Protein phosphorylation is a post-translational modification (PTM) that orchestrates a diverse array of cellular processes. Because this modification serves as a rapid and reversible means to modulate protein activity and transduce signals, the regulation of phosphorylation is a central mechanism in cell health and disease. The addition and removal of phosphoryl modifications via kinases and phosphatases, respectively, makes the landscape of phosphorylation particularly dynamic. Understanding the complex networks and functions coordinated by phosphorylation requires knowledge of specific amino acid modifications with both spatial and temporal resolution, a task that remains a challenging analytical endeavor. Mass spectrometry (MS) has emerged as the premier tool for global PTM analysis, boasting high sensitivity, considerable throughput, and the capacity to localize modifications to a single residue. Indeed, MS-centric phosphoproteomics has become a standard approach for investigating protein phosphorylation in laboratories worldwide.

Phosphoproteomics in the Age of Rapid and Deep Proteome Profiling

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Analytical Chemistry last reviewed the contribution of MS and related technologies to phosphoproteomics in 2011. Since that time, MS methodology has developed at an impressive pace. While routine proteomic experiments can now analyze thousands of proteins in just a few hours, rather than days or weeks, characterizing the global phosphoproteome is significantly more challenging than measuring nonmodified proteins. The relative low abundance of phosphorylated peptides and the need for residue-specific information require special considerations in sample handling, data acquisition, and postacquisition processing that constrain reproducibility, quantitative efficacy, throughput, and depth in phosphoproteomic workflows. Advances in MS-based approaches have remarkably improved our abilities to investigate the many roles of protein phosphorylation across a diverse set of biological contexts, but many technical obstacles still exist. Poor run-to-run overlap, challenges in confident phosphosite assignment, and complications inherent to various quantitative strategies limit biological insight, despite ever-increasing numbers of detected phosphopeptides. Focusing on work from the past two years (2013–2015), this review examines major developments in MS technology that have enabled the characterization of tens of thousands of phosphopeptides in a given experiment, and considers the contribution of this analytical power to translational research. We also discuss how future innovation can address technical challenges of today’s methods, and we offer our perspective on how phosphoproteomics will continue to mature.

SAMPLING THE PHOSPHOPROTEOME

As much as a third of eukaryotic proteins are estimated to be phosphorylated. However, because phosphorylation is a low stoichiometry modification, phosphopeptides (or phosphorylated proteins) must be enriched from complex mixtures that have high backgrounds of nonphosphorylated moieties. Typically, phosphoproteomic experiments involve LC–MS/MS analysis (i.e., chromatographic separations coupled to tandem MS) of phosphopeptides that have been isolated from an enzymatic digestion of proteins from lysed cells (Figure 1). Ongoing optimization efforts have focused on sample preparation protocols, especially phosphopeptide enrichment strategies and fractionation techniques to reduce complexity and maximize sampling depth. Proper steps must be taken in...
sample collection, as well, to avoid unintentional alteration of the phosphoproteome.\textsuperscript{13,14} Beyond sample handling, advancements in MS instrumentation have greatly improved the speed and sensitivity of routine phosphoproteome interrogation. These topics have been reviewed in the past,\textsuperscript{15−19} but here we focus on how these techniques have addressed challenges in run-to-run reproducibility and how they have contributed to improvements in throughput and/or depth for phosphoproteomic experiments.

**Generating Phosphopeptides.** Thanks to its high cleavage specificity C-terminal to lysine and arginine residues, and to its proclivity for producing peptides amenable to MS analysis, trypsin is the most commonly used protease in proteomics and phosphoproteomics. However, proximity of cleavage sites to phosphorylated amino acids can impair tryptic digestion,\textsuperscript{20} a problem which has inspired the evaluation of multiple protease approaches for large-scale phosphoproteomics.\textsuperscript{21−24} Studies have described varying degrees of success, but generally have demonstrated that utilizing two proteases improved both protein sequence coverage and phosphoproteomic depth, i.e., the number of identified phosphosites. Wisniewski and Mann found that consecutive use of LysC and trypsin to generate phosphopeptides allowed them to identify up to 40% more proteins and phosphorylation sites than a one-step tryptic digestion. Subsequent experiments by others confirmed the efficacy of this approach,\textsuperscript{25} and combinations of GluC and trypsin have also proven beneficial.\textsuperscript{26} Furthermore, Heck and co-workers recently published a thorough multiple-enzyme study, compiling a human phosphopeptide atlas composed of 37 771 unique phosphopeptides that correspond to 18 430 unique phosphosites.\textsuperscript{27} The overlap of sites detected by the five proteases accounted for only a third of the total number of sites. Clearly, the use of several orthogonal proteases can significantly enhance phosphoproteomic sampling depth, enabling detection of thousands phosphosites that may be inaccessible in traditional trypsin-only approaches (Figure 2).

**Enrichment Strategies.** Phosphopeptide enrichment arguably introduces the most variation of any step into a standard phosphoproteomic workflow. A variety of enrichment strategies have emerged as the field has evolved, with metal-based affinity enrichment leading in popularity. The two most prevalent metal-based methods are immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC). An established enrichment strategy in phosphoproteomics for over two decades, IMAC uses transition metal cations (Fe$^{3+}$, Ga$^{3+}$, Zr$^{4+}$, etc.) as affinity agents for negatively charged phosphate groups. These cations are immobilized via chelation on a substrate, such as magnetic beads or silica-based resins, which enables selective retention of phosphopeptides over nonphosphorylated peptides. A recently described titanium(IV) substrate (Ti$^{4+}$-IMAC) has been growing in popularity in many laboratories.\textsuperscript{28} MOAC, which has seen nearly a decade of broad use, similarly leverages the affinity of oxygen in phosphoryl groups for metals in metal oxide matrices. Titanium dioxide (TiO$\text{x}$) is the most commonly employed MOAC agent, although zirconium dioxide and magnetite

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**Figure 1.** Typical phosphoproteomic workflow. Each step in a phosphoproteomic experiment can contribute to limitations in reproducibility and phosphoproteomic depth, which can ultimately restrict the biological insight obtained from an experiment. Concerted efforts in the phosphoproteomics community to improve each step in this workflow continue to advance our ability to sample the phosphoproteome with greater speed and depth, but comprehensive phosphoproteome coverage remains out of reach.

**Figure 2.** Phosphoproteomics using complementary proteases. Phosphosite intensities correlate strongly ($r > 0.8$, yellow) when data sets are obtained following digestion with the same protease but correlation between data sets originating from different proteases is low ($r \sim 0.25−0.55$, blue). This difference indicates that using multiple proteases provides access to different regions of the phosphoproteome. Reprinted with permission from Giansanti, P.; Aye, T. T.; van den Toorn, H.; Peng, M.; van Breukelen, B.; Heck, A. J. R. *Cell Rep.* 2015, 11, 1834−1843 (ref 27). Copyright 2015 Cell Press.
Review

Phosphoproteomic depth can be achieved by batching replicate measurements. Combining replicate enrichments from the two methods also boosts phosphoproteomic depth can be achieved by batching replicate measurements. Combining replicate enrichments from the two methods also boosts phosphoproteomic depth. For example, gallium IMAC and TiOx have been used to enrich di-decoy amino acids (e.g., asparagine and glutamine), to improve the capture capacity and specificity of phosphopeptide identification. Reprinted with permission from Matheron, L.; van den Toorn, H.; Heck, A. J. R.; Mohammed, S. Anal. Chem. 2014, 86, 8312−8320 (ref 37). Copyright 2015 American Chemical Society.

(Fe₃O₄) are also used. Both IMAC and MOAC generally enrich phosphopeptides with phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) residues.

The success and popularity of IMAC and MOAC enrichment methods derive from sustained, widespread efforts to compare phosphoproteomic depth: IMAC, MOAC, or a combination thereof. A popular focus of recent years has been on optimization of sequential enrichment strategies or combinations of metal cations or enrichment substrates. Combinations of iron and gallium IMAC,29 iron and titanium IMAC,30 and gallium IMAC and TiOx31 have been used to enrich different classes of phosphopeptides with moderate to considerable success. Hunt and co-workers used complementary metal cation chelation groups like nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA) in Fe³⁺-IMAC enrichment columns to identify hundreds of phosphopeptides from nanograms or even picograms of starting material, an order of magnitude less than other contemporary methods.32 Several groups have utilized solution additives, like glycerol, bis-Tris propane, citric acid, or decy amino acids (e.g., asparagine and glutamine), to improve TiOx and other MOAC enrichment efficiencies.33−35 Even different particle sizes of MOAC resins have been investigated to compare phosphopeptide capture capacity and specificity.36 Many of these studies attempt to mitigate issues with reproducibility and sampling depth that challenge single-stage enrichment strategies. In general, however, these approaches only partially address the problem. They often introduce additional steps in the sample handling workflow, which can increase rather than alleviate variation. Furthermore, these protocols demonstrate a high degree of enrichment orthogonality through the combination and optimization of two strategies, but the overall result is often just as variable from experiment to experiment as single-stage enrichment.

