EXPERT REVIEWS

Recent advances in mass spectrometry: data independent analysis and hyper reaction monitoring

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New mass spectrometry (MS) methods, collectively known as data independent analysis and hyper reaction monitoring, have recently emerged. These methods hold promises to address the shortcomings of data-dependent analysis and selected reaction monitoring (SRM) employed in shotgun and targeted proteomics, respectively. They allow MS analyses of all species in a complex sample indiscriminately, or permit SRM-like experiments conducted with full high-resolution product ion spectra, potentially leading to higher sequence coverage or analytical selectivity. These methods include MS^E, all-ion fragmentation, Fourier transform-all reaction monitoring, SWATH Acquisition, multiplexed MS/MS, pseudo-SRM (pSRM) and parallel reaction monitoring (PRM). In this review, the strengths and pitfalls of these methods are discussed and illustrated with examples. In essence, the suitability of the use of each method is contingent on the biological questions posed. Although these methods do not fundamentally change the shape of proteomics, they are useful additional tools that should expedite biological discoveries.

KEYWORDS: AIF • DIA • FT-ARM • HRM • MRM^{HR} • MS^E • MSX • PRM • pSRM • SAWTH-MS

The analysis of peptides generated by proteolytic digestion of proteins, known as bottom-up proteomics, serves as the basis for many of the protein research undertaken by mass spectrometry (MS) laboratories today. In bottom-up proteomics, three different approaches are commonly used [1]: discovery-based approach (or shotgun proteomics); directed approach; and targeted approach (or targeted proteomics).

Discovery-based or shotgun proteomics employs data-dependent acquisition (DDA). Herein, a hybrid mass spectrometer first performs a survey scan, from which the peptide ions with the intensity above a predefined threshold value, are stochastically selected, isolated and sequenced by product ion scanning. In selecting the precursor ions, there is a preference toward the ions having the highest ion intensity. Other additional selection criteria, such as dynamic exclusion, background subtraction, charge state selection, etc. are also used to prevent redundant acquisition of the most abundant peptides, or to avoid acquiring product ion spectra of the interferences. Recently, precursor ion selection is also used to determine the most appropriate fragmentation techniques that are accessible on the same instrumental platform [2].

In direct approach, besides the signal intensity of the ions, certain characteristic fragmented ions produced are chosen as prerequisites, to trigger product ion scanning. This approach is generally executed using either precursor ion scan or neutral loss scan. Phosphorylated serine and tyrosine containing peptides, and acetylated peptides are examples of molecules that are often monitored by such kind of approaches [3–5].

In targeted proteomics, selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), is used to monitor a number of selected precursor-fragment transitions of the targeted peptides. The selection of the SRM transitions is normally calculated on the basis of the data acquired previously by product ion scanning, repository data in the public databases or based on a series of empirical rules predicting the enzymatic

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Box 1. Emerging SRM-associated MS techniques.

- DMS/FAIMS is a technique separating gas-phase ions based on the difference between ion mobility in high and low electric fields at or near atmospheric pressure. By adjusting the separation voltages and the compensation voltage, the trajectory of a particular ion can be modulated. Interfering ions are set to collide with one of the electrodes (and neutralized) whereas the targeted ions migrate toward the orifice of the DMS device and enter the mass spectrometer [91]. The technique acts effectively as an ion filter thereby enhancing signal-to-noise.
- MRM^3 increases the specificity of SRM detection by using resonant excitation to fragment primary product ions trapped in the Q3 linear ion trap. The detector eventually scans the secondary product ions produced. A two-stage SRM transition is used to reconstruct an MRM^3 ion chromatogram [31] and therefore the method has a high specificity.

MRM: Multiple reaction monitoring; SRM: Selected reaction monitoring.

cleavage sites [6]. The latter is referred as *in silico* digestion. The signal of the SRM transitions can further initiate a product ion scan when the SRM experiment is performed on hybrid quadrupole linear ion trap (Q-TRAP) or other fast scanning tandem quadrupole instruments to provide a confirmatory product ion spectrum. This method was first reported by Cox *et al.* [7] and Uwin *et al.* [8] and was termed by the authors MRM-initiated detection and sequencing (MIDAS).

In the past decade, most of the MS-based proteomic studies were carried out using shotgun proteomics to maximize the amount of information acquiring in an experiment. However, it is now apparent that DDA has a number of limitations including instrumental scanning speed [9], stochastic selection of ions for fragmentation and poor repeatability [10], a relatively narrow dynamic range [11] and the issues of chimericy (co-fragmentation of two or more ions) [12,13], etc. Furthermore, the number of peptides presented in a biological digest may be many times larger than the number of ions that can be sequenced in an experiment despite the recent advances in instrumentation. Consequently, and as shown by the work of Michalski *et al.* [14], most of the information would still be inaccessible (referred as under-sampling) even though the experiment was performed on a LTQ-Orbitrap Velos mass spectrometer. Due to the bias nature of DDA for the most abundant species, low abundance peptides are unlikely being sequenced in a complex biological sample. Similarly, closely eluted isobaric species, such as phosphorylated peptides that differ only in the sites of phosphorylation, may not be sequenced completely owing to the typical dynamic exclusion setting used in DDA. On the other hand, precursor ion scan and neutral loss scan have a limited applicability and they are normally used as complimentary methods.

