homogeneous cellular populations and allow easier labelling than animal models for quantitative proteomics (see below), but do not recapitulate complex functions or processes such as behaviour. In contrast, animals provide the most physiological models of *in vivo* functions but are often too complex to allow thorough proteomic analyses. Thus, depending on the question under study, either one or a combination of these models needs to be used. We have outlined below important steps and parameters that need to be considered when using cellular or animal models, and discuss some of the data that has been generated with these models.

Fractionation of brain tissue and isolation of nuclear sub-proteomes

An essential primary step in the analyses of biological processes by proteomic methods is the preparation of samples of maximal quality. Because cellular proteomes are complex and contain proteins with a wide range of abundance, fractionation is usually necessary to optimise the identification of these proteins (Liao et al. 2009). This is particularly true for tissues like the brain, which is rich in lipids, and whose cells have distinct anatomical components such as dendrites and synaptic terminals (Andersen et al. 2005; Andersen et al. 2002; Andersen and Mann 2006; Gauthier and Lazure 2008; Tweedie-Cullen et al. 2007; Yates et al. 2005) (see Fig. 1). To analyse histones in the nucleus it is common to first isolate nuclei by density gradient centrifugation before extracting the histones

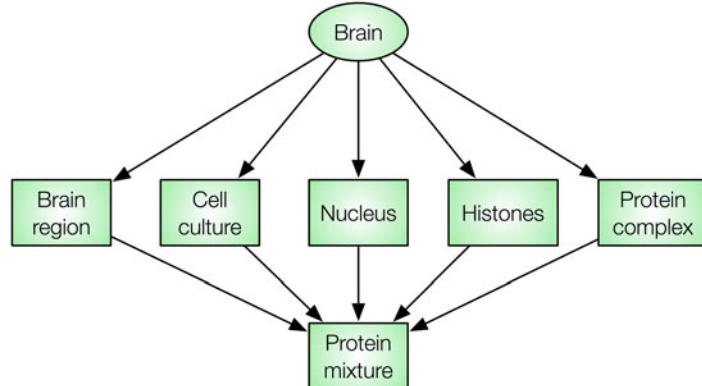
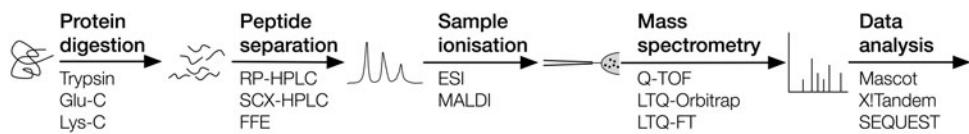
(A) Sample preparation**(B) MS Workflow**

Fig. 1 Flow of a typical MS-based proteomic experiment. **a** Neuroproteomic experiments generally begin with the processing of tissue to obtain a mixture of proteins. The methods employed include dissection, homogenisation, primary cell culture, cellular/protein fractionation and affinity purification of protein complexes. **b** The MS workflow begins by digesting protein samples with an enzyme such as Glu-C or trypsin, and the resulting peptides are fractionated using reversed-phase (RP), strong cation exchange (SCX) HPLC or

free-flow electrophoresis (FFE) to reduce sample complexity. Peptides are ionised and introduced into the MS via ESI or MALDI, and their mass is determined by an MS precursor scan. Specific ions are randomly selected and further fragmented to generate sequence information that can be compared to sequences from in silico digested protein sequence databases using search engines such as Mascot and SEQUEST, for peptide and hence protein identification

themselves (Shechter et al. 2007). Several proteomic studies have therefore used purified sub-cellular fractions from the nucleus and nucleoli, the chromatin and chromosome fractions, macromolecular complexes, enriched preparations of interchromatin granule clusters, nuclear envelope and pore clusters, or the nuclear matrix (Albrethsen et al. 2009; Andersen et al. 2005; Beausoleil et al. 2004; Saitoh et al. 2004; Schirmer and Gerace 2005; Tweedie-Cullen et al. 2009).

In addition to sub-cellular fractionation, separation of proteins or peptides after proteolytic digestion (see below) is also usually necessary. Such separation can be achieved by SDS-PAGE or high-pressure liquid chromatography (HPLC) (Tannu and Hemby 2006). However, reversed-phase (RP) chromatography, a method that fractionates peptides via their differing hydrophobicity, is the most common (Sandra et al. 2008). With highly complex samples, strong cationic exchange (SCX) chromatography can also be used prior to RP-HPLC. SCX separates peptides based on charge through the use of an increasing salt gradient (typically KCl). It simplifies peptides mixtures and thereby allows the identification of more peptides (Peng et al. 2003). Imaging-MS techniques have also recently been developed (McDonnell et al. 2009) and can provide highly relevant information on the physiological context of

identified peptides and PTMs as tissue sections are directly analysed in the mass spectrometer.

