

REVIEW

Towards functional phosphoproteomics by mapping differential phosphorylation events in signaling networks

Sergio de la Fuente van Bentem¹, Wieslawa I. Mentzen², Alberto de la Fuente² and Heribert Hirt^{1,3}

¹ Department of Plant Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Vienna, Austria

² CRS4 Bioinformatica, Pula, Italy

³ URGV Plant Genomics, Evry, France

Protein phosphorylation plays a central role in many signal transduction pathways that mediate biological processes. Novel quantitative mass spectrometry-based methods have recently revealed phosphorylation dynamics in animals, yeast, and plants. These methods are important for our understanding of how differential phosphorylation participates in translating distinct signals into proper physiological responses, and shifted research towards screening for potential cancer therapies and in-depth analysis of phosphoproteomes. In this review, we aim to describe current progress in quantitative phosphoproteomics. This emerging field has changed numerous static pathways into dynamic signaling networks, and revealed protein kinase networks that underlie adaptation to environmental stimuli. Mass spectrometry enables high-throughput and high-quality analysis of differential phosphorylation at a site-specific level. Although determination of differential phosphorylation between treatments is analogous to detecting differential gene expression, the large body of statistical techniques that has been developed for analysis of differential gene expression is not generally applied for detecting differential phosphorylation. We suggest possible improvements for analysis of quantitative phosphorylation by increasing the number of biological replicates and adapting statistical tests used for gene expression profiling and widely implemented in freely available software tools.

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1 Introduction

Reversible protein phosphorylation in multicellular organisms is mediated by the opposite action of large families of protein kinases and protein phosphatases. The MS-based

studies have given great insight into the number and diversity of phosphorylation events. A revolution was caused by the development of large-scale, gel-free approaches that enabled the identification of multiple phosphorylation sites from small protein amounts by MS [1]. The number of sites identified in single studies almost double each year, revealing the complexity of the phosphoproteome of multicellular organisms by reporting more than 10 000 phosphorylation sites on several thousand proteins in a single cell line [2]. Such analyses are enabled by the development of protocols for highly selective phosphopeptide isolation from complex starting samples [3]. Although phosphopeptide sequencing by MS has met some difficulties [4], new technological developments have considerably facilitated this process [5].

Correspondence: Professor Heribert Hirt, Department of Plant Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Dr.-Bohr-Gasse 9, 1030 Vienna, Austria

E-mail: heribert.hirt@univie.ac.at

Fax: +43-1-4277-9546

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; FDR, false discovery rate; RTK, receptor tyrosine kinase; SILAC, stable isotope labeling by amino acids in cell culture; TiO₂, titanium dioxide

During the last years, MS-based studies have provided a wealth of information on the protein phosphorylation events in organisms ranging from bacteria to humans. One of the outcomes is that protein phosphorylation can occur extremely rapid: For example, initiation of receptor tyrosine kinase (RTK) phosphorylation starts within seconds after stimulus application [6]. Moreover, it turned out that protein phosphorylation is an abundant process: multicellular eukaryotes contain hundreds to more than a thousand of protein kinases that collectively target an immense number of sites. Another result of these studies is that protein phosphorylation is an extremely complex mechanism: it was found that a given phosphoprotein is often targeted by distinct kinases at different sites. Moreover, some sites are targeted by several different kinases [7]. Phosphorylation events can have major impacts on the conformation [8] or charge of a protein [9, 10], thereby regulating protein activity, localization and stability. In addition, phosphorylation sites can connect proteins to downstream events by direct interaction with signaling proteins that contain specific phosphomotif-binding protein domains [11]. The analysis of the abundance of phosphorylation sites revealed that the large majority occurs on serine and threonine residues. Despite the fact that tyrosine phosphorylation is of low abundance, it is very dynamic [12–14] and can build extensive networks with interaction partners through their specific binding domains, such as Src homology 2 domains [15–18] and phosphotyrosine-binding domains [11].

Given the large number of phosphorylation events and phosphoproteins identified, the next question to tackle is how kinases choose between potential substrates [7]. Although it was originally thought that protein kinases should work like typical enzymes, *i.e.* that they should phosphorylate a large number of substrates in a minimum of time, it became apparent that most protein kinase-substrate interactions do not obey this rule, and are mostly occupied in excluding unwanted phosphorylations. The spatiotemporal regulation of components of signaling networks is extremely important for coordinating kinase specificity [19]. Therefore, the compartmentalization of protein kinases with their substrates, for instance by targeting to specific membranes, contributes to this process [7, 20].

Quantitative phosphoproteomic analyses have provided great insight into signaling networks and allowed to generate models for functional testing. In this review, we excluded the discussion of quantitative proteomics methods using antibody microarrays [21], protein chips [22, 23], or gel-based approaches [24, 25]. Instead, we focus on quantitative phosphoproteomic analysis by gel-free methods coupled to MS. For subsequent data analysis, we propose to apply statistical methods that are already well established in transcriptome research.

