EXPERT REVIEWS

Quantitative phosphoproteomics strategies for understanding protein kinase-mediated signal transduction pathways

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Protein phosphorylation is a central regulatory mechanism of cell signaling pathways. This highly controlled biochemical process is involved in most cellular functions, and defects in protein kinases and phosphatases have been implicated in many diseases, highlighting the importance of understanding phosphorylation-mediated signaling networks. However, phosphorylation is a transient modification, and phosphorylated proteins are often less abundant. Therefore, the large-scale identification and quantification of phosphoproteins and their phosphorylation sites under different conditions are one of the most interesting and challenging tasks in the field of proteomics. Both 2D gel electrophoresis and liquid chromatography-tandem mass spectrometry serve as key phosphoproteomic technologies in combination with prefractionation, such as enrichment of phosphorylated proteins/peptides. Recently, new possibilities for quantitative phosphoproteomic analysis have been offered by technical advances in sample preparation, enrichment, separation, instrumentation, quantification and informatics. In this article, we present an overview of several strategies for quantitative phosphoproteomics and discuss how phosphoproteomic analysis can help to elucidate signaling pathways that regulate various cellular processes.

Keywords: 2D-DIGE • cell signaling • iTRAQ/TMT • liquid chromatography-mass spectrometry • phosphoproteomics • phosphorylation • prefractionation • protein kinase • SILAC • two-dimensional gel electrophoresis

Protein phosphorylation on serine, threonine and tyrosine residues is one of the most widespread types of post-translational modifications used in cell signaling and can regulate diverse properties of proteins [1]. In cells, numerous proteins are continuously and dynamically phosphorylated and dephosphorylated at specific sites under the control of complex signaling networks [2]. This reversible phosphorylation is mediated by the opposite action of large families of protein kinases and phosphatases. Protein kinases are major components of signal transduction pathways and are encoded by over 500 genes in the human genome [3], emphasizing the important and complicated roles of phosphorylation. In fact, many pathologies such as cancer [4], autoimmune diseases [5], metabolic disorders [6] and pathogenic infections [7] are associated with protein kinase-mediated signaling pathways.

To fully understand the complex signaling networks, it is essential to develop analytical strategies for the global identification of phosphorylated proteins and the characterization of phosphorylation sites under different biological conditions. Furthermore, the global identification and characterization of *in vivo* substrates of an individual protein kinase would be useful for a thorough and therapeutically applicable understanding of its cellular functions. However, the low abundance of most signaling proteins, the low stoichiometry of phosphorylation and the highly dynamic regulation of phosphoproteins put great demands on these analytical strategies. Recent advances in various proteomic technologies have enabled more sensitive, comprehensive and quantitative analysis of protein phosphorylation in cells and tissues, as reviewed previously [8–13].

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Two-dimensional gel electrophoresis (2DE) and gel-free, liquid chromatography-tandem mass spectrometry (LC-MS/MS) are two major proteomic technologies. Both methods require prefractionation of proteins or peptides to reduce sample complexity and detect low-abundance phosphorylation events. In this article, we will first focus on these two proteomic methods coupled with various prefractionation procedures for the quantitative characterization of *in vivo* phosphorylation. Quantitative proteomic methods that are based on the analysis of *in vitro* phosphorylation, such as the kinase substrate tracking and elucidation (KESTREL) [14], analog-sensitive kinase alleles [15], expression library screening [16] and protein chips [17] are excluded. In addition, prediction of the responsible kinases, validation of the obtained proteomic data and functional analysis of the kinase-mediated cell signaling pathways will be discussed.

Strategies for quantitative phosphoproteomics using 2DE

Two-dimensional gel electrophoresis is a classical and powerful analytical method in proteomics that can separate complex mixtures of proteins based on charge (by isoelectric focusing) and apparent molecular mass (by sodium dodecyl sulfate polyacrylamide gel electrophoresis) [18]. In contrast to LC-MS/MS that analyzes digested peptides, 2DE delivers a map of intact proteins, which reflects changes in protein expression, isoforms or post-translational modifications [19]. These changes can be confirmed by 1D or 2D Western blot analysis. Some forms of post-translational modification such as phosphorylation, glycosylation or limited proteolysis are easily located in 2DE gels as they appear as distinct spot trains along the horizontal and/or vertical axis. In 2DE, stoichiometry of phosphorylation can be readily determined by quantifying the spot intensity of each phosphorylated form. Furthermore, the combination of 2DE with ^{32}P labeling, staining of phosphoproteins on gels or immunoblotting with phospho-specific antibodies makes it suitable for proteomics analysis of phosphorylation-mediated signaling pathways [20,21].

For 2DE analysis of signaling pathways, the signal-dependent changes in the 2D separation patterns between samples must be compared. However, the major disadvantage of 2DE is the inherent lack of reproducibility between gels, and labor-intensive staining procedure and image analysis are usually required for gel matching and removal of artifacts. Fluorescent 2D-DIGE was developed to overcome this problem by labeling proteins from two to three samples with spectrally resolvable fluorescent dyes before running all the samples on the same gel [22,23]. The fluorescent dyes used in 2D-DIGE are cyanine-based, size- and charge-matched, and reactive with lysine or cysteine residues in proteins. These characteristics reduce spot pattern variability and the number of gels in an experiment and thus allow simple and accurate spot matching [24,25]. It should be noted that 2D-DIGE is well suited to detect quantitative changes in phosphorylation, which cause quantitative shifts of protein spots in the isoelectric point (Figure 1). The use of highly sensitive dyes and large-format gels increases the number of spots on the gel and detects low-abundance signaling proteins [26]. Furthermore, 2D-DIGE can be followed by phosphoprotein staining or 2D-Western blotting with phospho-specific antibodies, both of which can be fluorescently detected and accurately compared with the fluorescent 2D-DIGE image [27,28].