Protein identification in classical ‘shot-gun’ proteomics

Several methods can be used to identify proteins in fractionated samples. The most classical approach conceptually known as ‘bottom-up’ or ‘shot-gun’ proteomics consists of firstly digesting the proteins into peptides using a protease such as trypsin. The resulting peptide mixture is then ionised and analysed by MS. Ionisation is usually performed by electrospray (ESI), for which the sample is directly sprayed into the mass spectrometer, or by MALDI in which peptides are spotted onto a solid phase matrix, and ionised via laser irradiation. The analysis of the ionised samples then uses tandem MS (MS/MS), which in most cases, allows the unambiguous identification of peptide sequences and the precise localisation of PTMs on specific residues (Steen and Mann 2004). Tandem MS has two stages of data acquisition. First, the instrument determines the mass to charge ratio (m/z) of all ions injected into the spectrometer, then precursor ions are selected semi-randomly based on their signal intensity, and are further fragmented via a process known as collision-induced

dissociation (CID). Fragmentation via CID results in the preferential cleavage of adjacent amino acids at the peptide bond. By measuring the mass of peptide fragments, it is then possible to determine their sequence computationally (Fig. 1).

Whilst ‘bottom-up’ analyses are most widely used, a parallel ‘top-down’ approach can also be employed. It involves the direct analysis of intact proteins without any prior proteolytic digestion, and therefore allows the identification of combinatorial PTMs on individual proteins (as long as these proteins do not exceed 70–100 kDa) and the order in which PTMs occur (Siuti and Kelleher 2007; Zabrouskov et al. 2006). Another methodological variant known as ‘middle-down’ also exists, in which large proteolytic peptide fragments are generated using enzymes such as Glu-C or Asp-N (as opposed to trypsin which generates short fragments). It combines the benefits of ‘top-down’ and ‘bottom-up’ approaches, but allows the analysis of very large proteins not suitable for top-down approaches alone.

One feature of ‘top-’ and ‘middle-down’ approaches is their reliance on new methods of peptide fragmentation based on electrons. When transferred to peptides, electrons trigger highly selective, rapid and extensive fragmentation of N-C α bonds along the peptide/protein amide backbone. Electron capture dissociation (ECD) is one of these methods, first reported in 1998 and subsequently developed into electron transfer dissociation (ETD) (Syka et al. 2004). ECD and ETD have been instrumental for the analysis of large peptides (>30 amino acids) because they circumvent the usually poor sequence coverage of such peptides by CID. Thus, while peptide fragmentation by CID results in a low number of fragment ions and limited sequence information, ETD favours extensive fragmentation of the protein/peptide backbone and does not suffer from this limitation (Everitts et al. 2010; Udeshi et al. 2008). ETD has thereby enabled the application of ‘middle-down’ MS to the analysis of histones, and the differentiation of histone protein isoforms and their PTMs, which is difficult to achieve by CID approaches (Garcia et al. 2007b; Mackay et al. 2008). The extensive backbone fragmentation by ETD/CID also improves the ability to localise PTMs to specific residues, which is not possible without a large number of fragment ions. Furthermore, ETD methods have the advantage of retaining labile PTMs such as phosphorylation or glycosylation, often cleaved off by CID, and thereby making these PTMs more easily identifiable (Garcia 2009; Kelleher et al. 1999).

Relative and absolute quantitative MS

An important limitation of most qualitative and mapping proteomic studies is that they only show a static view of the

proteome, and do not provide any information about the abundance of each protein, or about dynamic changes in protein stoichiometry. Quantitative proteomic approaches (see below) have, however, been developed in the past few years to circumvent this limitation. They use two major methods: differential isotopic labelling and label-free quantification (Fig. 2) (for review see Bantscheff et al. 2007; Ong and Mann 2005). By measuring differences in protein expression between samples, they allow comparative analyses of molecular phenotypes and the detection of changes in the relative and absolute protein abundance resulting from manipulations such as cellular activation, gene overexpression or knockout (Fig. 2).