2 Complexity and regulation of phosphoproteomes: How many sites make up a phosphoproteome?

Because of the low abundance of phosphopeptides in starting samples, phosphopeptides must be isolated specifically from such complex mixtures. Most commonly, phosphopeptides are captured from complex peptide mixtures by IMAC [1], titanium dioxide (TiO₂) [26, 27] or phosphoramidate chemistry (PAC) [28, 29]. Each of these methods has a bias towards a subfraction of the proteome [30, 31], and the combined use of these three methods recently allowed the identification of more than 10 000 sites in a single fruit fly (*Drosophila melanogaster*) cell type [2]. Although this study combined phosphosites detected in samples isolated during different growth conditions of the cells, this suggests that the estimation that around 30% of all proteins can become phosphorylated at any given time holds true [2]. However, it is likely that this number could be as high as 50%, because the use of a single protease to digest the proteins and the intrinsic limitations of mass spectrometry restricted the coverage of the proteome in this study [2]. The phosphoproteome of a multicellular eukaryote may thus consist of 50 000–100 000 sites. Large-scale studies suggest that tyrosine phosphorylation represents an estimated 1–3% of the total phosphoproteome in animals [2, 32]. If this were true, the identified 4551 phosphotyrosine sites from lung cancer cells [33] suggest that 100 000 sites is a reasonable estimation. Whether plants, with a protein kinase repertoire twice as large as humans [34], contain even more complex phosphoproteomes remains to be determined.

In contrast to the highly complex phosphoproteomes of eukaryotic organisms, the phosphoproteome of the bacteria *Bacillus subtilis* and *Escherichia coli* are far less complex. In comparable approaches, when 6600 phosphorylation sites were identified on human proteins [32], only 78 and 81 sites were detected on a similar number of *B. subtilis* and *E. coli* proteins [35, 36]. These studies revealed that protein phosphorylation on serine, threonine and tyrosine residues is ancient, but that diversification of protein kinases and their targets is a hallmark of eukaryote evolution.

3 Experimental approaches for quantitative MS-based phosphoproteomics

Now that different techniques allow automated large-scale mapping of phosphorylation sites [37], research shifted towards a more functional approach: to quantify changes in the phosphoproteome. Techniques used for quantitative (phospho)proteomics [38, 39] and software programs to support analysis of data generated by quantitative proteomic approaches [40] have been described elsewhere and will not be discussed in much detail in this review.

Since quantitative analysis of the entire phosphoproteome is not yet feasible, pre-fractionation of samples is required. In addition to the specific phosphopeptide isolation methods such as IMAC and TiO₂, protein samples have been pre-fractionated to focus on cytosolic, plasma membrane or nuclear compartments. In addition, peptide mixtures have been simplified by different methods such as strong cationic exchange chromatography, immunoprecipitation with anti-phosphotyrosine antibodies [12, 38] and hydrophilic-interaction chromatography [5]. By these means, early events were unraveled for many signaling networks. Time courses have allowed the separation of phosphorylation events into (very) early *versus* intermediate and late processes [6, 32, 41].

3.1 Techniques for quantitative proteomics

Workflows for MS-based quantitative proteomics and their benefits and pitfalls have recently been described [38, 40]. Here, we give a short overview of several commonly and successfully used methods that allow relative quantification of peptides. Approaches to absolutely quantify peptides [42, 43] are much more complicated, but targeted [44] and large-scale [45] absolute quantification determined that the stoichiometry of phosphorylation sites lies between 5 and 100%. This information is crucial to interpret quantitative analysis of individual sites, as a twofold increase can indicate an increase from 2 to 4%, but also from 50 to 100%. Label-free quantification is also possible, albeit more time-consuming [46]. Stable isotope labeling usually gives the most reliable results for quantification. *In vivo* labeling excludes the need to handle the samples separately since samples are pooled before protein purification.

Stable isotope labeling by amino acids in cell culture (SILAC) is an *in vivo* labeling technique that labels proteins with isotopic amino acids such as ¹³C₆-Arg or -Lys and ¹⁵N₄-Arg or -Lys [47, 48]. Additionally, the isotopes ¹³C₆-¹⁵N₄-Arg or -Lys can be used to obtain an extra time-point sample. SILAC has been extensively used for (phospho)-protein quantification and already more than 80 articles were published at the beginning of 2008 (<http://www.silac.org/publications>). SILAC with ¹³C₆-Arg suffers from metabolic conversion of Arg to Pro [49], but depending on the amount used for labeling this does not always occur extensively [50]. This method was believed to be only applicable to dividing cell culture systems, but non-dividing, differentiated cells have now been successfully SILAC-labeled and used to study brain-derived neurotrophic factor-induced signaling [51]. SILAC-based studies have provided the greatest numbers in terms of phosphopeptide quantifications in independent studies, but demanded many MS analyses [13, 32, 52–55].