To address the need for a simple yet robust enrichment strategy, several studies have offered head-to-head comparisons of single-stage methods using state-of-the-art enrichment protocols. Matheron et al. compared Ti⁴⁺-IMAC and TiOx enrichments on HeLa cell digests and on >23 000 synthetic phosphopeptides (pSer, pThr, and pTyr) and their non-modified counterparts.37 Although overlap was only ∼42% between Ti⁴⁺-IMAC and TiOx enrichments of the phosphopeptide libraries, they found no clear differences between the phosphopeptides enriched with the two methods when considering peptide length, site position, isoelectric point, hydrophobicity, motif analysis, and relative abundance of phosphopeptides (Figure 3); they did observe, however, a minor bias toward multiply phosphorylated peptides in Ti⁴⁺-IMAC versus TiOx. When enrichments on HeLa cells were combined, the results from both methods showed an increased number of localized phosphosites, which indicates that tandem enrichment strategies for titanium-based methods may still be valuable to increase phosphoproteomic depth. Ultimately, the lack of bias between the two methods demonstrated that biological origin, rather than methodological artifact, is largely responsible for observed differences in comparisons of studies using the two approaches.

As a complement to this study, Ruprecht et al. reported a comprehensive and reproducible enrichment using Fe³⁺-IMAC in HPLC column format.38 When they compared this strategy to Ti⁴⁺-IMAC and TiOx, they found that the Fe³⁺-IMAC column performed best, allowing identification of ∼5800 unique phosphosites in triplicate 4 h analyses and as many as 15 000 phosphopeptides in 48 h of analysis of fractionated samples. Moreover, they showed that the orthogonality of the Fe³⁺-IMAC, Ti⁴⁺-IMAC, and TiOx methods was greatly reduced when the phosphoproteomic depth was increased via hydrophilic strong anion exchange fractionation. They thus dismissed the concept of orthogonality between the methods and attributed most of the previously reported complementar-
ity to artifacts of nonoptimized analytical methods, e.g., limited binding capacity of the enrichment material, biased or incomplete elution, compromised enrichment scaffold (tips, beads, etc.), and limited analytical capacity of the mass spectrometer. This observation suggests that focusing on increasing sampling depth is key to improving reproducibility in phosphoproteomic experiments. The Hummon group performed a similar analysis with multistep enrichments but showed the converse result: that TiOx and Fe\textsuperscript{3+}-IMAC are indeed complementary.\textsuperscript{50} Not employing offline fractionation, however, their work achieved less phosphoproteome depth than the Fe\textsuperscript{3+}-IMAC column study. In light of this difference, there may be more support for Ruprecht et al.’s argument that perceived orthogonality diminishes as phosphoproteomic depth improves; however, a combination of enrichment strategies as reported by the Hummon group can provide a low-cost, time-efficient strategy to achieve greater depth when access to HPLC fractionation is limited.

Even as IMAC and MOAC methods dominate the field, alternative strategies for affinity-based phosphopeptide enrichment have also continued to mature. Immunoprecipitation, a canonical route for protein enrichment, is mainly limited to phosphotyrosine studies in phosphoproteomics. Nevertheless, combinations of metal-based and antibody-based affinity enrichments have proven useful for general and pTyr-specific phosphoproteomics experiments.\textsuperscript{40,41} Motif-based immune-affinity purification, affinity enrichment based on polyhistidine tags, and polymer-based enrichment substrates have also been successfully employed as alternative enrichment strategies.\textsuperscript{42–45} Although they may align with more traditional biochemical methods of purification, these approaches still struggle with reproducibility due to nonspecific binding, batch-to-batch variability of antibody production, and/or lack of dedicated effort from the field to refine protocols for global phosphoproteomic experiments.

Affinity-based methods can enrich intact phosphoproteins rather than digested phosphopeptides, as well. The Ge group demonstrated that phosphoproteins could be selectively enriched from complex cell and tissue lysates using superparamagnetic Fe\textsubscript{3}O\textsubscript{4} nanoparticles that were functionalized via a glutaric acid linker with a zinc(II)-dipicolylamine coordination complex to specifically bind phosphate groups.\textsuperscript{46} These nanoparticles were designed for multivalent interaction with phosphoproteins, which provided significantly higher enrichment specificity than Fe\textsuperscript{3+}-IMAC for intact phosphoproteins. Liu et al., employing a hydrophilic antacid aluminum glycinate functionalization for phosphate group affinity, also described nanoparticles for phosphoprotein enrichment.\textsuperscript{47} Interestingly, Hoehenwarter et al. combined intact phosphoprotein enrichment with aluminum oxide (Al(OH))\textsubscript{3} with subsequent tryptic digestion and standard TiOx phosphopeptide enrichment to study mitogen-activated protein kinase substrates in Arabidopsis.\textsuperscript{48} Because proteomics of intact proteins, especially of phosphoproteins, in complex mixtures is still a maturing field, many of these protocols have yet to see widespread use that could provide insight into their reproducibility or utility in routine experiments.

In all, a major challenge to establishing orthogonality or complementarity of various enrichment methods comes from poor run-to-run reproducibility in phosphoproteomic experiments. Analyzing back-to-back technical replicates of the same sample often yields only 60–75\% overlap in identified phosphopeptides, and comparing technical replicates of multiple enrichments further exacerbates this problem. Figure 3g exemplifies this prevalent, discipline-wide phenomenon. Starting at \(\sim 2000\) phosphopeptides per a single enrichment, each additional replicate contributed a significant increase to the cumulative total of phosphopeptides until \(\sim 3500\) phosphopeptides were identified with inclusion of the fourth replicate. Also, this with phosphopeptide analysis from only a single enrichment strategy! Results were similar in the Ruprecht et al. data, where technical triplicate Fe\textsuperscript{3+}-IMAC enrichments (without fractionation) identified \(\sim 7500\) unique phosphopeptides but less than 50\% of those (\(\sim 3600\)) were detected in all three replicates.\textsuperscript{38} Clearly, if not all phosphopeptides in a given sample are identified, comparisons of observed phosphopeptides between different enrichment methods can be misleading, hence, the great value of technical replicate measurements in these studies. Although populations of phosphopeptides enriched by a given method appear to be more similar that previously thought, adequate sampling depth, whether it comes from multidimensional chromatography, faster and more sensitive mass spectrometers, or more reproducible strategies for data acquisition (all discussed in the following sections), is imperative to understanding the degree of overlap between enrichment methods for optimization of single-stage and combinatorial approaches.

**Chromatographic Separations.** To increase sampling depth of the phosphoproteome, multidimensional chromatography has become a common practice for simplifying samples across many fractions. Ubiquitously used in proteomics and phosphoproteomics, reversed phase liquid chromatography (RPLC) is the online chromatography of choice for LC–MS/MS experiments but several studies have also explored various other chromatography modalities for both online and offline fractionation and enrichment of phosphopeptides.

Ion exchange chromatographies, especially strong cation exchange (SCX), are widely used separations approaches. SCX is usually combined with metal-based phosphopeptide enrichment for large-scale phosphopeptide enrichment, but Hennrich et al. demonstrated that two-dimensional SCX using complementary basic and acidic buffers could isolate phosphopeptides with no further enrichment required.\textsuperscript{59} In this case, one-dimensional SCX with basic buffers provided only 537 phosphopeptides from a HeLa cell digest. By contrast, the two-dimensional approach using basic then acidic SCX separations enabled identification of more than 10 000 phosphopeptides, 480 of which were also seen in the one-dimensional separation and most of which were basic phosphopeptides with two or more basic residues. SCX has also been successfully combined with TiOx for improved enrichment of phosphotyrosine, although it did not perform as well as a combination of pTyr antibodies and TiOx.\textsuperscript{60} In addition, rather than enrich phosphopeptides, ion exchange chromatographies can deplete undesired populations of phosphopeptides; for example, acidic phosphopeptides can be removed with strong anion exchange (SAX) to enhance detection of motifs associated with basophilic kinases.\textsuperscript{61}

In hydrophilic interaction chromatography (HILIC), which uses a polar stationary phase and an organic-to-polar mobile phase gradient, peptide retention is based on hydrophilicity (the opposite of RPLC). Several groups have recently explored HILIC as an orthogonal dimension of separation for phosphopeptides, coupling it with metal-based affinity enrichments and SCX separations with varying success.\textsuperscript{52,53} Another approach that has gained favor in phosphoproteomics is...
electrostatic repulsion-hydrophilic interaction liquid chromatography (ERLIC). Combining the principles of HILIC and anion exchange, ERLIC has been used both as an enrichment strategy and in multidimensional chromatography approaches.\(^5\)\(^4\)\(^5\) Alpert and co-workers recently published a comparison between ERLIC, weak anion exchange (WAX), and SAX for fractionation in phosphoproteomic experiments. ERLIC enriched and identified more than double the number of phosphopeptides achieved by the anion exchange chromatographies.\(^6\) This study also offered insight into the benefits of solvent additives for ERLIC and the performance of WAX and SAX at different pH values. Two-dimensional ERLIC in combination with other modes of separation and enrichment has been shown to increase phosphoproteomic depth as well.\(^6\)\(^7\) Other interesting alternatives for fractionation include chromatographic separations of intact proteins prior to digestion and subsequent phosphopeptide enrichment. Several groups have explored these approaches,\(^5\)\(^8\)\(^−\)\(^6\(^0\) but they are less common than the peptide separations described above.