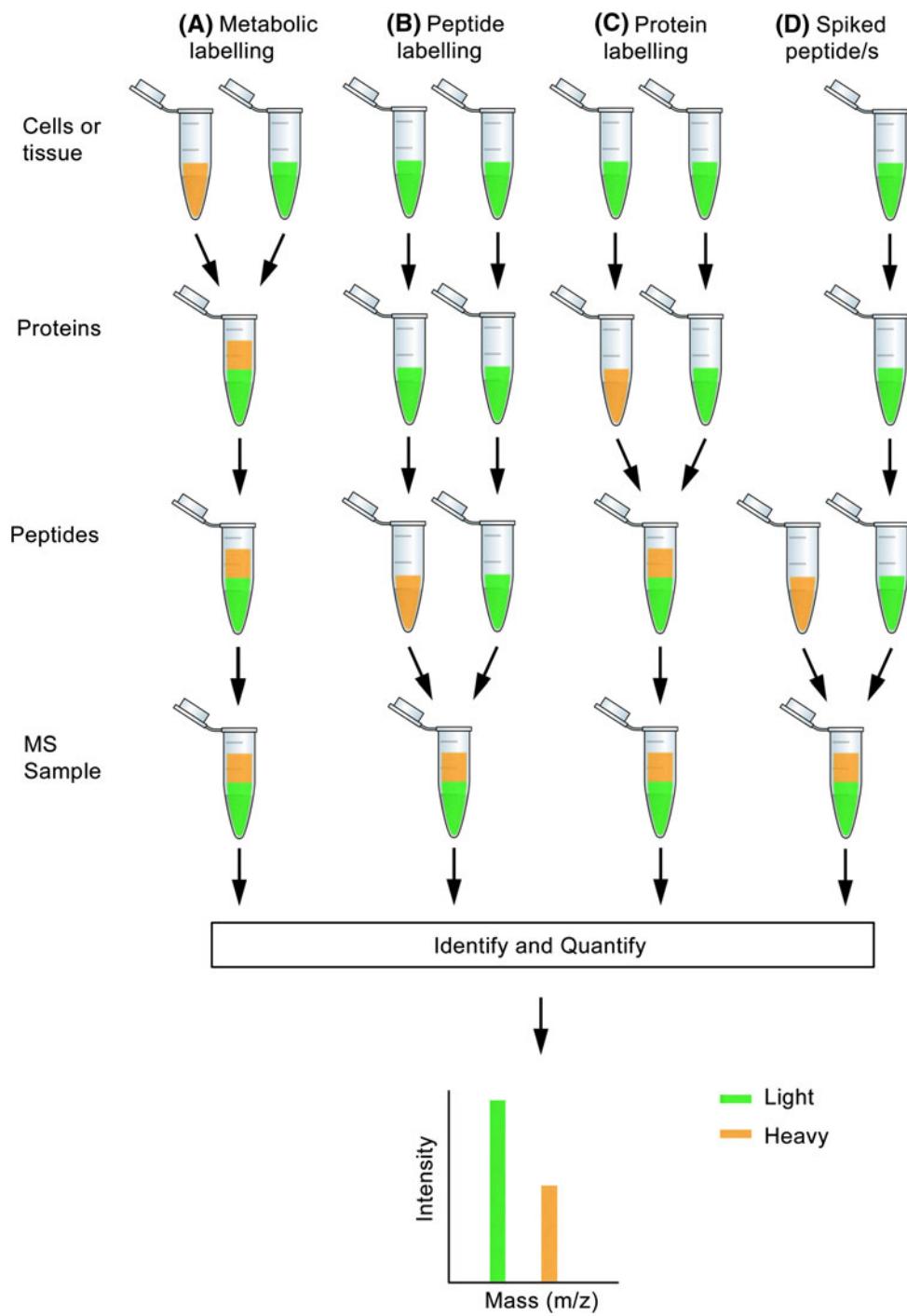
Differential isotopic labelling

Differential isotopic labelling is currently the method of choice for quantitative proteomics. It involves the chemical labelling of isolated proteins and peptides with chemical tags such as isobaric tag for relative and absolute quantitation (iTRAQ), isotope-coded affinity tags (ICAT), methyl-esterification/propionylation, or isotope-coded protein labelling (ICPL) (Leroy et al. 2010; Ong and Mann 2005; Pierce et al. 2007), or the *in vivo* incorporation of isotope-labelled amino acids by metabolic labelling using stable isotope labelling of amino acids in mammals (SILAM) or stable isotope labelling of amino acids in cell culture (SILAC) (Kruger et al. 2008; McClatchy et al. 2007; Ong et al. 2002). Once labelled, samples are then combined and analysed together (Fig. 2). In general, isotopic labelling increases the peptide mass by a fixed amount, except for iTRAQ, for which different isobaric tags are added to peptides and are then released allowing for measurement upon MS/MS analysis (Fig. 5 bottom). Relative quantitation is performed by comparing the peak intensity between the ‘light’ and ‘heavy’ form of peptides in a MS precursor scan (Fig. 2). Absolute quantification can also be determined by adding calibrated amounts of isotopically labelled peptides in the sample before MS analysis (Gerber et al. 2003; Munton et al. 2007). Chemical labelling has the advantage of being applicable to any cellular or tissue sample, unlike SILAC/SILAM, which requires that cells or animals be grown or fed with isotopically labelled medium/food.

Label-free quantitation

Label-free techniques do not make use of any isotopic labelling of peptides. One method is based on spectral counting (SC), which consists of determining the number of spectra acquired for peptides derived from a given protein. SC is a semi-quantitative method since it only produces correlative data between spectra number and protein abundance, as it does not measure the amount of

Fig. 2 Stable isotope labelling approaches in quantitative MS studies. The scheme outlines typical labelling workflows in quantitative proteomics from the cell or tissue stage through purification and protein digestion to MS analysis. *Green* (light) and *orange* (heavy) solutions represent the two different cell/tissue states that have been differentially labelled and can then be combined. The stage where samples are combined is indicated by both solutions in one tube. When samples have to be processed in parallel, uncompensated quantitation errors can occur. Metabolic labelling (**a**) strategies have the advantage of fewer handling steps as samples can be combined at an early stage and processed together and hence reduce uncompensated losses. It can however only be applied to cell lines or organisms that can be metabolically labelled. Peptide (**b**) and protein (**c**) labelling methods allow samples to be combined at the peptide and protein level, respectively, and can be applied to all samples. In addition, synthetic peptides (**d**) can also be spiked into the sample to obtain absolute quantitation of the level of specific peptides/proteins. Isotopic labelling results in a mass difference (except for iTRAQ tags: see Fig. 5) between peptides from each sample. Upon MS analysis the relative and/or absolute quantitation of peptides in each sample can be determined by comparing their peak intensities



protein directly (Liu et al. 2004). An alternative approach to SC thought to be more accurate is global isoform percentage (GP). It consists of integrating the area under the curve of each peptide and expressing it as a percentage of the total integrated area for all peptides from the same protein (America and Cordewener 2008; Phanstiel et al. 2008). Both methods, however, depend on a high level of consistency between each MS run (Ong and Mann 2005), which is often difficult to obtain. Thus at present, methods

based on stable isotopes are thought to be better and more accurate than label-free methods.