Another commonly used method is based on isobaric tags for relative and absolute quantitation (iTRAQ), which is a four- or eight-plex reaction to label N termini of peptides *in vitro* after digestion [56, 57]. A main benefit is that the multiplex iTRAQ reagents allow time-course profiling

of phosphorylation-dependent signaling by applying a single reaction to a mass spectrometer. A drawback is that labeling occurs at a late step during the protocol (after protein digestion), which can introduce some technical variation.

In vivo labeling of proteins with ¹⁴N/¹⁵N pairs can be achieved by supplying ¹⁴N- and ¹⁵N-containing amino acids (in case of SILAC) or K¹⁵NO₃ and (¹⁵NH₄)₂SO₄. The latter two compounds can be used for labeling of plant cells [58] or entire plants [59], but in this case hampers automated assignment of heavy and light peptide pairs in MS analysis because the number of N atoms *per* peptide differs [60, 61]. The ¹⁴N/¹⁵N labeling has been successfully used for yeast, plants and animals [62], and used for instance for quantitative phosphoproteomic profiling of the tumor necrosis factor pathway [63] and for plant-defense signaling [58].

3.2 Changes in the phosphoproteome induced by stimuli

Large-scale quantitative analysis has shown that 10–20% of the phosphorylation sites change in abundance within 30 minutes after application of one specific stress [32, 52, 64, 65]. Most changes are less than tenfold, suggesting that phosphoproteome dynamics is expressed in changes of threshold levels rather than on-off phosphorylation. Phosphorylation cascades are activated within seconds after perception of signals [6], and most changes occur transiently within 30 min [13, 32].

In one of the first studies using SILAC, changes in the yeast phosphoproteome in response to pheromone were reported [64]. The use of a mass spectrometer with high mass accuracy enabled the identification of 139 differentially regulated phosphopeptides, of which 89 were induced and 42 down-regulated after 2 h of pheromone treatment. Several known pheromone-induced phosphorylation events in the canonical pheromone signaling pathway were observed, such as tyrosine phosphorylation of the mitogen-activated protein kinase Fus3p and its substrate Far1p. Moreover, many novel events in this already well-studied pathway were discovered [64].

Benschop *et al.* [58] aimed to unravel the immediate signaling responses to two pathogen-derived elicitors that are both recognized by plasma membrane receptors. Distinct but overlapping sets of phosphorylation events were triggered by these signals, suggesting that signaling specificity is rapidly translated into different phosphorylation profiles [58]. A complementary study focused on differential phosphorylation of plasma membrane proteins induced by the bacterial pathogen-derived elicitor flagellin in the plant *Arabidopsis thaliana* [66]. Despite the differences in experimental setup and analysis (a combination of *in vitro* iTRAQ labeling and IMAC *versus in vivo* ¹⁴N/¹⁵N labeling and TiO₂), five of the 11 non-redundant differential phosphopeptides were also quantified by Benschop *et al.* [58] and all were found to be induced as well.

4 Integrating information to construct phosphorylation networks

To establish phosphorylation-dependent signaling cascades one needs to identify both the phosphorylation events and link these to the upstream kinases and phosphatases. Whereas traditionally substrates were identified from the kinase as starting point, now phosphorylation sites are described without having direct knowledge of the responsible kinases. A daunting task remains to enable high-throughput discovery of direct links between substrates and protein kinases and phosphatases.

4.1 MS-based screening for protein kinase targets

The phosphosite with one or more surrounding amino acids together constitute the kinase motif, which is an essential piece of information to link a phosphosite to the upstream kinase. These usually consist of one or a few required residues in close vicinity of the phosphorylated amino acid [7]. Kinase motifs are, however, not enough for the assignment to the responsible kinase, and recently it was found that contextual information is essential [67]. Large-scale analyses such as gene co-expression and protein-protein interactions provide such information and in many cases allow linkage of a particular kinase isoform to specific phosphorylation sites [67]. The *networKIN* algorithm takes context into account, thereby enabling the construction of protein phosphorylation networks [67]. Using data obtained from protein and peptide chips aids in this analysis [23, 34, 38, 68]. Optimization of this algorithm promises to contribute significantly to connect the continuously increasing number of phosphorylation sites to specific kinases.

Two experimental approaches have recently identified ataxia-telangiectasia, mutated (ATM) and ATM and Rad3-related (ATR) kinase networks involved in DNA damage signaling. A first study described 700 putative ATM and ATR targets by using 68 phosphomotif (pSQ/pTQ)-specific antibodies and subsequent MS-based identification of the target sites [69]. This elegant approach shows that antibodies against phosphomotifs, in addition to phosphotyrosine antibodies, enable the selection of subpopulations of the phosphoproteome specific to a kinase (group). In a second study, the phosphorylation network induced by UV light was examined and overlapped with pSQ/pTQ sites identified from UV-treated cells by a peptide affinity approach using antibodies [70]. In addition, UV-induced phosphorylation was measured in control and ATR-deficient cell lines. The 19 UV-induced SQ/TQ sites were found to decrease in the ATR mutant line and are thus good ATR candidate target sites [70]. Moreover, these studies provide a rich source of information to train the *NetworKIN* algorithm [67].

