



## Review

## Sample preparation and analytical strategies for large-scale phosphoproteomics experiments

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## ABSTRACT

Reversible protein phosphorylation is an important post-translational modification that controls a wide range of protein functions including enzyme activity, subcellular localisation, protein degradation, intra- and inter-molecular protein interactions. Significant advances in both phosphopeptide enrichment methods and sensitive mass spectrometry instrumentation have been achieved over the past decade to facilitate the large-scale identification of protein phosphorylation in humans and different animal and microbial model systems. While mass spectrometry provides the ability to identify thousands of phosphorylation sites in a single experiment, the further understanding of the functional significance of this modification on protein substrates requires detailed information on the changes in phosphorylation stoichiometry and protein abundance across experimental paradigms. This review presents different sample preparation methods and analytical strategies used in mass spectrometry-based phosphoproteomics to profile protein phosphorylation and unravel the regulation of this modification on protein function.

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## Contents

1. Introduction.....	843
2. Analytical strategies for phosphoproteomics.....	845
2.1. General considerations.....	845
2.2. Affinity purification of phosphopeptides.....	846
2.2.1. Immunoaffinity chromatography.....	846
2.2.2. Metal oxide affinity chromatography (MOAC).....	847
2.2.3. Immobilized metal affinity chromatography (IMAC).....	848
2.3. Fractionation of phosphopeptides using liquid chromatography methods.....	848
2.3.1. Ion exchange chromatography.....	848
2.3.2. Hydrophilic interaction chromatography (HILIC) and electrostatic repulsion–hydrophilic interaction chromatography (ERLIC).....	849
3. Quantitative phosphoproteomics.....	849
4. Conclusions.....	851
Acknowledgements.....	851
References.....	851

**Abbreviations:** ERLIC, electrostatic repulsion–hydrophilic interaction chromatography; HILIC, hydrophilic interaction chromatography; IMAC, immobilized metal affinity chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MOAC, metal oxide affinity chromatography; PTM, post-translational modification; LC, liquid chromatography; SCX, strong cation exchange; SAX, strong anion exchange chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; IDA, iminodiacetic acid.

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## 1. Introduction

Protein phosphorylation plays a major role in the regulation of protein activity and controls a wide range of important cellular functions such as cell signalling, cell differentiation and proliferation and progression through the cell cycle [1]. Many diseases including cancer are known to have aberrant activation of kinase signalling pathways that impart significant changes on the dynamic regulation of protein phosphorylation [2,3]. The misregulation of signalling pathways can be attributed to different factors that include mutations in genes or changes in their expression due to chromosome translocation (e.g. rearrangements of the *BCR* and *ABL*

genes in chronic myelogenous leukemia and acute lymphocytic leukemia [4]), mutations (e.g. somatic gain-of-function mutations in *RAS* genes [5]), defects in negative regulatory mechanisms (e.g. PKB/Akt pathway [6] or ErbB receptor tyrosine kinase [7]), or over-expression of kinases (e.g. HER-2/Neu tyrosine kinase receptor in breast cancer [8]). Detailed information on the nature of the target substrates to determine site-specific changes in phosphorylation, variation in protein abundance, and the impact on interacting partners and their associated functions are required to understand the complex regulation of protein phosphorylation in human diseases.

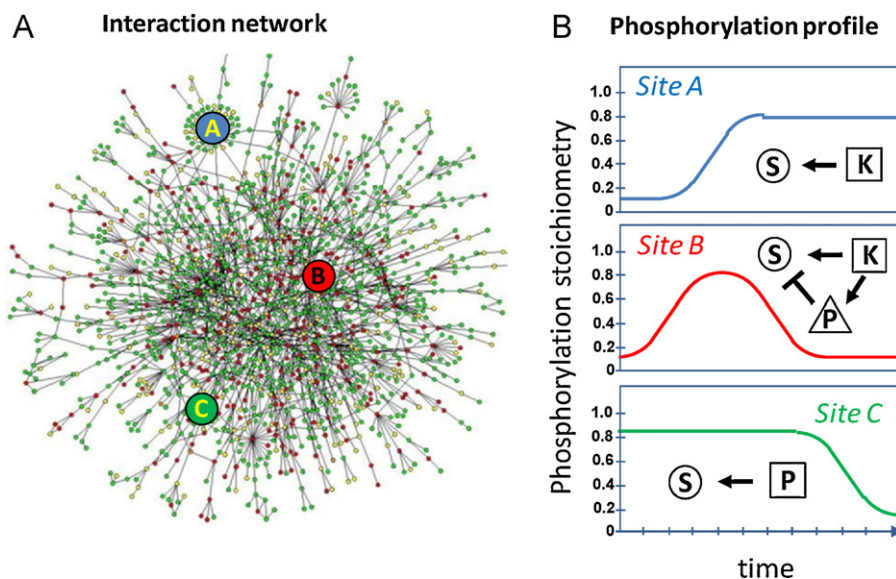
Phosphorylation of proteins is finely orchestrated by a network of protein kinases and phosphatases that add or remove phosphate groups from specific substrates [9,10]. This level of specificity is achieved in part, through the recognition of unique sequence motifs by one of the 518 putative kinases and also through physical localisation by scaffold and adaptor proteins [11,12]. In contrast, protein phosphatases catalyse the dephosphorylation of their substrates with little similarity in amino acid sequence. They also contain a small number of catalytic and hundreds of non-conserved regulatory subunits with degenerate docking motifs that confer enzyme specificity [13]. The combined action of kinases and phosphatases provides a convenient mechanism to relay information through the cell in response to extrinsic or intrinsic stimuli. Recent studies also indicated a high degree of connections among kinases and phosphatases suggesting that many proteins outside of the canonical pathway can influence the phosphorylation status of protein substrates (Fig. 1) [14,15]. This level of cooperation amongst reciprocal enzymes of this interconnected network could facilitate the integration of several inputs to generate complex cellular responses. The highly dynamic nature of protein phosphorylation gives rise to patterns of modification that vary in terms of stoichiometry and duration according to substrates and experimental paradigms (Fig. 1). It is, however, possible that a significant number (perhaps greater than 50%) of observed phosphorylations are non-functional, having no consequence to the organism [16]. A general trend observed is that the conservation and stoichiometry of phosphorylation vary inversely with protein abundance and this may reflect random phosphorylation of abundant proteins.

Protein phosphorylation is widely distributed in eukaryotic cells, and recent evidence suggests that this modification is also

present in several prokaryotes [17,18]. A large proportion of protein phosphorylation in eukaryotes takes place on hydroxylated amino acids such as serine, threonine, and tyrosine (*O*-phosphorylation) with a relative distribution of 88:10:2 [19,20]. However, other residues such as histidine, lysine, cysteine, aspartic or glutamic acid can be phosphorylated, though they are seldom encountered and the corresponding bonds are less stable than those of *O*-phosphorylation [21]. It is estimated that phosphoproteins account for at least 30% of eukaryotic proteomes [22], and that approximately 100,000 sites could be present in human proteins [22,23]. Furthermore, the extent to which a site is phosphorylated is highly variable, and a large majority of sites identified in *Saccharomyces cerevisiae* have occupancies less than 30% [24].

Traditional methods for studying protein phosphorylation have used metabolic labelling with radioactive phosphate, phosphospecific antibodies or in vitro kinase assays. However these methods are slow, laborious and often require prior knowledge on the sites under study. Mass spectrometry (MS) has rapidly emerged as a powerful tool for unbiased protein analysis, and its application has been expanded to large-scale identification of phosphorylation sites from different cell model systems. MS-based phosphoproteomics provides qualitative and quantitative analyses to identify and profile the abundance of thousands of phosphopeptides in a single experiment using microgram amounts of sample. Improvements in both MS sensitivity and affinity media have rapidly expanded the repertoire of protein phosphorylation and resulted in a dramatic increase in the number of identified phosphorylation sites over the past decade [25,26,27,28,29,30]. Specialized database resources, including PhosphositePlus [31], Phospho.ELM [32], PHOSIDA [33], PhosphoPep [34], LymphOS [35] and ProteoConnections [36] are now available in support to these large phosphoproteomics datasets.

When compared to proteomics analyses, the identification of phosphopeptides by MS presents additional challenges that are not normally encountered when performing sequencing of their non-phosphorylated peptide counterparts. These include low abundance of phosphopeptides and variable levels of site occupancy [37,38], uncertainty in the location of modified residues, suppression effects associated to sample complexity or ionisation efficiency, prompt loss of phosphate moiety and uneven



**Fig. 1.** Network organisation of protein phosphorylation. (A) Protein interactions are modulated by substrate phosphorylation that depends on the connectivity between kinases and phosphatases. (B) The phosphorylation profiles of protein substrates display different temporal profiles depending on interactions between regulating kinases and phosphatases. Abbreviation. S, substrate; K, kinase; P, phosphatase.

fragmentation compared to non-phosphorylated peptides [39,40]. In addition to these instrumental challenges, the successful identification of phosphopeptides by MS also relies on the use of appropriate sample preparation protocols to provide sufficient enrichment for their reliable detection. This is particularly true given the relatively small proportion of phosphopeptides obtainable from protein digests (typically less than 0.2%), and the variable stoichiometry of phosphorylated sites. Thus, specific precautions must be taken to maintain sample integrity during the isolation procedures.