Targeted proteomics: MRM/SRM

Classical ‘shot-gun’ proteomics is limited by the fact that it only samples a fraction of the proteome usually biased towards the higher end of the abundance scale (Picotti et al.

2007). Recently, new ‘targeted’ MS approaches have been developed in which the mass spectrometer is directed to select and fragment specific ‘proteotypic’ peptides (PTPs), which are unique to a specific protein and therefore, represent the most ‘informative’ peptides in a sample mixture (Schmidt et al. 2009). Targeted proteomics can be thought of as the MS equivalent of the traditional Western blot, in that specific proteins are targeted and quantitated. Pioneering studies have shown the ability of selected reaction monitoring (SRM)-based MS for the detection and quantitation of proteins over the whole range of cellular concentrations in unfractionated *S. cerevisiae* digests (Picotti et al. 2009; Picotti et al. 2010). This study demonstrated that comparative analyses of the protein amount of entire but relatively simple proteomes or organelles across multiple samples are now possible. SRM provides a powerful workflow for the development and use of quantitative assays to monitor protein signalling networks and their dynamics in sub-cellular compartments across multiple samples and replicates (Fig. 3). Furthermore, SRM can also be applied to specifically target and quantify peptides

with PTMs like phosphorylation (Unwin et al. 2005; Williamson et al. 2006), ubiquitination (Mollah et al. 2007) and acetylation (Griffiths et al. 2007). It is aided by the increasingly comprehensive and high quality peptide repositories, in which experimentally observed peptides are made accessible (Fig. 3). Targeted MS studies therefore, have the potential to generate more consistent and reproducible datasets and enlarge the known proteome.

Analysis of changes in the proteome

Proteomic approaches now allow the routine monitoring of thousands of proteins and their dynamics in different sub-cellular compartments, tissues and brain regions (Baumgartel et al. 2008; Olsen et al. 2010; Trinidad et al. 2008). Methods like SILAC, based on the incorporation of stable isotopes into proteins through pulse labelling, or the feeding of cells or animals, are available to trace proteins and determine their turnover and movement through specific sub-cellular organelles. Thus, SILAC was successfully used to reveal changes in the nuclear proteome during apoptosis (Hwang et al. 2006), DNA damage (Bennetzen et al. 2010), basal protein turnover in the nucleolus (Lam et al. 2007), after adenovirus infection (Lam et al. 2010), or treatment with inhibitors of transcription, proteasome activity or protein kinases (Andersen et al. 2005, 2002). SILAC also helped identify the target proteins of micro-RNA-1 (mRNA-1), and demonstrated that it regulates the level of several different proteins (Winther et al. 2006). iTRAQ was also used to monitor proteome changes in the amygdala resulting from overexpression of the transcription factor Zif268 (Baumgartel et al. 2009).

The characterisation of the flux of proteins in specific cellular organelles has become an important question in biology. So-called ‘spatial proteomics’ using whole-cells has therefore been developed for this purpose. It has been used to study the relative steady-state distribution of proteins in the cytoplasm, nucleus and nucleolus, and their changes upon DNA damage (Boisvert et al. 2010), complementing data generated in other studies on changes in phosphorylation during the same process (Bennetzen et al. 2010).

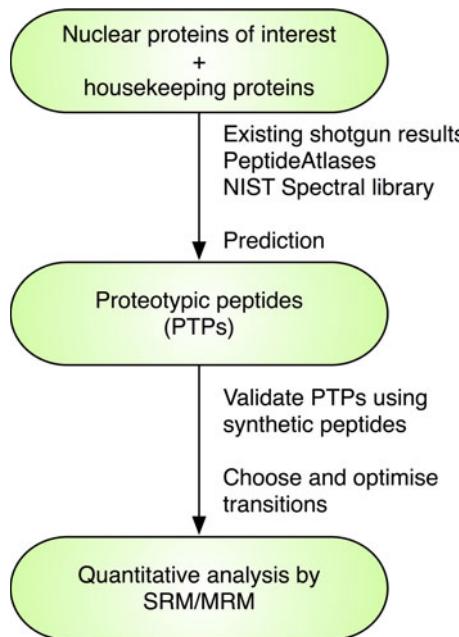


Fig. 3 Workflow to quantitate signalling pathways in the nucleus using MRM/SRM. Proteotypic peptides are selected for the protein/s of interest and for housekeeping proteins needed for normalisation of the data. Optimal proteotypic peptides are selected using pre-existing databases of MS detectable peptides (NIST/PeptideAtlas), or alternatively can be predicted using software tools (PeptideSieve). Transitions are best optimised and selected by analysing synthetic versions of all selected proteotypic peptides. Once established, an MRM assay can be applied to peptide samples derived from different experimental conditions to quantitate the pathway/proteins of interest. Absolute quantitation can be achieved by spiking isotopically labelled variants for each peptide into the sample (scheme adapted from Lange et al. 2008)