Prefractionation procedures have been shown to be effective for proteomic analysis of proteins of low abundance, such as signaling phosphoproteins. One of these methods, immobilized metal ion-affinity chromatography (IMAC), can enrich not only phosphopeptides (see later) but also phosphoproteins from lysates of cells and tissues [29–31]. The authors previously developed a quantitative phosphoproteomics approach using phosphoprotein enrichment by IMAC followed by 2D-DIGE, which resulted in the detection of multiple protein spots involved in the p38 MAPK pathway [32]. One of these spots was identified as the cochaperone BAG2, which was shown to be directly phosphorylated by MAPK-activated protein kinase 2 *in vitro* and *in vivo*. This approach has been successfully applied by different researchers to study other signaling systems [33,34]. On the other hand, subcellular fractionation coupled with 2D-DIGE is particularly useful for the analysis of signaling events at specific intracellular locations. Huber *et al.* performed 2D-DIGE after purification of endosomes from EGF-treated mouse epithelial cells and identified 23 endosomal targets of EGF receptor signaling, such as R-Ras [35]. Another group, led by Wang, performed 2D-DIGE analysis of phosphoprotein and plasma membrane fractions from brassinosteroid-treated *Arabidopsis* [36] and identified homologous protein kinases as key transducers of this steroid hormone signaling in plants [37]. Thus, the combination of prefractionation and 2D-DIGE is a powerful proteomics approach for unraveling protein kinase-mediated signaling networks.

Extracellular signal-regulated kinase (ERK)/MAPK is an evolutionally conserved and well-studied signaling kinase that regulates various cellular functions [38,39]. Although many ERK substrates have been identified to date [40], the diverse roles of the ERK pathway in cells suggest that more substrates remain to be identified. Therefore, the ERK signaling pathway has provided an excellent model system for phosphoproteomic identification of kinase targets by both *in vitro* and *in vivo* studies [16,41–43]. Ahn *et al.* succeeded in identifying novel ERK targets by comparing 2DE gels where total proteins from ERK-activated or -inhibited cells were separately loaded and silver-stained [44,45]. However, the core components of this kinase cascade, such as MAP kinase/ERK kinase 1, ERK1 and ERK2, could not be identified, and validation of direct substrates was not carried out. Recently, the authors have improved the previous phosphoproteomic approach [32] by incorporating the estrogen receptor fusion system and phosphomotif-specific antibodies to globally identify ERK substrates *in vivo* (Figure 2) [28]. This approach used cultured cells expressing a kinase domain of B-Raf fused to the estrogen receptor, which was treated with tamoxifen to selectively activate the Raf–MEK–ERK pathway. To detect low-abundance ERK pathway components, lysates from ERK-activated or -inhibited cells were enriched for phosphoproteins by IMAC. Phosphoproteins obtained from these cells were subjected to 2D-DIGE, which allowed the detection of many protein spots with quantitatively different fluorescence intensities following ERK activation. 2D Western blotting and matching with the 2D-DIGE image showed that a number of these spots were reactive with ERK

phosphomotif antibodies, indicating the possibility of direct ERK substrates. From this screen, 38 candidate targets were identified by mass spectrometry (MS), including 13 new substrates that were verified by *in vitro* ERK phosphorylation. Further studies of one of them, the nucleoporin Nup50, revealed an unexpected role of ERK signaling in the regulation of nucleocytoplasmic transport [46]. Thus, quantitative phosphoproteomics strategies combining 2D-DIGE with various methods can provide new insights into certain cellular processes.

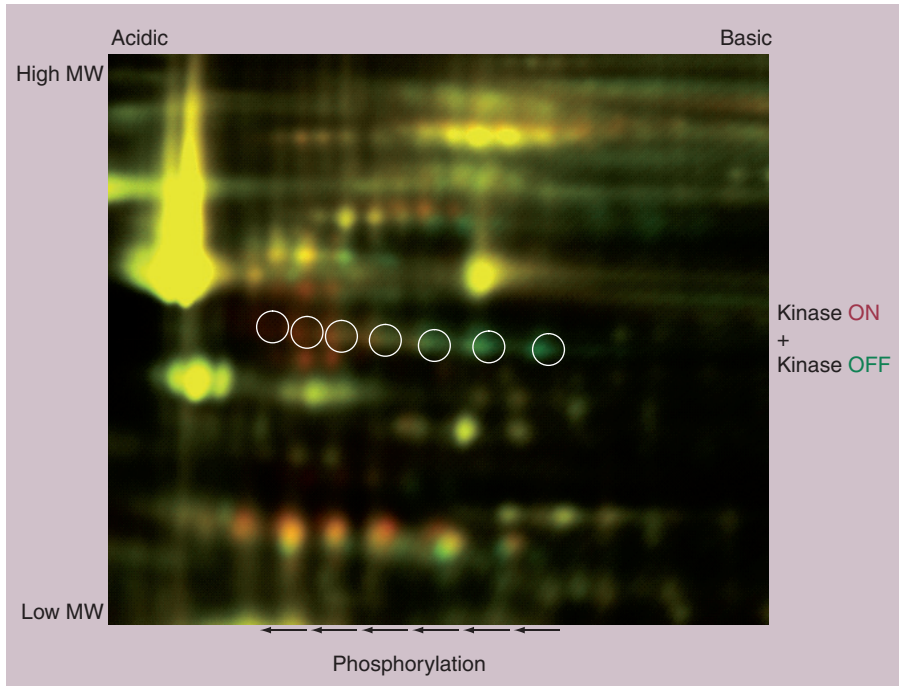

Figure 1. Detection of changes in phosphorylation by 2D difference gel electrophoresis. Phosphoprotein fraction from kinase-activated or -inhibited cells was labeled with Cy5 (shown in red) or Cy3 (shown in green), respectively. The paired samples were combined and separated on the same 2D gel, which was scanned at different wavelengths to visualize spot patterns. The overlaid image clearly shows spots of kinase targets with phosphorylation-dependent acidic shifts. Various stoichiometries of phosphorylation give multiple spots in a row with short intervals (circles).
MW: Molecular weight.
Data from [Kosako H, Unpublished Data].

Strategies for quantitative phosphoproteomics using LC-MS/MS

Shotgun proteomics, where a peptide mixture from a sample is analyzed by LC-MS/MS without separating proteins on gels prior to the analysis, is a robust and high-throughput method and enables the identification of thousands of proteins in a single analysis. LC-MS/MS analysis can detect and characterize various post-translational modifications, including phosphorylation [47]. Together with the improvement in phosphopeptide enrichment methods, LC-MS/MS analysis has been widely used for large-scale phosphoproteomic analysis. Furthermore, a variety of quantitative methods for LC-MS/MS analysis have been developed and successfully applied to quantify phosphopeptides from various cells and tissues. Thus, quantitative phosphoproteomics using LC-MS/MS has become the method of choice to analyze changes in phosphorylation at specific sites upon ligand stimulation and/or drug treatment instead of analyzing phosphoproteins, where the phosphorylation sites are undetermined. Strategies for quantitative phosphoproteomic analysis using LC-MS/MS are depicted in Figure 3.

