Proteomics by Mass Spectrometry: Approaches, Advances, and Applications

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Abstract

Mass spectrometry (MS) is the most comprehensive and versatile tool in large-scale proteomics. In this review, we dissect the overall framework of the MS experiment into its key components. We discuss the fundamentals of proteomic analyses as well as recent developments in the areas of separation methods, instrumentation, and overall experimental design. We highlight both the inherent strengths and limitations of protein MS and offer a rough guide for selecting an experimental design based on the goals of the analysis. We emphasize the versatility of the Orbitrap, a novel mass analyzer that features high resolution (up to 150,000), high mass accuracy (2–5 ppm), a mass-to-charge range of 6000, and a dynamic range greater than 10^3. High mass accuracy of the Orbitrap expands the arsenal of the data acquisition and analysis approaches compared with a low-resolution instrument. We discuss various chromatographic techniques, including multidimensional separation and ultra-performance liquid chromatography. Multidimensional protein identification technology (MudPIT) involves a continuum sample preparation, orthogonal separations, and MS and software solutions. We discuss several aspects of MudPIT applications to quantitative phosphoproteomics. MudPIT application to large-scale analysis of phosphoproteins includes (*a*) a fractionation procedure for motif-specific enrichment of phosphopeptides, (*b*) development of informatics tools for interrogation and validation of shotgun phosphopeptide data, and (*c*) in-depth data analysis for simultaneous determination of protein expression and phosphorylation levels, analog to western blot measurements. We illustrate MudPIT application to quantitative phosphoproteomics of the beta adrenergic pathway. We discuss several biological discoveries made via mass spectrometry pipelines with a focus on cell signaling proteomics.

Contents

1. INTRODUCTION

Whole-genome sequencing efforts of the past century have produced many fully sequenced genomes, punctuated by the completion of the Human Genome Project (1, 2). Genomics provides sequence information of the full complement of genes in an organism, and to date, there are more than 180 fully sequenced genomes. Transcriptomics uses DNA microarray (3–6) technologies to study gene expression by measuring transcriptional regulation of genes via their messenger levels. In many cases, however, it is proteins that act as the cellular building blocks that directly assert the potential function of genes via enzymatic catalysis, molecular signaling, and physical interactions. This third downstream "omics" of science is known as proteomics (7), and it studies the protein complement of cells, including identification, modification, quantification, and localization. Mass spectrometry (MS) uses mass analysis for protein characterization, and it is the most

comprehensive and versatile tool in large-scale proteomics. Whereas DNA microarray technology is based on a highly sensitive and specific hybridization reaction between nucleic acid fragments, inherent limitations of biological MS (8) require several different approaches to protein analysis. Implementation of these strategies (e.g., sample preparation, front-end separation, ionization, data acquisition, and data analysis) differs depending on the sample complexity and the goals of the analysis (9).

MS: mass spectrometry

ESI: electrospray ionization

2. IONIZATION TECHNIQUES

Protein MS has enjoyed rapid growth in the past two decades owing to important developments in experimental methods, instrumentation, and data analysis approaches. One of the most important developments in instrumentation is the introduction of soft ionization methods that allow for proteins and peptides to be analyzed by MS. Proteins and peptides are polar, nonvolatile, and thermally unstable species that require an ionization technique that transfers an analyte into the gas phase without extensive degradation. Two such techniques paved the way for the modern bench-top MS proteomics, matrix-assisted laser desorption ionization (MALDI), (10–13) and electrospray ionization (ESI) (14).

2.1. MALDI

The MALDI matrix absorbs laser energy and transfers it to the acidified analyte, whereas the rapid laser heating causes desorption of matrix and $[M+H]^+$ ions of analyte into the gas phase. MALDI ionization requires several hundred laser shots to achieve an acceptable signal-to-noise ratio for ion detection (15). MALDI-generated ions are predominantly singly charged. This makes MALDI applicable to top-down analysis of high-molecular-weight proteins with pulsed analysis instruments. The drawbacks are low shot-to-shot reproducibility and strong dependence on sample preparation methods (16, 17). Matrix-free MALDI techniques, such as SALDI (18) and DIOS (19), substitute matrix lattice for porous graphite and silicon, respectively, have higher tolerance toward detergents and salts, and do not suffer from matrix effects. An important development in MALDI ionization is atmospheric pressure MALDI (AP-MALDI) (20). This interface allows easy interchange between MALDI and ESI sources. The concept of MALDI has led to techniques such as surface-enhanced laser desorption ionization (SELDI) (21) that introduce surface affinity toward various protein and peptide molecules.

2.2. ESI

Unlike MALDI, the ESI source produces ions from solution. Electrospray ionization is driven by high voltage (2–6 kV) applied between the emitter at the end of the separation pipeline and the inlet of the mass spectrometer. Physicochemical processes of ESI involve creation of electrically charged spray, Taylor cone (22), followed by formation and desolvation of analyte-solvent droplets. Formation and desolvation of the droplets is aided by a heated capillary, and in some cases, by sheath gas flow at the mass spectrometer inlet. There are several physical models of ESI ion formation (23–25), but some of the practical features are the multiply charged species and sensitivity to analyte concentration and flow rate. An important development in ESI technique includes micro- and nano-ESI (26, 27), in which the flow rates are lowered to a nanoliter-per-minute regime to improve the method's sensitivity. Nano-ESI is compatible with capillary reverse phase (RP) columns (27) that offer higher sensitivity than the 2.1 and 1.0 mm analytical columns (28, 29). An ESI source is usually coupled to the continuous analysis instruments.

3. INSTRUMENTATION

LTQ: Thermo Scientific version of linear ion trap

Mass spectrometers usually consist of the following parts: the ion source and optics, the mass analyzer, and the data processing electronics. Mass analyzers are an integral part of each instrument because they can store ions and separate them based on the mass-to-charge ratios. Ion trap (IT), Orbitrap, and ion cyclotron resonance (ICR) mass analyzers separate ions based on their *m/z* resonance frequency, quadrupoles (Q) use *m/z* stability, and time-of-flight (TOF) analyzers use flight time. Each mass analyzer has unique properties, such as mass range, analysis speed, resolution, sensitivity, ion transmission, and dynamic range. Hybrid mass spectrometers have been built that combined more than one mass analyzer to answer specific needs during analysis. An in-depth analysis of different types of mass analyzers is out of the scope of this review because there are already many excellent texts (30, 31) and reviews of the instrumentation (32–37).

3.1. Mass Analysers

There are two broad categories of mass analyzers: the scanning and ion-beam mass spectrometers, such as TOF and Q; and the trapping mass spectrometers, such as IT, Orbitrap, and FT-ICR. The scanning mass analyzers like TOF are usually interfaced with MALDI to perform pulsed analysis, whereas the ion-beam and trapping instruments are frequently coupled to a continuous ESI source. The following instrument configurations are the most widely used solutions in the field of proteomics: ion traps [QIT: three-dimensional (3D) ion trap, LIT: linear ion trap] (38), triple quadrupoles (TQ), LTQ-Orbitrap (39–42) hybrid instrument (Thermo Scientific), LTQ-FTICR (43–46) (Thermo Scientific), and the TQ-FTICR hybrid instruments Q-TOF (47, 48) and IT-TOF (Shimadzu) (49–52). **Table 1** highlights comparative features and applications of the instruments most commonly used in proteomics.