4.2 Differential phosphorylation profiling enables mapping of signaling cascades

Globally oriented approaches have identified numerous known and novel phosphorylation events in classical signaling pathways, even in well-studied cascades. A global approach using quantitative MS recently showed aspects of cell migration [71]. Spatial organization of signaling networks was unraveled by phosphopeptide isolation from non-motile and motile compartments of chemotactic cells. Subsequent relative quantification of 3509 proteins and 228 phosphorylation sites revealed great insight into cell motility and spatial regulation of signaling networks.

Large-scale temporal analysis of tyrosine phosphorylation events during immune responses in mast cells was conducted [72]. Relative phosphorylation ratios of hundreds of sites were determined across nine time points in two different cell lines. Phosphorylation sites were clustered using fuzzy *k*-means according to their dynamic behavior during the immune response [72]. Others have clustered phosphorylation profiles using self-organizing maps [13] or fuzzy *c*-means [32].

One of the best-described signaling pathways is the one downstream of the epidermal growth factor receptor (EGFR) [73, 74]. Dimerization, trafficking through endosomal compartments and turnover is critical for regulation of EGFR signaling to downstream effectors [75, 76]. This highlights the complexity and stringency of regulation of EGFR signaling to execute proper physiological responses. Activation of the EGFR leads to differential phosphorylation on serine, threonine and tyrosine residues of more than a thousand proteins [13, 32, 52–55].

4.3 Modeling of phosphorylation-dependent signaling pathways

Proteomics data are very suitable to be analyzed by systems biology approaches to establish and model pathways [77]. Protein phosphorylation events in the ERBB2/HER2 and EGFR signaling network were combined with protein-protein interaction data and modeled, revealing novel links [78].

In two elegant studies, Wolf-Yadlin *et al.* [79] and Kumar *et al.* [80] showed the effect of ERBB2/HER2 overexpression on the tyrosine phosphoproteome after EGF or heregulin (HRG) application. By a quantitative MS-based approach, they identified a large number of regulated phosphosites, and they analyzed the relationship between specific phosphorylation sites and downstream biological behavior. This showed that EGF and HRG activate multiple or single pathways, respectively, to stimulate the same biological response (migration). Moreover, using partial least-squares regression (PLSR) analysis, a reduced number of phosphosites most strongly correlating with proliferation and migration were discovered and termed a “network gauge” [79, 80]. Such an approach might prove to be extremely important for selecting candidates for targeted experiments in future research.

4.4 Using knowledge of the phosphoproteome for cancer therapy

The challenge to use obtained networks for novel drug development has been initiated. Mutations in RTKs are involved in many cancers and are successful drug targets [81]. This allows dissection of drug actions and perhaps aid in further drug optimization and development. EGFR and c-MET are RTKs that share part of an extensive signaling network involving many tyrosine phosphorylation events, which collapse by application of their inhibitors [53]. EGFRvIII is a hyperactive EGF receptor mutant that plays an important role in glioblastoma (aggressive brain tumor) behavior, and confers resistance against the EGFR inhibitor gefitinib. Huang *et al.* [54] dissect the phosphotyrosine signaling network triggered by EGFRvIII. The authors carefully examined the network and identified c-MET as being activated in EGFRvIII-carrying glioblastomas. The c-MET amplification bypasses inhibition of hyperactive EGFR mutants by gefitinib through activating a cell survival pathway [82]. Resistance against EGFR inhibitors occurs in most glioblastomas and lung cancers with activating EGFR mutations, but can be defeated by a combinatorial application of both EGFR and c-MET inhibitors [54, 82]. Thus, chemoresistance can be caused by coexpression of different active RTKs, and phosphorylation profiling of RTKs in cancer cells can reveal targets for such therapies [33, 54, 82]. These systematic approaches are thus capable of providing handles to combat cancer [83].

Many chemical kinase inhibitors have been used to treat cancers. Although several have been used successfully for many years now, major questions have remained about the mechanisms underlying side effects and drug resistance. Quantitative analysis of kinase inhibitors has recently shown that most clinically used kinase inhibitors are aspecific [84, 85]. A large-scale, semiquantitative approach to profile tyrosine phosphorylation showed the expression of active RTKs in different lung cancers, implicating these RTKs in the disease [33]. As mentioned above, these kinases are attractive targets for therapeutic use. In conclusion, these studies gave first insights into the specificities of these cancer drugs and how they affect downstream signaling pathways and may ultimately serve the treatment of cancers.

