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Advances in Phosphoproteomics: New Methods, Techniques, and Neurological Applications

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Abstract: ***As one of the most prevalent and functionally critical post-translational modifications, protein phosphorylation governs virtually every aspect of cellular signalling, making its comprehensive analysis indispensable to modern biology. In this paper, we review the principal methodologies underpinning phosphoproteomic analyses, including phosphopeptide enrichment strategies, advanced mass spectrometry workflows, and quantitative approaches. We further highlight the application of phosphoproteomics to neuroscience, emphasising its role in dissecting neuronal signalling networks, understanding synaptic plasticity, and revealing phosphorylation-driven mechanisms in neurodegenerative and psychiatric disorders. Future directions include single-cell and spatial phosphoproteomics, which promise improved resolution of neuronal signalling networks and disease mechanisms.***

Keywords: Phosphoproteomics, Protein Phosphorylation, Mass Spectrometry, Neuronal Signalling, Post-translational Modifications, Neurodegenerative Disease, Single-cell Phosphoproteomics, Spatial Phosphoproteomics, Kinase Signalling

1. Introduction

Protein phosphorylation – the reversible addition of a phosphate group to serine, threonine, or tyrosine residues – is one of the most abundant and versatile post-translational modifications (PTMs) in eukaryotic cells. Catalysed by a superfamily of over 500 kinases in the human genome and reversed by approximately 150 phosphatases, phosphorylation acts as a universal molecular switch, toggling protein activity, localisation, stability, and interaction networks [1], [2]. It has been estimated that up to one-third of all cellular proteins are phosphorylated at any given time, underscoring the scale and centrality of this modification to cell biology [3].

Phosphoproteomics has emerged as the systematic discipline for cataloguing and quantifying phosphorylation events on a proteome-wide scale. By coupling selective enrichment of phosphorylated peptides with high-resolution mass spectrometry (MS), phosphoproteomics enables the simultaneous identification of thousands of phosphorylation sites and the quantification of their dynamic changes in response to stimuli, drugs, or disease [4], [5]. This systems-level perspective is transforming our understanding of signal transduction, moving beyond individual kinase-substrate relationships toward a holistic view of entire signalling networks.

Despite its power, phosphoproteomics faces formidable technical challenges. Phosphopeptides are typically present in sub-stoichiometric amounts relative to unphosphorylated counterparts, and the dynamic range of phosphorylation occupancy can span several orders of magnitude [5], [6]. Furthermore, the labile nature of phosphate groups under standard mass spectrometric fragmentation historically impeded confident site localisation. Significant advances in enrichment chemistry, instrument sensitivity, and computational data analysis have progressively overcome these obstacles, enabling ever-deeper coverage of the phosphoproteome [6], [7].

Among the many biological systems amenable to phosphoproteomic investigation, the nervous system stands out for its exceptional reliance on phosphorylation-mediated signalling. Neurons integrate diverse extracellular signals through tightly regulated phosphorylation cascades that control synaptic transmission, neuronal excitability, gene expression, and synaptic plasticity [8]. Dysregulation of these pathways underlies a wide spectrum of neurological and psychiatric conditions, from Alzheimer's disease to schizophrenia [9]. Phosphoproteomics therefore provides an exceptionally powerful lens for neuroscience research. In the following sections, we review the methodological landscape of phosphoproteomics and examine its most significant contributions to neuroscience and neurological disease research.

2. Methodologies

2.1 Sample Preparation and Enrichment

Effective phosphopeptide enrichment is the cornerstone of any phosphoproteomic workflow. Because phosphopeptides can be present at only a fraction of the level of their unmodified counterparts, direct analysis of complex tryptic digests without prior enrichment results in severe suppression of phosphopeptide signals by abundant unphosphorylated peptides [5], [10]. A variety of complementary enrichment strategies have therefore been developed, each with distinct selectivity and coverage characteristics.

2.2 Metal Oxide and Immobilised Metal Affinity Chromatography

Metal oxide affinity chromatography (MOAC) and immobilised metal affinity chromatography (IMAC) are the selectively retain phosphopeptides from complex mixtures [10]. Ficarro *et al.* pioneered the use of IMAC for large-scale phosphoproteomics, demonstrating the identification of hundreds of phosphopeptides from yeast cell lysates and establishing IMAC as the dominant enrichment paradigm of the field [11].

Titanium dioxide (TiO_2) MOAC, introduced by Pinkse *et al.* and subsequently optimised by Larsen *et al.*, emerged as a highly effective alternative and complement to IMAC [12], [13]. TiO_2 exhibits strong and selective affinity for phosphate groups under acidic conditions, and its performance can be substantially improved by the inclusion of competing ligands such as 2,5-dihydroxybenzoic acid (DHB) or glutamic acid, which suppress the non-specific binding of acidic non-phosphorylated peptides [13]. Comparative studies have demonstrated that TiO_2 and IMAC exhibit partially complementary selectivity, and sequential or parallel combination of both methods substantially increases the depth of phosphoproteome coverage [14]. Zirconium dioxide (ZrO_2) has also been explored as an alternative metal oxide with comparable performance characteristics [15].