Much like the search for the best metal-based phosphopeptide enrichment strategy, optimal fractionation methods are still open to debate, with recent discussion centered on the comparison of high pH reversed phase (RP) fractionation versus SCX. In 2014, Batth et al. evaluated offline high-pH RPLC fractionation head-to-head with SCX fractionation, both with TiOx enrichment. They demonstrated a surprising advantage to the RP approach.\(^6\)\(^1\) In four biological replicates of mouse embryonic cells, high-pH RPLC facilitated the identification of an average of 17 566 (±3 737) phosphopeptides, compared to an average of 6 215 (±1 759) phosphopeptides for SCX fractionation. Moreover, optimization of high-pH RPLC conditions and MS acquisition parameters more than doubled the number of phosphopeptides identified in biological replicates (>37 000 in individual replicates, 27 712 localized phosphosites in total, Figure 4). Corroborating this result, Yue et al. reported a similar advantage for high-pH reversed phase separation. They employed a multistep Fe\(^3\) IMAC approach in combination with high-pH RP cartridges that not only fractionated the phosphopeptides but also desalted the samples.\(^6\)\(^2\) The multistep IMAC-RP cartridge workflow lessened starting material requirements, reduced sample preparation time, and eliminated the need for HPLC instrumentation while identifying 8 969 phosphopeptides (6 337 phosphosites) from 3 mg of human epithelial cells, compared to 5 519 phospho-peptides (3 686 phosphosites) from 15 mg of starting material with the traditional SCX-Fe\(^3\) IMAC approach. Others have reported that the addition of solvent additives, such as EDTA, can further improve RPLC fractionation.\(^6\)\(^3\)

From our perspective, recent data lends clear support for high-pH RP fractionation over SCX. Additionally, the RP approach is generally more flexible than SCX because the buffers require no additional cleanup to be MS compatible. In increasing sampling depth, the combination of phosphopeptide enrichment and fractionation for extensive sample characterization has the potential to improve reproducibility issues in routine phosphoproteomic experiments. However, this benefit incurs significant cost in data acquisition time, a balance we discuss further below.

**Mass Spectrometry Instrumentation.** Many hundreds or thousands of phosphopeptides may be introduced into a mass spectrometer at any given moment of an LC–MS/MS experiment. The speed and sensitivity of mass spectrometers thus play critical roles in successful and reproducible phosphopeptide identification. Nearly all phosphoproteomic experiments in recent years have been conducted on hybrid MS systems that couple multiple mass analyzers for gains in sensitivity, acquisition speed, and efficacy of tandem MS (MS/MS). Such hybrid systems include quadrupole-time-of-flight (qTOF), linear ion trap (LIT)-Orbitrap, quadrupole-Orbitrap, and ion trap-Fourier transform ion cyclotron resonance (FTICR) mass spectrometers.\(^6\)\(^4\)–\(^6\)\(^7\) The Orbitrap Fusion, a “tribrid” quadrupole-Orbitrap-LIT mass spectrometer, has been introduced within the past 2 years as a powerful and versatile new platform. Through the parallelization of many scan functions, this instrument can operate with ion trap MS/MS acquisition rates at 22 Hz or greater, nearly double those of previous ion trap-Orbitrap hybrids.\(^6\)\(^8\) Its acquisition speed has driven improvements in throughput and depth in proteomics experiments\(^6\)\(^9\)\(^,\)\(^7\)\(^0\) and recent work has highlighted its analytical power and throughput capabilities for phosphoproteomics. In a study of mouse brain and liver tissue, Gygi and co-workers quantified >38 000 phosphopeptides (11 015 phosphosites) across 10 samples using a multiplexed isobaric labeling strategy on the quadrupole-Orbitrap-LIT platform.\(^7\)\(^1\) They achieved this quantitative phosphoproteomic depth, typically the result of a week or longer of acquisition time on previous instruments, in only 2 days of analysis.

Enhanced throughput capabilities for phosphoproteomic experiments have also come through improvements on an alternative Orbitrap platform, a quadrupole-Orbitrap hybrid called the Q-Exactive HF. This instrument, which relies entirely on high-resolution Orbitrap data acquisition for MS and MS/
MS scans, is equipped with a segmented quadrupole for more robust precursor selection and transmission. Its high field Orbitrap, capable of achieving a sequencing speed above 20 Hz, permits faster acquisition times for a given resolution. The Olsen group, in addition to leveraging the strengths of this instrument for the in-depth characterization of offline high-pH RPLC studies described above, showed that the Q-Exactive HF can identify more than 7600 unique phosphopeptides (6831 of which were localized) from a HeLa cell digest in an hour of acquisition time. Interestingly, they also explored optimal instrument parameters to show that acquisition speed may not always be the most important metric for phosphoproteomic experiments. Rather, the collection of high-quality fragmentation spectra, which can come at the cost of scan speed, permitted modification localization for nearly all phosphopeptides (∼97%) detected in a given experiment.

Advances on other hybrid instrument platforms also promise to broaden horizons for phosphoproteomics. The newest q-TOF instrument, the Impact II described by Mann and co-workers, offers high transmission efficiencies and improvements in resolution/mass accuracy that benefit shotgun proteomics on complex samples. In their work, the Impact II ultimately led to the characterization of ∼5200 human proteins and ∼3600 yeast proteins in triplicate single-shot analyses. The latest in ion trap-FTICR instruments feature the highest field superconducting magnet ever used for FTICR (21 T), which has enabled ultrahigh resolution/mass accuracy on the order of a resolving power of 300 000 at 400 m/z for a 0.76 s detection period and 2 000 000 resolving power for the z = 48+ charge state of bovine serum albumin (∼1385 m/z) for a 12 s detection period. Although to the best of our knowledge no phosphoproteomics studies have been reported on these new systems to date, they are poised to contribute to improved analysis of phosphopeptides and phosphoproteins in the coming years.

Data Acquisition Strategies. Data-dependent acquisition (DDA), or automated selection and fragmentation of precursor ions using predetermined criteria and real-time decision making, is the most widely used data acquisition strategy in LC–MS/MS proteomic and phosphoproteomic experiments, including those reported in this review. Typically, precursors are selected based on their relative abundance, biasing experiments toward highly abundant species, including those that may simply ionize more favorably than others. In hopes of improving run-to-run reproducibility and sampling of low abundance precursors, data-independent acquisition (DIA), which collects data largely independent of precursor ion information, has come into vogue. One of the most popular approaches involves repeated sampling of successive isolation windows using discrete m/z ranges over the course of chromatographic elution (i.e., SWATH-MS). The major potential benefit of DIA strategies is their reproducibility: in theory, fragmentation spectra are collected for every precursor ion in every experiment, as opposed to the stochastic precursor selection in DDA approaches.

Aebersold and co-workers used SWATH-MS in combination with affinity purification to study protein–protein interactions of the 14-3-3 system, a family of seven abundant cellular scaffolds with diverse regulatory functions that bind phosphorylated residues on ligand proteins. Providing quantitative data to follow dynamic phosphorylation-related changes in protein–protein interactions in perturbed systems, SWATH-MS offered data consistency similar to targeted approaches but with reduced overhead time in assay development and with increased peptide observation. Parker et al. used DIA to quantify the effect of insulin on phosphorylation of 86 protein targets and demonstrate 14-3-3 binding effects in insulin signaling. Improvements in postacquisition data analysis in DIA experiments, such as the ability to differentiate phosphopeptide isomers that may be missed using DDA methods, have also benefited phosphoproteomic applications. Used in combination with DIA, ion mobility has enhanced precursor fragmentation efficiency for improved reproducibility and proteome coverage. Targeted methods that use directed/inclusion list methods using a predefined set of precursor ion masses have also proven useful in reproducibly measuring quantitative changes in specific signaling cascades.