Sample preparation for phosphoproteomics analyses has evolved significantly over the past decade and previous reports have provided detailed accounts of different affinity methods for the identification of phosphopeptides by MS [21,41,42,43,44]. This review builds upon emerging trends in sample preparation for phosphoproteomics and presents practical advice on analytical strategies for the identification and quantitation of phosphopeptides from different cell model systems.

## 2. Analytical strategies for phosphoproteomics

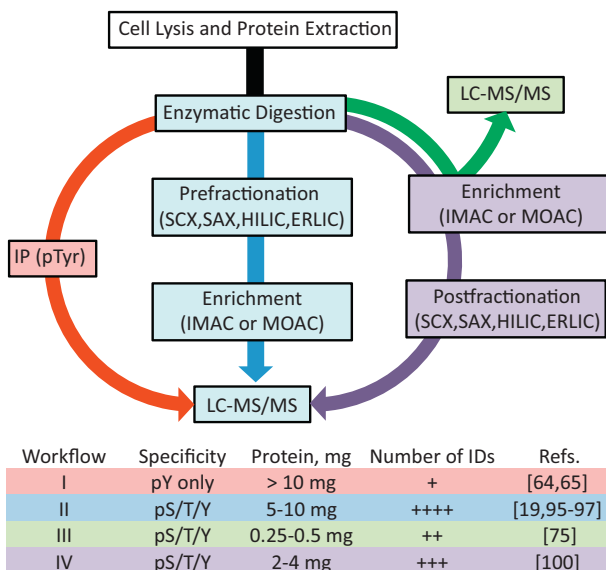
Phosphoproteomics analyses can extend far beyond a simple cataloguing of phosphorylation sites. Indeed, the further understanding of cell signalling requires quantitative measurements to profile the phosphorylation stoichiometry and protein abundance over time or across different biological conditions. Appropriate protein isolation and extraction protocols must be considered to maintain the integrity and phosphorylation status of phosphoproteins from cell extracts and obtain meaningful measurements. Current strategies for phosphoproteomics analyses typically involve four important steps that consist of cell fractionation and protein extraction, protein and peptide separation, phosphopeptide enrichment and mass spectrometry analyses (Fig. 2). These steps can be tailored according to the experimental paradigms under study to gain additional information on protein translocation events or subset of phosphopeptides comprising a specific consensus motif or modified residues (e.g. phosphotyrosine). The section below outlines key aspects that must be

considered as part of the experimental design in phosphoproteomics analyses.

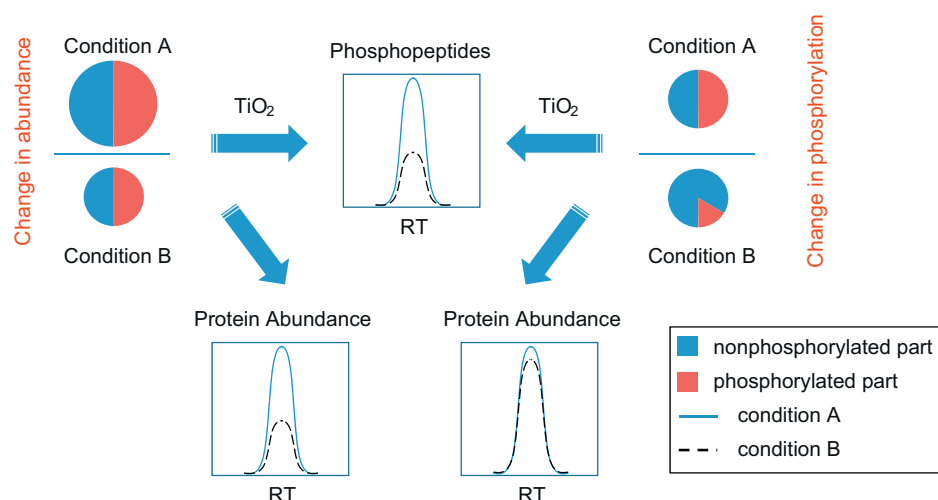
### 2.1. General considerations

Protein phosphorylation is a highly dynamic modification regulated by an intricate network of kinases and phosphatases. Upon extrinsic and intrinsic stimuli, proteins can be phosphorylated and dephosphorylated within minutes, and protease and phosphatase inhibitors must be used during cell lysis and protein extraction to preserve the integrity of proteins and their phosphorylation status [45]. Cocktails of phosphatase inhibitors generally contain sodium orthovanadate (tyrosine phosphatases), imidazole (alkaline phosphatase), sodium tartrate (acid phosphatase), EDTA (alkaline phosphatase, protein phosphatase 2), sodium pyrophosphate or sodium fluoride (Ser/Thr phosphatases). Also, appropriate precautions must be taken to avoid unintentional isolation of protein contaminants that could be introduced depending on the cell types and culturing conditions used. For example, adherent cells should be scraped rapidly under ice rather than using trypsin to avoid the release of cell surface components. Proteins present in the cell culture media could be co-isolated (e.g. serum proteins) if cells have not been properly washed prior to cell lysis and protein extraction. Care must be taken during cell harvesting to avoid triggering phosphorylation events associated with changes in osmolarity, temperature, or nutrient availability. It is thus recommended that cell harvesting and lysis be conducted rapidly at low temperature and that cell pellets be snap-frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  prior to protein extraction and digestion.

A common objective of large-scale phosphoproteomics studies is the quantitative comparison of phosphorylation profiles upon cell stimulation, across multiple cell states or between normal versus pathological, for example cancer tissues. It cannot be stressed more that attention to the type of sample preparation is crucial to making meaningful interpretations of differential phosphoproteome data. For example, all of the cell preparation and lysis procedures described above, can themselves induce signal transduction pathways massively and even if cells are prepared on ice, these pathways will be activated to some extent. This becomes a problem when comparing two cell or tissue populations. For instance, when comparing a cancer and normal cell line, the cancer cell with an adherent functioning signal transduction might produce a synergistic change in protein phosphorylation that may only be observed when cells are stressed during preparation. This could lead to a serious misinterpretation of the nature of the pathology. Among the preparation procedures described above, the snap freezing in liquid nitrogen is recommended to assure unambiguous results. Equally, while these studies, even with ideal sample preparation, can provide valuable insights on phosphorylation dynamics, appropriate attention must be placed on the experimental design to distinguish changes imparted by differential phosphopeptide abundances from those associated with protein expression [46]. This is particularly true when profiling phosphorylation events over long stimulation periods ( $>1$  h) or for extracts obtained from separate cell culture conditions. Accordingly, quantitation of both protein expression and phosphorylation must be performed from the same extracts to determine precise changes in differential phosphorylation (Fig. 3). However, these measurements cannot be obtained simultaneously from the same LC-MS analysis due to the variable levels of phosphorylation stoichiometry and the wide dynamic range of protein expression. Methods enabling the enrichment of the cell phosphoproteome are thus required to facilitate the detection of phosphopeptides that would otherwise be invisible to proteome analyses. This requirement places additional constraints on sample availability since comprehensive phosphoproteomics analyses typically require 50–100 times more material than



**Fig. 2.** Phosphoproteomics workflow. Cell extracts are digested, and the corresponding proteolytic peptides can be separated by LC and/or affinity chromatography to enrich phosphopeptides prior to their analyses by LC-MS/MS. Using a label-free or stable-isotope approach, the relative and absolute abundances of these peptides can be determined.



**Fig. 3.** Changes in protein phosphorylation and abundance. Variations in phosphopeptide abundance can be associated with either change in phosphorylation stoichiometry (right) or changes in protein abundance (left). When profiling phosphorylation events over extended stimulation periods (>1 h), phosphoproteomic results should be normalized to account for relative changes in protein abundances.

quantitative proteomics. For example, 250  $\mu\text{g}$  of protein digests from yeast cell extracts enabled the identification of 3000–4000 phosphorylation sites from 1500 proteins using LC–MS/MS, whereas a comparable number of peptides can be identified from only 2–5  $\mu\text{g}$  of protein digest. More elaborated workflows using peptide pre-fractionation and subsequent phosphopeptide enrichment can be utilized to enhance identification beyond 10,000 phosphorylation sites, though cell extracts of 5–10 mg are often necessary.

Two major approaches are commonly used to convert proteins from cell extracts to peptides suitable for MS-based proteomics analyses. The first approach involves the solubilisation of proteins with detergents, separation of proteins by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and digestion of the gel-embedded proteins. The second approach is detergent-free and consists of extracting proteins with strong chaotropic reagents such as urea and thiourea prior to their digestion under denaturing conditions ('in-solution' digestion). The second approach is frequently followed by two-dimensional liquid chromatography separation of peptides, commonly referred to as the 'MudPit' strategy.