Phosphopeptide enrichment

Because of the small amounts of phosphopeptides in digested peptide mixtures from total cell/tissue lysates, they need to be enriched prior to LC-MS/MS analysis to make the identification more efficient. Although many different methods for phosphopeptide enrichment have been reported, IMAC and metal oxide chromatography (MOC) are now widely used. The IMAC method is based on the high affinity of phosphates to certain trivalent metal ions [48] and has been further developed and improved by many others [49–53]. Posewitz and Tempst compared the capacity and selectivity of trivalent ions, including Fe^{3+}, Ga^{3+}, Al^{3+} and Zr^{3+}, for phosphopeptide binding and concluded that iminodiacetate columns coupled with Ga^{3+} resulted in the best selectivity [52]. However, the main problem with IMAC is the nonspecific binding of acidic peptides. To prevent this, Ficarro *et al.* performed methylation of all acidic groups in peptides prior to IMAC enrichment [53]. As a result, the selectivity of IMAC for phosphopeptides was increased and more than 216 phosphopeptides and 383 phosphorylation sites in a yeast lysate were successfully identified [53]. Different phosphopeptide enrichment kits are now available from several companies, including Waters, Sigma, Perkin Elmer, BD Biosciences and Thermo Fisher Scientific, among others [54–57]. The authors compared four commercially available kits and found that MassPREP™ from Waters was the most suitable for our purposes in terms of sensitivity, specificity and feasibility [57]. This kit enabled the routine identification of 1000 phosphopeptides by LTQ-Orbitrap™ in a single analysis from 500 µg cell lysate.

The MOC using titanium dioxide (TiO_2) or zirconium dioxide (ZrO_2) has emerged as an alternative for phosphopeptide enrichment [58–60]. Pinkse *et al.* developed a selective and robust technology for large-scale phosphoproteomics using TiO_2 [58], although acidic peptides still caused contamination during enrichment. To prevent this, competitive binders such as aromatic modifiers (2,5-dihydroxybenzoic acid [DHB] and phthalic acid [59]) or aliphatic hydroxy acids (lactic acid and 3-hydroxypropanoic acid [61]) were used in the buffers during enrichment. These competitive binders significantly improved the selectivity of TiO_2 for phosphopeptides.

Strong cation exchange (SCX) chromatography is another approach to enrich phosphopeptides. A typical tryptic peptide has

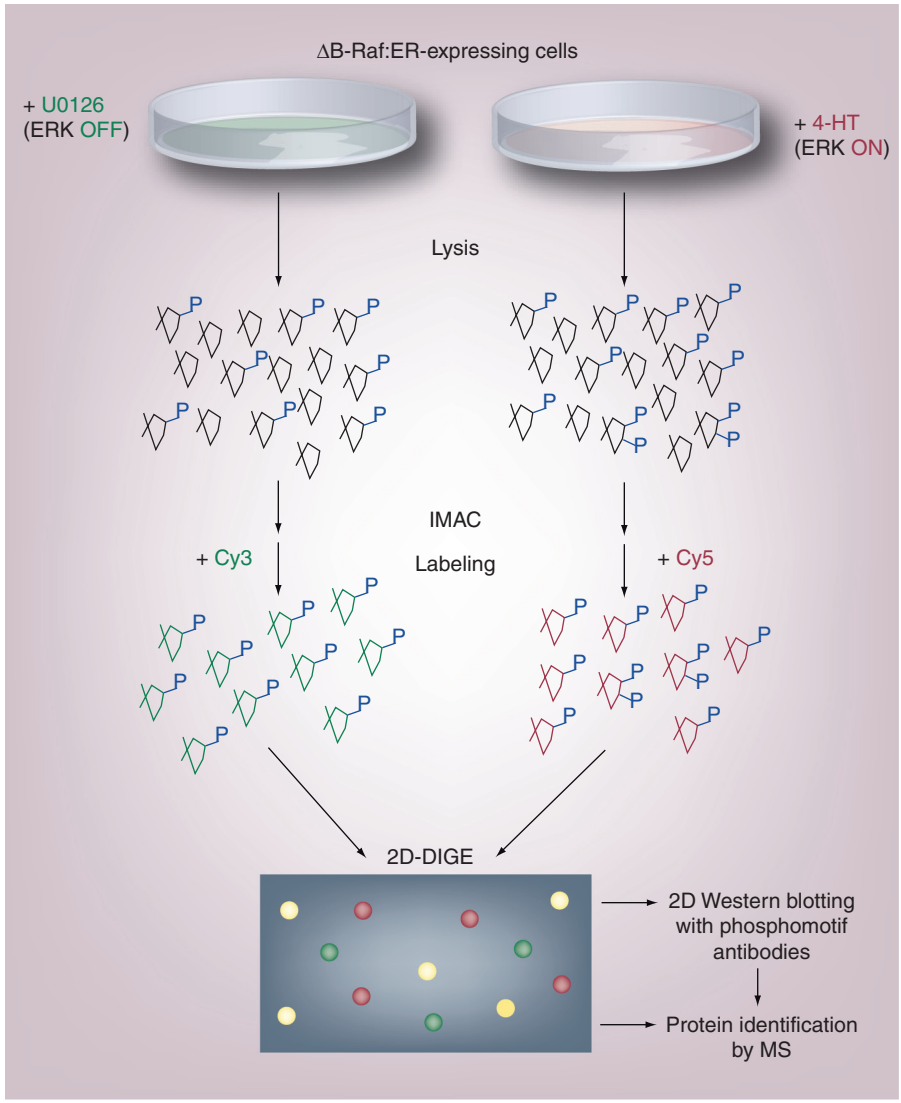

Figure 2. Phosphoproteomics strategy for identifying ERK substrates.
Fibroblastic cell lines expressing ΔB-Raf:ER were treated with 4-hydroxy-tamoxifen to activate the Raf–MEK–ERK pathway or with the MEK inhibitor U0126 to inhibit the pathway. Cell lysates were subjected to IMAC to enrich phosphoproteins, followed by labeling with different fluorophores. After 2D-DIGE analysis, changed protein spots were characterized for reactivity to ERK phosphomotif antibodies and identified by MS. Various types of phosphomotif-specific antibodies are available from Cell Signaling Technology, and this strategy may be applicable to other protein kinases.
IMAC: Immobilized metal ion-affinity chromatography; MS: Mass spectrometry.

a net charge of +2 at pH 2.7, because the N-terminal amino group and the C-terminal arginine or lysine side chain are protonated. When the peptide is phosphorylated, the negatively charged phosphate group reduces the charge state by one. Thus, phosphopeptides can be enriched using an ion-exchange column. Indeed, the +1 SCX fractions were highly enriched in phosphopeptides [62], and the analysis of these fractions by LC-MS/MS on an ion trap led to the identification of more than 2000 phosphopeptides from a HeLa cell nuclear extract. Combination of SCX with IMAC and/or MOC was successfully applied to several large-scale phosphoproteomic analyses [63–67].