More recently, polymer-based materials and novel metal-organic frameworks (MOFs) have been introduced as phosphopeptide sorbents. Qing *et al.* demonstrated that specific MOF materials could achieve highly selective phosphopeptide enrichment with low non-specific binding, suggesting these emerging materials may complement or eventually supersede conventional metal oxide approaches in high-throughput settings [16].

2.3 Phosphopeptide Fractionation Strategies

To maximise phosphoproteome depth, enrichment is typically combined with upstream or downstream peptide fractionation. Strong cation exchange (SCX) chromatography was among the first fractionation strategies applied to phosphoproteomics; phosphopeptides elute at lower salt concentrations than their non-phosphorylated counterparts due to the negative charge of the phosphate group [17]. Subsequent adoption of high-pH reverse-phase fractionation (basic RP-HPLC) has become increasingly common, as it provides superior orthogonality to the acidic RP-LC used in the final analytical step and results in more even peptide distribution across fractions [18].

Electrostatic repulsion hydrophilic interaction chromatography (ERLIC) represents a particularly elegant single-step approach. Alpert demonstrated that phosphopeptides are selectively retained under ERLIC conditions due to the interplay of electrostatic repulsion and hydrophilic interactions, enabling their enrichment in a single chromatographic step without separate metal oxide or IMAC capture [19]. While ERLIC has not fully replaced MOAC-based workflows, it offers an attractive simplification for certain sample types and remains in active use.

most widely employed techniques for phosphopeptide enrichment. Both exploit the affinity of phosphate groups for positively charged metal ions. In IMAC, metal ions such as Fe^{3+}, Ga^{3+}, or Ti^{4+} are chelated to a solid support and

2.4 Mass Spectrometry Analysis

Mass spectrometry is the analytical engine driving phosphoproteomics. The standard workflow involves nano-scale liquid chromatography coupled to a high-resolution tandem mass spectrometer (nLC-MS/MS), with data-dependent acquisition (DDA) being the dominant acquisition strategy for discovery phosphoproteomics [4], [6]. Data-independent acquisition (DIA) has emerged as a powerful alternative that systematically fragments all precursors within defined m/z windows, providing more reproducible and comprehensive quantitative data [20].

Fragmentation methodology is particularly important in phosphoproteomics. Collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) generate b- and y-type fragment ions but are prone to neutral loss of the phosphate group (loss of H_3PO_4, 98 Da), which can impede confident site localisation [21]. **Electron transfer dissociation (ETD)** and electron capture dissociation (ECD) preserve the phosphate modification on fragment ions by cleaving N-Cα bonds through radical-driven mechanisms, substantially improving phosphorylation site localisation on multiply phosphorylated peptides [21], [22]. Modern instruments implementing supplemental activation during ETD – such as **EThcD** – provide complementary fragment ions in a single spectrum and represent the current state-of-the-art for phosphopeptide characterisation [22].

The adoption of Orbitrap-based instruments, which provide mass accuracy below 5 ppm and resolving powers exceeding 100,000, has been transformative for phosphoproteomics [6]. Recent platforms, including the Orbitrap Astral and timsTOF series instruments incorporating trapped ion mobility spectrometry (TIMS), offer additional gas-phase separation of phosphopeptides, further reducing spectral complexity and improving identification rates [23].

2.5 Quantitative Phosphoproteomics

Quantitative comparison of phosphorylation states across conditions is central to most biological applications of phosphoproteomics. **Stable isotope labelling with amino acids in cell culture (SILAC)** is widely regarded as one of the most accurate quantitative approaches, as it introduces isotope labels at the metabolic level, minimising sample handling variability [24]. The limitation of SILAC to cell culture systems has driven development of chemical labelling strategies, most notably **isobaric tandem mass tag (TMT) labelling**, which allows multiplexed quantification of up to 18 samples simultaneously and is compatible with tissue-derived material [25].

Label-free quantification (LFQ) based on extracted ion chromatogram areas represents an economical alternative applicable to any sample type without additional reagents [26]. For studies requiring absolute quantification of specific sites, parallel reaction monitoring (PRM) with isotopically

labelled synthetic phosphopeptide standards provides highly accurate targeted quantification, bridging discovery phosphoproteomics and clinical validation assays [27].