Table 1 Performance comparisons of the mass spectrometry instruments

Instrument	Applications	Resolution	Mass accuracy	Sensitivity	Dynamic range	Scan rate
LIT (LTQ)	Bottom-up protein identification in high-complexity, high-throughput analysis, LC-MS^n capabilities	2000	100 ppm	Femtomole	1e4	Fast
TQ (TSQ)	Bottom-up peptide and protein quantification; medium complexity samples, peptide and protein quantification (SRM, MRM, precursor, product, neutral fragment monitoring)	2000	100 ppm	Attomole	1e6	Moderate
LTQ-Orbitrap	Protein identification, quantification, PTM identification	100,000	2 ppm	Femtomole	1e4	Moderate
LTQ-FTICR, Q-FTICR	Protein identification, quantification, PTM identification, top-down protein identification	500,000	<2 ppm	Femtomole	1e4	Slow, slow
Q-TOF, IT-TOF	Bottom-up, top-down protein identification, PTM identification	10,000	2–5 ppm	Attomole	1e6	Moderate, fast
Q-LIT	Bottom-up peptide and protein quantification; medium complexity samples, peptide and protein quantification (SRM, MRM, precursor, product, neutral fragment monitoring)	2,000	100 ppm	Attomole	1e6	Moderate, fast

3.2. Ion Trap

Ion trap instruments (33, 53) are the high-throughput workhorses in proteomics. These versatile instruments feature fast scan rates, MS^n scans, high-duty cycle, high sensitivity, and reasonable resolution [2000 full width at the half height (FWHH)] and mass accuracy (100 ppm). The LTQ ion trap (54) from Thermo Scientific combines a tenfold-higher ion storage capacity than 3D traps and high resolution at a fast scanning rate (5555 Da s^{-1}). In addition, the LTQ radial ion ejection offers higher sensitivity than other two-dimensional (2D) ion-trap instruments (54). Stand-alone ion trap instruments are best suited for the bottom-up liquid chromatography (LC)/MS protein identification studies from complex samples and whole cell lysates for which the fast scanning rates and high sensitivity of LITs offer high proteome coverage. LIT is used as the front end of hybrid instruments, such as LTQ-Orbitrap and LTQ-FTICR, where it is used for trapping, ion selection, and ion reactions.

LC: liquid chromatography

3.3. LTQ-Orbitrap

Of several hybrid instruments, the LTQ-Orbitrap deserves a special mention because it uses a novel mass analyzer. Orbitrap uses orbital trapping of ions in its static electrostatic fields (39–42) in which the ions orbit around a central electrode and oscillate in axial direction. **Figure 1**

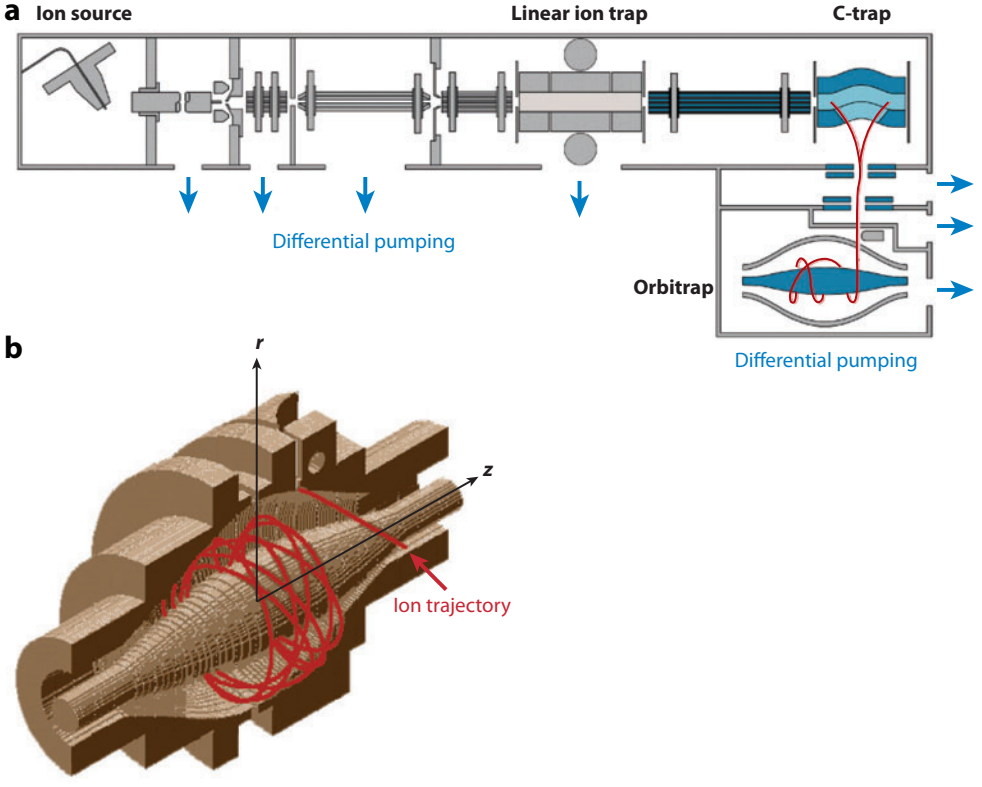


Figure 1

Schematic of the linear ion trap-orbitrap hybrid instrument by Thermo Scientific (LTQ-Orbitrap). (*a*) Overall diagram of the LTQ-Orbitrap (reprinted with permission from Reference 39). (*b*) Cross section of the Orbitrap analyzer (reprinted with permission from Reference 40).

illustrates an Orbitrap mass analyzer. Both Orbitrap and ICR instruments use a fast Fourier transform (FFT) algorithm (55) to convert time-domain signal into mass-to-charge spectrum. The Orbitrap mass analyzer features high resolution (up to 150,000), high mass accuracy (2–5 ppm), a mass-to-charge range of 6000, and a dynamic range greater than 10^3 (40, 42). When coupled to an LTQ ion trap, the hybrid instrument has the advantages of both high resolution and mass accuracy of the Orbitrap and the speed and the sensitivity of the LTQ. Furthermore, one can operate LTQ-Orbitrap in a parallel fashion: the Orbitrap acquires MS full scans while the LTQ carries out fragmentation reactions. There are several papers that review and benchmark the performance of the Orbitrap for bottom-up (32, 33, 35, 56, 57) and top-down (58, 59) proteomic applications. Some of the recent applications of the LTQ-Orbitrap highlight the benefits of high mass accuracy: It improves quantification of low-abundance peptides with S/N ratio of 10 (60), it aids in profiling very complex biological samples such as human plasma (61), and it furthers identification of proteins from the limited sequence proteomes (62). Orbitrap was recently used in a large-scale proteomic analysis of *Mycobacterium tuberculosis* in which the protein identification results were used to improve gene annotations in Sanger and The Institute for Genomic Research (TIGR) databases. The high mass accuracy of the Orbitrap allows for alternate data acquisition and data analysis approaches. A massive virtual Multiple Reaction Monitoring (MRM) approach (63) was carried out using a high-complexity sample. As for the data analysis, high mass accuracy allows integration of database search, de novo search, Peptide Mass Fingerprint (PMF) search, and the library lookup into a proteomic pipeline to achieve higher coverage and accuracy (64, 65). In conclusion, the LTQ-Orbitrap offers mass accuracy comparable to the LTQ-FTICR at a lower price tag and a lower maintenance cost for many proteomic applications. Although the LTQ-Orbitrap is used in top-down experiments (58, 66), one of the FTICR benefits is broader mass-to-charge range, which is best suited for top-down protein analysis and has the ability to carry out gas-phase reactions in the ICR cell (45, 67).

4. SEPARATION TECHNOLOGIES

Protein MS is tightly linked and highly dependent on separation technologies that simplify incredibly complex biological samples prior to mass analysis. Because proteins are identified by the mass-to-charge ratios of their peptides and fragments, sufficient separation is required for unambiguous identifications. Front-end separation is also required to detect low-abundance species that would otherwise be overshadowed by a higher abundance signal. Therefore, both accuracy and sensitivity of a mass spectrometric experiment rely on efficient separation. There is a very strong conceptual link between chemical separation and MS in which the latter is viewed as the mass-resolution dimension of separation of molecules (33). Selection of appropriate separation methods is often the first step in designing the proteomic application. Two major approaches to separation widely used in proteomics are gel based and gel free. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is the historic centerpiece of the gel-based separation methods (68–71). There are many excellent reviews that cover 2D PAGE and gel-based approaches to proteomics (72–75).