Hydrophilic interaction chromatography (HILIC) is also used to separate phosphopeptides. In this method, peptides are loaded onto the HILIC column in an organic solvent and eluted with a gradient of aqueous solvent. Using this method, more than 1000 phosphorylation sites from 300 µg of HeLa cell lysate were identified [68].

Anti-phosphotyrosine antibodies are well established and often used for enrichment of tyrosine-phosphorylated proteins. Immunoprecipitation with the anti-phosphotyrosine antibody followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis or direct LC-MS/MS analysis has been performed in many studies [69–71]. However, this analysis alone cannot determine the phosphorylation sites. Furthermore, many proteins are copurified by specific or nonspecific interaction, making it difficult to know whether the identified proteins are indeed tyrosine-phosphorylated or have been isolated by their interaction with phosphorylated proteins. Rush *et al.* and Rikova *et al.* performed immunoprecipitation of tyrosine-phosphorylated peptides instead of proteins, followed by LC-MS/MS [72,73]. This method enabled the identification of over 100 tyrosine-phosphorylated sites in a single LC-MS/MS analysis. The peptides identified included many phosphorylation sites of which the responsible kinases are known, such as autophosphorylation sites of tyrosine kinases, which are usually difficult to identify using IMAC or MOC.

In contrast to anti-phosphotyrosine antibodies, antibodies for phosphoserine and phosphothreonine suffer from their specificity. However, some anti-phosphomotif antibodies have sufficient specificity to immunoprecipitate proteins that contain a phosphorylated residue in the context of a specific kinase substrate, binding motif or other phosphorylation motifs. Matsuoka *et al.* immunoprecipitated phosphopeptides from DNA-damaged cells with antibodies for phospho-SQ/TQ motif and identified more than 900 regulated phosphorylation sites containing the consensus ATM/ATR substrate motif [74]. This approach is also considered to be useful to analyze other kinases-mediated signaling pathways when their phosphomotif antibodies are available.

Phosphopeptide analysis by MS

The major problem for phosphopeptide identification by MS/MS is that the O-phosphate bond in serine- and threonine-phosphorylated peptides is labile during collision-induced dissociation

(CID), resulting in a neutral loss of phosphoric acid from the precursor ion. This problem has been solved by introducing sequential fragmentation. In ion traps, the neutral loss precursor ion can be further analyzed by MS/MS/MS (MS3) [62,75]. In the neutral loss-dependent MS3 mode, when a neutral loss of phosphoric acid (-98 Da) is detected, MS3 fragmentation of the neutral loss precursor ion is triggered. This method has further evolved as multistage activation [76]. In this mode, ion species resulting from neutral losses by CID are subjected to MS/MS analysis (pseudo-MS3) without detecting the neutral loss ions. Thus, multistage activation significantly reduces analysis time and enhances the efficiency of phosphopeptide identification. However, it has recently been reported that transfer of the phosphate residue occurs in the gas phase during CID-MS/MS using ion trap, suggesting that CID-MS3 of neutral loss ions may not be used for unambiguous phosphorylation site localization [77]. More recent studies from two groups have compared the site assignments of phosphopeptides by CID spectra with those from electron transfer dissociation (ETD) [78] spectra where the phosphate group retains during analysis, and both studies conclude that the gas-phase phosphate transfer does not affect the reliability of phosphoproteomic data [79,80].