5 Statistical methods for deciding differential phosphorylation

With the introduction of highly accurate mass spectrometers, it is feasible to obtain statistically significant protein identifications. However, proteins are usually identified by at least two peptides, whereas phosphorylation sites only by single peptides. Since different phosphorylation sites on the same protein usually behave differently [32], each phosphosite has to be evaluated and quantified individually. In addition, quantification of multiply phosphorylated peptides has to be carefully evaluated because of the distinct behavior of

individual phosphosites. Here, we review and discuss statistical issues related to deciding when a phosphosite is differentially phosphorylated between two conditions. The basic question that we seek to answer here is whether there is a significant difference between the degrees of phosphorylation of the phosphosite at two conditions (*e.g.* stressed *vs.* non-stressed, mutant *vs.* wild type) with the goal to find out which phosphosites have changed due to stress or mutation.

As discussed in previous sections, phosphoproteomic techniques usually produce relative rather than absolute phosphorylation changes: ratios between the areas of two peaks are taken. This is analogous to cDNA microarray analysis, where two samples are hybridized with different color dyes and loaded on one spot. The analysis of differential gene-expression has grown into a whole new branch of statistic research in recent years, and resulted in the development of many new techniques. Because of the analogy, many results of this research should be directly applicable to the emerging area of phosphoproteomics for selecting differentially phosphorylated phosphosites.

In order to make reliable decisions if a phosphosite is differentially phosphorylated, repeated experimental measurements are necessary, followed by the following steps:

(i) Formulating a null-hypothesis: in this case, the hypothesis that the mean phosphorylation degree of the phosphosite in condition 1 is equal to its mean phosphorylation degree in condition 2. Because ratios between the phosphorylation degrees are taken, the hypothesis will thus be that the ratio is equal to 1 (or 0 in case log-ratios are considered, which is commonly done for gene expression because it usually makes the data more normally distributed, and would also be favorable for phosphorylation degree ratios). Rejecting the null-hypothesis means accepting that a phosphosite is differentially phosphorylated.

(ii) Choosing the suitable statistics. This will depend on the particular assumptions on the distributions, *i.e.* when the data are normally distributed particular tests are used; other tests when this is not the case.

(iii) Selecting the cut-off for significant statistics: this is a crucial step in which the actual decision is made when to consider a phosphosite to be differentially phosphorylated due to the change in condition and not just a fluctuation due to intrinsic biological variance. Usually this decision is made based on *p*-values obtained from the test statistics.

Without repeated measurements it is not possible to perform the required testing. Like in the early days of cDNA-expression data analysis, several recent works in phosphoproteomics make use of the concept of 'fold change' to decide if phosphosites are differentially phosphorylated. The observed fold change in a single experiment, no matter how large, is not a justified way to decide about differential phosphorylation, as one needs to measure the biological variability inside each of the conditions in order to assess if the change is due to the different conditions or just due to intrinsic variability. The fold-change approach might incorrectly select phosphosites with large fold change, and miss

truly differentially phosphorylated phosphosites with small fold changes. An improvement of the fold-change approach is applied by Benschop *et al.* [58] and Nühse *et al.* [66] in their studies on differential phosphorylation in *A. thaliana*. They select a fold-change cut-off and check if sites pass this threshold in several biological replicates, thereby excluding sites with large intrinsic variability. Benschop *et al.* [58] performed two biological replicates and selected only sites, which showed 1.5-fold change (up or down) in both replicates. This indeed emphasizes the need for replication: in one biological sample 88 differential sites were identified, while in the other only ten sites were identified and of these eight sites overlapped with the previous biological replicate. Nühse *et al.* [66] performed more than two independent biological experiments and accepted only two-fold changes that were seen in two or more experiments. Their approach still has limitations, since it will miss sites that change in response to the applied conditions, but with small fold change. In addition, no motivation is provided as to why the particular fold-change threshold is selected: what is a better number, 1.5 or 2? A recent method proposed for gene expression data [86] formalized the approach employed by Benschop *et al.* [58] and Nühse *et al.* [66]. In their method, genes are ranked according to their fold change and the consistent appearance of a gene in either the top (T) or bottom (B) of the ranking across the replicates is the criterion for differential expression. No arbitrary fold-change threshold is taken. Rather, the size of top T and bottom B is decided based on how many differential phosphorylation findings are made at a particular size of T and B and compared to the number of expected incorrect findings at that T and B. Evaluating this at different sizes of T and B one can select an acceptable fraction of incorrect findings [also referred to as false discovery rate (FDR), see below for more details]. The latter is a very strong aspect of this approach. Rather than choosing an arbitrary threshold that gives no indication of how confident one can be about the findings, now the threshold can be selected according to a tolerable percentage of wrong discoveries. The authors showed that at FDR level of 0.02 (meaning that out of 100 discoveries two are expected to be wrong) of the 131 discoveries made 111 corresponded to fold changes below 1.45! These results concerned cDNA gene-expression data, but it is likely that small fold changes in the phosphorylation state of a particular residue play an important role in signal transduction as well.