Analysis of PTMs

Signalling pathways in the cytoplasm and the nucleus are extremely complex, and their components are subjected to multiple PTMs and ‘cross-talk’ between these PTMs. Identifying these PTMs and their ‘cross-talk’ is a difficult task because PTMs are covalently attached, labile, and usually present at a sub-stoichiometric level. They often affect only a small fraction of target proteins on a given

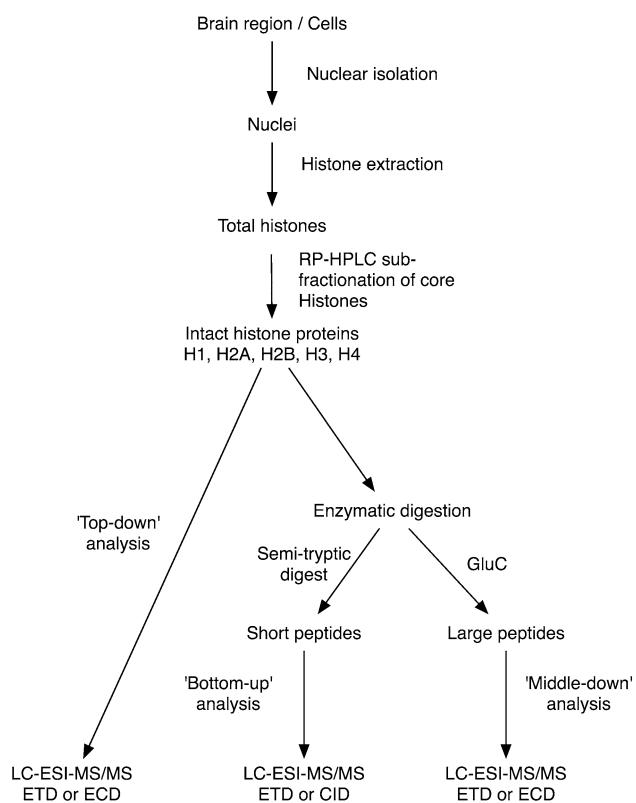


Fig. 4 Strategy to analyse histones and histone PTMs. After isolation of nuclei and acid extraction of bulk histones, histone sub-types can be separated using RP-HPLC. Individual histones can then be analysed by ‘bottom-up’, ‘middle-down’ or ‘top-down’ strategies. Typical ‘top-down’ methods employ ETD or ECD for intact protein fragmentation. ‘Bottom-up’ and ‘middle-down’ methods involve the enzymatic digestion of intact histones into peptides of varying lengths depending on the specificity of the enzyme used. These peptides can then be analysed by ETD/ECD or CID. In all methods, MS/MS data generated allows the determination of peptide sequence and the location of any PTMs. In addition, quantitation can be achieved through chemical/metabolic labelling of histone proteins, or alternatively through label-free means

residue, and are hard to detect in complex samples that contain thousands of proteins. They therefore need to be enriched, for instance by affinity purification or chromatography, especially in the case of phosphorylation (Tweedie-Cullen et al. 2007). While specific workflows need to be developed for analysing each PTM, they can often be applied to different PTMs with only small changes, for instance by using PTM-specific antibodies (anti-phospho, anti-acetyl or anti-ubiquitin) (Zhao and Jensen 2009). Finally, the identification using MS of previously unknown PTMs such as acetylation of serines and arginines (Mukherjee et al. 2007), or formylation (Wisniewski et al. 2008), butyrylation and propionylation (Chen et al. 2007) of lysines on histone proteins has opened new perspectives for important discoveries on cellular signalling in the future (Fig. 4).

Phosphorylation, acetylation and methylation

Although protein phosphorylation is thought to be a cell-wide regulatory mechanism for protein functions, recent quantitative proteomic studies have shown that it preferentially targets nuclear proteins (Olsen et al. 2006). Many protein phosphatases are enriched in the nucleus, and some are even present exclusively in this sub-cellular compartment (Moorhead et al. 2007). Several strategies have been developed in the past years to enrich and analyse phosphopeptides in different sub-cellular compartments (Bodenmiller et al. 2007; Tweedie-Cullen et al. 2007) including the nucleus (Beausoleil et al. 2004; Liao et al. 2008; Tweedie-Cullen et al. 2009; Villen et al. 2007). Approaches based on iTRAQ have quantified changes in phosphorylation in synaptic terminals from different brain regions (Coba et al. 2009; Trinidad et al. 2008) while SILAC in cell culture (Bennetzen et al. 2010; Olsen et al. 2006) or SILAM in young mice (Liao et al. 2008) revealed the extent of phosphorylation in the nucleus. Advancements in methodologies have allowed the simultaneous monitoring of thousands of phosphorylation sites and proteins, and greatly enlarged phosphorylation datasets. Notable recent studies have quantified 20,443 phosphorylation sites during mitosis (Olsen et al. 2010), 5,204 sites during DNA damage (Bennetzen et al. 2010), and demonstrated the ability to monitor phosphorylation dynamics at sub-millisecond timescales (Dengjel et al. 2007); all of which was inconceivable just a few years ago.