A recent evaluation of the value of DDA and DIA in analyzing phosphopeptides (albeit in a study limited to ∼10 phosphopeptides) showed that targeted DIA methods can improve sensitivity of phosphopeptide identification and quantification by 5–10-fold, and a coupling of global DDA and targeted phosphoproteomics proved useful in biomarker discovery in clinical breast cancer samples. Methods combining the strengths of DIA and DDA approaches have emerged in recent years, but they have yet to be widely applied to phosphoproteomic analyses. The major challenge of DIA lies in the complicated spectra it generates, making data extraction nontrivial and limiting the number of peptides detected in an experiment. In our view, the popularity of DIA (i.e., SWATH-like approaches) has produced many valuable new informatics tools that will continue to make it a viable alternative for proteomics and phosphoproteomics alike, particularly when reproducibility is more critical than phosphoproteomic depth. That said, consistent improvements in the speed and sensitivity of mass spectrometers favor well-established DDA methods, especially as the fastest instruments no longer struggle to select and fragment every available precursor above a desired signal-to-noise ratio across an LC–MS/MS experiment. In short, both DDA and DIA have valuable utility in phosphoproteomics, but DIA does not appear set to outpace DDA in global profiling or phosphoproteomic depth in the foreseeable future.

Balancing Throughput and Depth. Advances in technology have corresponded to increased numbers of phosphopeptide identifications per experiment. Recent work has shown that, provided adequate acquisition time, experiments can characterize tens of thousands of phosphopeptides from a sample. Sharma et al. identified 38 229 phosphosites from 81 098 unique phosphopeptides in a human cancer cell line (HeLa cells), which provided valuable insight into the extent of phosphorylation in the proteome and into the differences between serine/threonine phosphorylation and tyrosine phosphorylation (Figure S). A combination of SCX fractionation and both TiOx and pTyr antibody enrichment enabled this superb sampling depth. The price of this ultradepth phosphoproteomic coverage, however, came to the tune of approximately 270 LC–MS/MS experiments and 40 days of data acquisition time, not accounting for additional overhead in sample preparation and data analysis. Other studies have also reported impressive phosphoproteomic depth: 29 057 quantified phosphorylation sites in adipocytes, 31 480 quantified phosphorylation sites across 14 rat tissues and organs, and 15 004 quantified phosphosites from human embryonic stem cell
phosphorylation dynamics in Jurkat T Cells, requiring only 2 h of acquisition time per LC−MS/MS analysis.\textsuperscript{102} In the same vein, Humphrey et al. recently described their EasyPhos strategy, which combines a trifluoroethanol-based tryptic digestion and a 96-well plate format for TiOx phosphopeptide enrichment.\textsuperscript{103} This format facilitated high-throughput phosphoproteomic experiments without the need for fractionation, enabling as many as six or more biological replicates to be measured at multiple time points in time course experiments. They reported 20 000−24 000 phosphosites detected in 24 h or less of analysis time in various biological systems and described the method as a scalable platform to profile >10 000 phosphosites in hundreds of samples in a high-throughput fashion.

With the rising interest in high-throughput capabilities for phosphoproteomic measurements, several groups have explored sample preparation, enrichment, and fractionation methods that can offer both reproducibility and feasible labor requirements for large numbers of samples.\textsuperscript{104−106} Describing methods requiring only 45 min for enrichment in a 96-well format, Tape et al. reported high well-to-well reproducibility ($r^2 \geq 0.8$) and plate-to-plate reproducibility that remained robust over 5 days of independent enrichments.\textsuperscript{105} Lee and co-workers constructed a multifunctional LC system capable of standard one-dimensional separations in addition to online TiOx phosphopeptide enrichment and two-dimensional chromatography (SCX-RPLC).\textsuperscript{107} Such an approach clearly has potential benefits in reproducibility and reduced bench time for sample preparation, but the technology is still specialized to a small pool of researchers. Others have focused on development of cartridge-based enrichments and fractionation on solid phase extraction substrates. These relatively inexpensive alternative strategies are adaptable to microgram amounts of starting material, yet still offer many thousands of phosphopeptide identifications.\textsuperscript{62,108,109} Because removing the requirement for HPLC instrumentation enables rapid, flexible, and multiplexed phosphoproteomic sample preparation, these methods are valuable in many settings when fractionation is desired for increased phosphoproteomic coverage.

As with most experimental design, the balance between throughput and depth requires careful consideration. A dichotomy in the approach to sampling the phosphoproteome has emerged: either fractionate to achieve maximum phosphoproteomic depth at the cost of significant acquisition times or settle on moderate phosphoproteomic coverage at the benefit of only a few hours of analysis time per sample. (We do note that contemporary phosphoproteomics has matured greatly in the past decade; a few thousand phosphopeptides was once cutting edge, while 5 000 to even 10 000+ phosphosites is considered moderate phosphoproteomic coverage by today’s standards, depending on the biological system.) Despite all the efforts in sample preparation, enrichment, separations, and MS instrumentation and data acquisition, the number of phosphosites characterized per hour of instrument time has not drastically grown in the past 2−3 years. We expect that this will begin to change, however, as the newest generations of MS instrumentation become more ubiquitous in laboratories across the field, especially given the speed and sensitivity of the quadrupole-Orbitrap-LIT and newest q-TOF platforms. This anticipated improvement may shift considerations of the balance between throughput and depth in the coming years. Soon, single-shot analyses may be able to offer >15 000−20 000 phosphosites in just a few hours of instrument
time and thus render highly fractionated approaches relatively obsolete, except when ultra-deep phosphoproteomic coverage is paramount.

QUANTIFYING THE PHOSPHOPROTEOME

Quantitative proteomic tools have become universal and robust in recent years, making quantitative phosphoproteomics an ever more accessible undertaking (Figure 6). Cases in point: a significant proportion of the works cited in this review include quantitative components, even if not explicitly stated (Figure 7). Quantitation in phosphoproteomics is markedly more difficult than standard proteomics because quantitative information cannot be integrated over all peptides of a given protein. Quantitative values of specific phosphosites can differ even for different sites on the same protein. Thus, only quantitation from direct measurements of phosphopeptides with a specific site are useful, meaning measurements often come from relatively small sample sizes. Here we discuss recent advances in quantitation strategies as they pertain to phosphoproteomics. Additional, perhaps more extensive, information about these methods in a larger context can be found elsewhere.110−114

Stable Isotope Labeling. The incorporation of stable isotopes into proteomic samples via cell culture or chemical tagging regimes has been an active area of innovation in proteomic workflows for more than 15 years. Because they allow many intensity measurements to be taken over an elution profile, MS1 strategies, e.g., SILAC, stable isotopes are incorporated into samples during growth on a defined medium. Phosphopeptides from different samples vary in mass based on the incorporated isotopes, which can be seen by mass shifts in the MS1. Areas under the elution curve for the corresponding light and heavy phosphopeptides can then be compared for quantitative information. Chemical labeling (dimethyl, mTRAQ) works via the same mechanism, except that the mass shifts are achieved through a chemical label that is reactive with peptide functional groups (e.g., primary amines), rather than incorporation in growth media. Isobaric labeling also uses a reactive tag that labels peptide functional groups, but quantitation is achieved at the MS2 level. The intact mass of each label is the same based on the coupling of reporter and balance regions that have an equivalent number of total heavy isotopes. Upon phosphopeptide dissociation, the reporter ions fragment off, allowing comparison of relative reporter ion intensities for quantitative measurements between samples, all within the same scan that provides phosphopeptide identification.
amounts, thereby allowing characterization of several samples in a single LC–MS/MS analysis.

Developments in SILAC methods are providing new approaches for quantitative phosphoproteomics. Mann and co-workers reported the application of their in vivo labeling model, the SILAC mouse, to study tumor development in skin cancer at the proteome and phosphoproteome level. Their work provided a detailed molecular picture into skin carcinogenesis and a platform for future work in elucidating tumor progression mechanisms. The number of phosphosites reported here represents what was reported as confidently localized and quantified by each manuscript. An asterisk (*) indicates that localization confidence was not reported, and an octothorpe (#) indicates other PTMs were also enriched in the study. Some publications did not report quantitative information.