The advantages of in-gel digestion include pre-fractionation based on molecular weight, better solubilisation and denaturation of proteins using strong detergents (SDS). However, the entrapment of proteins within the gel matrix decreases peptide recovery upon digestion, and the method is time consuming and labour intensive. While trypsin is commonly used due to its specificity, enhanced stability and activity for in-gel digestion, the resulting peptides may be too small or too large to be recovered efficiently. In contrast, in-solution digestion minimizes sample handling, though protein digestion might be impeded by incomplete solubilisation or by the presence of co-isolated interferences. It is noteworthy that numerous MS-compatible detergents have been introduced for enhanced protein solubilisation and digestion. These include RapiGest, PPS [47], ProteaseMAX [48] or sodium deoxycholate [49]. A filter-aided sample preparation (FASP) that combines the advantages of in-gel and in-solution digestion for mass spectrometry-based proteomics was previously described, and enabled the exchange of chaotropic agents such as urea on a standard filtration device with remarkable proteome coverage [50]. This method is scalable to large (several mg) and submicrogram sample quantities and was described for unbiased identification of membrane proteins and mapping of phosphorylation and glycosylation [51,52] sites. Another advantage of in-solution digestion is

the possibility of using other enzymes (e.g., Asp-N, Glu-C, etc.) that can provide enhanced sequence coverage [53,54]. Also, alternate proteases such as lysyl endopeptidase (LysC) can be advantageous as they can produce fewer miscleavages and increase the number of identified phosphopeptides [55].

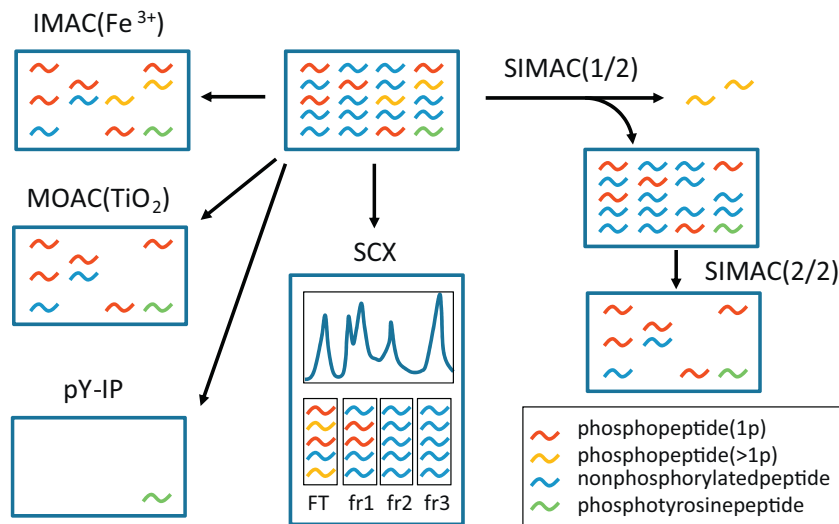
## 2.2. Affinity purification of phosphopeptides

As mentioned above, the variable phosphorylation stoichiometry and the wide dynamic range in protein expression represents a significant challenge for the identification of phosphopeptides, and the use of selective enrichment techniques are necessary for their detection in complex biological extracts. Most techniques are applied to protein digests, as they facilitate sample solubilisation and provide substantial depletion of non-phosphorylated peptides. Sample pre-fractionation such as ion exchange chromatography (see Section 2.3) can also be applied on tryptic digests or enriched phosphopeptides extracts to provide additional selectivity. Several approaches including immunoaffinity methods, immobilized metal affinity chromatography (IMAC) with multivalent cations (e.g.  $\text{Fe}^{3+}$  and  $\text{Ga}^{3+}$ ) [56,57], metal oxide affinity chromatography (MOAC) with  $\text{TiO}_2$ ,  $\text{ZrO}_2$ , or  $\text{Nb}_2\text{O}_5$  [21,41,42,43], and covalent modification [58,59] have been used in large-scale phosphoproteomics (Fig. 4). Methods that use covalent modifications via  $\beta$ -elimination or reactive phosphoramidate chemistry have been described in [58,59,60,61,62]. For convenience, we briefly describe below the most commonly used affinity techniques for phosphopeptide enrichment.

### 2.2.1. Immunoaffinity chromatography

Some antibodies are commercially available for the affinity purification of phosphoproteins, but only a limited number of them have been used in large-scale phosphoproteomics studies. These can be classified according to recognition motif or residue-specific binding. Antibodies against phosphotyrosine, first introduced by Frackelton et al. [63], are by far the most commonly used immunoaffinity approach for the enrichment of phosphotyrosine peptides in cell cultures and in tissue extracts. The low proportion of proteins phosphorylated on tyrosine residues (e.g. <2%) dictates the use of large amounts of starting material. A recent publication reported the detection of more than 700 phosphotyrosine sites from 10 mg of protein [64]. A remarkable application of phosphotyrosine-specific antibodies was described





**Fig. 4.** Common phosphopeptide enrichment methods. Peptides can be enriched by immunoaffinity methods using phosphospecific antibodies to purify peptides with specific phosphorylated residues (e.g. pTyr) or phosphomotifs. Affinity methods such as MOAC or immobilized metal affinity chromatography (IMAC), can be used to enrich populations of singly and multiply phosphorylated peptides. Ion exchange, hydrophilic interaction or electrostatic repulsion–hydrophilic interaction chromatography are typically used to enrich and/or separate pools of phosphopeptides prior or after affinity purification.

by Rikova et al. [65] for the identification of 4551 sites on 2700 different proteins from large panel of non-small cell lung cancer (NSCLC) cell lines. While the proportion of phosphorylated serine and threonine residues is significantly higher than that of phosphotyrosine, the lack of suitable antibody for their selective enrichment currently limits their practical application for large-scale phosphoproteomics studies. It is noteworthy that the use of a panel of phosphoserine and phosphothreonine antibodies has been reported for the enrichment of phosphoproteins from cells treated with the serine/threonine phosphatase inhibitor calyculin A and identified several unknown substrates such as poly(A)-binding protein 2 and Frigg [66]. Targeted identification of modified residues sharing a specific phosphorylation motif can be achieved using immunoaffinity enrichment [67]. This application has been previously demonstrated for the identification of putative kinase substrates of ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR) [68] and for the identification of phosphorylated proteins interacting with 14-3-3 (RSXpSXP and RXY/FXpSXP motifs, where X is any amino acid) [69].

### 2.2.2. Metal oxide affinity chromatography (MOAC)

Application of MOAC in phosphoproteomics is based on the ability of some metal oxides to form complexes with the phosphate group. The most common MOAC affinity medium is  $\text{TiO}_2$ , which was first described in 1990 for the selective retention of inorganic phosphate. Infrared spectroscopy revealed that the monosubstituted phosphoester bound was retained on  $\text{TiO}_2$  in a pH dependent manner through a bidentate interaction [70]. The use of  $\text{TiO}_2$  was described for the purification of phosphorylated amino acids [71] and subsequently for phosphopeptides with on-line enrichment prior to LC–MS/MS analysis [72]. The major drawback of all MOAC methods is the extent of nonspecific binding of acidic peptides, which decreases the enrichment efficiency and compromises the detection and identification of phosphopeptides by MS. To overcome this limitation, Larsen et al. [73], proposed the use of 2,5-dihydroxybenzoic acid (DHB) to compete with the binding of acidic peptides on  $\text{TiO}_2$  beads while maintaining the selective retention of phosphopeptides. This was first demonstrated for the enrichment of phosphopeptides from a tryptic digest of casein with different concentrations of DHB in 80% acetonitrile and 0.1% TFA. Selective phosphopeptides elution was achieved using

ammonium hydroxide (pH: 10.5). The increased concentration of DHB in the loading buffer provided a progressive enrichment of phosphopeptides and suggested that the binding of acidic peptides and phosphopeptides is mediated by different active sites on the  $\text{TiO}_2$  surface. While the use of DHB can be advantageous for matrix-assisted laser desorption ionisation (MALDI), its high concentration in the sample buffer can result in deleterious effects on the chromatographic separation and ionisation of phosphopeptides and its removal is required prior to LC–MS analysis. More recently, the substitution of DHB with hydrophilic hydroxylated modifiers such as lactic acid was proposed to improve selectivity and capacity of  $\text{TiO}_2$  towards phosphorylated peptides. Sugiyama et al. [74] evaluated different hydroxy acids in MOAC, and determined that lactic acid provided enhanced selectivity for the isolation of phosphopeptides from tryptic digests of HeLa cells. Numerous studies have reported the use of MOAC (mostly  $\text{TiO}_2$ ) in large-scale phosphoproteomics, and have greatly expanded the repertoire of phosphorylation sites from different cell model systems. For example, temporal changes in phosphorylation following EGF stimulation of HeLa cells enabled the identification of 6600 sites on 2244 human proteins [19]. A combined pharmacological and mutagenesis approach was used to determine system-wide responses in yeast and enabled the identification of more than 8800 regulated phosphorylation events [75]. Hilger et al. [76] used  $\text{TiO}_2$  with metabolic labelling and RNAi knockdown of the phosphatase Ptp61F to profile changes in phosphorylation of more than 6400 high confidence sites in *Drosophila melanogaster* cells.

The proportion of identified phosphopeptides from tryptic digests of mammalian cells often exceeds 80% using DHB and lactic acid in the loading buffers. While the number of acidic peptides is greatly reduced using these buffer modifiers, it is noteworthy that other types of non-specific peptides can be retained on  $\text{TiO}_2$  media under these conditions. For example, we obtained phosphopeptides enrichment levels of 83, 72, and 17% for HEK293, *Drosophila* S2 and *Dictyostelium* cells, respectively. Interestingly, we noted that a significant proportion of non-specific binders (>25%) identified in *Dictyostelium* extracts were peptides with segments of 5 up to 25 asparagine or glutamine residues. Similar observations were also noted for the enrichment of phosphopeptides from *S. cerevisiae* samples where up to ~45% of nonspecific binders corresponded to peptides with high

content of asparagine and glutamine residues. Interactions of poly-N/Q peptides with TiO<sub>2</sub> were unexpectedly strong and compromised phosphopeptide binding when enrichment is performed under high mass ratio of peptides/TiO<sub>2</sub> media. The addition of a washing step with free asparagine and glutamine facilitated the removal of these non-specific binders without compromising the phosphopeptide binding capacity of TiO<sub>2</sub> beads. This resulted in an increase of enrichment efficiency up to ~75% with a concurrent improvement in the detection of low abundance phosphopeptides [77].