4.1. HPLC

Gel-based methods have been traditionally used with pulsed ionization MALDI instruments in which the protein band can be excised, digested, and off-line sampled with MALDI source (76). In contrast, the high-pressure liquid chromatography (HPLC) is usually directly coupled to instruments with an ESI source. Continuous separation via HPLC is conceptually and technologically

compatible with a continuous ionization source such as ESI, and both are usually interfaced with scanning or trapping mass analyzers (LTQ, QqLIT, QqTOF, LTQ-Orbitrap, LTQ-FTICR). HPLC has become a standard front end for many biological applications and gave rise to several LC/MS setups (33, 37, 77). The following types of HPLC chromatographic materials are most commonly used in MS-based proteomics: ion exchange (IEX), reverse phase, hydrophilic-interaction chromatography (HILIC), affinity, and hybrid materials. However, the high-pressure reverse phase chromatography is as essential to LC/MS as 2D PAGE is to gel-based proteomics (27, 78).

UPLC: ultra performance liquid chromatography

4.2. RPLC

Reverse phase resins (RPLC or RP) separate compounds based on their hydrophobicity, and a significant advantage of RPLC is that the buffers used are compatible with ESI (79). Given high resolution, efficiency, reproducibility, and mobile phase compatibility with ESI, the analytical RPLC is used as the single phase and as the last dimension of multidimensional separation (80–82) before mass analysis. Significant effort goes into increasing peak capacity (83), sensitivity (84, 85), reproducibility, and analysis speed of reverse phase chromatography (86, 87). It has been shown that packing long, narrow capillary RP columns greatly improves loading capacity, sensitivity, and dynamic range of the RPLC (29, 79, 88). Shen et al. (88) have introduced long, small-particle-size (1.4 μm) RPLC columns with high peak capacity (1500 and higher, compared with an average of 400) operated in an ultrahigh pressure regime (20 kpsi). Using only RPLC, they have identified more than 2000 proteins that vary over six orders of magnitude from human plasma in a single experiment. The small particle size of RP material (2 μm and smaller) allows improved peak capacity, resolution, and reduced analysis time (89, 90) when using an ultrahigh pressure regime. The small particle size and the elevated temperature (65°C) ultra performance liquid chromatography (UPLC) approach was shown to improve separation of intact proteins (91). UPLC was also shown to double the number of identified proteins compared with HPLC (92). A recent comparison of regular HPLC with sub-2-μm-particle UPLC using human plasma samples highlighted improved resolution, sensitivity, and analysis time reduction (93). Therefore, increasing the column length while decreasing the particle size and using UPLC leads to improved peak capacity, resolution, sensitivity, and analysis time.

4.3. Multidimensional Separation

Another common way to address limited peak capacity (94, 95) is to integrate RPLC as part of a multidimensional separation approach. High-complexity large-scale proteomic samples contain thousands of proteins that can range upward of five orders of magnitude in their abundance (96). The complexity of the shotgun proteomic samples is even higher where each proteolytically digested protein yields multiple peptide products (97). Multidimensional separation is used to address this high sample complexity. By definition, the multidimensional separation approach combines several separation techniques coupled to improve the resolving power. An important consideration for multidimensional separation is the orthogonality of the individual separation methods (98) in which each dimension uses different (orthogonal) molecular properties of molecules as a basis for separation. Although there are recent review papers that cover historical and theoretical aspects of multidimensional separation (77, 99), we mention some of the milestones in addition to the current trends. One of the first 2D setups featured cation exchange chromatography coupled to a reverse phase column in line with a mass spectrometer (82) used for separation of *Escherichia coli* proteins. The overall peak capacity of the method was in excess of 2500, with femtomolar

sensitivity due to the high capacity of strong cation-exchange (SCX) resin and the high resolution of SCX and RP. The 2D SCX chromatography followed by RPLC has become a popular method in shotgun proteomics known as multidimensional protein identification technology (MudPIT) (80, 97, 100). High complexity sample is loaded onto an SCX column and eluted in a series of steps with increasing salt concentration. Each fraction is loaded onto an RP column either off-line or directly eluted into an ESI source with nonpolar buffer. ESI is incompatible with high detergent and salt concentrations, and there have been several technical improvements to MudPIT to circumvent this drawback (101–103). MudPIT setup with long RP UHPLC columns has yielded higher sensitivity (low Picogram amounts) (104) and twice the protein coverage (92) compared with the conventional MudPIT setup. **Figure 2** illustrates advantages of UHPLC in MudPIT. Other materials used as first dimension (1D) are size exclusion (97), anion-exchange, (105) and a mixed-bed approach (106). Recent work compares several 1D methods (107) within the 2D setup to separate proteins of various physicochemical properties.

MudPIT: Multidimensional Protein Identification Technology

IMAC: immobilized metal affinity chromatography

4.4. Affinity Chromatography

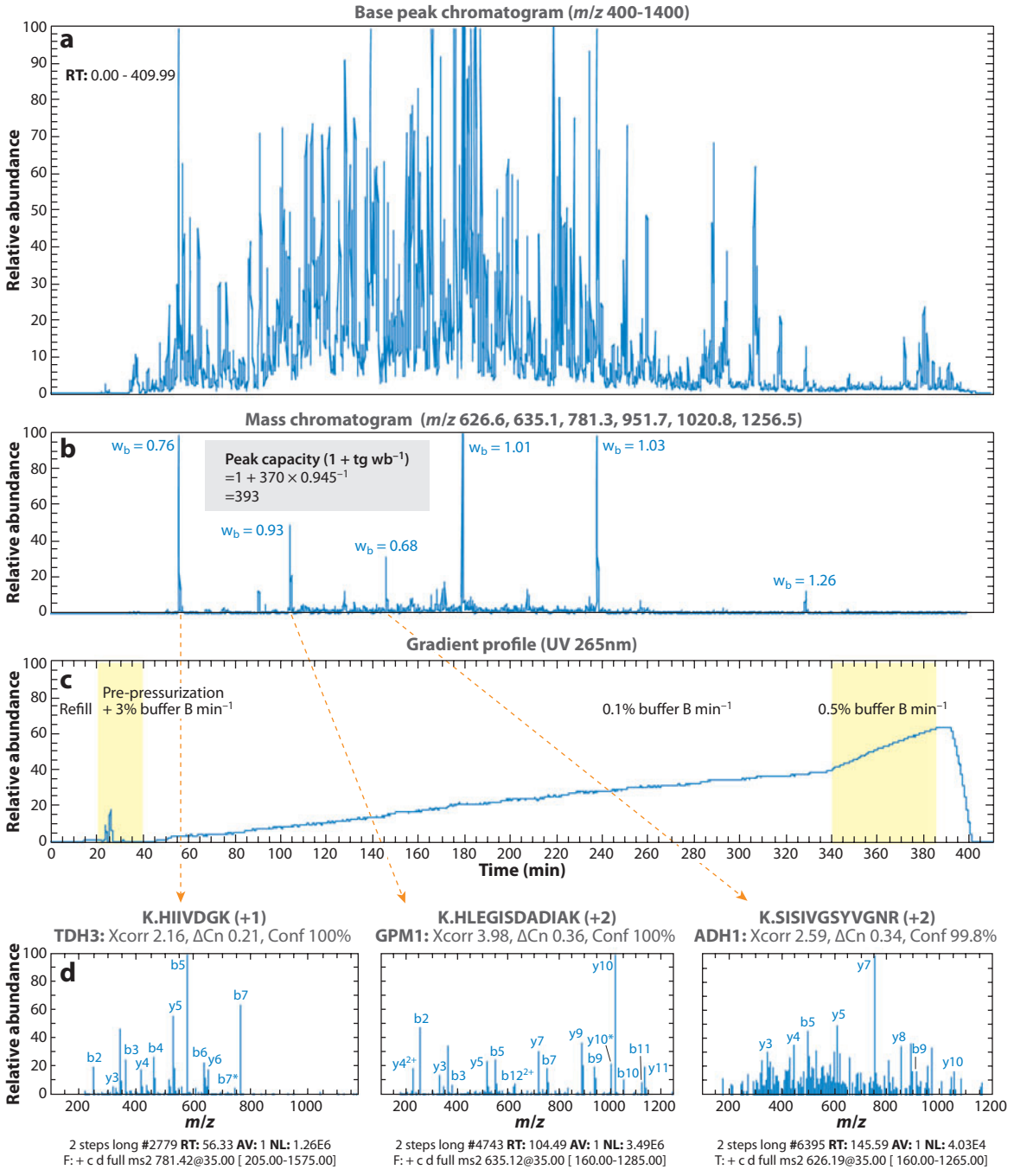
Another important category of chromatographic techniques is affinity chromatography. Affinity materials are often used to enrich posttranslationally modified (PTM) proteins and peptides to the levels measurable by mass spectrometers. Posttranslationally modified proteins often play a regulatory role in the cell and are often present in very small concentrations. In addition, dynamic nature and low stoichiometry of PTMs such as phosphorylation require enrichment prior to analysis. Affinity chromatography is usually a part of the multidimensional separation scheme directly or off-line coupled to the RP column.