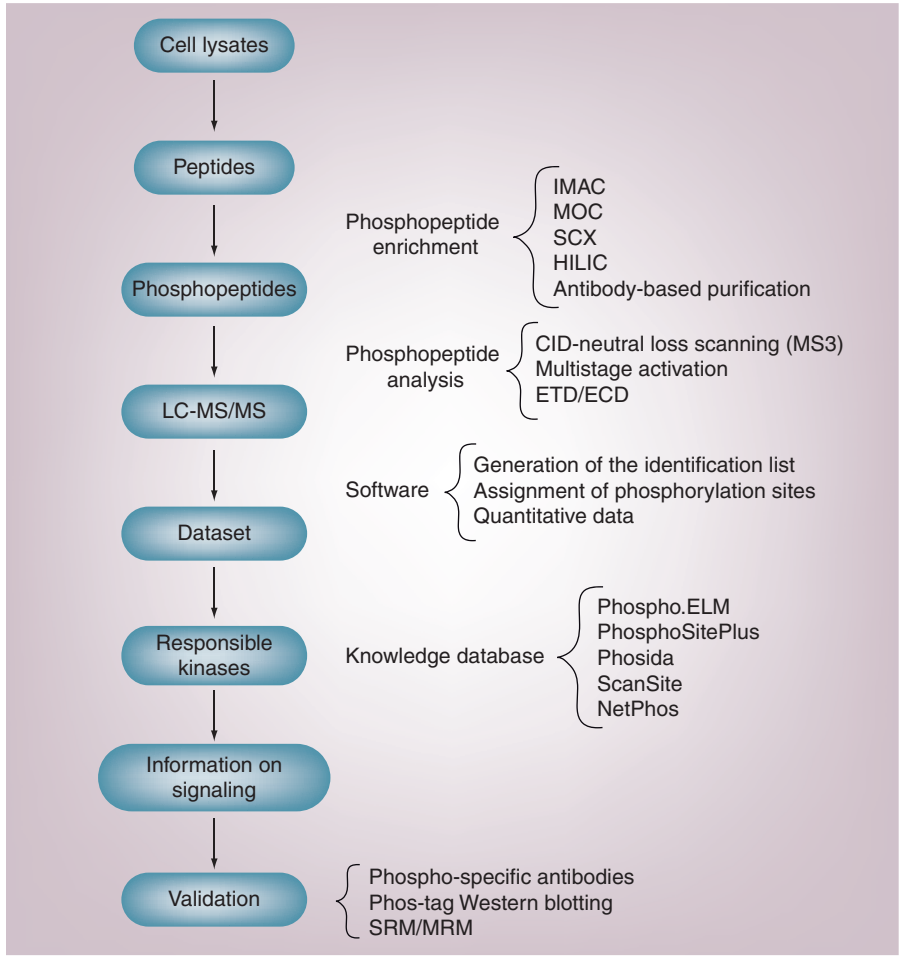

Figure 3. Strategies for quantitative phosphoproteomic analysis of cell signaling using liquid chromatography-tandem mass spectrometry. In shotgun proteomics, peptide mixtures are first prepared from cell/tissue lysates. Phosphopeptides are then enriched using various technologies and analyzed by LC-MS/MS. Large mass spectrometry datasets are then processed with software to generate the identification list with quantitative information. Because phosphoproteomics identifies the *in vivo* phosphorylation status of the substrates, knowledge databases for kinase–substrate relationships are used to predict the activation status of the corresponding kinases. To confirm the findings from quantitative phosphoproteomics, validation studies must be performed by other methods, such as phospho-specific antibodies, Phos-tag Western blotting or recently established mass spectrometry measurement, multiple reaction monitoring (also called SRM).
CID: Collision-induced dissociation; ECD: Electron capture dissociation; ELM: Eukaryotic linear motif; ETD: Electron transfer dissociation; HILIC: Hydrophilic interaction chromatography; IMAC: Immobilized metal ion-affinity chromatography; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; MOC: Metal oxide chromatography; MRM: Multiple reaction monitoring; MS3: Mass spectrometry/tandem mass spectrometry; SCX: Strong cation exchange; SRM: Selected reaction monitoring.

Alternative peptide-dissociation methods, termed ETD and electron-capture dissociation (ECD) [81], cleave the backbone N-Cα bond, generating c- and z-ions and preserve post-translational modifications that are labile upon CID, such as phosphorylation and glycosylation [78,81–86]. ECD has been equipped with Fourier transform ion cyclotron resonance, whereas more recently developed ETD comes equipped on LTQ-Orbitrap as well as other MS instruments, such as ion trap from Bruker Daltonics, Q-TOF (Waters) and FT-ICR. Although there are few studies demonstrating the impact of using ETD or ECD on phosphoproteomics, Sweet *et al.* demonstrated that the combination of ECD and CID allowed confident identification and localization of the phosphorylation sites in a large-scale phosphoproteomics experiment [87]. An optimal strategy is to combine CID of doubly charged peptides with ETD for the more highly charged peptides [88]. Swaney *et al.* performed a phosphoproteomics analysis of human embryonic stem cells using both CID and ETD with a data-dependent decision tree algorithm to select the fragmentation method based on the charge state and mass-to-charge ratio of the precursor ions, and determined 11,925 phosphopeptides in total [89,90]. They argue that ETD can identify a larger number of unique phosphopeptides than CID. Thus, both ETD and ECD have a great potential in the global analysis of phosphorylation, especially for multiply charged and multiply phosphorylated peptides.

Assignment of phosphorylation sites

Although phosphopeptides can be identified by LC-MS/MS analysis, the identified phosphorylation site is sometimes ambiguous. When an identified phosphopeptide contains consecutive serine/threonine/tyrosine residues, the MS/MS fragments generated by the cleavage of both N- and C-terminals of the identified phosphorylation site must be assigned in the MS/MS spectrum to define the position of phosphorylation precisely. However, a standard database search assigns phosphorylation sites without inspecting the MS/MS assignations around their sites, and thus manual inspection of each spectrum is often required for unambiguous determination of the phosphorylation sites. In large-scale proteomics experiments involving hundreds or thousands of phosphorylation sites, this is an extremely tedious task. Thus, algorithms have been developed to provide probability scores for each of the potential phosphorylation sites, which help in the confident assignation of those sites [75,91]. However, the use of these algorithms is limited to the peptides fragmented by CID. Although CID is the most efficient method for phosphopeptide identification, ECD or ETD is superior for localization of phosphorylation sites due to retention of phosphate groups from the peptides. Thus, the combination of ECD/ETD with CID enhanced the number of the identified phosphopeptides, as well as confidence of the site localization. Cooper *et al.* developed the algorithm termed Site Localization of Modification that allows analysis of data obtained from CID and ETD/ECD fragmentation methods [92].

Quantification methods

There are many quantification methods in LC-MS/MS analysis, as summarized in Figure 4. Which method should be selected depends on the accuracy required, the sample source (from cultured cells or tissues) and the number of samples to be compared. The easiest way is a label-free method based on the spectral counts of identified peptides. An abundant peptide is represented by a large LC peak eluted for a long time and has more chance of being analyzed by MS/MS. Thus, the number of observed spectra assigned to a particular peptide is a semiquantitative measure of the abundance of the peptide. Although the accuracy of quantification using spectral counts is not high, it is convenient for analyzing large quantitative differences between samples. Rikova *et al.* performed profiling of tyrosine phosphorylation in 41 cell lines and 150 clinical samples of non-small-cell lung carcinoma (NSCLC) [73]. They compared the tyrosine phosphorylation status of cell lines and clinical samples using spectral counts of the identified phosphopeptides. Normalization was carried out by an internal control, a phosphopeptide containing GSK3β-pY279, which is ubiquitously identified in different cell lines or tissues. As a result, clinical samples of NSCLC were classified into five groups according to the activated tyrosine kinases. They also focused on the highly phosphorylated proteins in particular cell lines and identified known and novel tyrosine kinases not previously implicated as target molecules for NSCLC. Recently, the authors performed comparative phosphoproteomics among distinct tumor cell lines in the presence or absence of nocodazole, which blocks cells in mitosis [57]. Changes of phosphorylation upon nocodazole treatment were investigated by spectral counts using a low-resolution MS instrument, LTQ, and this analysis revealed common features of phosphorylation by nocodazole in distinct cell lines. Quantification by spectral counts has been further developed to account for the different physicochemical properties of peptides, such as protein size, sequence properties and ionizability [93,94], or to include normalization of run-to-run variations [95].