In addition to TiO<sub>2</sub>, different MOAC sorbents including ZrO<sub>2</sub>, Nb<sub>2</sub>O<sub>5</sub>, and Al<sub>2</sub>O<sub>3</sub> were also described for the enrichment of phosphopeptides from tryptic digests [74,78,79]. Comparison of data obtained using ZrO<sub>2</sub> or Nb<sub>2</sub>O<sub>5</sub> to those of TiO<sub>2</sub> revealed different populations of phosphopeptides, suggesting complementary selectivity for these metal oxide resins. However, no clear pattern of selectivity has been obtained for the chemical properties of phosphopeptides retained by each resin. MOAC methods are characterized by high affinity for phosphopeptides and by high enrichment efficiency using loading buffers containing high concentrations of DHB or lactic acid. These methods are tolerant to many buffer additives such as salts, detergents and denaturing agents. To date, TiO<sub>2</sub> remains the most popular sorbent for phosphopeptide enrichment and can be implemented in an automated fashion for on-line LC–MS analyses [72].

### 2.2.3. Immobilized metal affinity chromatography (IMAC)

The immobilisation of multivalent cations on affinity resin has found numerous applications in protein chemistry; one of the most popular formats represented by Ni (II) is extensively used for the purification of His<sub>6–9</sub> tag recombinant proteins. In 1987, the use of IMAC was extended to the enrichment of phosphorylated proteins by Andersson and Porath [80], where they reported the use of Fe (III) immobilized via iminodiacetic acid (IDA) onto a sepharose matrix. Ion pair formation between Fe (III) and the negatively charged phosphate group enabled the selective retention of ovalbumin phosphoisoforms. Since then, IMAC has been applied to a wide range of application in phosphoproteomics.

The selective retention of phosphopeptides using IMAC resins uses similar binding conditions to those of MOAC. Electrostatic interactions between phosphorylated residues and the immobilized cations favour the selective retention of phosphopeptides while unretained peptides are removed during the washing steps. Phosphopeptides are loaded on IMAC columns using acidic buffers and subsequently eluted with high pH, EDTA or inorganic phosphate buffers. IMAC methods are utilized for the enrichment of peptides phosphorylated on serine, threonine, and tyrosine residues. Although Fe<sup>3+</sup> is predominantly used with IMAC, other coordinating metal ions such as Ga (III), Zr (IV), and Al (III) have also been described for the selective enrichment of phosphopeptides [56,81]. Ga (III) was previously shown to have a higher selectivity toward phosphorylated proteins [82].

To reduce the extent to which acidic peptides that can bind non-specifically to IMAC resins, solutions containing 0.1% TFA in 50% acetonitrile are used as loading buffers [83]. The protonation of carboxyl groups at pH 3 reduces the propensity of acidic peptides to bind to multivalent cations, whereas the phosphate group remains negatively charged. The use of acetonitrile also reduces hydrophobic interactions between peptides and the IMAC resin. This affinity medium is entirely suited for large-scale phosphoproteomics experiments as recently described by Huttlin et al. [84] for nine mouse tissues where they reported the identification of 284,000 phosphopeptides matching nearly 36,000 phosphorylation sites from 6296 proteins. The application of IMAC was also presented for the quantitation of more than 8000 phosphosites in

wild type and Ppt1-deficient yeast strains to identify Ser/Thr sites regulated by this phosphatase [85].

Methyl esterification of acidic residues has also been proposed to enhance the selectivity of IMAC with no apparent loss in sensitivity [86]. This was successfully demonstrated for the yeast phosphoproteome and enabled the identification of 383 phosphorylation sites, a remarkable achievement in 2002. The esterification of carboxylic group can also be achieved using isotopically labelled CD<sub>3</sub>OH to facilitate the comparison of differentially abundant phosphopeptides between sample sets [87]. A disadvantage of the esterification procedure is the occurrence of side reaction products (partial hydrolysis of peptides, deamidation of asparagine and glutamine residues) that can increase sample complexity.

In contrast to MOAC which favours the isolation of monophosphorylated peptides, IMAC was reported to yield a higher proportion of multiply phosphorylated peptides [88]. The complementary distribution of phosphopeptides obtainable by TiO<sub>2</sub> and Fe (III)-IMAC can be used advantageously for the combined separation of monophosphorylated and multiply phosphorylated peptides from cell digests. The sequential use of IMAC and TiO<sub>2</sub> also termed SIMAC (sequential elution from IMAC) gave a 2-fold increase in phosphopeptide identification from lysates of human mesenchymal stem cells compared to TiO<sub>2</sub> alone [89].

IMAC resins are available commercially from different suppliers. The resin specificity toward phosphopeptides is typically improved through the modification of the solid support or the chelating linker. For example, we typically obtained enrichment efficiency of 85–95% (ratio of phosphorylated peptides to total peptides) using PhosSELECT affinity gel.

### 2.3. Fractionation of phosphopeptides using liquid chromatography methods

Numerous groups have exploited the negatively charged phosphate moiety of phosphopeptides to enrich them using ion exchange or mixed modes chromatography separation. The analytical merits of these approaches are briefly outlined below.

#### 2.3.1. Ion exchange chromatography

Ion chromatography separation of phosphopeptides has been reported for both strong anion (SAX) and strong cation (SCX) exchange resins. This type of chromatography exploits the strong electrostatic interactions taking place between the ionised groups of the stationary phase and the peptide counter ions present in the sample at a given pH. The elution of target analytes is obtained by modulating the strength of the interactions using salts, pH and/or organic buffers. The low pK<sub>a</sub> values of phosphorylated residues can be used advantageously to specifically retain phosphopeptides using SAX resins. The application of SAX for the fractionation of phosphopeptides was first demonstrated for the tryptic digest of β-casein [90], and enabled the separation of phosphopeptides from their non-phosphorylated peptide counterparts. The application of SAX was soon followed by different groups for the analyses of complex cell extracts including human liver tissue [91] and HeLa cells [92]. SAX fractionation has been mostly described with on-line reverse phase (RP) LC–MS analysis, although a recent report described the use of an on-line RP-SAX-RP configuration to enhance the peak separation and the number of unique phosphopeptides from μg-scale cell lysates [93]. The later application was demonstrated for the identification of phosphopeptides from activated CD8<sup>+</sup> T-cells purified from a single mouse. Since SAX normally requires that alkaline solutions are used for sample and elution buffers, precautions must be taken to avoid the formation of β-elimination products from phosphorylated serine and threonine residues under these conditions.

A larger number of reports have described the application of SCX for on-line and off-line separation of phosphopeptides. The separation of peptides on SCX resins is typically performed at low pH, and the large majority of tryptic peptides containing at least one basic amino acid have an overall charge higher than two. The presence of a phosphate group reduces their effective charge and their interactions with the SCX resin resulting in a relative enrichment of phosphopeptides in early fractions. Beausoleil et al. [94] were the first to take advantage of this feature in a phosphoproteomic study on tryptic digests of HeLa cells where they identified more than 2000 phosphorylation sites using off-line SCX fractionation. It is noteworthy that SCX fractionation alone is not sufficient to enrich phosphopeptides from complex cell extracts and this technique is typically used to decrease sample complexity. Further enrichment of phosphopeptides from SCX fractions is achieved using IMAC [55,95,96] or MOAC [19,97]. The combination of the SCX-IMAC enrichment strategy provided up to 30-fold increase in the proportion of phosphopeptides observed from *S. cerevisiae* compared to SCX alone [98]. The application of the SCX-TiO<sub>2</sub> approach was recently used to profile the dynamic changes of the proteome and phosphoproteome of HeLa cells during mitosis and enabled the quantitation of 6027 proteins and 20,443 unique phosphorylation sites [99]. In situation where sample availability is limited, SCX can be applied in a multi-dimensional separation format with on-line RP LC-MS to enhance sample loading and increase the number of identified phosphopeptides previously enriched by IMAC or MOAC [100].

### 2.3.2. Hydrophilic interaction chromatography (HILIC) and electrostatic repulsion-hydrophilic interaction chromatography (ERLIC)

HILIC can also provide an orthogonal separation to RP chromatography and phosphopeptides can be selectively enriched due to their increased hydrophilicity [101]. The sample is typically loaded on a polar stationary phase with a high concentration of organic solvent (typically 95% ACN) favouring the retention of polar phosphopeptides. Their subsequent elution is achieved by increasing the proportion of aqueous buffer leading to desorption of phosphopeptides with increasing hydrophilicity. IMAC enrichment of phosphopeptides from HILIC fractions provided 99% selectivity, as demonstrated by McNulty et al. for 300 µg equivalent of HeLa cell lysate where more than 1000 unique phosphorylation sites were identified [101]. The high organic content of these fractions precludes its direct coupling to on-line RP LC and HILIC is generally preferred as a pre-fractionation technique prior to LC-MS analysis of phosphopeptides. More recent applications of HILIC have been demonstrated in combination with size exclusion chromatography to identify low abundance phosphoproteins from immunodepleted plasma samples from prostate cancer patients [102] or with IMAC and stable isotope labelling to profile the abundance of 2857 unique phosphorylation sites in 1338 phosphoproteins from 1 mg of cell lysate [103].