4.5. Phosphoproteomics

Phosphoproteomics is geared toward the identification and quantification of phosphorylated proteins and the identification of phosphorylation sites (108, 109). Several selective enrichment techniques take advantage of the chelating properties of some metals toward the phosphate group of phosphorylated peptides. Immobilized metal affinity chromatography (IMAC) (110) has become a popular method whereby immobilized Fe^{3+} ions (111) are used to selectively bind phosphorylated peptides. The IMAC selectivity and specificity can be altered with different buffer conditions, such as pH, salt concentration (112), buffer composition, and the presence of detergents (113). Also, other metals such as Zr^{4+} (114) and Ga^{3+} (115) are used with IMAC, yielding different specificities and improved coverage. Other metal oxide affinity resins are TiO_2 (116), Fe_3O_4 (117), and ZrO_2 (118). Some nonaffinity techniques such as anion-exchange chromatography (119), mixed-bed chromatography (106), and HILIC (120) offer additional strategies for enrichment. For more detailed discussion of phosphoproteomic enrichment methods see Section 6.

Figure 2

High-resolution separation demonstrated by the UPLC-MudPIT system. (*a*) A base peak chromatogram of tryptic peptides from yeast lysate separated by a 60-cm triphasic column with a 350-min gradient. (*b*) A mass chromatogram of six typical peptides used to estimate a peak capacity. (*c*) A gradient profile monitored by UV. (*d*) Representative fragmentation scan (MS/MS) spectra and their assignments. A triphasic column composed of 5-cm C18 trap (5 μm)/5-cm SCX/60-cm C18 analytical (3 μm) was operated at 125 μL min^{-1} constant flow (system pressure, ~15 kpsi; column flow rate, ~0.16 μL min^{-1}). A 10-μg of yeast Lys-C + tryptic digest was injected in the system. Peptides were eluted by a two-step UHP-MudPIT (i.e., the chromatogram shown in *a* is of the second step eluted with 500 mM ammonium acetate). In *b*, six mid-intensity peaks distributed nearly evenly across the chromatogram were picked. wb, a peak width at the base line, given in minutes. The estimated peak capacity was ~400. Reprinted with permission from Reference 92.



4.6. Glycoproteomics

Glycoproteomics is another large area in which affinity chromatography is applicable. Glycosylation plays a significant role in the immune response in which many glycosylated proteins are displayed on the cell surface and serve as extracellular receptors. N-linked glycosylated peptides are selectively bound to the solid support (121) and are subsequently enzymatically released. Another affinity enrichment method of N-linked glycopeptides involves immobilized lectin binding site chemistry (122). Enzymatic release from a solid support combined with $H^{18}O$ enzymatic labeling led to the development of IGOT, the isotope-coded glycosylation site–specific tagging (123, 124). Immobilized lectin chemistry is also used for O-linked glycoprotein analysis in a technique called serial lectin affinity chromatography (SLAC) (125).

5. PROTEOMIC APPROACHES

Given the many technical options available for proteome analysis, several general strategies of protein identification have emerged. Gel-based or chromatographic separation is used to reduce sample complexity prior to mass analysis. Mass spectrometric data acquisition is usually implemented in a data-dependent manner in which information from a current mass spectrometric scan determines the parameters of subsequent scans. Another feature of proteomic analysis is tandem MS, whereby mass analysis is carried out on intact molecular ions (full-scan MS) or on fragmented precursor ions (MS^n scans). In most cases, full scans produce masses of the proteins or peptides, and fragmentation scans yield the primary sequence information.

A proteomic analysis begins with the sample preparation in which proteins are either enzymatically digested into peptides (bottom-up analysis) (126–128) or analyzed intact (top-down analysis) (45, 129–131). **Table 2** outlines some of these approaches.

5.1. The Bottom-Up Approach

The bottom-up approach is the most popular method when tackling high-complexity samples for large-scale analyses. The term shotgun proteomics (33, 80, 97) is the protein equivalent to shotgun genomic sequencing in which the DNA is sheared and sequenced in smaller overlapping contigs. Bottom-up proteomics is an approach in which proteins are proteolytically digested into peptides prior to mass analysis, and the ensuing peptide masses and sequences are used to identify corresponding proteins. Most bottom-up applications require tandem data acquisition in which peptides are subjected to collision-activated dissociation (CAD or CID). The most widely used method for bottom-up tandem MS data identification is the database search (132, 133) in which experimental MS^n data are compared with the predicted, in silico–generated fragmentation patterns of the peptides under investigation. Since the original publication of SEQUEST (132) in 1994, many methods have been developed that address some of the computational challenges associated with bottom-up proteomics. Some of these developments include using probabilistic scoring schemes (133–138), incorporating additional search criteria (139, 140), and storing previously identified spectra to bootstrap the database search (141). Substantial efforts are being made to establish public repositories of proteomic data aimed at promoting data format standardization and increasing data availability for independent analyses (142–147). The bottom-up approach is also well suited for chemical modification of peptides, with the aim of peptide and protein quantification. Techniques such as ICAT (148), O^{18} labeling (149), and Hamon tandem mass tags (150) work best with flexible and accessible peptides. Some of the advantages of the bottom-up approach include better front-end separation of peptides compared with proteins and higher sensitivity than

Table 2 Approaches in mass spectrometry proteomics[a]

	Prerequisite	Advantages	Drawbacks	Applications	Front end	Analysis
Top down	High mass accuracy instruments	High sequence coverage	Precursor ion charge state limitations	Single-protein characterization	Ion-exchange chromatography	EST
	LTQ-Orbitrap	PTM and protein-protein complexes information	Separation limitations	Proteome analysis	RP chromatography	De novo
	LIT-ICR	Multiple PTM identification	Low sensitivity	Alternative splicing	Two-dimensional separation	
	Large sample amount	Soft fragmentation with ECD, ETD	Protein identification issues related to the charge-state umbiguity	Multiple PTM analysis	ESI ionization	
		Better quantification compared with bottom up			Sample infusion	
Bottom up	Wide variety of instruments	Large-scale data acquisition	Narrow mass range	Protein identification via peptide analysis	Gel based	PMF
	Q-TOF, LIT, LTQ-Orbitrap, etc.	High-complexity samples	Front-end separation required	Protein quantification, PTM analysis	Gel free	Database search
	Sample digest prior to analysis	High sensitivity	Oversampling of high-abundance peptides		RP chromatography	De novo
		Good front-end separation	Mass of the intact protein is not accessible directly		Ion-exchange chromatography	Library search
		Chemical derivatization	Loss of labile PTMs		Affinity chromatography	

[a]EST, expressed sequence tag; PTM, posttranslational modifications; RP, reverse phase resins; ICR, ion cyclotron resonance; LIT, linear ion trap; ECD, electron capture dissociation; ETD, electron transfer dissociation; ESI, electrospray ionization; PMF, peptide mass fingerprinting; Q-TOF, quadrupole time-of-flight.

the top-down method. Drawbacks of the bottom-up approach include limited protein sequence coverage by identified peptides, loss of labile PTMs, and ambiguity of the origin for redundant peptide sequences.