While the global approach by Zhou *et al.* [86] has its strengths, it is more common to evaluate differential expression for each gene one at a time. The simplest way of formally correct testing for differential expression/phosphorylation is the *t*-test, employed for example by Zhang *et al.* [13] to test for differential tyrosine phosphorylation between different EGFR stimulations. Over a set of biological replicates, the mean phosphorylation ratio and its standard deviation can be calculated. Using these two quantities, a one-sample *t*-test can be performed, which accounts for the specific variability of each phosphosite:

$$t = \frac{\mu_R}{\sigma/\sqrt{n}}$$

where μ_R stands for the mean log-ratio, σ is its SD and n the number of replicates. The larger the *t*-statistic, the more confident one can be in the finding of a true differential phosphorylation. As is easily seen, the *t*-statistic will be large whenever the mean ratio is larger than the standard deviation, so a phosphosite can have a small mean ratio (fold change), but if the ratio is not very variable, this mean can still be considered significant. *Vice versa* for large mean ratios; high variance may cause such large ratios to be deemed insignificant. It is also clear that the more replicates are performed, the more confident one can be about the estimated values of μ_R and σ . An adequate number of replicates are essential to assess the intrinsic variance and tell it apart from the truly differential phosphorylation, and to make any significant statement about the test result. At this time, the analysis of replicates by MS-based quantitative phosphoproteomics is more demanding than by gene expression experiments, but we do need them in order to be able to make reliable decisions on which phosphosites are differentially phosphorylated. When there are only few replicates available, as is often the case with the data under consideration, variance estimation may be unreliable. To address this issue for gene-expression data, several modifications of the *t*-test and other tests have been proposed (regularized *t*-statistic [87]; SAM, Significance Analysis of Microarrays [88]; penalized *t*-statistic [88, 89]; B-statistic [90]). The many tests proposed could give widely different results and often it is not clear which test applies best. DEDS (Differential Expression by Distance Synthesis [91]) is an interesting step toward synthesis of many of the above statistics. It ranks the genes according to the multi-dimensional distance to the point with maximum value for each statistic.

The *t*-test assumes normally distributed data, but is known to be quite robust towards deviations from normality. If the distribution is far from normal, non-parametric tests are preferred. For example, Trinidad *et al.* [92] applied the Mann-Whitney *U* test to detect differential phosphorylation between samples from hippocampus and cerebellum. Interestingly, these authors do not test for 'differential phosphosite phosphorylation', but rather for 'differential protein phosphorylation' and then use several phosphosites as 'replicated observations' of the same protein. While from a statistical point of view this is favorable (because the number of 'replicates' of each protein is much higher than the actual experimental replicates), from a biological point of view it is not: it has been shown by many studies that the majority of phosphosites on a given phosphoprotein behave differently during stress reactions [32].

All above tests are implemented in the statistical software R, freely available at: <http://www.r-project.org/>.

5.1 Choosing a threshold for differentially phosphorylated sites

After the genes are ranked according to the chosen statistic, the decision must be made, which of them to declare significant. The graphical methods, like Q-Q (quantile-quantile) plot or volcano plot may be useful aid in identifying genes with unusual fold change or *t*-statistics [93].

Usually the final decisions are made based on the *p*-values obtained from the test statistics. The *p*-value threshold ‘classically’ used is 0.05, indicating the probability of observing a given value of fold change if the null-hypothesis (“the site is not differentially phosphorylated”) is right. Since 0.05 is a rather low probability, the alternative hypothesis (“the site is differentially phosphorylated”) is accepted. However, the probability of obtaining a significant result just by chance is growing along with the number of tests. Thus, when multiple tests are conducted in parallel, there is a need for adjustment against false positives (type I error; deciding that a site is differentially phosphorylated while it truly is not). This can be done either by adjusting *p*-values or by adjusting the significance threshold. There are two standard approaches to adjust for multiple testing. Deciding a threshold depends on selecting a trade off between False Positives and False Negatives.

False Positive = false discovery = wrongly rejected null hypothesis = type I error = deciding that a site is differentially phosphorylated while it truly is not

False Negative = missed discovery = wrongly accepted null hypotheses = type II error = deciding that a site is not differentially phosphorylated while it truly is

Family-Wise Error Rate, or overall type I error rate (FWER) aims to control the probability of one or more false positive results. Bonferroni correction [94] adjusts *p*-values by dividing them by the total number of tests. It is the most conservative approach: it will miss many true differential phosphorylation events (True Positives) in order to strongly limit the number of incorrectly accepted differential phosphorylation events (False Positives). The less conservative Holm’s procedure [95] rejects the null hypotheses corresponding to the *k* smallest *p*-values if $p_{(i)} \leq \frac{\alpha}{m - i + 1}$ for all $i \leq k$, where *m* is the total number of tests and α is the desired level of FWER control.