Along with protein phosphorylation, acetylation is another extremely prevalent PTM that occurs on proteins in most sub-cellular compartments. Acetylation of lysine residues and of the N terminus is the most frequent PTM on histone proteins (Kouzarides 2007; Tweedie-Cullen et al. 2009). However, despite this prevalence, the identity and specificity of the enzymes that generate and regulate acetylation on selected residues are still poorly understood. Furthermore, it is only recently that the important regulatory role of acetylation has been recognised. Quantitative MS studies have revealed that it goes far beyond histone regulation and DNA repair and that, like phosphorylation, it is not only prevalent on nuclear proteins but it also affect major nuclear processes, and cytoplasmic macromolecular complexes (Choudhary et al. 2009). Intriguingly, recent MS studies have also demonstrated its presence on serine and threonine residues, suggesting the possibility that it may interfere with phosphorylation (Mukherjee et al. 2007).

Large modifications: ubiquitin, and related modifiers

Proteins can also be modified by conjugation to other proteins, in particular to ubiquitin and the small ubiquitin-like modifier (SUMO). Ubiquitin is a small protein of

76 amino acids often attached to lysine by ubiquitin ligases (Kirkpatrick et al. 2005a). Although classically associated with protein degradation (Ciechanover 2005), ubiquitination is also essential for cellular signalling. MS studies have shown that it is relatively abundant on histone proteins (Tweedie-Cullen et al. 2009), supporting the idea that it is a key component of the histone code (Briggs et al. 2002). Ubiquitinated substrates in proteomic experiments can be isolated via affinity purification of tagged substrates (Kirkpatrick et al. 2005b), as was done in a transgenic mouse line engineered to express poly-histidine-tagged ubiquitin (Tsirigotis et al. 2001). Nonetheless, ubiquitin can also be detected in simple histone samples without prior enrichment using MS (Beck et al. 2006; Tweedie-Cullen et al. 2009). Its size does make its analyses by MS more difficult than with other smaller PTMs. However, it can be cleaved by trypsin and therefore be examined by ‘bottom-up’ proteomic studies (Denis et al. 2007). Nonetheless when studying ubiquitin by MS, workflows must be adjusted because commonly used reagents such as iodoacetamide can generate chemical artefacts that are indistinguishable from ubiquitin (Nielsen et al. 2008).

In addition to ubiquitin, other ubiquitin-like proteins such as SUMO can be added to proteins, in particular in the nucleus. Sumoylation exclusively modulates non-proteasomal endpoints. It is thought to be primarily a nuclear or perinuclear reaction because sumoylation enzymes and their substrates are predominant at the nuclear membrane and within the nucleus (Hay 2005). Proteomic analyses based on various tagged versions of SUMO-1 and SUMO-2 in human cells have identified multiple SUMO substrates, involved in chromatin organisation, transcription, and RNA metabolism (Andersen et al. 2009; Li et al. 2004; Vassileva and Matunis 2004; Vertegaal et al. 2004; Zhao et al. 2004). SUMO-1 was reported to be primarily nuclear whilst SUMO-2/3 can occur in the nucleus and the cytoplasm (Manza et al. 2004; Melchior et al. 2003). Nuclear proteins such as the transcription factor NF- κ B can be sumoylated or ubiquitinated on the same residue, with SUMO acting as a protein stabiliser and preventing its degradation by the proteasome (Destero et al. 1998). Several studies have indeed demonstrated the tight regulatory link between these two PTMs (Schimmel et al. 2008).

Proteomics has greatly expanded the knowledge on PTMs in the nucleus, in particular their abundance and dynamic regulation in different conditions and during development. Their preponderance on nuclear proteins suggests that they do not work in isolation and multiple signalling pathways may use all or most of these PTMs in combination. Therefore, quantitative MS studies are increasingly required to take into account all PTMs when analysing signalling pathways.

Combinatorial PTMs: the ‘histone code’

The histone code hypothesis proposes that histone PTMs co-occur in specific combinations and patterns, and are linked by multiple reciprocal and controlled ‘cross-talks’ (Latham and Dent 2007; Wang et al. 2008). Histone PTMs contribute to chromatin remodelling and serve in part, to bind effector proteins. In the nucleus, the combinatorial assembly of chromatin regulatory complexes is critical for reading and maximising the information provided by histone PTMs (Wu et al. 2009). The histone code is thus one mode of the epigenetic regulation of gene expression (Campos and Reinberg 2009; Jenuwein and Allis 2001).

Because of this code’s complex and dynamic nature, which makes it rapidly and specifically changing in different cells, it is difficult to study and remains not fully understood. However, specific aspects of the histone code can be examined by MS approaches, and several studies have demonstrated the potential of these approaches for investigating the histone code. ‘Bottom-up’ MS methods allowed the successful generation of comprehensive maps of isolated histones (Garcia et al. 2006, 2007c; Tweedie-Cullen et al. 2009; Wisniewski et al. 2007). Likewise, MS/MS based on ETD led to the analyses of intact histones or large proteolytic fragments (Garcia et al. 2007b), providing a map of PTMs on individual histones, essential to fully capture the dynamic interactions and functions of histones. It also distinguished PTMs on variants of histones such as H2A, H2B (Boyne et al. 2006; Eliuk et al. 2010; Siuti et al. 2006), H3.2 and H4, and their potential combinatorial code (Garcia et al. 2007b; Young et al. 2009).