5.2. Top-Down Methods

Top-down methods use masses of intact proteins and their fragments for successful identifications. Alternative fragmentation reactions, such as electron capture dissociation (ECD) (67) and electron transfer dissociation (ETD) (151, 152), that yield a more complete backbone sequencing and retain labile PTMs (153) are the preferred fragmentation methods of the top-down approach.

ETD: electron transfer dissociation

Top-down data are usually analyzed using the expressed sequence tag (EST) method (154, 155) or the de novo method (156). Some of the benefits of the top-down approach include higher sequence coverage of target proteins (157) and better characterization of the posttranslational modifications (158, 159). Compared with bottom-up approaches, the higher sequence coverage of top-down experiments reduces the ambiguities of the peptide-to-protein mapping, which allows for identification of the specific protein isoforms (160, 161). Another reported advantage of the top-down approach is improved reliability of protein quantification (66, 162, 163) when protein abundances are measured directly instead of using abundances of peptides. However, there are several technological limitations to the top-down method, which keeps it from widespread use. Front-end separation of intact proteins is more challenging than the separation of peptide mixtures. This means that larger quantities of protein and higher mass accuracy instruments such as FTMS (130) and LTQ-Orbitrap (58, 66) are required to resolve isotopic envelopes of coeluting proteins. Furthermore, generic and efficient methods to fragment large proteins are not available yet. Owing to these technical limitations, the scope of the top-down approach has been limited to the analysis of single proteins and simple protein mixtures. However, some of the recent studies (164, 165) have extended the top-down approach to complex mixture analysis. In addition to discussing the overall workflow, we emphasize below two proteomic applications: protein quantification and phosphoproteome analysis.

6. PROTEOMIC APPLICATIONS—QUANTITATIVE PROTEOMICS

A key advantage of the large-scale proteomic for systems biology is the capability to quantify functional entities of the cell, the proteins. The overall goal of such measurements is to obtain a snapshot of concentrations of proteins associated with different states. Quantitative measurements of protein concentrations represent one of the key components toward building a functional network. There are two broad groups of quantitative methods in MS-based proteomics, (*a*) relative quantitative proteomics and (*b*) absolute quantitative proteomics. Relative quantitative proteomics can compare two or more samples using either stable isotope–labeling methods or label-free methods.

Isotope labels can be introduced (*a*) metabolically, (*b*) chemically, or (*c*) enzymatically (166). Metabolic labeling represents the earliest point of marking proteins with the stable isotopes of elements (^{15}N) or stable isotopes of amino acids (heavy Arg, Lys, Leu, and Ile). In early studies, total labeling of yeast has been achieved using ^{15}N-enriched cell culture media (167). For the stable isotope labeling by amino acids in cell culture (SILAC) approach, cell media contain $^{13}C_6$-Lys and $^{13}C_6$, and $^{15}N_4$-Arg for comprehensive labeling of tryptic cleavage products (166). In vivo metabolic ^{15}N labeling of model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster* (168), and rat (169) has been reported. The relative-abundance ratio of peptides is experimentally measured by comparing heavy/light peptide pairs, and then protein levels are inferred from statistical evaluation of the peptide ratios. Examples of chemical derivatization techniques for quantitative proteomics include isotope-coded affinity tags (ICAT), used for the labeling of free cysteine (148), and isobaric tags for relative and absolute quantification (iTRAQ), used for the labeling of free amines (170). Chemical derivatization procedures can be applied to any sample at either the protein or the peptide level. Enzymatic labeling usually incorporates ^{18}O either during or after digestion. Absolute measurements of protein concentrations can be achieved with spiked synthetic peptides (171), artificial proteins derived from detected peptides, as in QconCAT (172), and SILAC (173). In this review, we describe some of the applications of quantitative methods to cell signaling proteomics.

SILAC: stable isotope labeling with amino acids in cell culture

6.1. Isobaric Tags for Relative and Absolute Quantification

Multiplexing tagging chemistry for iTRAQ affords monitoring of four to eight samples in a single experiment (174). In a typical iTRAQ experiment, peptide levels are inferred from MS/MS spectra. Initially, iTRAQ experiments were carried out on Q-TOF instruments (170, 175) because quadrupole (176) and TOF instruments are capable of detecting low *m/z* fragment ions in the region of the iTRAQ reporter ions, unlike ion traps in which recovery of ions in the mass range below 30% of the precursor's ion mass is very poor. Introduction of pulsed Q dissociation (PQD) (177) in the ion trap facilitates detection of iTRAQ reporter ions, bridging the gap between the linear ion trap with PQD and a quadrupole TOF instrument (178). Emerging reports show applicability of ETD for peptide (32) and protein (179) quantification with an ETD-enabled LTQ-Orbitrap.

6.2. Stable Isotope–Labeling by Amino Acids in Cell Culture

The SILAC approach labels proteins with one or more heavy amino acids: Leu (180), Arg, Lys (166), and/or Tyr (181). SILAC-labeled peptides are quantified from full-scan mass spectra. Numerous studies applied SILAC to studying dynamic changes in response to stimuli (182, 183). Recently, SILAC has been applied to labeling of primary cells (184) and mice (185). Quantitative accuracy, however, requires complete incorporation of the labeled amino acids, as metabolic conversion of arginine to proline results in tryptic peptides containing heavy prolines (186). Experimental or bioinformatics solutions can be used to minimize interference from incompletely labeled peptides (187–189).

When isotopic labeling is not applicable, the label-free techniques can be used in abundance-based proteomics. Label-free methods use either spectral counting or peptide signal intensity to estimate abundance of proteins (37). Spectral sampling is directly proportional to the relative abundance of the protein in the mixture (190). Comparison of spectral counting methods with $^{14}N/^{15}N$ metabolic labeling showed strong correlations between these two approaches for quantitative proteomics by MudPIT (191).

6.3. Software for Quantitative Proteomics

Automated quantification of complex proteomes necessitates additional software solutions (192). One such solution is Census, a software tool for analyzing quantitative MS data (189). Census is a flexible tool that can handle quantitative proteomic data, including ^{15}N, SILAC, iTRAQ, and label-free experiments (**Figure 3*a***). Briefly, for isotopically labeled analyses, Census incorporates the following steps. For high-resolution MS data acquired with a LTQ-Orbitrap, Census employs an algorithm that extracts individual isotopes using a mass accuracy tolerance. This method is very effective in excluding noise peaks, and it results in high correlation for chromatograms. Census calculates peptide ion-intensity ratios for each peptide pair using a linear least squares correlation (i.e., slope of the line) and closeness of fit [i.e., correlation coefficient (r)] between data points of labeled and unlabeled ion chromatograms (193).

Census determines the protein ratios by calculating a weighted average of all peptide ratios quantified for a specific protein. Weights are determined by considering the errors associated with each peptide ratio measurement or, more precisely, the inverse square of the standard deviation of the measurement. A similar approach for calculating protein ratios has been reported (194). Census removes statistical outliers for proteins with more than three quantified peptides. Standard deviations are calculated for all proteins using their respective peptide ratio measurements. Finally, a Grubbs test (195) is applied with a user-defined *p*-value to remove outlier peptides.