The interpretation of accepting adjusted *p*-value of, say, 0.05, is that if the method were applied 100 times and produced 100 lists of significant discoveries, only five of these lists would be expected to contain any single false positive. This leads to significant increase in the error II rate (the probability of missing a true positive result, *i.e.* deciding that a site is not differentially phosphorylated while it truly is). Such a strong control is often found unnecessary and impractical in microarray experiments analysis. In practice, many researchers would trade the risk of including false discovery for a stronger power of a test.

The concept of False Discovery Rate (FDR), introduced by Benjamini and Hochberg [96], is a less stringent alternative to FWER and has completely revolutionized the field of multiple hypothesis testing. FDR controls the expected proportion of type I errors in a list of rejected hypotheses, or proportion of false positives in tests declared positive. The concept of FDR is very practical, since it provides a way to select a threshold simply at a desired percentage of false discoveries. Instead of deciding on *p*-values that tell how many mistakes are made in the hypothetical situation that all null-hypothesis are true, the FDR level simply tells how many discoveries are expected to be false. Usually researchers are quite satisfied with FDR levels of 0.1, meaning that 1 out of 10 discoveries is false, or even higher levels.

FDR methods set a cut-off for *p*-values. The Benjamini and Hochberg [96] procedure sorts *p*-values in decreasing order and rejects the hypotheses whenever their *p*-value is no larger than $p_{(k)}$, where $\frac{mp_{(k)}}{k} \leq \alpha$ (α is a desired level of FDR control and *m* is the fraction of true null hypotheses). Modifications of this procedure use an estimate of the null-distribution by fitting the observed distribution of *p*-values [89, 97–103] or by bootstrapping [104].

Several methods for multiple hypothesis testing corrections are implemented in the R-software. The Optimal Discovery Procedure [105] implemented in the software EDGE [106] maximizes expected true positives for a fixed level of expected false positives.

5.2 Multiple conditions

In certain cases, it may be of interest to perform comparisons between more than two groups. Differential phosphorylation across multiple conditions could be tested using linear models. Linear models could represent the expression value as a linear combination of effects from different sources of variation [107]. The simplest, two conditions linear model is equivalent to a *t*-statistic. For more than two conditions, fixed-effects ANOVA (ANalysis Of VAriance) and F-test [93, 97, 107, 108] could be employed. As with *t*-test, several variants of the F-test (*e.g.* global, penalized) have been developed to improve the estimation of the variance. Mixed-effects ANOVA is appropriate when there are many experimental factors with potential interactions between them [109]. Freely available software can be used to perform these analyses, such as the MAANOVA package for R and the SAM software [88].

5.3 Time-course experiments

The responses in signal-transduction pathways after elicitation proceed dynamically over time. Measuring and analyzing time responses in signal transduction may therefore be more important than the static data discussed above. Several phosphoproteomic data are in a form of a time course, and more such datasets can be expected to appear in the future.

While the analysis of time-series data is different from static data, the main issue remains the same: identifying which phosphosites significantly change in their degree of phosphorylation. Also in case of time-series, the fold-change approach has been used [32]. However, several statistical methods for significance analysis of time-series data have been developed.

Like in the analysis of static data, differential phosphorylation could be tested between two or more groups, *e.g.* testing if a protein is differentially phosphorylated over time in an elicited series *versus* an un-elicited control time series. Unlike static samples, time series provide an ordering of observations and this allows testing for differential phosphorylation even within a single group, *e.g.* simply testing if a certain protein's phosphorylation state changes over time after elicitation. Statistical analysis of a time-course experiment poses several difficulties arising from the facts that such experiments are usually short, have limited number of replicates, and that measurements from the same sample are correlated over the time. Unlike in the static data, in time-course analysis not only the magnitude of the activity, but also its pattern over time is of importance. The approaches that have been applied fall in two categories: (i) Methods that test for differential expression between pairs of time points, and (ii) Profile based methods that explicitly consider the whole series.

ANOVA (with time-variable) has been used for detecting differential expression between each time-point in the time-series [110]. While performing well, the drawback of ANOVA is that it requires many replicates [111, 112]. In addition, it does not take into account the global profile of the time series. Profile-based models [113, 114] do not rely on the data for particular time point only, but rather take the overall shape of the time-course curve under account. The approach developed by Storey *et al.* [113] is based on fitting a curve to the time series, first separately for each treatment group (*e.g.* elicited and control), and then using combined data from both groups (corresponding to the null-hypothesis that both series share the same fit). If the individual fits are significantly better than the simultaneous fit (based on sum of squares using F-test), the time-series profiles are decided to be different and thus, the gene is declared differentially expressed between the treatment groups. To detect differential expression within a single group, it is tested whether the mean expression level in time is significantly different from constant. This approach is very attractive, as it does not require any replicated measurements of the time series! However, recently this method was compared to two methods that do require replicated time series [111] and it was shown using simulated data that for sparsely sampled time series (about ten time points) the two methods completely outperform the approach by Storey *et al.* [113]. For denser time series the differences get smaller and for time series with many points (50 time points) the approach by Storey *et al.* [113] outperformed the other two. It seems that there is no way