![Table](https://example.com/table.png)

**Figure 7.** Cross section of recent phosphoproteomic literature. This graphic shows relevant information for 30 recent and impactful phosphoproteomic methodology publications. Although not comprehensive, it gives a snapshot of popular methods in current studies. General details about the biological system, enrichment method, fractionation approach, quantitative strategy, and number of phosphosites characterized are provided. The number of phosphosites reported here represents what was reported as confidently localized and quantified by each manuscript. An asterisk (*) indicates that localization confidence was not reported, and an octothorpe (#) indicates other PTMs were also enriched in the study. Some publications did not report quantitative information.
strategy when metabolic labeling is not ideal, e.g., with primary tissues. SILAC is not highly compatible with in vivo models that require larger numbers of animals, so Wilson-Grady et al. demonstrated the utility of reductive dimethylation protocol at low-pH conditions to quantify hepatic phosphoproteome changes in tissue from fasted and refed mice. Of the 8500 phosphosites identified in this study, nearly 7400 of them were reliably quantified, with 390 phosphosites found to be changing between the fasted and refed conditions (2-fold change cutoff). Dimethyl labeling has been used in combination with enzymatic kinase reactions, as well, providing large-scale determination of absolute phosphorylation stoichiometries. Chemical labeling strategies have also been coupled with single-step enrichment platforms to enable robust yet straightforward methods for quantitative phosphoproteomic experiments.

MS/MS quantitation strategies provide an alternative approach for multiplexed quantitation, one that eliminates the MS\(^1\) spectral complexity of the approaches described above, which can limit sampling depth. Generally, these methods employ isotopic labels, e.g., tandem mass tags (TMT) and isobaric tag for relative and absolute quantification (iTRAQ), for the quantitative comparison of six to ten samples in a single experiment. Largely used for relative quantitation in global phosphoproteomic experiments, isobaric labels have also proven useful in recent studies of study phosphopeptide stoichiometry and absolute quantitation.

The key limitation in isobaric labeling strategies is the cofragmentation of peptides in the same precursor isolation window. This well-known phenomenon impairs quantitative accuracy by compressing ratios used in comparing reporter ion intensities. MS\(^3\)-based approaches and precursor charge reduction via proton transfer reactions have been introduced to address precursor interference. Another approach to mitigate precursor interference, called synchronous precursor selection (SPS), has built off of the MS\(^3\) strategy. SPS uses a multinoth waveform to isolate and cofragment multiple product ions in an CAD MS/MS scan to increase the number of reporter ions in the MS\(^3\) spectrum 10-fold over the standard MS\(^3\) method. These improvements translate to gains in the dynamic range of reporter ion quantitation and reduction in reporter ion signal variance, which in turn provides high-quality quantitative measurements. The SPS method has been commercially implemented on the quadrupole-Orbitrap-LIT platform and has enabled accurate, multiplexed quantitation of >38 000 phosphopeptides (discussed above).

Increases in theplexing capacity of isobaric labels have arisen from the manipulation of subtle mass differences caused by nuclear binding energy variation in stable isotopes. When coupled with high-resolution MS/MS scans, these ~6 mDa mass differences can be discriminated for quantitative measurements. These so-called neutron-encoded signatures have also been leveraged in the design of NeuCode, a new quantitation strategy that provides the quantitative accuracy of MS\(^1\)-based quantitation approaches without sensitivity-limiting increases in spectral complexity. The compatibility of NeuCode with both metabolic and chemical labeling methods makes it a flexible platform for protein and PTM quantitation in a variety of samples and experimental designs, including DIA approaches. Recently, Rhoads et al. implemented an in vivo labeling strategy with NeuCode to study the phosphoproteome in Caenorhabditis elegans. This study provided one of the largest phosphoproteomic data sets to date for C. elegans (6 620 phosphorylation isomers), revealing a post-translational signature of pheromone sensing in the organism.

Traditional stable isotope labeling methods use \(^{13}\)C and \(^{15}\)N, as well as deuterium when necessary, but \(^{18}\)O isotope labeling approaches have also proven valuable for phosphoproteomics. Xue et al. described a novel stable isotope labeled kinase reaction approach to study direct substrates of kinases, in which a whole cell extract was moderately dephosphorylated and subjected to in vitro kinase reactions using \(^{18}\)O-ATP as the phosphate donor. Similarly, Molden et al. employed \([\gamma-^{18}\text{O}]\text{ATP}\) to label amino acids with heavy phosphate to determine global site-specific phosphorylation rates. This strategy boasts direct labeling of phosphosites, the ability to measure phosphorylation rates, improved confidence in phosphopeptide identification due to the presence of heavy isotopes, and the identification of actively phosphorylated sites in a cell-like environment. Approximate rate constants for >1 000 phosphosites were calculated based on labeling progress curves, with phosphorylation rate constants ranging from 0.34 min\(^{-1}\) to 0.001 min\(^{-1}\).

Label-Free Strategies. Label-free approaches, namely, spectral counting and spectral intensity (also known as area under the curve, AUC) measurements, offer relative quantitative comparisons between samples without the use of isotopic labels. Label-free quantitation is popular in the proteomic and phosphoproteomic communities due to its lack of implementation costs and its flexibility in experimental design. Opposed to stable isotope labeling approaches, label-free strategies are not multiplexed and samples are never mixed prior to LC–MS/MS analysis. Thus, the number of conditions and replicates compared by label-free quantitation is theoretically unrestrained, although practical limitations apply; because each sample is analyzed individually, data acquisition times can be significantly higher in label-free experiments compared to isotopic labeling quantitative experiments, where samples can be multiplexed in one analysis. However, the cost in acquisition time has not deterred widespread use of label-free quantitation in large-scale phosphoproteomic experiments, as indicated by the selected publications in Figure 7. The straightforward nature of label-free strategies makes them easy to couple with the various enrichment and fractionation strategies used in phosphoproteomics and, as they do not introduce additional workflow steps, they are simple to implement in high-throughput and automated sample preparation. Thus, the majority of the challenges with label-free quantitation come in postacquisition data analysis.

Key improvements in label-free quantitation software, most notably Skyline and MaxQuant, have made quantitation in phosphoproteomic experiments more robust. Nevertheless, the stochastic nature of phosphopeptide data acquisition (see above) still presents a significant challenge to label-free phosphoproteomic strategies. If a given phosphopeptide evades detection in any single LC–MS/MS analysis within a given set of experiments, quantitation of that phosphopeptide becomes difficult. A variety of missing value imputation methods can be used for label-free data; a popular approach uses retention time alignment and accurate mass to assign sequences to unidentified spectral features based on MS/MS identification from other LC–MS/MS files in an experi-
Popular as the “match-between-runs” feature in MaxQuant,149 this valuable tool can salvage many peptide identifications in large-scale studies, but it may also introduce ambiguity in phosphoproteomic data. Different phosphopeptide isoforms, i.e., peptides that have phosphoryl modifications on different residues, have the same intact mass but not the same phosphosite identity, meaning confident phosphosite assignment can be lost when using the match-between-runs approach. Additionally, perturbations that cause large unidirectional changes in biological systems can challenge the ability to reproducibly detect and quantify phosphopeptides with label-free strategies. New strategies are emerging to combat this difficulty, such as pairwise normalization approaches that adjust normalization based on phosphopeptide abundances before and after enrichment.150

Label-free strategies will continue to be popular in phosphoproteomics, even though stable isotope labeling approaches may offer more confident quantification and multiplexed data acquisition. We view label-free strategies as especially powerful in standard proteomic experiments; they are practical and suitable for phosphoproteomics, as well, but they must be used with consideration and awareness of their challenges in throughput, phosphosite assignment, and reproducibility.

### CONFIDENT PHOSPHOSITE ASSIGNMENT

One of the principal advantages of MS-based phosphoproteomics is the ability to offer site-specific resolution for systems-level phosphorylation events. Determination of specific phosphosites permits the functional characterization of the modifications observed. Thus, identification and quantification of tens of thousands of phosphopeptides becomes far less powerful if an experiment cannot provide unambiguous phosphosite assignment for the phosphopeptides detected. A sizable percentage (20−40%) of identified phosphopeptides in typical phosphoproteomic experiments is lost because confident localization of a phosphosite (or phosphosites) cannot be assigned to the peptide. Phosphosite localization data comes directly from MS/MS fragmentation spectra of phosphopeptides, so advances in the fragmentation methods and informatics tools used to generate and extract this information are incredibly valuable to the field. The success of these methods is felt in experimental reproducibility, as well: if phosphopeptides are detected, but phosphosites cannot be localized due to inconsistent fragmentation spectra or inadequate analysis software, the overlap in useable information is diminished.