In contrast, ERLIC makes use of the properties of HILIC and ion exchange chromatography whereby the selectivity is modulated by changing the pH, organic content of mobile phase or by applying a salt gradient [104]. Anionic phosphopeptides are preferentially retained on weak anion exchange column at pH ~2 while neutral and protonated peptides are eluted. A comparison of ERLIC with SCX-IMAC for the analysis of human epithelial carcinoma A431 cells where a total of 2058 unique phosphopeptides were identified revealed that both techniques yielded complementary identifications [105]. SCX-IMAC alone provided approximately 50% of unique phosphopeptide identifications compared to 33% using ERLIC. The small overlap observed suggests that both techniques enriched for distinct populations of phosphopeptides.

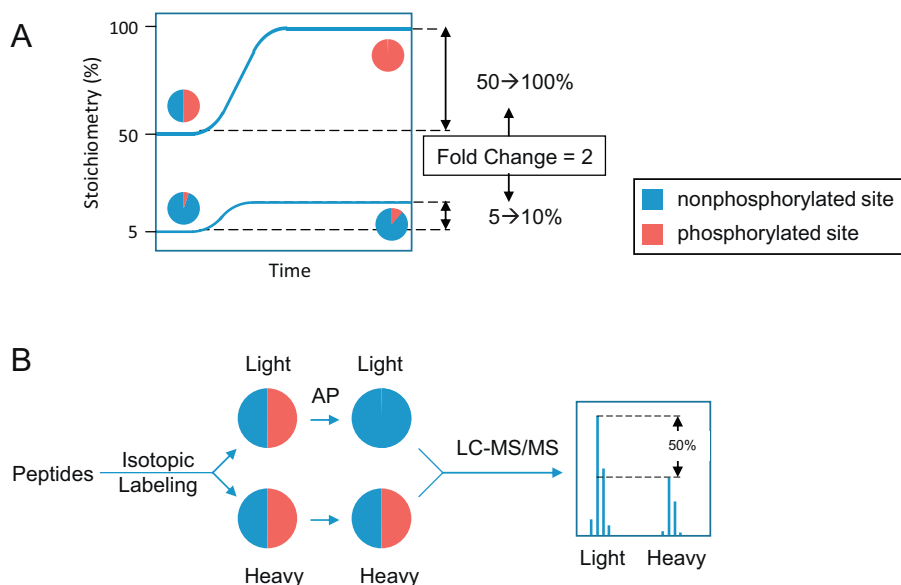
### 3. Quantitative phosphoproteomics

Changes in protein phosphorylation can be determined with methods similar to those used in quantitative proteomics. However, these changes must be clearly distinguished from those arising from variations in protein abundance that can occur when comparisons are performed across different conditions, cell types or for extended time periods. Quantitative phosphoproteomics measurements can be regrouped into two types of analysis to profile changes in the relative abundance of phosphoproteins or to determine relative changes of phosphorylation stoichiometry (Fig. 3). The former analysis is typically performed over short time periods (<1 h) to profile the phosphorylation dynamics following cell perturbation. No significant change in protein synthesis or degradation is expected across conditions, and the concentration of protein substrate is assumed constant over the duration of the experiment. The ratio of protein phosphorylation of a given condition to its reference sample serves as a proxy for relative changes in phosphorylation stoichiometry. However, when protein abundance is known to change across experimental paradigms, variations in phosphorylation stoichiometry must be normalized to account for protein expression. This task represents a sizable challenge in view of the requirements to acquire both proteome and phosphoproteome datasets to accurately determine genuine differential phosphorylation.

Another important consideration comes from the spatiotemporal distribution of phosphoproteins resulting from translocation events or rapid dephosphorylation of protein substrates. Indeed, the phosphorylation of protein substrates does not take place randomly within the cell, but is rather regulated by kinases and phosphatases that have different abundances in organelles or protein complexes. Obviously, changes in phosphorylation stoichiometry taking place on substrates differentially localized across subcellular compartments are lost when cells are homogenized and measurements are inferred from total cell lysates. Uneven distribution in phosphorylation can only be determined efficiently when quantitative phosphoproteomics analyses are performed on subcellular fractions, and additional details can be obtained in a recent review on this topic [106].

The following paragraphs highlight different methods for quantitative phosphoproteomics using native peptides (label-free), and stable isotope incorporation via metabolic labelling or specific reagents. Methods used for phosphopeptide quantitation are similar to those used for protein expression, except that changes in phosphorylation relies on abundance measurements performed on a single phosphopeptide for each site. In contrast, changes in protein expression can be obtained from multiple peptide ions of the same cognate protein which reduces variability associated with ions of decreasing abundances. More detailed description on these methods can be obtained from recently published reviews on quantitative proteomics [107,108,109,110].

In label-free quantitative proteomics, samples are analysed in a parallel fashion to generate maps of all detected peptides (e.g. mass, elution time, abundance) that can be correlated with identifications obtained by MS/MS [110,111]. Peptide maps are then clustered together and normalized to identify candidates showing statistically significant changes in abundance across conditions. Normalisation of peptide abundance is required to correct for unequal protein amounts across conditions or to compensate for variations in MS response over the course of the entire experiment. This is achieved by normalizing peptide ratios to ensure that log median value is zero. Also, samples are typically analysed in an interleave fashion across conditions and replicates to minimize variability in LC-MS measurements over time. While label-free can provide a higher number of quantifiable peptides due in part to lower sample complexity, larger variability in abundance



**Fig. 5.** Determination of phosphorylation stoichiometry. (A) The stoichiometry of phosphorylation at a specific residue corresponds to the relative proportion of a site to be phosphorylated and fold change (FC) are associated to relative changes in phosphorylation between conditions. A 2-fold increase in phosphorylation at a specific site could come from proteins with different abundances (e.g. 50–100% or 5–10%). (B) Experimental design in to measure phosphorylation stoichiometry [24]. After tryptic digestion, peptides are divided into 2 pools, labelled using reductive methylation with light and heavy isotopically labels, and one pool is dephosphorylated by alkaline phosphatase (AP). Samples are combined and fractionated before LC–MS/MS analysis.

measurements are typically observed compared to stable isotope labelling methods described below. It is thus necessary to maintain reproducible LC–MS performance through regular quality control checks.

Improvements of abundance measurements in LC–MS experiments can be obtained using isotopic labelling methods where samples are mixed and analysed together to minimize experimental variability. This is achieved by labelling proteins or peptides from different samples with distinct stable isotopes of the same elements (e.g.  $^{12}\text{C}/^{13}\text{C}$ ,  $^{14}\text{N}/^{15}\text{N}$ ,  $^1\text{H}/^2\text{H}$ ) that provide comparable separation and MS response for peptides with the same sequence. Stable isotope labelling of amino acids in culture (SILAC) is a common labelling approach applicable to cell cultures where the media contains either “light” or “heavy” isotopic forms of arginine and lysine residues [108,109]. Since protein isotope labelling is performed during cell culture, samples can be combined immediately after treatment and then processed together to minimize variability in sample processing. SILAC can be used to compare either 2 or 3 (triple SILAC with “light”, “medium” and “heavy” isotopic forms of lysine and arginine) conditions [112]. A recent report described the use of SILAC to profile both phosphorylation dynamics and protein expression in wild type and yeast mutants with *FUS3* or *STE7* deletions, and yielded quantitative ratios for more than 10,500 unique phosphopeptides [46]. Non-redundant phosphopeptide ratios were normalized based on protein levels to distinguish differential phosphorylation from altered protein expression. Also, the combination of isotopic labelling and enzymatic dephosphorylation was used to determine absolute phosphorylation stoichiometry from large-scale proteomics analyses of yeast extracts [24]. This was achieved by measuring single ratio associated to phosphatase-treated and mock-treated samples, and provided stoichiometry measurements for 5033 phosphorylation sites from *S. cerevisiae* growing through mid-log phase (Fig. 5). Interestingly, this study indicated that low stoichiometries were generally observed for cytoplasm, ribosome and mitochondria phosphoproteins whereas nucleus and mitotic bud phosphoproteins displayed high phosphorylation stoichiometries. While SILAC has been mostly used for cell cultures, different

reports also described spike-in SILAC or super SILAC for quantitative proteomics measurements in tissues and different cell types [113,114,115].

Alternative approaches to the incorporation of stable isotopes into peptides include the use of  $^{16}\text{O}$ - or  $^{18}\text{O}$ -water during the proteolytic digestion, and tryptic digests are combined prior to their MS analyses [116]. Specific digestion conditions must be used to ensure complete  $^{18}\text{O}_2$ -labelling while minimizing back exchange [117]. Stable isotopes can also be introduced via chemical modifications of proteins or peptides first described for cysteine containing peptide and more recently for the derivatisation of amino groups [118,119]. The most common reagents are iTRAQ and TMT that comprise an amine-reactive functionality, a spacer arm and an MS/MS reporter group [120,121,122]. The latter group provides traceable fragment ions unique to each sample while a spacer arm confers the same mass to peptides sharing identical sequences. This allows for simultaneous determination of peptide abundance and identity of peptide isotopomers. The main advantage of these reagents is the capability to compare different samples together (up to 8 different conditions) thereby reducing significantly instrument time. A recent example of combined labelling reagent and SILAC was presented by Dephore and Gygi where they used triplex metabolic labelling and six-plex isobaric tags to profile dynamic response to rapamycin in yeast [123].