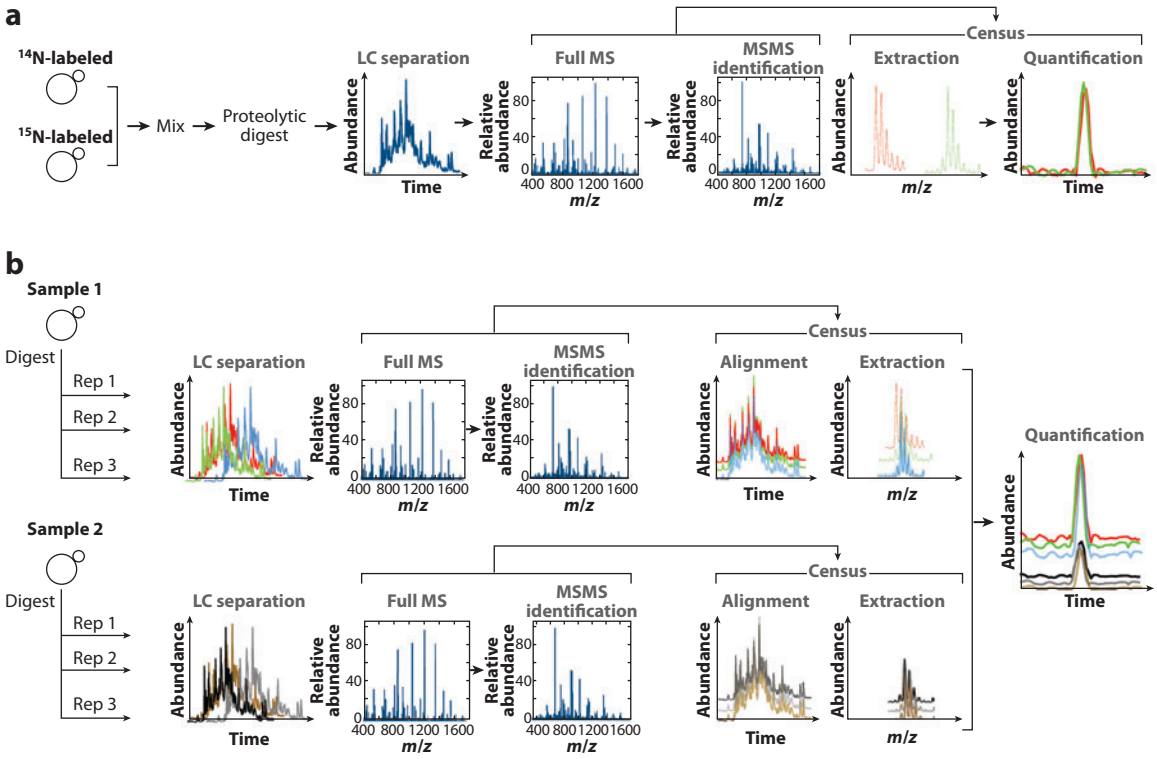


Figure 3

Schematic detailing the quantitative analysis capabilities of Census. (*a*) Use of Census with isotopic labeling (see text). (*b*) Use of Census with label-free analysis. For chromatogram alignment, Census uses a Pearson correlation between mass spectra and dynamic time warping (255). After alignment, chromatograms are extracted as described. LC, liquid chromatography. Reprinted with permission from Reference 189.

Census is capable of achieving en masse quantification of proteins for high-complexity samples analyzed with MudPIT.

7. PROTEOMIC APPLICATIONS—PHOSPHOPROTEOMICS

7.1. Enrichment Techniques

Protein kinases control every basic cellular process, including metabolism, growth, division, differentiation, motility, organelle trafficking, immunity, learning, and memory via regulated protein phosphorylation (196). MS is the method of choice for accurate identification and quantification of phosphorylation sites.

One of the most common strategies for enrichment of phosphoproteomes is a combination of chromatography with affinity-based enrichments. These chromatography techniques include IMAC, immunopurification, metal oxide affinity chromatography (MOAC), and strong cation-exchange chromatography. In addition to chromatographic methods, the antibodies against phosphoamino acid epitopes, magnetic materials, and nanoparticles (197) as well as metal ion-phosphopeptide precipitations are part of the toolbox for the affinity-based enrichment of phosphopeptides. Another route is to enrich phosphopeptides using chemical derivatization

techniques of phosphate groups (198). Comparison of IMAC, MOAC, and phosphoramidate chemistry methods highlights the complementary nature of enrichment (199).

IMAC uses metal chelators such as iminodiacetic acid and nitrilotriacetic acid linked to chromatographic support to immobilize metal ions. Available coordination sites of positively charged ions interact with negatively charged groups of phosphate and carboxylate moieties. Either low pH buffer or chemical derivatization increases the specificity of IMAC for phosphopeptides. Peptide IMAC is one of the techniques for second-round enrichment of phosphopeptides, following strong cation exchange chromatography (200, 201) or protein IMAC (202, 2003); however, MOAC does not require a charging step because the metal ions are part of a solid metal bead, and consequently, material such as titanium dioxide is widely used for enrichment of phosphopeptides (116). By limiting nonspecific interactions, 2,5-dihydroxybenzoic acid (DHB) (204) and aliphatic hydroxyl acids (205) increase selectivity of TiO_2.

In solution, interaction of phosphopeptides with certain metal ions can be used for enrichment. Both Ba^{2+} and Ca^{2+} ions have been shown to enrich phosphopeptides (206, 207). In addition to the enrichment, Ba^{2+}/acetone precipitation was shown to further separate phosphorylated peptides based on the number of phosphate groups, using a stringent false-positive rate (206). Complexes of metal-phosphopeptides can be further separated by IMAC, RP (207), and MudPIT (206).

An alternative enrichment method for phosphopeptides is HILIC. HILIC partitions peptides between a hydrophilic layer and the hydrophobic elution buffer. HILIC fractionation with an IMAC compatible buffer (salt-free TFA/acetonitrile) constitutes an attractive alternative for screening phosphoproteomes (120).

Compared with the serine and threonine phosphorylation, investigation of the phosphotyrosine proteome relies almost exclusively on immunoaffinity purifications (208). Proteome-scale screening of phosphotyrosines has been extended to the identification of oncogenic kinases (209), identification of core proteins responding to drug treatment (210), and definition of the organ-specific phosphorylation (201). An alternative to immunopurification of phosphotyrosine peptides is dendrimer conjugation chemistry (211). In this strategy, a solution polymer that has functional groups only at its surface (a dendrimer) reacts with any phosphorylated peptide. Following the spin-column filtration and acid hydrolysis, a considerable number of previously unidentified phosphotyrosine proteins were described.

7.2. Fragmentation Methods for Identification of Phosphopeptides

In bottom-up proteomics, phosphopeptides are traditionally fragmented by CID (212). To account for the ubiquitous presence of phosphate neutral losses, a neutral loss–triggered MS^3 data–acquisition method has been introduced for phosphoproteome analysis (213). ECD and ETD help to identify different segments of phosphoproteomes (214, 215). Successive acquisition of CID and ECD fragmentation spectra has been shown to aid in localizing phosphorylation sites (216). Another alternative for structural characterization of phosphopeptides using high mass accuracy may be high-energy collision-induced dissociation (HCD) (217) carried out in a separate octapole collision cell added to the LTQ-Orbitrap. One of the features of HCD spectra is the presence of both b- and y-series ions in addition to immonium ions, which highlights the presence of a modified residue.

7.3. Identification of Phosphopeptides and Phosphorylation Sites

Identification of phosphopeptides is based on database searches. As with any database search, the decoy database is used to filter out false-positive identification (218). Lu et. al have developed a

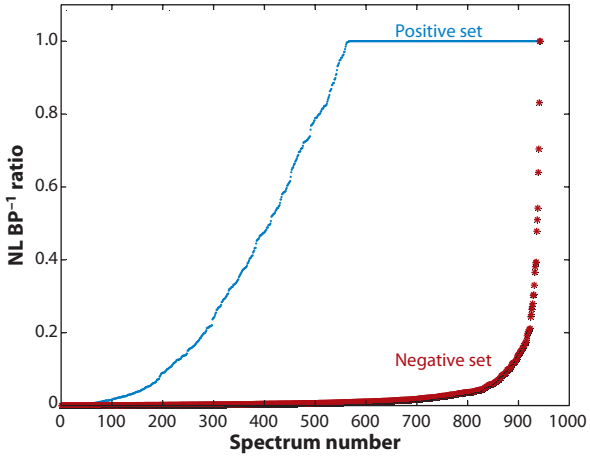


Figure 4

Distribution of extracted feature-precursor neutral loss (NL)/base peak (BP) ratio for the training set. The plots show the distributions of features for 944 positive spectrum/peptide identifications and 944 negative spectrum/peptide identifications (randomly selected from the 1064 negative training spectrum/peptide matches). Reprinted with permission from Reference 219.

suite of algorithms for automatic analysis of phosphopeptides. An SVM-based supervised classification method trained on neutral loss features from ion trap tandem mass spectra is used to provide high-confidence identifications. They showed that phosphate neutral loss from the base peak is the most informative SVM feature in validating the identification of Ser/Thr-containing phosphopeptides (219) (**Figure 4**). For shotgun phosphoproteomic experiments, algorithms with SVM-trained features can filter potential phosphopeptide spectra before a database search (220). This is followed by phosphorylation site localization that describes positions of phosphorylated residues (221, 222).