2.6 Bioinformatics and Computational Analysis

The informatic analysis of phosphoproteomic datasets presents significant challenges beyond standard proteomics. Accurate phosphorylation site localisation requires both high-quality MS/MS spectra and sophisticated scoring algorithms. The **Ascore algorithm** introduced by Beausoleil *et al.* and related probabilistic frameworks such as PhosphoRS assess the probability that a given site assignment is correct based on diagnostic fragment ions observed in the spectrum [28]. More recent frameworks implemented in MSFragger have further improved both sensitivity and accuracy of site localisation [29].

Downstream biological interpretation requires dedicated computational tools. **Kinase-substrate enrichment analysis (KSEA)** and related approaches infer the activities of upstream kinases from changes in the phosphorylation of their known substrates, translating phosphoproteomic observations into network-level insights [30]. Curated databases including **PhosphoSitePlus**, Phospho.ELM, and NetworKIN provide essential reference resources for such analyses [31], [32]. Network-level visualisation platforms including Cytoscape and STRING enable integration of phosphoproteomic data with protein-protein interaction networks, facilitating identification of signalling hubs and disease-relevant network perturbations [33].

3. Applications of Phosphoproteomics in Neuroscience

3.1 Neuronal Signalling and Synaptic Plasticity

The nervous system is among the most phosphorylation-dependent biological systems. Early applications of phosphoproteomics to neurons demonstrated the exceptional complexity of the neuronal phosphoproteome: Bhanu *et al.* identified thousands of phosphorylation sites in primary neuronal cultures, establishing that neuronal phosphorylation networks are considerably more extensive than had been appreciated from earlier targeted studies [8].

Synaptic plasticity- the activity-dependent strengthening or weakening of synaptic connections underlying learning and memory- is intimately governed by phosphorylation. Bhattacharyya *et al.* employed quantitative phosphoproteomics to map changes in the synaptic phosphoproteome following chemically induced long-term potentiation (LTP) and long-term depression (LTD), providing a systems-level view of phosphorylation events accompanying synaptic strengthening and weakening [34]. Their findings identified novel phosphorylation events on scaffolding proteins including PSD-95 and Shank family members.

The postsynaptic density (PSD) has been a particularly productive target for phosphoproteomic analysis. Calcium/calmodulin-dependent protein kinase II (**CaMKII**), a master regulator of synaptic plasticity whose own activation is phosphorylation-dependent, emerged as a particularly prominent node in these analyses, with numerous novel substrates identified through phosphoproteomic approaches [35], [36].

3.2 Neurotransmitter and Receptor Signalling

Phosphoproteomics has provided remarkable insights into signalling cascades downstream of neurotransmitter receptors. Dopaminergic signalling, which governs reward, motivation, and motor control, has been extensively studied using quantitative phosphoproteomics. Nishi *et al.* demonstrated that dopamine D1 receptor activation triggers coordinated phosphorylation of numerous striatal proteins through cAMP-dependent protein kinase A (PKA) and the downstream effector DARPP-32, a phosphoprotein acting as an integrative hub for dopaminergic and glutamatergic signalling [37]. Phosphoproteomic analyses revealed that the network of phosphorylation changes extends far beyond classically studied PKA substrates, encompassing hundreds of proteins involved in cytoskeletal regulation, vesicular trafficking, and gene expression [37].

Quantitative phosphoproteomic profiling of neurons treated with NMDAR antagonists or agonists revealed bidirectional phosphorylation changes at thousands of sites, many previously unknown, providing a comprehensive picture of signalling changes accompanying NMDAR activation [38]. These datasets have proven invaluable for identifying novel drug targets; the rapid antidepressant effects of ketamine have been linked to specific phosphorylation changes at synaptic signalling proteins identified through phosphoproteomic analyses [38], [39].

3.3 Neurodegenerative Diseases

Aberrant protein phosphorylation is a defining molecular hallmark of many neurodegenerative diseases. In Alzheimer's disease (AD), hyperphosphorylation of the microtubule-associated protein tau is a pathological hallmark, with over 80 tau phosphorylation sites identified in AD brain tissue [9]. Phosphoproteomic analyses of post-mortem AD brain have revealed system-wide dysregulation of kinase activities, including hyperactivation of CDK5 and GSK-3β, which are implicated in tau hyperphosphorylation and neuronal death [9], [40].

Jiang *et al.* conducted a large-scale quantitative phosphoproteomic study of AD brain across disease stages, identifying hundreds of differentially phosphorylated proteins involved in synaptic function, cytoskeletal integrity, and energy metabolism [40]. Notably, many phosphorylation changes preceded the accumulation of amyloid plaques or neurofibrillary tangles, suggesting that phosphorylation dysregulation is an early event in AD pathogenesis, highlighting the potential of phosphoproteomics for identifying early disease biomarkers.