As described above, different approaches can be used for quantitative proteomics and phosphoproteomics experiments. Importantly, careful considerations must be placed on the nature of the sample (e.g. biological tissue, cell cultures), sample complexity and workflow, and the requirement for sample multiplexing. SILAC is generally preferred in experimental paradigms that can be evaluated in cell model systems where metabolic labelling is feasible. Label-free and chemical labelling methods can be used in other situations including the profiling of phosphopeptides from animal models or human biofluids. Simple sample workflow must be considered to minimize the number of steps required for sample preparation to reduce variability in abundance measurements done with label-free techniques.



#### 4. Conclusions

The availability of high resolution and high sensitivity MS instruments combined with affinity media such as IMAC and MOAC have greatly expanded the repertoire of protein phosphorylations that can be observed from different cell and animal model systems. Several large-scale phosphoproteomics studies have reported the identification of approximately 25,000 unique phosphorylation sites on mammalian phosphoproteins. While significant progress has been made toward comprehensive phosphoproteome analyses, the number of identified sites also underscores the practical limitations of current methods and instrumentation. Indeed, the wide dynamic range of protein expression across different cell types, the variable stoichiometry of protein phosphorylation and the instrumental biases in detecting phosphopeptides of different size and phosphorylation state represent significant challenges that cannot be addressed simultaneously with present phosphoproteomics approaches. The development of subcellular fractionation methods may offer a potential avenue to enrich phosphoproteins that remain difficult to identify from total cell lysates alone.

Changes in phosphorylation have an important impact on protein function and regulation, and comprehensive phosphoproteomics strategies must consider methods to assess site-specific variation of stoichiometry. Important steps towards this goal were described recently using SILAC and alkaline phosphatase [24], and by profiling changes in phosphorylation from separate proteome- and phosphorylation-based analyses [12,99]. Information on site stoichiometry and conservation can be used to prioritise identified phosphorylation sites for subsequent functional studies. It is noteworthy that low phosphorylation stoichiometry can be disproportionately found on high abundance proteins though they may show higher conservation. Background protein conservation rates must be considered to correctly interpret the relationship between phosphorylation stoichiometry, protein abundance and site conservation [124].

The modulation of protein phosphorylation in response to different cellular cues provides important information on protein interactions through network of kinases and phosphatases affecting the faith and functions of different protein substrates. This modification can also influence interactions with other enzymes giving rise to interplay between phosphorylation and other types of PTMs. Histone represent a classical case where different residues can be modified in a combinatorial fashion to regulate chromatin accessibility. Recent evidences suggest cross talk between O-GlcNAc and phosphorylation where the former modification can act as a nutrient/stress sensor to modulate cell signalling and transcription [125]. Also, phosphorylation can be a pre-requisite for substrate recognition by ubiquitin-like modifiers such as E3 ubiquitin ligase Skp1/Cullin/F-box (SCF) [126] or substrates containing phospho-dependent SUMO motifs [127]. While different affinity methods are available to generate comprehensive repertoires of protein modifications and probe their potential interplay, the confirmation of connectivity will ultimately rely on their direct detection. This aspect also highlights a current limitation of bottom-up proteomics where connectivity is limited to modifications located on the same peptides. Advances in top-down (or middle-down) proteomics may provide new opportunities to integrate protein interactions and underlying cross-talk between protein modifications.

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#### References

- [1] Hunter T. Signaling—2000 and beyond. *Cell* 2000;100:113–27.
- [2] Lim YP. Mining the tumor phosphoproteome for cancer markers. *Clinical Cancer Research* 2005;11:3163–9.
- [3] Harsha HC, Pandey A. Phosphoproteomics in cancer. *Molecular Oncology* 2010;4:482–95.
- [4] Clark SS, McLaughlin J, Timmons M, Pendergast AM, Ben-Neriah Y, Dow LW, et al. Expression of a distinctive BCR-ABL oncogene in Ph1-positive acute lymphocytic leukemia (ALL). *Science (New York, NY)* 1988;239:775–7.
- [5] Schubert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. *Nature Reviews* 2007;7:295–308.
- [6] Lincova E, Hampl A, Pernicova Z, Starsichova A, Krcmar P, Machala M, et al. Multiple defects in negative regulation of the PKB/Akt pathway sensitise human cancer cells to the antiproliferative effect of non-steroidal anti-inflammatory drugs. *Biochemical Pharmacology* 2009;78:561–72.
- [7] Sweeney C, Miller JK, Shattuck DL, Carraway 3rd KL. ErbB receptor negative regulatory mechanisms: implications in cancer. *Journal of Mammary Gland Biology and Neoplasia* 2006;11:89–99.
- [8] Wilson KS, Roberts H, Leek R, Harris AL, Geradts J. Differential gene expression patterns in HER2/neu-positive and -negative breast cancer cell lines and tissues. *The American Journal of Pathology* 2002;161:1171–85.
- [9] Bourne HR. GTPases: a family of molecular switches and clocks. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 1995;349:283–9.
- [10] Bourne HR. Signal transduction. Team blue sees red. *Nature* 1995;376:727–9.
- [11] Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science (New York, NY)* 2002;298:1912–34.
- [12] Ubersax JA, Ferrell Jr JE. Mechanisms of specificity in protein phosphorylation. *Nature Reviews Molecular Cell Biology* 2007;8:530–41.
- [13] Roy J, Cyert MS. Cracking the phosphatase code: docking interactions determine substrate specificity. *Science Signaling* 2009;2:re9.
- [14] Breikreutz A, Choi H, Sharom JR, Boucher L, Neduva V, Larsen B, et al. A global protein kinase and phosphatase interaction network in yeast. *Science (New York, NY)* 2010;328:1043–6.
- [15] Levy ED, Landry CR, Michnick SW. Cell signaling. Signaling through cooperation. *Science (New York, NY)* 2010;328:983–4.
- [16] Landry CR, Levy ED, Michnick SW. Weak functional constraints on phosphoproteomes. *Trends in Genetics: TIG* 2009;25:193–7.
- [17] Gnad F, Forner F, Zielinska DF, Birney E, Gunawardena J, Mann M. Evolutionary constraints of phosphorylation in eukaryotes, prokaryotes, and mitochondria. *Molecular and Cellular Proteomics* 2010;9:2642–53.
- [18] Miller M, Donat S, Raketts S, Stehle T, Kouwen TR, Diks SH, et al. Staphylococcal PknB as the first prokaryotic representative of the proline-directed kinases. *PLoS ONE* 2010;5:e9057.
- [19] Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, et al. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 2006;127:635–48.
- [20] Sickmann A, Meyer HE. Phosphoamino acid analysis. *Proteomics* 2001;1:200–6.
- [21] Reinders J, Sickmann A. State-of-the-art in phosphoproteomics. *Proteomics* 2005;5:4052–61.
- [22] Kalume DE, Molina H, Pandey A. Tackling the phosphoproteome: tools and strategies. *Current Opinion in Chemical Biology* 2003;7:64–9.
- [23] Johnson SA, Hunter T. Kinomics: methods for deciphering the kinome. *Nature Methods* 2005;2:17–25.
- [24] Wu R, Haas W, Dephoure N, Huttlin EL, Zhai B, Sowa ME, et al. A large-scale method to measure absolute protein phosphorylation stoichiometries. *Nature Methods* 2011;8:677–83.
- [25] Ham BM, Yang F, Jayachandran H, Jaitly N, Monroe ME, Gritsenko MA, et al. The influence of sample preparation and replicate analyses on HeLa Cell phosphoproteome coverage. *Journal of Proteome Research* 2008;7:2215–21.
- [26] Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, et al. Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Molecular and Cellular Proteomics* 2005;4:310–27.
- [27] Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends in Biotechnology* 2002;20:261–8.
- [28] Nagaraj N, D'Souza RC, Cox J, Olsen JV, Mann M. Feasibility of large-scale phosphoproteomics with higher energy collisional dissociation fragmentation. *Journal of Proteome Research* 2010;9:6786–94.
- [29] Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101:9528–33.
- [30] Swaney DL, McAlister GC, Coon JJ. Decision tree-driven tandem mass spectrometry for shotgun proteomics. *Nature Methods* 2008;5:959–64.
- [31] Hornbeck PV, Chabra I, Kornhauser JM, Skrzypek E, Zhang B. PhosphoSite: a bioinformatics resource dedicated to physiological protein phosphorylation. *Proteomics* 2004;4:1551–61.