7.4. Quantification of Phosphorylation Sites

Several quantities related to site-specific phosphorylation are usually measured in an MS assay: changes in relative abundance in response to stimuli, stoichiometry of phosphorylation sites, and the site occupancy. Quantitative phosphoproteomics is used to depict temporal profiles of activated pathways (222). Mitotic phosphorylation events were shown to involve more than 1000 proteins described by 14,000 phosphopeptides (223). An AbsoluteQUAntification (AQUA)-based approach quantified inhibitory phosphorylation of adjacent sites of cyclin-dependent kinases (224). Alternatively, absolute quantification of protein phosphorylation can be achieved by inductively coupled plasma (ICP)-MS (225). Distinct labeling of phosphorylation sites can be achieved through either Tyr-SILAC (181) or $\gamma[^{18}O_4]$-ATP (226).

Occupancy of phosphorylated sites describes the level of phosphorylation compared with the unmodified site. Different methods have been introduced to characterize occupancy of phosphorylation sites (227–229). Determination of occupancy rates of modified sites can shed light on mechanisms of signal transduction. For example, two or more modifications can directly compete for the same single residue in a negative cross talk (230). New developments in top-down and quantitative proteomics could provide insights into proteotyping (231) of phosphorylation site occupancy (232) and into competition of different modifications for the same residue (233).

7.5. Motifs Present in Phosphoproteomes

Kinases recognize short linear sequences around the preferred site of phosphorylation, known as phosphorylation motifs. In proteomic-based discovery, phosphorylation motifs can function as affinity reagents for ligands such as monoclonal antibodies or free-metal ions. In one application, an antibody toward the protein kinase C (PKC) recognition sequence has been used to enrich for a phosphomotif-specific subproteome (234). More recently, a panel of 68 antibodies specific for SQ/TQ motifs enriched the subproteome involved in the response to DNA damage (235). A total of 905 phosphorylation sites from 700 proteins changed more than fourfold in irradiated cells as compared with nonirradiated cells. The role of the newly identified substrates of ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) kinases in DNA-damage response was further investigated by depleting these proteins with small interfering RNA (siRNA). From an interrogated subset of 37 substrates, a large fraction (35 substrates) contributed to at least one phenotype of DNA-damage response. Therefore, quantitative measurements of the phophorylation state of motif-specific subproteomes proved useful in selecting protein candidates for further functional studies.

Complementary to immunoaffinity techniques, customized bioinformatics tools, such as Motif-X, can mine for phosphorylation motifs potentially preferred by kinases (236). Motif-X statistically analyzes sequences of amino acids bracketing phosphorylation sites localized in MS assays. On subtraction of background sequences, Motif-X output represented proportional frequencies of preferred amino acids for a total of 13 positions. Alternatively, a peptide-oriented library and a position-scoring matrix can outline strongly favored and strongly disfavored residues in sequences around sites phosphorylated by different kinases (237). Yet other bioinformatics algorithms, such as artificial neural networks (238), can further refine the list of kinase-preferred sites.

Phosphorylated motifs can also be selected from complex proteome mixtures. We have shown that sequences around phosphosites can act as an affinity molecular reagent in metal-phosphopeptide interaction modulated by the solution pH (206). This interaction was sufficient to facilitate precipitation of metal-phosphopeptide complexes in solvents with low organic concentration. Direct application of sequence logos (239) identified strongly favored residues precipitated by Ba^{2+}/acetone (**Figure 5**). Acidic amino acids and Pro-directed motifs were enriched at different pH values. We have also outlined a subset of doubly phosphorylated motifs that contained acidic residues around phosphorylation sites (**Figure 5**).

Functional characterization of phosphorylation motifs involves a combination of the bioinformatic and experimental approaches. Motifs were, in silico, extracted from detected phosphotyrosine proteomes, and corresponding peptides were synthesized (240). Quantitative pull-down assays (241) tested motif-peptides for binding to Src Homology 2 (SH2) and phosphotyrosine binding (PTB) domains. The authors found 15 novel phosphorylation-dependent interactions and identified a new hydrophobic N-terminal motif as a binding motif for the SH2 domain.

7.6. Connecting Phosphoproteome and Kinome

Mapping of phosphoproteomes identifies an ever-increasing number of phosphorylation sites in systems as diverse as *D. melanogaster* (199) and HeLa cell lines (223). A daunting challenge is to map connections between phosphorylated substrates and their respective kinases (242). Recent technological developments could translate into routine assays of kinase substrates through direct labeling (243). One such example is the engineering of analog-specific (AS) kinase alleles that use ATP-γ-S, resulting in thiophosphorylation of protein substrates (244). Alkylation of thiophosphorylated residues creates a unique epitope, leading to immunopurification of direct substrates