Although technically more difficult and challenging, quantitative proteomic analyses of multi-site PTMs have also been performed. Quantitation of histones cannot be done by chemical labelling based on iTRAQ because it targets the free amine of the N terminus and lysines, which also carry multiple PTMs and thus interfere with labelling. Alternative methods based, for instance, on SILAC (Bonenfant et al. 2007; Zee et al. 2010), propionylation of unmodified lysines (Garcia et al. 2007a; Plazas-Mayorca et al. 2009), or label-free methods (Beck et al. 2006; Fraga et al. 2005; McKittrick et al. 2004) can however be used. Recent quantitative and semi-quantitative studies have exploited these methods to examine the effect on histone PTMs of HDAC inhibitors (Beck et al. 2006), the cell cycle (Bonenfant et al. 2007), differences between mouse strains (Jung et al. 2010), and the interplay between neighbouring PTMs (Fischle et al. 2005; Garcia et al. 2007b).

Overall, the knowledge of the histone code gained by MS is expected to greatly advance the understanding of the epigenetic mechanisms of gene regulation. Interesting extensions have already emerged that further exploit MS methodologies or the data generated. For instance, the

nature of chromatin-binding proteins associated with histone tails has been examined. Using a combinatorial peptide library containing 5,000 PTM-randomised peptides derived from H3, a study probed the interaction between six binding modules reading H3K4 methylation and chromatin, and identified potential additional PTMs regulating these interactions (Garske et al. 2008, 2010). Workflows used for histone studies (see Fig. 4) have also been utilised for the analysis of other proteins rich in PTMs. ‘Top-’, ‘middle-down’ and ‘bottom-up’ MS methods have thus been used to determine the combinatorial code of PTMs on the chromatin-associated high mobility group protein HMG1a (Young et al. 2010).

Analysis of the composition and alterations of protein complexes

Vital cellular functions such as DNA replication, transcription and mRNA translation require the coordinated action of multiple proteins assembled in complexes with different compositions and structures (D’Alessio et al. 2009; Hager et al. 2009). Such multi-protein complexes also underlie the development of complex organs like the central nervous system (Ryan and Grant 2009), and their disruption can lead to brain diseases (Emes et al. 2008; Laumonnier et al. 2007). The analysis of protein complexes and protein–protein interaction networks, and the dynamics of these networks across time and in different cells, are therefore of central importance in biological research (Fig. 5).

Different approaches have been used to characterise multi-protein complexes but typical workflows have combined affinity purification with MS. Here, multi-protein complexes are isolated directly from cell lysates or subcellular compartments through one or more affinity purification steps, and their components are analysed by MS. The advantage of this approach is that it can be performed in near-physiological conditions directly from nuclear extracts, which preserves proteins and biochemical modifications such as PTMs (Gingras et al. 2007; Pflieger et al. 2008; Ranish et al. 2003). Whilst the large-scale analysis of chromatin-associated proteins has proven technically difficult, proteomic workflows have successfully investigated protein complexes in the nucleus, for instance chromatin-remodelling complexes such as the SWI/SNF-like (Lessard et al. 2007), complexes bound to histone H4 and H3 tail (Chan et al. 2009; Choi et al. 2007), the RNA Pol II holoenzyme (Jeronimo et al. 2004), the transcription factor GATA-1 (Rodriguez et al. 2006), and the protein phosphatase PP1 (Trinkle-Mulcahy et al. 2006). Cell culture-based studies have also looked at binding partners of the