- [32] Diella F, Gould CM, Chica C, Via A, Gibson TJ. Phospho.ELM: a database of phosphorylation sites—update 2008. *Nucleic Acids Research* 2008;36:D240–4.
- [33] Gnad F, Ren S, Cox J, Olsen JV, Macek B, Oroschi M, et al. PHOSIDA (phosphorylation site database): management, structural and evolutionary investigation, and prediction of phosphosites. *Genome Biology* 2007;8:R250.
- [34] Bodenmiller B, Campbell D, Gerrits B, Lam H, Jovanovic M, Picotti P, et al. PhosphoPep—a database of protein phosphorylation sites in model organisms. *Nature Biotechnology* 2008;26:1339–40.
- [35] Ovelleiro D, Carrascal M, Casas V, Abian J. LymPHOS: design of a phosphosite database of primary human T cells. *Proteomics* 2009;9:3741–51.
- [36] Courcelles M, Lemieux S, Voisin L, Meloche S, Thibault P. ProteoConnections: a bioinformatics platform to facilitate proteome and phosphoproteome analyses. *Proteomics* 2011;11:2654–71.
- [37] Steen H, Jebaranthirajah JA, Rush J, Morrice N, Kirschner MW. Phosphorylation analysis by mass spectrometry: myths, facts, and the consequences for qualitative and quantitative measurements. *Molecular and Cellular Proteomics* 2006;5:172–81.
- [38] Hunter T. When is a lipid kinase not a lipid kinase? When it is a protein kinase. *Cell* 1995;83:1–4.
- [39] Boersema PJ, Mohammed S, Heck AJ. Phosphopeptide fragmentation and analysis by mass spectrometry. *Journal of Mass Spectrometry* 2009;44:861–78.
- [40] Palumbo AM, Smith SA, Kalcic CL, Dantus M, Stemmer PM, Reid GE. Tandem mass spectrometry strategies for phosphoproteome analysis. *Mass Spectrometry Reviews* 2011;30:600–25.
- [41] D'Ambrosio C, Salzano AM, Arena S, Renzone G, Scaloni A. Analytical methodologies for the detection and structural characterization of phosphorylated proteins. *Journal of Chromatography* 2007;849:163–80.
- [42] Dunn JD, Reid GE, Bruening ML. Techniques for phosphopeptide enrichment prior to analysis by mass spectrometry. *Mass Spectrometry Reviews* 2009;29:29–54.
- [43] Morandell S, Stasyk T, Grosstessner-Hain K, Roitinger E, Mechtler K, Bonn GK, et al. Phosphoproteomics strategies for the functional analysis of signal transduction. *Proteomics* 2006;6:4047–56.
- [44] Schmelzle K, White FM. Phosphoproteomic approaches to elucidate cellular signaling networks. *Current Opinion in Biotechnology* 2006;17:406–14.
- [45] Thingholm TE, Larsen MR, Ingrell CR, Kassem M, Jensen ON. TiO<sub>2</sub>(2)-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. *Journal of Proteome Research* 2008;7:3304–13.
- [46] Wu R, Dephoure N, Haas W, Huttlin EL, Zhai B, Sowa ME, et al. Correct interpretation of comprehensive phosphorylation dynamics requires normalization by protein expression changes. *Molecular and Cellular Proteomics* 2011;10:M111.009654.
- [47] Chen EI, Cociorva D, Norris JL, Yates 3rd JR. Optimization of mass spectrometry-compatible surfactants for shotgun proteomics. *Journal of Proteome Research* 2007;6:2529–38.
- [48] Carayon K, Leh H, Henry E, Simon F, Mouscadet JF, Deprez E. A cooperative and specific DNA-binding mode of HIV-1 integrase depends on the nature of the metallic cofactor and involves the zinc-containing N-terminal domain. *Nucleic Acids Research* 2010;38:3692–708.
- [49] Zhou J, Zhou T, Cao R, Liu Z, Shen J, Chen P, et al. Evaluation of the application of sodium deoxycholate to proteomic analysis of rat hippocampal plasma membrane. *Journal of Proteome Research* 2006;5:2547–53.
- [50] Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nature Methods* 2009;6:359–62.
- [51] Zielinska DF, Gnad F, Wisniewski JR, Mann M. Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints. *Cell* 2010;141:897–907.
- [52] Wisniewski JR, Nagaraj N, Zougman A, Gnad F, Mann M. Brain phosphoproteome obtained by a FASP-based method reveals plasma membrane protein topology. *Journal of Proteome Research* 2010;9:3280–9.
- [53] Wisniewski JR, Mann M. Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Analytical Chemistry* 2012;84:2631–7.
- [54] Bian Y, Ye M, Song C, Cheng K, Wang C, Wei X, et al. Improve the Coverage for the Analysis of Phosphoproteome of HeLa Cells by a Tandem Digestion Approach. *Journal of Proteome Research* 2012.
- [55] Dephoure N, Gygi SP. A solid phase extraction-based platform for rapid phosphoproteomic analysis. *Methods* 2011;54:379–86.
- [56] Nuhse T, Yu K, Salomon A. Isolation of phosphopeptides by immobilized metal ion affinity chromatography. *Current protocols in molecular biology* 2007 [edited by Ausubel FM et al., Chapter 18:Unit 18.13].
- [57] Thingholm TE, Jensen ON. Enrichment and characterization of phosphopeptides by immobilized metal affinity chromatography (IMAC) and mass spectrometry. *Methods in Molecular Biology* (Clifton, NJ) 2009;527:47–56, xi.
- [58] Tao WA, Wollscheid B, O'Brien R, Eng JK, Li XJ, Bodenmiller B, et al. Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. *Nature Methods* 2005;2:591–8.
- [59] Zhou H, Watts JD, Aebersold R. A systematic approach to the analysis of protein phosphorylation. *Nature Biotechnology* 2001;19:375–8.
- [60] McLachlin DT, Chait BT. Improved beta-elimination-based affinity purification strategy for enrichment of phosphopeptides. *Analytical Chemistry* 2003;75:6826–36.
- [61] Chen M, Su X, Yang J, Jenkins CM, Cedars AM, Gross RW. Facile identification and quantitation of protein phosphorylation via beta-elimination and Michael addition with natural abundance and stable isotope labeled thiocholine. *Analytical Chemistry* 2010;82:163–71.
- [62] Bodenmiller B, Mueller LN, Pedrioli PG, Pflieger D, Junger MA, Eng JK, et al. An integrated chemical, mass spectrometric and computational strategy for (quantitative) phosphoproteomics: application to *Drosophila melanogaster* Kc167 cells. *Molecular BioSystems* 2007;3:275–86.
- [63] Frackelton Jr AR, Ross AH, Eisen HN. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor-stimulated cells. *Molecular and Cellular Biology* 1983;3:1343–52.
- [64] Jedrychowski MP, Huttlin EL, Haas W, Sowa ME, Rad R, Gygi SP. Evaluation of HCD- and CID-type fragmentation within their respective detection platforms for murine phosphoproteomics. *Molecular and Cellular Proteomics* 2011;10:M111.009910.
- [65] Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007;131:1190–203.
- [66] Gronborg M, Kristiansen TZ, Stensballe A, Andersen JS, Ohara O, Mann M, et al. A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies: identification of a novel protein, Frigg, as a protein kinase A substrate. *Molecular and Cellular Proteomics* 2002;1:517–27.
- [67] Zhang H, Zha X, Tan Y, Hornbeck PV, Mastrangelo AJ, Alessi DR, et al. Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *Journal of Biological Chemistry* 2002;277:39379–87.
- [68] Matsuoaka S, Ballif BA, Smogorzewska A, McDonald 3rd ER, Hurov KE, Luo J, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* (New York, NY) 2007;316:1160–6.
- [69] Jin J, Smith FD, Stark C, Wells CD, Fawcett JP, Kulkarni S, et al. Proteomic, functional, and domain-based analysis of in vivo 14-3-3 binding proteins involved in cytoskeletal regulation and cellular organization. *Current Biology* 2004;14:1436–50.
- [70] Connor PA, McQuillan AJ. Phosphate adsorption onto TiO<sub>2</sub> from aqueous solutions: an in situ internal reflection infrared spectroscopic study. *Langmuir* 1999;15:2916–21.
- [71] Ikeguchi Y, Nakamura H. Determination of organic phosphates by column-switching high performance anion-exchange chromatography using on-line preconcentration on titania. *Analytical Sciences* 1997;13:479–83.
- [72] Pinkse MW, Uitto PM, Hillhorst MJ, Ooms B, Heck AJ. Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-nano-LC-ESI-MS/MS and titanium oxide precolumns. *Analytical Chemistry* 2004;76:3935–43.
- [73] Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Molecular and Cellular Proteomics* 2005;4:873–86.
- [74] Sugiyama N, Masuda T, Shinoda K, Nakamura A, Tomita M, Ishihama Y. Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. *Molecular and Cellular Proteomics* 2007;6:1103–9.
- [75] Bodenmiller B, Wanka S, Kraft C, Urban J, Campbell D, Pedrioli PG, et al. Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. *Science Signaling* 2010;3:rs4.
- [76] Hilger M, Bonaldi T, Gnad F, Mann M. Systems-wide analysis of a phosphatase knock-down by quantitative proteomics and phosphoproteomics. *Molecular and Cellular Proteomics* 2009;8:1908–20.
- [77] Kanshin E, Michnick SW, Thibault P. Decoy amino acids enhance the depth and coverage of large-scale phosphoproteomics studies. In: Proc 60th ASMS Conference on Mass Spectrometry and Allied Topics. Vancouver: ASMS; 2012.
- [78] Ficarro SB, Parikh JR, Blank NC, Marto JA. Niobium(V) oxide (Nb2O5): application to phosphoproteomics. *Analytical Chemistry* 2008;80:4606–13.
- [79] Kweon HK, Hakansson K. Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Analytical Chemistry* 2006;78:1743–9.
- [80] Andersson L, Porath J. Isolation of phosphoproteins by immobilized metal (Fe<sup>3+</sup>) affinity chromatography. *Analytical Biochemistry* 1986;154:250–4.
- [81] Posewitz MC, Tempst P. Immobilized gallium(III) affinity chromatography of phosphopeptides. *Analytical Chemistry* 1999;71:2883–92.
- [82] Collins MO, Yu L, Husi H, Blackstock WP, Choudhary JS, Grant SG. Robust enrichment of phosphorylated species in complex mixtures by sequential protein and peptide metal-affinity chromatography and analysis by tandem mass spectrometry. *Science STKE* 2005;2005:pl6.
- [83] Kokubu M, Ishihama Y, Sato T, Nagasu T, Oda Y. Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Analytical Chemistry* 2005;77:5144–54.
- [84] Huttlin EL, Jedrychowski MP, Elias JE, Goswami T, Rad R, Beausoleil SA, et al. A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* 2010;143:1174–89.
- [85] Schreiber TB, Mausbacher N, Soroka J, Wandinger SK, Buchner J, Daub H. Global analysis of phosphoproteome regulation by the Ser/Thr phosphatase Ppt1 in *Saccharomyces cerevisiae*. *Journal of Proteome Research* 2012;11:2397–408.
- [86] Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, et al. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nature Biotechnology* 2002;20:301–5.