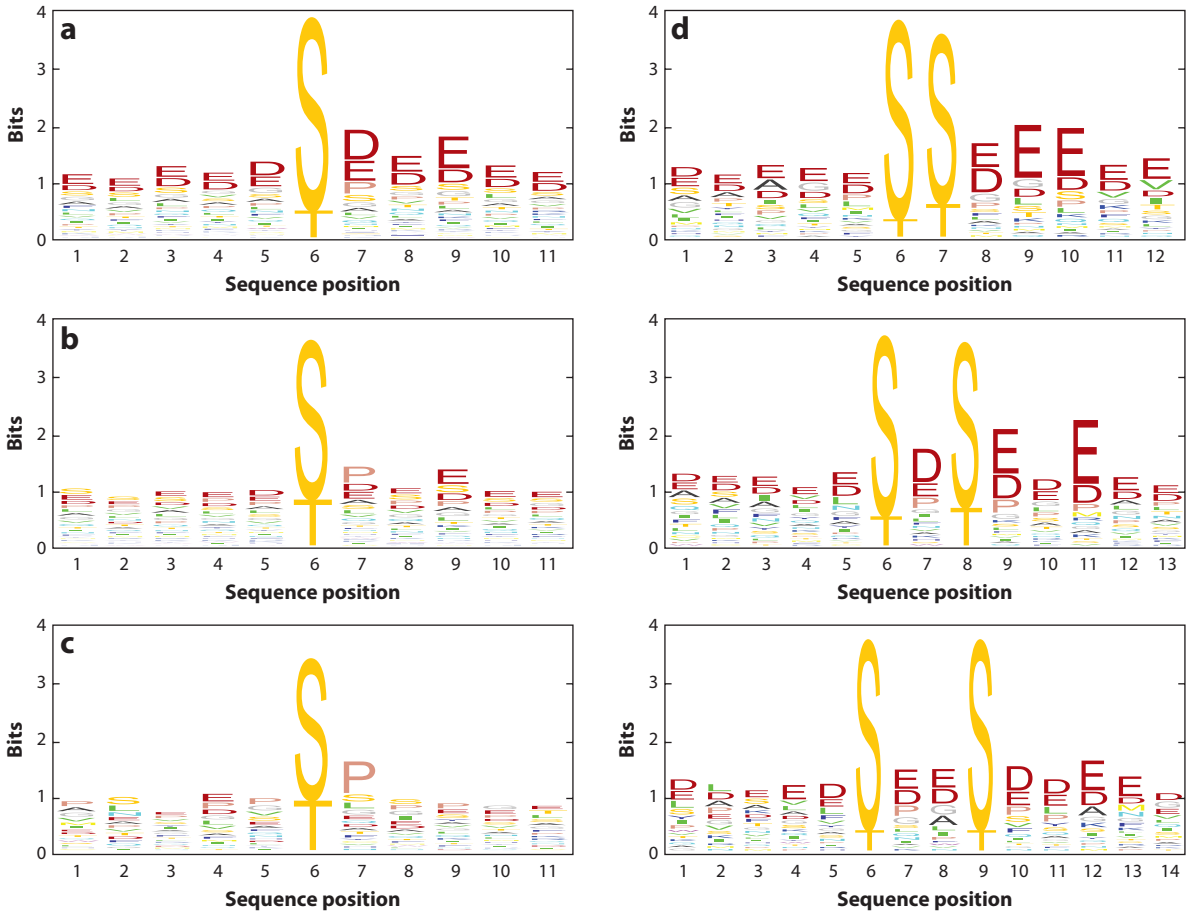


Figure 5

Sequence logos of phosphopeptides from nuclear extract of HeLa cells. (*a*) pH 3.5, (*b*) pH 4.6, and (*c*) pH 8.0°. Sequence logos were built for amino acids forming the binding pockets of Ba^{2+} ions (−5 to +5 AAs). Sequence logos statistically evaluate the binding properties of a population of phosphopeptides by measuring the information (in bits) required at each position around the phosphosite for interaction with barium ions. (*d*) Sequence logo profiles of doubly phosphorylated peptides identified in a pH 3.5 Ba^{2+}/acetone fraction of HeLa cell nuclear extract. Reprinted with permission from Reference 206.

accompanied by MS identification (245). Currently, a repertoire of 40 AS kinases exists, and it is estimated that most kinases can be engineered in AS alleles (246).

7.7. Kinases and Signaling Pathways

Effective drug discovery can benefit from a set of simple molecular rules that can substitute for a distinct link in diseases with complex etiologies. Intracellular molecular signaling pathways represent one such set of rules (247). By different definitions, there are between 16 and 200 signaling pathways (247, 248). Understanding the role of signaling pathways for a given biological context (e.g., stimuli, diseases) requires measurement of (*a*) pathway outputs, (*b*) dynamic changes, and (*c*) cross talk. Signaling proteins (i.e., kinases, phosphatases, scaffold proteins) are usually present in low amounts as compared with housekeeping proteins. Therefore, different approaches that specifically interrogate a subproteome or classes of proteins are particularly useful. Affinity

techniques facilitate analyses of subproteomes associated with pathways or low-molecular-weight chemicals.

Several studies investigated activation of the epidermal growth factor receptor (EGFR) signaling pathway (175, 249). Using LTQ-Orbitrap (41) and applying sequential enrichment by SCX and TiO_2, researchers monitored changes in as many as 6600 phosphorylation sites over five time points (222). Mapped phosphorylation sites and their temporal profiles were deposited in a phosphorylation-site resource database (250). An alternative to large-scale quantitative phosphoproteomics is to target a subset of identified phosphorylation sites by using multiple-reaction monitoring of stable isotope-labeled peptides (176). Although only a few hundred proteins can be profiled with a targeted approach, almost 90% of the targets were reproducibly quantified across four time points. To achieve high sensitivity detection of phosphorylation sites, these discovery or targeted-mode quantitative studies required high specificity enrichment. Consequently, quantification of protein expression levels, even if possible, has not been performed. Recent studies quantified both phosphorylation levels and protein levels for mitotic phosphorylation (223), cell cycle kinases (251), and the beta-adrenergic pathway (206) (**Figure 6**).

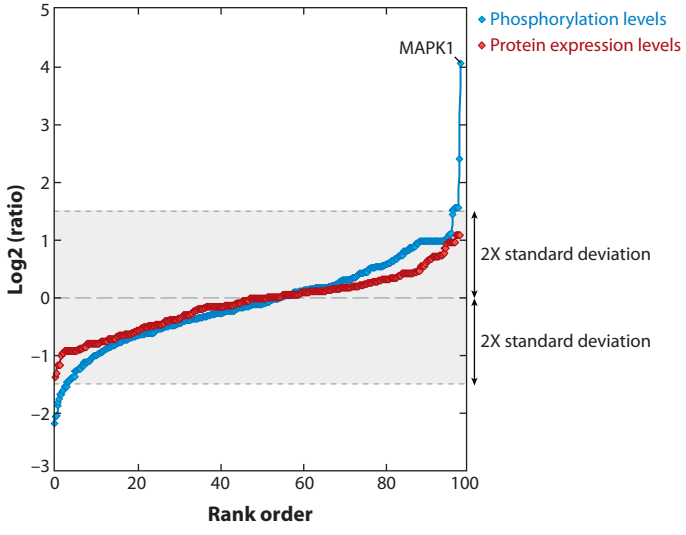


Figure 6

Log2 ratio of protein expression levels and their corresponding phosphorylation levels during isoproterenol stimulation from 30 sec to 2 min. Protein expression levels were derived from 2066 peptide ratios. Phosphorylation levels were derived from 398 phosphopeptide ratios. Protein expression levels were calculated from both peptides and phosphopeptides signals. Up- and down-regulation were considered significant for protein expression levels with a ratio of 2 or 0.5, respectively. If phosphopeptide signals were to be substracted from protein ratios, expression levels would distribute closer to a ratio of 1. Therefore, protein expression levels were essentially unchanged during 90 s stimulation with isoproterenol. For a ratio more than two standard deviations away from the mean, we considered peptides to be differentially phosphorylated (ratio <0.33 and ratio >2.66). The highest increase in phosphorylation levels (16-fold) belonged to phosphorylation of MAPK1(IPI00376295.1). Thus, we detected changes in phosphorylation levels of MAPK1, a critical kinase for cross talk with the β-adrenergic pathway. Knowledge analysis of quantified phosphoproteins identified protein modules from the following canonical pathways: ERK/MAPK (eight molecules), cardiac beta-adrenergic (four molecules), calcium signaling (four molecules), insulin receptor signaling (four molecules), and cAMP-mediated signaling (three molecules). The MAPK1-centered module showed the highest degree of interconnectivity, with seven modules from a total of nine possible modules. Reprinted with permission from Reference 206.

ACE-MudPIT: Anion-Cation-Exchange Multidimensional Protein Identification Technology

Immobilized cAMP-selected nucleotide-binding proteins yielded a highly enriched fraction of cAMP interactors using sequential elutions (252). Subsequent MS proteomic assays identified all known regulatory protein kinase A isoforms, including their phosphorylation states and 11 different A-kinase anchoring proteins. Immobilized low-molecular-weight inhibitors and phosphopeptide enrichment were used to characterize cell cycle kinases (251). Several broad-selectivity inhibitors immobilized on beads captured ~200 kinases and a subproteome of 600 proteins. This approach combined with iTRAQ and MS revealed new drug targets selected from cell lysates (253).

8. OUTLOOK

It is conceivable that discovery-based MS proteomics can be a complement to the conventional Western blot without the requirement to generate antibodies (254). Simultaneous quantitative measurements of both protein expression levels and phosphorylation levels are key to this advancement. Orthogonal multidimensional chromatography is at the forefront of these developments. Emerging applications, such as fractionation procedures and mixed-bed anion-cation-exchange multidimensional protein identification technology (ACE-MudPIT), already focus the classes of phosphopeptides and peptides during the same analysis.

High mass accuracy measurements expanded the application range for shotgun phosphoproteomics and facilitated coherent interrogation of large-scale data. Software solutions are already available for quantifying protein expression levels and up/down regulation of phosphorylation.

MS has tremendous potential for broad-based proteomic discovery and should contribute to important biological discoveries.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Cristian I. Ruse and Aleksey Nakorchevsky contributed equally to this manuscript.

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