Another label-free method measures the intensity of MS chromatograms. A number of methods have been developed to quantify peptides/proteins from peak heights in shotgun proteomics using an internal control [96–98]. Using high-resolution MS instruments, a peptide ion can be analyzed accurately in the low parts per million mass unit range, and it facilitates the peptide signal mapping across a few or multiple LC-MS measurements, using their mass to charge and retention time dimension. Thus, this method depends on the mass resolution, the mass precision and the consistency of the retention time to match the same peptides among different LC-MS analyses. It is essential to use a high-resolution MS, as well as a sensitive and reproducible nano-LC where the retention time of a particular peptide in crude extract behaves exactly the same. To help this quantification method, there are various matching software products that are commercially and publicly available, such as SpecArray [99], MsInspect [100], MSight [101], TOPP [102], PEPPeR [103], SuperHirn [104], MaxQuant [105], QuanLynx™ (Waters), SIEVE™ (Thermo Scientific), Elucidator® (Rosetta Biosoftware) and Expressionist® (Genedata) [106]. Ahn *et al.* performed label-free profiling of B-Raf–MEK–ERK signaling in human melanoma cells [107]. Without enrichment of phosphopeptides, their profiling was carried out in negative ionization mode using a triple quadrupole linear ion trap instrument. The -79 Da (PO^{3-}) precursor ion scanning enabled quantitative comparison between samples by measuring -79 Da signal intensity. Of 90 phosphorylation sites identified by sensitivity to the MEK inhibitor, multisite phosphorylation of a previously uncharacterized protein was further investigated, and its ERK-dependent phosphorylation was shown to be involved in melanoma cell invasion.

Relative quantification based on differential stable isotope labeling is frequently used for quantitative phosphoproteomic analyses by MS. Although many techniques have been developed, only a few methods have been used in multiple laboratories. These include isotope-coded affinity tags [108], stable isotope labeling by amino acids in cell culture (SILAC) [109] and the recently introduced chemical labeling by tandem mass tags, such as isobaric tag for relative and absolute quantitation (iTRAQ) [110] and tandem mass tags (TMT) [111]. SILAC and iTRAQ/TMT are currently the most frequently used techniques in quantitative MS-based phosphoproteomics. In SILAC, cell cultures to be compared are differentially labeled with amino acids containing stable isotopes, usually $^{13}C_6$-Lys and/or $^{13}C_6$-Arg, and normal amino acids. Lysates from differentially labeled cells are then mixed, digested with protease and analyzed by LC-MS/MS. As a result, differentially labeled peptides (light and heavy) with the same amino acid sequence are detected in the MS spectrum, and the relative abundance of the peptides derived from different samples can be compared by calculating their ratio. Mann intensively analyzed phosphorylation changes caused by stimulation and cell cycle progression using SILAC [112].