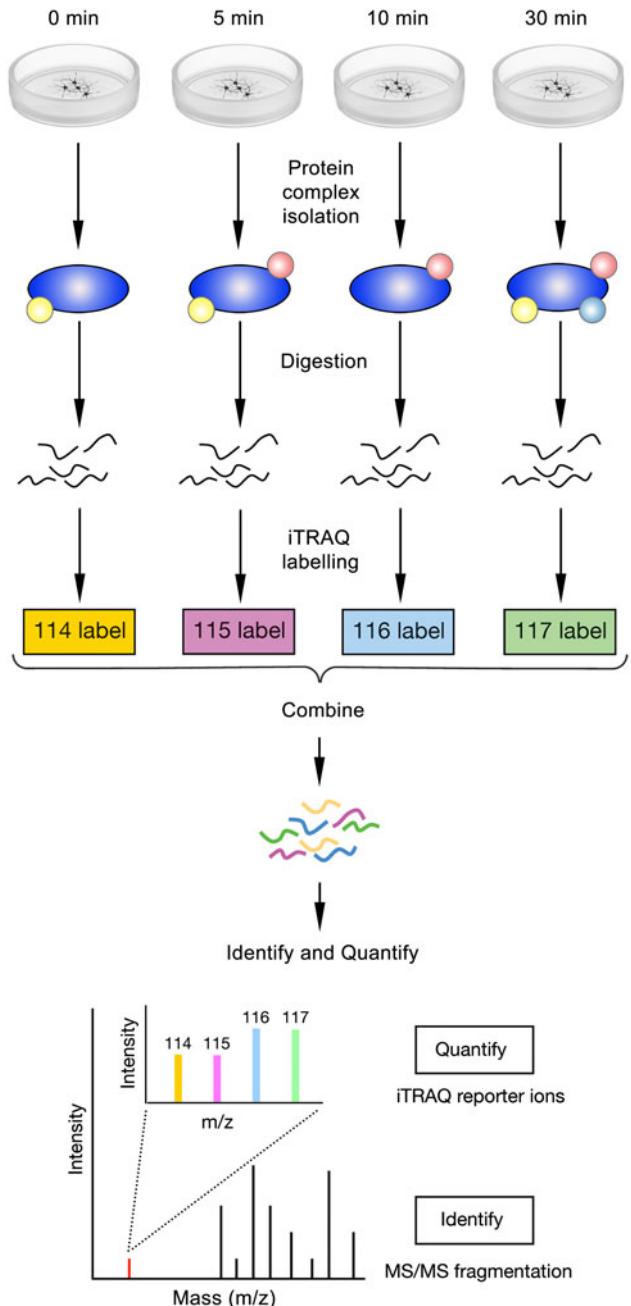


Fig. 5 Quantitative analysis of changes in protein complex stoichiometry using iTRAQ. Desired treatment of cells or extraction of tissue from different conditions is followed by isolation of protein complexes using affinity purification and proteolysis using trypsin. Isobaric iTRAQ tags are chemically added to the N terminus and lysines of every peptide and samples from different times or conditions are labelled with a different iTRAQ tag (4 shown, up to 8 possible), and then combined. Samples can then be analysed via ESI- or MALDI-MS. MS/MS analysis of iTRAQ labelled samples generates a spectrum that yields the sequence of the peptide, and iTRAQ reporter ions (red peak: expanded in close-up), which can be seen in the low mass range ($114\text{--}121\text{ }m/z$). Comparison of the peak intensities for each reporter ion allows quantitation. In the example shown, iTRAQ analysis would allow the elucidation of changes in the proteins attached to the bait protein (shown as a blue oval) over time

protein phosphatases PP2A, PP4 and PP6, which play important roles in the nucleus, in whole cell lysates (Chen and Gingras 2007; Goudreault et al. 2009; Mumby 2007).

A major limitation of many of these approaches however, is that the resulting view of the protein complexes is static and does not take into account dynamic changes. The use of quantitative MS approaches based on multiplex tags can however allow the determination not only of the specific composition of complexes, but also changes in their composition, and in the abundance of their components (Gingras et al. 2007; Ranish et al. 2003). Another important aspect often not addressed in high-throughput studies is the stoichiometry of these components, important for understanding the structural organisation of protein assemblies. One strategy to determine stoichiometry is to combine protein complex isolation with isotope-based absolute quantitative proteomics. If all components of a complex are known, synthetic peptides can be generated to monitor the abundance of each protein in the complex. These can either be synthesised with heavy isotopes and then mixed in with the unlabelled sample using an absolute quantitation (AQUA) approach (Gerber et al. 2003), or labelled with iTRAQ or a similar reagent in parallel to the samples (Munton et al. 2007). Such an approach was successfully used in the nucleus to determine the stoichiometry of the human spliceosomal U1 small nuclear ribonucleoprotein complex (Hochleitner et al. 2005). Quantitative studies have also enabled the analysis of the protein dynamics of the kinetochore during mitosis (Akiyoshi et al. 2009), H2AX-associated proteins during DNA damage and repair (Du et al. 2006), and the Mediator complex during transcriptional activity (Paoletti et al. 2006). High-throughput approaches that quantitatively analyse protein interactions promise to accelerate the understanding of protein complexes in the future (Wepf et al. 2009). Their application in combination with methods like chromatin immunoprecipitation (ChIP) will be instrumental to the identification of the binding location of protein complexes to the DNA, and of the nature and number of binding partners (Collas and Dahl 2008; Le Guezennec et al. 2005).

Bioinformatics and the integration of proteomic data

High-throughput studies of biological systems are providing a rapidly accumulating wealth of highly accurate qualitative and quantitative data. The visualisation and integration of this data is the key to their analysis and comprehension. Many new tools now exist or are being developed to help process, integrate and use these large-scale datasets (for a review see Gehlenborg et al. 2010). In proteomic studies, visualisation has been instrumental to the understanding of biological systems such as signalling

via PTMs (Choudhary et al. 2009; Olsen et al. 2006, 2010), the role of multi-protein complexes in evolution and disease (Pocklington et al. 2006; Ryan and Grant 2009), and for the integration of disparate data (Leung et al. 2003). The tools available are diverse and range from the optimisation and automation of common workflows, through to specifically dealing with, and aiding, the analysis of protein interaction networks, protein expression and PTM profiling, and modelling cellular pathways (Gehlenborg et al. 2010). The availability of large-scale proteomic datasets has also had the added benefit of providing training sets for improving the accuracy of prediction tools, e.g. for PTMs such as acetylation (Basu et al. 2009) and phosphorylation (Schwartz and Gygi 2005). Increasingly, data generated in biology is being used in meta-models of entire systems such as the whole brain in the Blue Brain Project (Markram 2006).

Conclusions

Proteomics is leading a new way in the interpretation of molecular studies in organelles such as the nucleus, moving away from single genes to networks of molecules, thereby providing a logical and innovative framework to study physiology and behaviour. ‘Shot-gun’ and emerging proteomic techniques have proven to be a driving force in neuroscience research. The advantages of analysing thousands of proteins in a single experiment have led to the identification of novel proteins and PTMs involved in cellular functions. Ultimately, the rapid ascension of systems biology and bioinformatics will potentially allow us to model biological processes and determine in silico how drugs and manipulations affect them in their entirety.

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