**Tandem MS Approaches.** The labile nature of phosphoryl groups on modified peptides has often put phosphopeptide fragmentation at center stage. Challenges in investigating phosphorylation with collisional activation dissociation (CAD) have inspired the development of many alternative fragmentation strategies for phosphoproteomic applications,151–160 although its relative simplicity still makes collision-based dissociation a popular option. Though CAD’s complications have been well documented, current research continues to offer insight into its utility in phosphoproteomic experiments. Citing increased rearrangement under nonmobile or partially mobile protonation conditions, Cui and Reid recently described the challenges of localizing phosphosites during CAD of phosphopeptides due to competing fragmentation and rearrangement reactions occurring upon activation.161 Brown et al. have also reported the proclivity for neutral losses in CAD with increased proximity of the phosphorylated residue to the peptide N-terminus. However, neutral loss activity is reduced when basic groups are directly N-terminal to the phosphate, which they accounted to steric hindrances in catalyzing neutral loss.162 Eyers and co-workers reported improved phosphopeptide fragmentation and phosphosite localization with CAD through enzymatic removal of basic lysine or arginine residues from the C-terminus of tryptic phosphopeptides.163 This strategy promoted the formation of sequence-informative b- and y-type fragment ions over the typical neutral loss of phosphoric acid (H3PO4) that can dominate CAD spectra. Other groups continue to use neutral loss ions from CAD fragmentation to inform data acquisition for phosphoproteomics, combining CAD with alternative fragmentation methods like electron transfer dissociation (ETD) to improve phosphopeptide identification and phosphosite localization.164−167

ETD technologies for phosphoproteomic analyses have steadily matured.168−170 Commercial developments, like the introduction of a more stable front-end ETD source on quadrupole-Orbitrap-LIT instruments,171 have improved accessibility of ETD for routine use in the phosphoproteomic community. Although ETD can provide extensive peptide backbone fragmentation while retaining the labile phosphoryl modification, it can also suffer from poor dissociation efficiencies when precursor ion charge density is low. Introducing additional energy to the ETD reaction can improve dissociation efficiencies to provide more sequence-informative product ions. Recent work has shown that concurrent photoactivation with infrared photons172 and combinations of ETD with ultraviolet photons173 can improve phosphopeptide identification phosphosite localization.

Collisional activation of ETD products has also shown significant benefit for phosphopeptide fragmentation.174−176 In 2012, the Heck lab introduced EThcD, a hybrid fragmentation method that utilizes beam-type collisional activation of ETD products after the ion−ion reaction. EThcD has compellingly improved identification of phosphopeptides and localization of phosphosites.177,178 In 2013, Frese et al. showed that EThcD, although identifying fewer phosphopeptides than HCD, improved peptide sequence coverage and percentage of localized phosphosites over both HCD and ETD fragmentation.178 For endogenous peptides and phosphopeptides presented by HLA class I, biomolecules that have been traditionally difficult to analyze via conventional fragmentation methods, EThcD improved identification rates by ~15% over ETD and nearly 30% over collisional dissociation methods.179

EThcD can also improve fragmentation of whole proteins and improve localization of phosphosites on phosphorylated proteoforms.180 Intact phosphoprotein interrogation provides a holistic picture of all modifications on a given protein, and extensive backbone fragmentation can provide single residue specificity for each modification. Because the increased chemical complexity of intact proteins makes them more difficult to analyze than peptides, top-down approaches for phosphoprotein characterization are far less common that bottom-up approaches that target phosphopeptides. Still, the combinatorial patterns of PTMs that decorate proteins are lost in the popular peptide-centric approaches. Recent improvements in alternative intact protein fragmentation methods therefore help drive top-down phosphoproteomics to better understand the role of PTMs on multiply modified proteins.181−183

DOI: 10.1021/acs.analchem.5b04123
Anal. Chem. 2016, 88, 74−94
Fragmentation of peptide anions is another alternative approach gaining traction in the proteomics community, as it can provide access to new information not seen by traditional methods. The vast majority of proteomics workflows use positive electrospray ionization with LC–MS/MS to fragment peptide cations with collisional activation, which limits detection of species that prefer deprotonation over protonation. While collision-based dissociation approaches do not generate reproducible sequence-informative product ion spectra of negatively charged peptides, several photodissociation and electron-driven fragmentation methods have emerged to facilitate high-throughput proteomics in the negative mode.184–190 Because the negative charge of phosphoryl groups can lead to preferential ionization of phosphopeptides as anions,191,192 these negative mode approaches have the potential to provide a new dimension to phosphoproteomic experiments. Holistically, there are certainly many interesting avenues to explore with phosphopeptide fragmentation. That said, the majority of the phosphoproteomic experiments use higher-energy collisional dissociation (HCD) for routine experiments because it is relatively straightforward to implement and the neutral loss problems of CAD are largely overcome by the higher energy deposition of the collisions.193

**Post-Acquisition Processing and Informatics.** Informatics tools to reliably extract tandem MS data for phosphosite localization and functional annotation are crucial to biological interpretation of phosphoproteomic experiments. Recently developed libraries of synthesized phosphopeptides of known sequence and their fragmentation spectra have provided excellent resources for evaluating search algorithms, fragmentation schemes, enrichment and separation strategies, and prediction tools.194–197 Perhaps their most powerful application, these libraries help researchers develop new informatics tools for MS/MS spectral interpretation and phosphosite localization. One popular and relatively recent algorithm for phosphosite localization is PhosphoRS, which assigns individual site probabilities for phosphopeptides (Figure 8).198 PhosphoRS is compatible with multiple fragmentation types and a range of mass accuracy measurements. It has improved upon previously available algorithms, with 3470 unique localized phosphosites from HeLa cells compared to 3107 with A-score,5349 and 2763 with Mascot Delta score.200 A generic approach for obtaining a single confidence score for PTM localization, called the D-score, has been developed from the established Mascot Delta score algorithm for compatibility with multiple search engines.201 The D-score is calculated by translating search engine scores into posterior error probability (PEP) and estimating the PEP distribution and the neutral loss problems of CAD are largely overcome by the higher energy deposition of the collisions.193

![Figure 8. Phosphosite localization.](image)

Many informatics platforms are being developed with flexibility and accessibility in mind, with an ultimate aim for universal tools that perform well for diverse types of spectral data sets. The MS-GF+ search algorithm has demonstrated that its robust probabilistic model works well across a variety of data sets, including spectra generated using diverse configurations of MS instruments and experimental protocols.203 Described as a truly universal MS/MS database search tool, MS-GF+ performed more favorably for phosphopeptides than older tools like Mascot-Percolator and InsPecT. Other more broadly applicable PTM spectral matching approaches have also been developed; these include wide precursor tolerance (±500 Da) database searches to identify peptide modifications without a priori knowledge on a proteome-wide scale,205 and directed database searching to match modifications like phosphorylation based on previous observations at specific amino acid residue positions.206 With the increasing availability of informatics tools, recent studies have aimed to equip researchers with the knowledge to choose the best tools for their work through evaluation of other open-source applications for phosphoproteomic data analysis with multiple-search-engine compatibility.207,208 Overall, the maturation and availability of robust phosphosite localization tools has greatly increased the information density of phosphoproteomic data sets, providing
residue-specific data for further biological interpretation. Continual development in making these tools compatible with high-volume data and cutting edge phosphopeptide fragmentation techniques will only improve the efficacy and reproducibility of phosphosite characterization.

Beyond postacquisition processing and phosphosite localization, informatics tools are required to translate large-scale data sets to a biologically relevant context, including spatial and temporal information about signaling networks. In a recent subcellular phosphoproteomics study, support vector machines were used to determine compartment-specific phosphosites, which provided spatial resolution to more than 10,000 human phosphoproteins with experimentally verified information on subcellular localization. A cluster evaluation approach used to study temporal dynamics of signaling cascades in two time-series phosphoproteomics data sets identified key kinases associated with human embryonic stem cell differentiation and insulin signaling pathway. This approach used prior knowledge, annotated kinase-substrate relationships mined from literature, and curated databases to generate biologically meaningful partitioning of phosphorylation sites. It then determined key kinases associated with each cluster based on temporal kinetics of similar substrates of a given kinase. Deriving and training logic models to handle high-content phosphoproteomic data using prior knowledge of kinase/phosphatase-substrate interactions has also been utilized to investigate targets and effects of kinase inhibitors and reconcile conclusions obtained from multiple data sets.

A pipeline for systematic elucidation of signaling networks has also been developed to identify key proteins in specific pathways, discover protein—protein interactions, and infer signaling networks. Using quantitative phosphoproteomic experiments, this informatics approach performed phosphopeptide meta-analysis, correlation network analysis, and causal relationship discovery to study stress responses in budding yeast. Follow-up experiments validated the discovery of 5 high-confidence proteins from meta-analysis and 19 hub proteins from correlation analysis. Ultimately, this pipeline provides a comprehensive tool for systematically discovering signaling networks and candidate proteins for further investigation. PhosphoSitePlus, a publically available database that contains ∼260,000 reported phosphosites, is another valuable tool for network analysis in phosphoproteomic experiments, although many of the phosphosites do not have a known function or associated kinase.