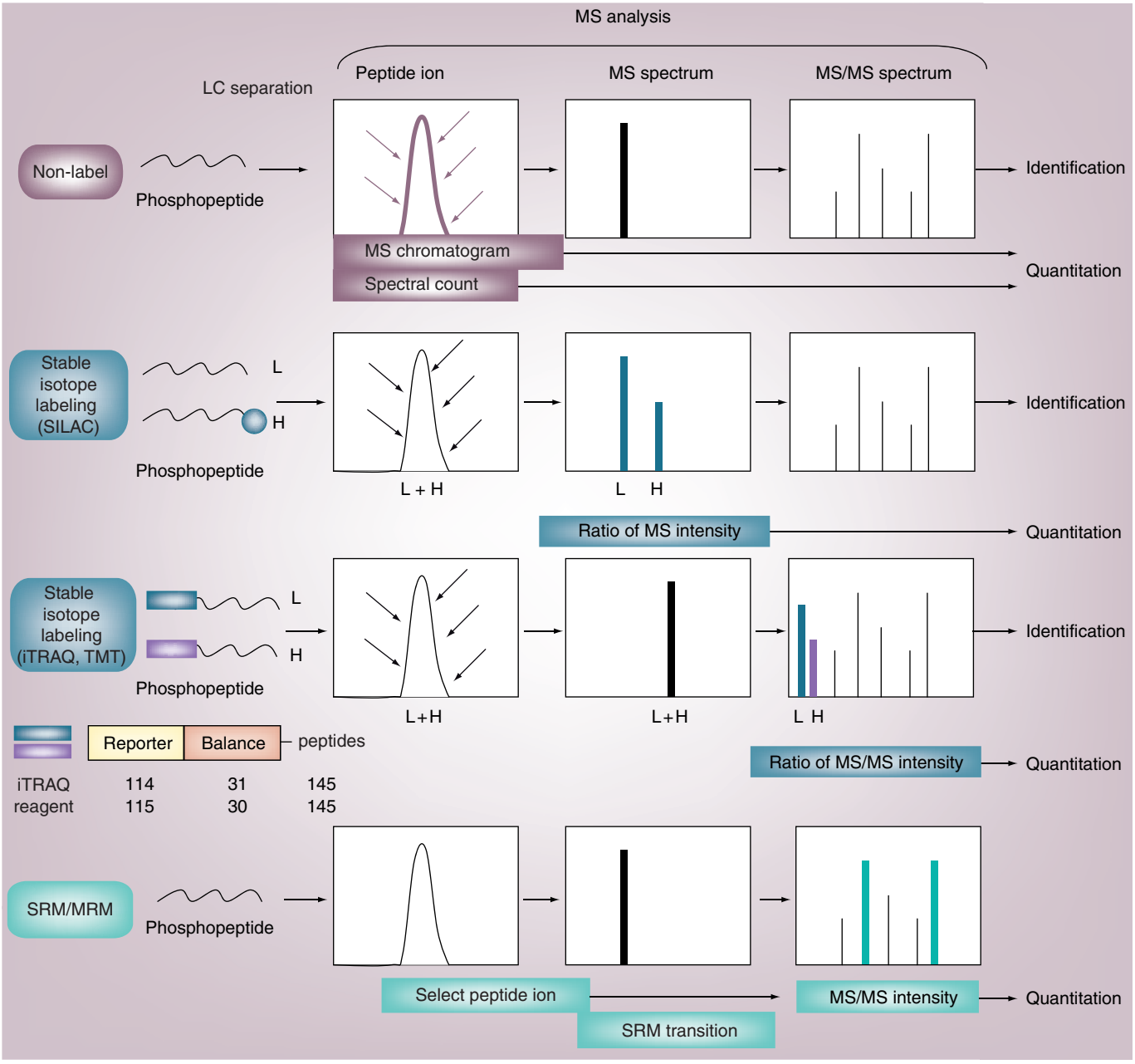

Figure 4. Quantification methods for liquid chromatography-tandem mass spectrometry analysis. There are three major methods for quantification: non-labeling (maroon), labeling with stable isotopes (teal) and SRM/MRM (turquoise). The two main non-labeling methods are based on the intensity of MS chromatograms and the spectral counts of identified peptides. Labeling methods are classified into two major groups: metabolic labeling and *in vitro* labeling. The representative of the metabolic labeling is SILAC. In SILAC, two cell cultures to be compared are differentially labeled with heavy amino acids containing stable isotopes (heavy) and normal amino acids (light). Lysates from differentially labeled cells are mixed, digested with protease and analyzed by LC-MS/MS. Differentially labeled peptides having the same amino acid sequence are detected in MS spectrum, and the relative abundance of the peptides can be compared by calculating their ratio. The representative of the *in vitro* labeling is performed using isobaric amine-specific tandem mass tags, such as iTRAQ and TMT. The iTRAQ reagent consists of reporter regions with 1 Da difference (molecular weight: 114, 115, 116…) and balance regions that adjust the molecular weight of the labeled parent ions (molecular weight: 31, 30, 29…). Each tag generates a unique reporter ion in the MS/MS spectra, and the relative abundance of the peptides can be compared by calculating their ratio. In SRM/MRM, the selected precursor to fragment ion transition is continuously monitored to diagnose for the presence of the desired ion in the sample. The abundance of the peptides is quantified by the intensity of the selected product ions in the MS/MS spectra.
H: Heavy; iTRAQ: Isobaric tag for relative and absolute quantitation; L: Light; LC: Liquid chromatography; MRM: Multiple reaction monitoring; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; SILAC: Stable isotope labeling by amino acids in cell culture; SRM: Selected reaction monitoring; TMT: Tandem mass tag.

For example, the yeast α-factor pheromone response pathway was analyzed. Phosphopeptides were enriched by a combination of SCX chromatography and IMAC, and more than 700 unique phosphorylation sites were identified [63]. This unbiased phosphoproteomic approach identified many pheromone-regulated phosphorylation sites on RNA-processing and RNA transport proteins, suggesting that the pheromone pathway has a previously unappreciated role in regulating RNA metabolism. They also applied the SILAC-based method to study phosphorylation changes in EGF-stimulated HeLa cells. After enrichment of phosphopeptides with SCX and TiO_2, temporal profiles of 6600 unique phosphorylation sites on 2244 proteins were determined, including many known members of the EGF receptor signaling pathway [64]. More recently, the cell cycle profiles of 20,443 phosphorylation sites in 6027 proteins have been determined and the site-specific stoichiometry of more than 5000 sites has been achieved by combining the results from corresponding non-phosphorylated peptides [67]. Although SILAC has mostly been limited to analyzing samples from cell culture, it has recently been extended to *in vivo* experiments by comparing tissue samples with cell lines and calculating the ratio of ratios between tissue samples [113]. Furthermore, mice were completely labeled with a diet containing stable isotope-labeled amino acid (SILAC mice), and differentially regulated proteins in knockout mice were successfully identified [114].

Isobaric tagging for relative and absolute quantitation/tandem mass tags is a recently developed protein quantification method that uses isobaric amine-specific tandem mass tags and quantification in MS/MS instead of MS spectra [110,111]. In MS spectra, the differentially labeled peptides possess the same mass by using the balance region in the tag and are represented in a combined single peak (Figure 4). However, each tag generates a unique reporter ion, and the intensities of the reporter ions in the MS/MS spectra are compared for protein quantification. iTRAQ can comparatively analyze four or eight different conditions in one experiment, whereas TMT can compare six conditions. These chemical labeling methods are suitable for the phosphoproteomic analysis of tissue and clinical samples [115]. White *et al.* performed phosphoproteomics using iTRAQ and obtained temporal changes of hundreds of tyrosine phosphorylation sites after EGF and insulin treatment [116]. Other groups also reported the identification and quantification of 700–1600 phosphopeptides using iTRAQ [117,118]. However, there are still limited reports of quantitative phosphoproteomics using iTRAQ. The main reason for this is that MS using ion traps, including LTQ-Orbitrap, which is widely used in large-scale proteomics studies, cannot detect the reporter ions of iTRAQ or TMT by conventional CID fragmentation in the MS/MS spectra. The studies successfully identifying and quantifying peptides were mainly performed by using MS instruments such as ESI-Q-TOF and MALDI-TOF/TOF. For quantification by iTRAQ/TMT using LTQ-Orbitrap, other fragmentation methods, such as higher-energy C-trap dissociation (HCD) or pulsed q-dissociation, must be used. In fact, Nilsson *et al.* succeeded in detecting quantitative changes in the IL-6 signaling pathway in glioblastoma stem cells by using TMT and HCD [119]. Although HCD provides good fragmentation of the reporter ions, the efficiency of protein identification is generally poor compared with CID. Dayon *et al.* have developed a method for detecting reporter ions of iTRAQ by HCD and identifying proteins by CID [120]. The efficiency of protein identification was significantly enhanced by this method compared with HCD alone.

Prediction of the responsible kinases

Although the current LC-MS/MS-based phosphoproteomics has enabled the identification and quantification of thousands of phosphopeptides, the kinases responsible for the majority of the identified phosphorylation sites have been uncharacterized, and thus it is difficult to obtain information on the activation status of the kinases and signaling pathways from the dataset. Therefore, it is particularly important to map kinase–substrate relationships in the phosphoproteomics dataset. In recent years, several databases that collate proteomically identified and/or biochemically verified phosphorylation sites have been generated, such as Phospho.ELM [201], PhosphoSitePlus® [202] and Phosida [121,203]. Thus, the responsible kinases may be predicted using these databases. On the other hand, consensus amino acid sequences for phosphorylation by various kinases have been intensively analyzed by *in vitro* phosphorylation assays using a combinatorial peptide library. Based on this information, several kinase prediction tools for a given substrate have been developed, which include ScanSite [204] and NetPhos [205], based on matrix motifs and neural networks, respectively. Using these databases, the large amount of phosphoproteomic data can be applied to *in silico* predictions of phosphorylation sites and their corresponding kinases [13]. Pawson *et al.* have also shown that the kinase–substrate relationship can be inferred by bioinformatic mining of linear sequence motifs in combination with other large-scale datasets [122].