out: many observations are needed to make reliable statistical decisions, either by obtaining replicates or by obtaining single, but densely sampled time series. The time-course analysis using the algorithm by Storey *et al.* [113] is implemented in the user-friendly software EDGE [106]. Other methods to detect differential expression in time series include the use of a multivariate empirical Bayes statistic [115], Wald test, accounting for autocorrelation [116], Hidden Markov Models [117], and GSVD (Generalized Singular Value Decomposition) [118].

5.4 Analysis of differential phosphorylation in signaling pathways

While the analysis tools that we have discussed so far are oriented toward finding individual phosphosites that are differentially phosphorylated (expressed) between conditions, the underlying questions usually aim to identify the biological processes involved in these conditions. Ideally then, one performs tests to find out which signal-transduction pathways are differentially active between conditions. It may be of interest to test whether a particular kinase is differentially phosphorylated with all its known targets (or subsets thereof). Besides the biological relevance, such tests are also appreciable from a statistical point of view: the pathway-level change in activity may be missed by the individual protein kinase level analysis if the changes in individual activities are too small to be found significant in the limited number of experiments [119]. Considering the whole pathway, the small changes add up and may provide enough power to establish significance. Several tools have been developed to test for differentially expressed gene sets; these might be applied either to the list of genes declared significant (*e.g.* tools for analysis of the overrepresentation of Gene Ontology terms; EASE, [120]), to the whole list of genes ranked by their *p*-values (GSEA [119, 121]; SAM-GS, [122]) or to the original expression data [123]. Again, these algorithms can be equivalently used on phosphoproteomics data for mapping differential phosphorylation events in signaling networks.

6 Conclusions and challenges

At present, tens of thousands of phosphorylation sites and for many their behavior during different cues have been revealed. However, for most of the sites the responsible protein kinase has not been identified. Consequently, phosphorylation networks are still fragmented. A challenge remains to couple these sites to the responsible protein kinases, but novel algorithms and approaches have been developed to discover kinase-substrate links [67, 68]. The experimental verification of the *in vivo* kinase is, however, still a tedious task.

Combining quantitative information on changes in phosphoproteomes induced by kinase inhibitors, mutated kinase forms and environmental cues, phosphorylation

in protein complex components [44], drug action on protein kinase pathways [53, 54], kinase deletion or over-expressing strains with bioinformatics approaches will eventually lead to comprehensive knowledge of the dynamic behavior of phosphorylation networks [67, 70, 124]. Cross-talk between different PTMs exists [125], and therefore knowledge of different modifications should be combined. This may ultimately allow computational modeling of signaling networks on a systems-wide scale [126–128].

Where is the limit? With the current progress, the first full phosphoproteome (MS limitations not taken into account) could be solved within the next few years. Quantitative aspects of stimulus-induced changes in the phosphoproteome will continue to grow and modeling of the resulting pathways will uncover many novelties. Comprehensive profiling of physiologically relevant signaling cascades could be possible by metabolic labeling of tissues or whole multicellular organisms such as animals [129–131] and plants [59].

Proteins usually occur in many isoforms that differ in PTMs. Some of these PTMs, such as phosphorylation and ubiquitylation, show intricate relationships [125]. Knowledge of modifications of protein isoforms rather than of peptides derived from these isoforms is therefore crucial for understanding the action of individual PTMs. Top-down proteomics, *i.e.* the analysis of entire proteins, to decipher the concerted action of PTMs on single proteins, will be a further challenge [132]. Although such analysis is difficult and needs further improvement, it offers a promising method for future proteomic research [133].

Quantitative approaches have provided unbiased views on signaling pathways and show that it is feasible to generate working hypotheses from these. High-quality data that conform to MIAPE standards [134], and the introduction of general rules for analysis will increase the confidence of MS data [133]. Consistency in data processing of quantitative MS-based phosphoproteomic approaches will likewise increase the quality and allow comparison of datasets generated by different methods. Large-scale, MS-based approaches have already begun to reveal the specificities of protein kinases, their downstream signaling networks and how inhibitors affect these. Functional phosphoproteomics [68] with selective testing of obtained candidates [54, 135] may aid in understanding and treating diseases like cancer and neurological disorders. As described above, the first functional tests of such hypotheses have already shown promising results in the combat against cancer. The analysis of individual phosphorylation sites is still tremendously complicated, but the time is ripe for biological verification of modeled signal-transduction pathways.

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7 References

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