Figure 9. Interaction networks built from a phosphosite-centric perspective. PhosphoPath is a Cytoscape-based tool that aids visualization and analysis of quantitative phosphoproteomic data. Displayed here is a quantitative interaction network of members of the MAPK pathway, with blue and red representing down- and up-regulation, respectively. Straight lines show protein—protein interactions from Biogrid while arrows visualize kinase-substrate interactions from PhosphoSitePlus. Multiplicity is indicated by the color bar for each protein, and edges can be added manually, such as the red edge at the top of the figure showing inhibition of NF1 on NRAS. Reprinted with permission from Raaijmakers, L. M.; Giansanti, P.; Possik, P. A.; Mueller, J.; Peper, D. S.; Heck, A. J. R.; Altena, A. F. M. J. Proteome Res. 2015 (ref 213). Copyright 2015 American Chemical Society.
Building on these options, PhoSigNet was designed to be a phosphorylation-centric database and analysis platform that can store and display human phosphorylation-mediated signal transduction networks, taking kinase–substrate regulatory pairs into account and also extending regulatory relationships up- and downstream.214 PhosphoPath takes visualization one step further as a PTM-specific tool, focusing on displaying protein–protein interactions, kinase–substrate interactions, and pathway enrichments at a phosphosite-centric level (Figure 9).215 Integrating data from three public databases, PhosphoPath is a Cytoscape plug-in that offers accessibility and phosphosite-directed data analysis for quantitative information for multiple conditions or time points at protein and PTM levels. Given the development of analytical and informatics tools across a diverse range of species and sample types, cross-species mapping of PTMs can be a valuable informatics approach to understanding key signal transduction mechanisms. Many functionally important modification sites are more likely to be evolutionarily conserved, and new tools like PhosphOrtholog are facilitating the comparison of data sets derived from multiple species.216

**BIOLOGICAL INSIGHTS VIA PHOSPHOPROTEOMICS**

The development of analytical methods for robust and quantitative phosphoproteomics over the past several years has led to significant impact in translational science in human health and disease. Even with the challenges in reproducibility and phosphosite assignment discussed here, the field actively contributes knowledge to the greater scientific community at an impressive pace, continually accelerated by improvements in throughput and depth. The contribution of phosphoproteomics to molecular biology is far too immense to review extensively here. Instead, we discuss a cross section of studies that capture the breadth of phosphoproteomics’ impact on biological research. We direct readers desiring a greater context for the biological implications of phosphoproteomics to recent and more thorough reviews on subjects including cancer biology,217,218 clinical applications,219 cell and tissue analysis,220 metabolism,221 and systems biology.222

The long- appreciated role of phosphorylation signaling in cancer cells remains one of the most active areas of research in phosphoproteomics. Recent work from the Cutillas group showed that tumors from different hematological cancer cell lines, including acute myeloid leukemia, three lymphoma, and three multiple myeloma cell lines, can be distinguished by their phosphoproteomes and their phenotypic responses to inhibitors.223 This group also used phosphoproteomic analyses of acute myeloid leukemia cells to systematically infer the activation of kinase pathways, providing a computational approach to profile dysregulation of signaling pathways in an untargeted fashion.224 Conserved oncogenic signaling pathways can also distinguish mouse models of breast cancer on the basis of tyrosine phosphorylation signatures and signaling networks.225

Protein kinase B (Akt) is known to play key roles in cell proliferation and metabolism, and aberrant hyperactivation of the mTORC2 (mechanistic target of rapamycin complex 2)—Akt pathway can facilitate tumorigenesis.226 Using MS-based phosphoproteomic methods in combination with other approaches, Liu et al. showed that phosphorylation of Akt at serine 477 and threonine 479 is an essential layer of its activation mechanism in the regulation of its physiological functions, providing a mechanistic link between Akt hyperactivation in cancer and aberrant cell cycle progression.227 Quantitative phosphoproteomics also facilitated identification of apoptosis-modifying kinases that are highly connected to regulated substrates downstream of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This study offers a resource of potential targets for the development of TRAIL combination therapies to selectively kill cancer cells.228 Mechanistic insights into novel kinase activity, such as enzymes involved in coenzyme Q biosynthesis, have established a molecular foundation for further investigation of how classes of proteins affect cancer and other diseases through diverse biological pathways as well.229

Nontraditional approaches have been integrated with more canonical bottom-up phosphoproteomic techniques to study phosphorylation signaling in cancer. In order to study combinatorial PTM patterns related to the progression of breast cancer through the cell cycle, top-down methods were leveraged to identify and quantify phosphorylation of histone H1 proteoforms (a potential clinical biomarker of breast and other cancers).230 Peptide-centric bottom-up phosphoproteomics was then integrated with the intact proteome data to ultimately show progressive H1 phosphorylation across the cell cycle, suggesting specific phosphorylation events may serve as markers for proliferation. Quantitative phosphoproteomics has also provided more insight into cell-cycle regulation via histone H2A. One study showed that autophosphorylation of Bub1 kinase, which phosphorylates H2A at tyrosine 120 to promote centromere sister chromatid cohesion, is mitosis specific, and that Bub1 activation is primed in interphase but only fully achieved in mitosis.231 Moreover, phosphorylation of H2A at tyrosine 57, a conserved modification from yeast to mammals, is involved in regulation in transcriptional elongation based on the unsuspected tyrosine kinase activity of casein kinase 2.232 Very recently, a “multi-omics” approach that incorporated data from metabolomics, lipidomics, and phosphoproteomics on multiple myeloma cells revealed that kinase inhibitors may not only downregulate phosphorylation of their targets but also induce metabolic events via increased phosphorylation of other cellular components.233

Phosphorylation of proteins involved in nuclear activity is central to many cellular processes. In 2013, Kirkpatrick et al. used large-scale phosphoproteomics to uncover extensive signaling among proteins in the DNA damage pathway when cell death was initiated in melanoma cells through treatment with small molecule inhibitors against MAP/ERK kinase (MEK) and phosphatidylinositol 3-kinase (PI3K).234 Their work provided further insight into short- and long-term sensitivity of tumor cells to MEK- and PI3K-targeted therapies, in addition to the broader impacts of combinatorial therapeutic approaches for intervention in many cancers. Regulation of transcription in the nucleus by stretches of consecutive phosphoserine residues (3 to >10 in a row) has recently been shown in the human phosphoproteome, with the majority of the phosphoproteins with pSer stretches functioning in macromolecular, nucleotide, or metal ion binding.235 Interestingly, stretches of consecutive pThr and pTyr are almost absent. Phosphorylation can also play an important role in nuclear activity during the viral life cycle. Phosphorylation of the monomeric nucleoprotein in large ribonucleoprotein (RNP) complexes from negative-sense RNA viruses regulates oligomerization of the monomer into the complex, an essential step for virus replication.236
Appreciation for the role of reversible mitochondrial phosphorylation in signaling and energy utilization continues to grow. Using isobaric labeling-based quantitative phosphoproteomics, Grimsrud and co-workers demonstrated that phosphorylation is widespread in mitochondria and is a key mechanism for regulating ketogenesis during the onset of obesity and type 2 diabetes. Mitochondria play a key role in the cell’s adaptation to metabolic demands, and Ferreira et al. used a label-free quantitative approach to show that reprogramming of the phosphoproteome reflects the response of heart mitochondria to metabolic demands of long exercise programs, which are associated with improvement in cardiac function and lifespan extension. The plasticity of mitochondrial response to acute exercise signaling via phosphorylation has also been shown in skeletal muscle, where many previously undescribed roles for phosphorylation modifications exposed the unexplored complexity of signaling in acute exercise. Unexpected roles in signaling in the secreted phosphoproteome have emerged as well. Extracellular phosphoproteins have been recognized for over a century, for example, but challenges in measuring them have limited the development of substantial knowledge. As it turns out, Fam20C generates the majority of the extracellular phosphoproteome, and its substrates suggest roles for the kinase beyond biomineralization, including lipid homeostasis, wound healing, and cell migration and adhesion.

Beyond the focus on mammalian phosphoproteomics in translational research, the importance of global phosphorylation in plants and microbial systems has garnered considerable attention. Recent descriptions of the roles of phosphorylation in Arabidopsis thaliana (a model organism for flower development) have included responses to hormone signaling, DNA damage, and circadian clock cycles. Large-scale experiments have linked phosphorylation-based signaling in Medicago truncatula, a model legume used for studying nitrogen fixation, to the formation and association of symbiotic relationships with rhizobia that assist in the nitrogen fixation process.