Validation of findings from quantitative phosphoproteomics analysis

The proteomically identified phosphorylation changes have to be validated by other methods. Phospho-specific antibodies are useful to detect targeted phosphorylation sites and visualize their intracellular distribution [28,123]. So far, commercially available phosphospecific antibodies have been limited, and generation of these antibodies is costly and time-consuming, and sometimes results in failure. However, the number of phosphorylation sites to be verified are often in the hundreds. Therefore, the majority of phosphoproteomic data have not been validated or utilized effectively for functional analysis of phosphorylation-mediated cell signaling.

Recently, phosphate-affinity polyacrylamide gel electrophoresis has been developed to detect stoichiometric protein phosphorylation [124]. The phosphate-affinity site is a polyacrylamide-bound dinuclear Mn^{2+} complex (Mn^{2+}-Phos-tag) that can enhance mobility shifts of phosphorylated forms of many proteins. Phosphorylation levels of cellular proteins of interest can be assessed by subsequent Western blotting [125]. This Phos-tag Western blotting would be useful to evaluate the obtained phosphoproteomic data when antibodies are available.

As an alternative to the data-dependent operation of MS, an operation mode termed selected reaction monitoring or multiple reaction monitoring, which continuously monitors a selected precursor to fragment ion transitions, has emerged as a highly reproducible and sensitive method for quantifying targeted peptides [126–128]. Although full-scan MS/MS spectra are not acquired in this mode, which is often operated on a hybrid triple quadruple/linear ion trap instrument, the method enables the quantification of hundreds or thousands of targeted peptides with an approximately tenfold increase in sensitivity over pre-existing methods, such as neutral loss scanning or precursor ion scanning. White *et al.* applied this to quantify temporal profiles of 222 tyrosine-phosphorylated peptides across seven time points following EGF treatment [127]. With this approach, 88% of the targeted signaling molecules were reproducibly quantified; a greatly improved reproducibility compared with conventional data-dependent analysis of 34%. Other studies also used selected reaction monitoring/multiple reaction monitoring for validation of the quantitative changes of a specific phosphorylation [129–132].

Expert commentary

Comparative studies have revealed that different proteomic strategies are complementary to each other. For example, different phosphopeptide enrichment methods show distinct and partially overlapping preferences in phosphopeptide recovery [133]. Furthermore, phosphoproteomic profiling of the ERK pathway using 2D-DIGE [28], label-free precursor ion scanning [107] and SILAC [134] identified surprisingly different subsets of ERK targets. Thus, a combination of various phosphoproteomic strategies, such as LC-MS/MS, 2DE and protein chips, can increase the reliability and comprehensiveness of the data obtained. The unique advantages and drawbacks of different strategies are summarized in **Box 1.**

Despite the vast amount of quantitative phosphoproteomic data generated in recent studies, validation of these data has been quite limited. Furthermore, most of these studies revealed minimal mechanistic insight into the functional role of the kinase-regulated phosphorylation sites. Further advances in bioinformatics and computational biology that can integrate genome, transcriptome, proteome, metabolome, interactome and phenome data will extract useful information about functional phosphorylation. Detailed biochemical and cell biological analyses of selected phosphorylation sites and their responsible kinases are clearly important for elucidating the regulatory networks of cellular functions.

Five-year view

Recent dramatic advances in quantitative phosphoproteomics using LC-MS/MS have provided huge amounts of information about signal-dependent phosphorylation events [135]. Further advances in MS instrumentation and other related analytical tools will provide much more information. In particular, absolute quantification of phosphopeptides and determination of phosphorylation stoichiometry will progress in the future. Such LC-MS/MS-based phosphoproteomic analysis can be complemented by 2DE-based analysis, which has been strengthened by recent technologies including 2D-DIGE, phosphoprotein-specific staining and anti-phosphomotif antibodies.

Box 1. Comparative summary of the main advantages and limitations of quantitative phosphoproteomics based on the respective technologies.

2D-DIGE

Advantages
- Easy determination of phosphorylation stoichiometry
- Validation by matching with 2D Western blot
- Simple and accurate quantification
- Widely adaptable

Limitations
- Low throughput
- Relatively small number of identified proteins
- No information about phosphorylation sites

SILAC

Advantages
- High throughput
- Large number of identified peptides and phosphorylation sites
- Accurate quantification

Limitations
- Generally limited to cell culture
- Unintended metabolic interconversion of isotopic amino acids
- Difficulty in determining phosphorylation stoichiometry

iTRAQ/TMT

Advantages
- High throughput
- Large number of identified peptides and phosphorylation sites
- Widely adaptable

Limitations
- Possible errors during the prefractionation process
- Difficulty in determining phosphorylation stoichiometry

iTRAQ: Isobaric tag for relative and absolute quantitation; SILAC: Stable isotope labeling by amino acids in cell culture; TMT: Tandem mass tag.

Thus, databases that collect the obtained phosphoproteomic data will expand further, and *in silico* analysis may accurately predict kinase–substrate relationships in specific cell signaling. Of course, biochemical validation and cell biological examination are required to elucidate new molecular mechanisms in the kinase-mediated signaling pathways. In addition, collected phosphoproteomic data can be used for systems biology to understand spatiotemporal regulation of cell signaling at the network level.

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Key issues

- The large-scale identification and quantification of phosphoproteins and their phosphorylation sites under different biological conditions are essential to fully understand complex signaling networks.
- Classical 2DE and multiplexing 2D-DIGE remain powerful technologies for phosphoproteomics by combining them with prefractionation and other methods.
- Gel-free liquid chromatography-tandem mass spectrometry analysis has become a robust and high-throughput method for quantifying numerous phosphopeptides with continuous improvement of various tools.
- Prefractionation of proteins or peptides to reduce sample complexity is important for both 2DE- and liquid chromatography-tandem mass spectrometry-based phosphoproteomics to globally detect low-abundance phosphorylation events.
- Bioinformatic analysis using several databases may predict responsible kinases for the identified phosphorylation sites.
- The obtained phosphoproteomic data should be validated by phospho-specific antibodies, Phos-tag Western blotting and selected reaction monitoring/multiple reaction monitoring, among others.
- Further biochemical and cell biological analysis of the identified phosphorylation events can unravel novel mechanisms in protein kinase-mediated cell signaling.

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