- [87] Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, et al. Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. *Journal of Biological Chemistry* 2003;278:11579–89.
- [88] Jensen SS, Larsen MR. Evaluation of the impact of some experimental procedures on different phosphopeptide enrichment techniques. *Rapid Communications in Mass Spectrometry* 2007;21:3635–45.
- [89] Thingholm TE, Jensen ON, Robinson PJ, Larsen MR. SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Molecular and Cellular Proteomics* 2008;7:661–71.
- [90] Zhang K. From purification of large amounts of phospho-compounds (nucleotides) to enrichment of phospho-peptides using anion-exchanging resin. *Analytical Biochemistry* 2006;357:225–31.
- [91] Han G, Ye M, Zhou H, Jiang X, Feng S, Jiang X, et al. Large-scale phosphoproteome analysis of human liver tissue by enrichment and fractionation of phosphopeptides with strong anion exchange chromatography. *Proteomics* 2008;8:1346–61.
- [92] Dai J, Wang LS, Wu YB, Sheng QH, Wu JR, Shieh CH, et al. Fully automatic separation and identification of phosphopeptides by continuous pH-gradient anion exchange online coupled with reversed-phase liquid chromatography mass spectrometry. *Journal of Proteome Research* 2009;8:133–41.
- [93] Ficarro SB, Zhang Y, Carrasco-Alfonso MJ, Garg B, Adelmant G, Webber JT, et al. Online nanoflow multidimensional fractionation for high efficiency phosphopeptide analysis. *Molecular and Cellular Proteomics* 2011;10.0111.011064.
- [94] Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, et al. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101:12130–5.
- [95] Villen J, Beausoleil SA, Gerber SA, Gygi SP. Large-scale phosphorylation analysis of mouse liver. *Proceedings of the National Academy of Sciences of the United States of America* 2007;104:1488–93.
- [96] Zhai B, Villen J, Beausoleil SA, Mintseris J, Gygi SP. Phosphoproteome analysis of *Drosophila melanogaster* embryos. *Journal of Proteome Research* 2008;7:1675–82.
- [97] Sui S, Wang J, Yang B, Song L, Zhang J, Chen M, et al. Phosphoproteome analysis of the human Chang liver cells using SCX and a complementary mass spectrometric strategy. *Proteomics* 2008;8:2024–34.
- [98] Villen J, Gygi SP. The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nature Protocols* 2008;3:1630–8.
- [99] Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, Jensen LJ, et al. Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Science Signaling* 2010;3:ra3.
- [100] Boulais J, Trost M, Landry CR, Dieckmann R, Levy ED, Soldati T, et al. Molecular characterization of the evolution of phagosomes. *Molecular Systems Biology* 2010;6:423.
- [101] McNulty DE, Annan RS. Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. *Molecular and Cellular Proteomics* 2008;7:971–80.
- [102] Garbis SD, Roumeliotis TI, Tyrirtzis SI, Zorpas KM, Pavlakis K, Constantinides CA. A novel multidimensional protein identification technology approach combining protein size exclusion prefractionation, peptide zwitterion-ion hydrophilic interaction chromatography, and nano-ultraperformance RP chromatography/nESI-MS2 for the in-depth analysis of the serum proteome and phosphoproteome: application to clinical sera derived from humans with benign prostate hyperplasia. *Analytical Chemistry* 2011;83:708–18.
- [103] Wu CJ, Chen YW, Tai JH, Chen SH. Quantitative phosphoproteomics studies using stable isotope dimethyl labelling coupled with IMAC-HILIC-nanoLC-MS/MS for estrogen-induced transcriptional regulation. *Journal of Proteome Research* 2011;10:1088–97.
- [104] Alpert AJ. Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides. *Analytical Chemistry* 2008;80:62–76.
- [105] Gan CS, Guo T, Zhang H, Lim SK, Sze SK. A comparative study of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) versus SCX-IMAC-based methods for phosphopeptide isolation/enrichment. *Journal of Proteome Research* 2008;7:4869–77.
- [106] Trost M, Bridon G, Desjardins M, Thibault P. Subcellular phosphoproteomics. *Mass Spectrometry Reviews* 2010;29:962–90.
- [107] Nilsson CL. Advances in quantitative phosphoproteomics. *Analytical Chemistry* 2012;84:735–46.
- [108] Pimienta G, Chaerkady R, Pandey A. SILAC for global phosphoproteomic analysis. *Methods in Molecular Biology* 2009;527:107–16, x.
- [109] Ong SE, Foster LJ, Mann M. Mass spectrometric-based approaches in quantitative proteomics. *Methods* 2003;29:124–30.
- [110] Neilson KA, Ali NA, Muralidharan S, Mirzaei M, Mariani M, Assadourian G, et al. Less label, more free: approaches in label-free quantitative mass spectrometry. *Proteomics* 2011;11:535–53.
- [111] Zhu W, Smith JW, Huang CM. Mass spectrometry-based label-free quantitative proteomics. *Journal of Biomedicine & Biotechnology* 2010;2010:840518.
- [112] Pan C, Olsen JV, Daub H, Mann M. Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. *Molecular & Cellular Proteomics: MCP* 2009;8:2796–808.
- [113] Monetti M, Nagaraj N, Sharma K, Mann M. Large-scale phosphosite quantification in tissues by a spike-in SILAC method. *Nature Methods* 2011;8:655–8.
- [114] Geiger T, Cox J, Ostasiewicz P, Wisniewski JR, Mann M. Super-SILAC mix for quantitative proteomics of human tumor tissue. *Nature Methods* 2010;7:383–5.
- [115] Zanivan S, Krueger M, Mann M. In vivo quantitative proteomics: the SILAC mouse. *Methods in Molecular Biology* 2012;757:435–50.
- [116] Reynolds KJ, Yao X, Fenselau C. Proteolytic 18O labelling for comparative proteomics: evaluation of endoprotease Glu-C as the catalytic agent. *Journal of Proteome Research* 2002;1:27–33.
- [117] Fenselau C, Yao X. 18O2-labelling in quantitative proteomic strategies: a status report. *Journal of Proteome Research* 2009;8:2140–3.
- [118] Jones AM, Nuhse TS. Phosphoproteomics using iTRAQ. *Methods in Molecular Biology* 2011;779:287–302.
- [119] Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labelling for quantitative proteomics. *Nature Protocols* 2009;4:484–94.
- [120] Evans C, Noirel J, Ow SY, Salim M, Pereira-Medrano AG, Couto N, et al. An insight into iTRAQ: where do we stand now? *Analytical and Bioanalytical Chemistry* 2012.
- [121] Mertins P, Udeshi ND, Clauser KR, Mani DR, Patel J, Ong SE, et al. iTRAQ labelling is superior to mTRAQ for quantitative global proteomics and phosphoproteomics. *Molecular and Cellular Proteomics* 2011.
- [122] Sinclair J, Timms JF. Quantitative profiling of serum samples using TMT protein labelling, fractionation and LC-MS/MS. *Methods* 2011;54:361–9.
- [123] Dephoure N, Gygi SP. Hyperplexing: a method for higher-order multiplexed quantitative proteomics provides a map of the dynamic response to rapamycin in yeast. *Science Signaling* 2012;5:rs2.
- [124] Tan CS, Bader GD. Phosphorylation sites of higher stoichiometry are more conserved. *Nature Methods* 2012;9:317.
- [125] Hart GW, Slawson C, Ramirez-Correa G, Lagerlof O. Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annual Review of Biochemistry* 2011;80:825–58.
- [126] Vodermaier HC. APC/C and SCF: controlling each other and the cell cycle. *Current Biology* 2004;14:R787–96.
- [127] Hietakangas V, Anckar J, Blomster HA, Fujimoto M, Palvimo JJ, Nakai A, et al. PDSM, a motif for phosphorylation-dependent SUMO modification. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103:45–50.