Yeast has been a popular system for studying TOR signaling. Target of rapamycin signaling complex 1 (TORC1) is implicated in growth control/proliferation and aging from yeast to humans. Recent work has leveraged phosphoproteomics in combination with dynamic metabolomics data to infer the functional role of phosphorylation in the metabolic activity of 12 enzymes, including three candidate TORC1-proximal targets. This work ultimately helped resolve the temporal sequence of phosphorylation responses to nutritionally and chemically induced changes. A high temporal-resolution global phosphoproteomics experiment was evaluated recently in Schizosaccharomyces cerevisiae. This study indicated that putatively functional kinase- or phosphatase-substrate interactions occur more rapidly (within 60 s) than promiscuous interactions, allowing specific and functional kinase- and phosphatase-substrate interactions to be profiled. Measuring proteomic and phosphoproteomic changes over the four major cell cycles of Schizosaccharomyces pombe, Carpy et al. quantified cell-cycle-dependent fluctuations on a proteome-wide scale and showed that protein phosphorylation peaked in mitosis. This study also coupled measurements of copy numbers per cell with the phosphoproteomic data to estimate phosphosite stoichiometry with absolute amounts of protein-bound phosphate.

**PMT Cross-Talk.** Phosphorylation is one of many dozens of important PTMs in cellular function. Protein modification rarely, if ever, occurs in isolation, and the interaction of various PTMs can carry important biological information in prokaryotic and eukaryotic systems. Carr and co-workers introduced a robust serial enrichment strategy that enabled characterization of an average of 20 800 localized phosphosites, 15 408 ubiquitination sites, and 3 190 acetylation sites from 7.5 mg of a single sample, all with SILAC quantitation. This powerful platform revealed cross-talk among six interconnected protein networks that regulate cell cycle, replication, transcription, translation, and the proteasome in Jurkat cells. Swaney et al. studied cross-talk events between phosphosites and ubiquitination that regulate protein degradation via the ubiquitin-proteasome system. Phosphosites had greater conservation on ubiquitinated proteins, indicating a role in biological function and suggesting a global cross-talk directionality, in which phosphorylation more frequently precedes ubiquitination. Co-regulation of phosphorylation- and ubiquitination-dependent signaling networks has been shown in yeast treated with rapamycin, as well. PTM interaction in signaling networks aimed at cell survival or death in myocardial ischemia is also important, where lysine acetylation can activate protein kinases during ischemia and increase proximal dephosphorylation by as much as 10-fold. The interplay of phosphorylation and acetylation was also investigated in the strong correlation of maximal exercise-associated oxidative capacity to health and longevity. Cross-talk between phosphorylation and O-linked N-acetylglucosamine (O-GlcNAc), which modifies the same residues as phosphorylation, is widespread and important, as well, serving as a nutrient/stress sensor to modulate signaling, transcription, DNA damage response, and cytoskeletal functions.

Phosphorylation can also exhibit cross-talk with other phosphorylation-regulated pathways. Signal integration between mitogen-activated protein kinase cascades in budding yeast has shown that concurrent stimuli (high salt concentration and pheromones) affect multiple pathways previously thought to be specific to a given stimulus. This intra-phosphorylation cross-talk revealed that phosphorylation events in many pathways affect each other at more levels than anticipated, showing that the integration of a response to different stimuli requires complex interconnections between signaling cascades. Cross-talk is not limited to interactions between PTMs, either; recent work has shown that cross-talk can occur between phosphorylation and heat shock protein in brain tissue samples from Alzheimer’s patients.

### PHOSPHORYLATION BEYOND SERINE, THREONINE, AND TYROSINE

While the majority of the phosphoproteomics community focuses on O-phosphorylation (serine, threonine, and tyrosine), there is a growing appreciation for alternative sites of modification, namely, N-phosphorylation (lysine, arginine, and histidine) and S-phosphorylation (cysteine). These alternative phosphorylation events are known to function in important signaling mechanisms in bacterial systems, and more studies are emerging to suggest they may play a role in eukaryotic signaling as well. Analytical tools for the investigation of N- and S-phosphorylation, however, are lacking. Canonical LC–MS/MS approaches that utilize acidic buffers are less than ideal because both phosphoramidate and phosphothiolate bonds are acid labile. Similar to CAD on phosphopeptides with pSer, pThr, and pTyr, gas-phase rearrangements in MS/MS fragmentation for peptides...
containing phosphorylated histidine, arginine, and lysine have led to false localizations. Electron-driven fragmentation methods seem to hold more promise for these modifications than collisional dissociation.275–278

One alternative to O-phosphorylation in particular, phosphohistidine, has garnered increased interest in recent years.266 Phosphohistidine antibodies have been generated by the Muir and Hunter groups,276–278 and a large-scale phosphohistidine study was reported using a neutral loss fragmentation strategy.279 These studies certainly show progress in the analysis of this elusive PTM, but in all, investigations have remained limited. As specific enrichment strategies for phosphohistidine and other nontraditional phosphorylated residues emerge, larger scale and more systematic evaluations of their biological roles can be investigated. These investigations may require alternative separations, fragmentation, and informatics tools, but a more comprehensive understanding of nontraditional phosphorylated residues’ role in prokaryotic and eukaryotic systems makes the effort worthwhile.

■ LOOKING FORWARD

The optimal phosphoproteomic methodology for thorough phosphoproteome coverage with minimal sample preparation steps and data acquisition time remains elusive, but present-day techniques continue to progress toward more reproducible methods that offer considerable throughput and depth. Leveraging refinement of phosphopeptide enrichment protocols, improved sensitivity and speed of new-generation mass spectrometers, and more robust informatics tools, phosphoproteomic technology now can offer >10 000 localized and quantified phosphosites in only a few hours of data acquisition and tens of thousands of phosphosites in weeks of analysis. We remain far from complete phosphoproteome characterization, however. Confident reports of a complete phosphoproteome from even a single sample or cell line have yet to emerge, much less a generic platform where complete phosphoproteomes can be routinely profiled. While rapid and deep whole proteome-level characterization is currently within reach, comprehensive PTM-level characterization, such as a complete phosphoproteome, appears a decade or more away.

Phosphoproteomic technology will surely continue to advance hand-in-hand with improvements in MS instrumentation. Traditional shotgun phosphoproteomic experiments will benefit directly from gains in instrument scan rate and sensitivity, and alternative methods, such as SWATH-MS-like DIA approaches, promise to offer increased reproducibility, perhaps coupled to competitive phosphoproteomic sampling depths in coming years. We anticipate an increase in routine sampling depth to entail a greater appreciation for overlap, as opposed to orthogonality of sample preparation methods. On the basis of current data comparing state-of-the-art techniques, we expect the coming years will see a convergence of preparation and enrichment methods into a few reproducible but versatile options. Beyond traditional LC–MS/MS bottom-up strategies, advances in top-down proteomic tools have placed large-scale, quantitative intact phosphoprotein analysis within reach, and progress in alternative separations technologies, like capillary zone electrophoresis, may also provide more thorough phosphoproteome characterization.280–284

Ultimately, the future of phosphoproteomic research holds reproducible identification and quantification of tens of thousands of phosphosites in a few hours of analysis per sample. With such capability, phosphoproteomics can achieve even more significant impact in biological research and clinical platforms. In parallel with technological developments necessary for this level of analytical power, the field will require streamlined data-analysis and interpretation tools that can capitalize on the speed and sensitivity of state-of-the-art MS methodology. Success will rely on technology that can integrate large data sets into systems biology approaches while maintaining the flexibility of phosphoproteomic tools to address discovery-based hypothesis-driven questions.

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Notes

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Joshua J. Coon is a Professor of Chemistry and Biomolecular Chemistry at the University of Wisconsin-Madison. Coon earned his B.S. degree at Central Michigan University and received his Ph.D. at the University of Florida in 2002. At Florida, Coon studied ambient ionization processes under the guidance of Professor Willard Harrison. From 2003 to 2005 he was an NIH postdoctoral fellow with Professor Donald Hunt at the University of Virginia. During his time at Virginia he, with Hunt and John Syka, coinvented electron transfer dissociation (ETD). Coon’s research program at Wisconsin is focused on all aspects of biomolecular mass spectrometry.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge support from National Institutes of Health (NIH) Grants R01 GM114122 and R01 GM080148. NMR was funded through a National Science Foundation (NSF) Graduate Research Fellowship (DGE-1256259). The authors acknowledge the phosphoproteomic researchers whose original research work, both before and during the period covered by the article, could not be discussed in great detail due to space limitations. The authors also thank Anna Merrill for help in compiling references, Alicia Williams for valuable edits to the manuscript, and Alex Hebert, Greg Potts, Nick Kwieicen, Alicia Richards, and Tim Rhoads for helpful discussions about content and figures.

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