In Parkinson's disease (PD), phosphoproteomics has illuminated the role of leucine-rich repeat kinase 2 (**LRRK2**), the most commonly mutated gene in familial PD. Phosphoproteomic profiling of cells and mouse brain tissue expressing LRRK2 variants identified Rab GTPase family

members as major substrates of LRRK2, revealing an unexpected role in regulating vesicular trafficking [41]. These findings have substantially reshaped the understanding of PD pathobiology and opened new therapeutic avenues targeting the LRRK2-Rab signalling axis [41].

Amyotrophic lateral sclerosis (ALS) and TDP-43 proteinopathies have also been investigated by phosphoproteomics. Analyses of motor neurons and spinal cord tissue from ALS models have revealed perturbations in RNA processing, stress response signalling, and cytoskeletal dynamics that accompany TDP-43 pathology [42]. As phosphoproteomic technologies become applicable to cerebrospinal fluid and blood plasma, the prospect of using phosphorylation signatures as minimally invasive biomarkers for neurodegeneration is becoming more realistic [42], [43].

3.4 Psychiatric Disorders and Neuropharmacology

Phosphoproteomics is increasingly applied to psychiatric research. Quantitative phosphoproteomic analyses of post-mortem prefrontal cortex from individuals with schizophrenia have identified alterations in the phosphorylation of proteins involved in synaptic vesicle cycling, glutamatergic neurotransmission, and cytoskeletal organisation, consistent with the synaptic dysfunction hypothesis of schizophrenia [44].

Sakata *et al.* and related studies have used quantitative phosphoproteomics to map the intracellular signalling cascades activated by antipsychotic drugs, mood stabilisers, and antidepressants in rodent brain, providing mechanistic insights that complement transcriptomic and genetic approaches [45]. The identification of drug-induced phosphorylation changes on specific proteins provides both insights into drug mechanism and potential biomarkers of therapeutic response [44], [45].

4. Challenges and Future Directions

Despite remarkable progress, phosphoproteomics faces enduring challenges. The sub-stoichiometric nature of many phosphorylation events means that even state-of-the-art enrichment workflows miss a substantial fraction of low-abundance phosphosites, particularly on low-copy-number signalling proteins. The dynamic range problem is particularly acute in neurological research, where cellular heterogeneity of brain tissue means that signals from relatively rare neuronal subtypes can be swamped by contributions from more abundant glial populations [6], [46].

Single-cell phosphoproteomics represents a frontier that the field is beginning to explore, with recent studies demonstrating the feasibility of phosphoproteomic analysis from very small cell numbers using nanoscale enrichment and sensitivity-optimised MS platforms [46]. These advances hold particular promise for neuroscience, enabling dissection of cell-type-specific signalling in brain circuits. Spatial phosphoproteomics, combining phosphopeptide detection with spatial information from tissue sections, is another emerging area that could transform our understanding of region-specific phosphorylation in the brain [47].

On the computational side, machine learning and deep learning methods are increasingly being applied to predict kinase-substrate relationships de novo from sequence and structural features, and to integrate phosphoproteomic data with other omics layers in multi-modal analyses [33], [48]. The development of more comprehensive, regularly updated phosphorylation databases and standardisation of data reporting formats will be essential to realise the full potential of these computational advances.

5. Conclusion

Phosphoproteomics has evolved from a technically challenging niche discipline into a mature and indispensable branch of proteomics, enabling comprehensive characterisation of protein phosphorylation on a systems-wide scale. In this review, we have examined the principal methodologies that underpin phosphoproteomic workflows, from selective enrichment strategies such as IMAC and TiO_2 MOAC, through advanced fragmentation methods including EThcD, to quantitative labelling approaches and sophisticated bioinformatic pipelines. These technical advances have collectively enabled the routine identification and quantification of tens of thousands of phosphorylation sites in a single experiment.

In neuroscience, phosphoproteomics has proven to be a particularly powerful approach, revealing the extraordinary complexity of neuronal signalling networks and providing mechanistic insights into synaptic plasticity, neurotransmitter receptor signalling, and the pathological phosphorylation events that characterise neurodegenerative and psychiatric disorders. The identification of LRRK2 substrates, the mapping of synaptic plasticity-associated phosphorylation networks, and the characterisation of kinase dysregulation in Alzheimer's disease exemplify the biological discoveries uniquely enabled by phosphoproteomic approaches.

Looking ahead, the continuing development of single-cell and spatial phosphoproteomic methods, combined with increasingly powerful computational tools for network analysis and multi-omics integration, promises to further deepen our understanding of phosphorylation-regulated processes. As these technologies expand into clinical settings, phosphoproteomics is poised to deliver transformative insights into the molecular mechanisms of neurological disease and to identify novel biomarkers and therapeutic targets that could meaningfully advance patient care. Furthermore, the integration of phosphoproteomics with single-cell, spatial, and multi-omics technologies is expected to accelerate biomarker discovery, therapeutic target identification, and precision neuroscience applications.

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