Recently, there has been a renaissance of targeted proteomics using SRM method. It is because SRM offers several advantages, such as specificity, reproducibility, sensitivity, linearity and it ideally suits for quantitative analysis. Furthermore, SRM-based quantification can be coupled to different strategies for relative (differential) or absolute protein quantification. One of the absolute approaches is termed absolute quantification (AQUA) of proteins [15]. In this strategy, synthetic peptides incorporated stable isotopes are spiked to a protein digest as internal standards to mimic the native peptides formed by proteolysis. This method allows accurate quantification of a network of proteins in a biological system. A similar method, stable isotope dilution (SID), is routinely being used in quantification of pharmaceutical compounds and small molecules [16]. An additional advantage of SRM is that the experiment can be conducted on relatively low-cost triple quadrupole-type instruments.

Despite the advantages, targeted proteomics has not been the preferred method by many proteomic researchers. Given that it is a targeted approach, a prior knowledge of the targeted proteins in the sample is a requisite. Arguably, up to 6000 transitions can be monitored by an SRM experiment using triggered or intelligent selected reaction monitoring (iSRM) on the latest triple quadrupole mass spectrometer (e.g., TSQ Vantage) [17]. However, only a relatively small number of proteins (up to 100) are monitored by a typical SRM experiment in practise. The method also requires lengthy and labor-intensive development and optimization process [18]. Compared to SRM, other tag-based quantitative proteomic methods, such as isotope-coded affinity tags (ICATs) [19,20], isobaric tags for relative and absolute quantification (iTRAQ) [21–23], tandem mass tags (TMT) [24,25], isotope-coded protein label (ICPL) [26,27], etc. offer a much greater flexibility (in terms of what proteins and the number of proteins being measured in an experiment), whereas SILAC [28,29] offers good reproducibility and accuracy.

Furthermore, in SRM, the detection of a chromatographic peak, even with all the predicted SRM transitions detected, does not confirm the identity of the peptide. This is because the mass of interfering ions could fall within the tolerance of both quadrupoles and leads to a false positive identification. The problem of interfering or isobaric ions can be alleviated with the use of differential mobility separation (e.g., DMS or FAIMS) [30] or MRM^3 (**Box 1**) [31]. Additionally, both of these techniques lowers the lower detection limit and increases the dynamic range. However, since they either lead to ion neutralization or increase the length of duty cycle, one may have to either reduce the number of analytes that can be measured concomitantly in a complex sample or to have a lower absolute sensitivity of the measurement [32,33]. On the other hand, even if sequence information is acquired using the MIDAS approach, the product ion spectrum is usually of low quality (low mass resolution/accuracy or high interferences) for confident assignment of the peptide identity. The data generated will have to be validated with relatively expensive reference or isotopically labeled peptide standards [34]. In short, and as shown by the Aebersold lab, it is paradoxical that highly sensitive SRM assays have to be developed and validated by a

method that has a substantially lower sensitivity and dynamic range than the SRM assay itself, which has prevented the routine development of SRM assays for low-abundance proteins [35].

However, the situation for targeted proteomics might be changing. A major driving force is that MS-based proteomics is in a transitional phase from being largely a discovery-based analysis to emphasizing more on hypothesis driven analysis [36]. Hence there is an impeccable need for high analytical precision, accuracy and wide dynamic range of targeted proteomics. Much of the biomarker candidates identified by shotgun proteomics in the past were not being followed-up or validated. Our laboratory (and many other) performs validation and characterization of these candidates with biochemical techniques, such as western blotting. However, the required antibodies are often unavailable. Many commercially available antibodies do not work effectively, and the associated cost is normally very high. Should there be a large number of potential biomarkers, multiplexed targeted proteomic methods would be more time and cost efficient than biochemical investigations to rationale the biomarker candidates identified in the discovery-based approaches [37]. But the restraints and difficulties associated with SRM have not made the objectives of targeted proteomics a reality and this has fuelled the recent advances of MS methods and instrumentation that permit data-independent analysis (DIA) or hyper reaction monitoring (HRM) on high-resolution and accurate-mass hybrid systems. This potentially allows one to obtain sequence information for peptide identification while the SRM-like ion chromatographs can still be subsequently extracted or reconstructed from the raw data. This does not only allow comprehensive qualitative assay of the species in the sample but the method can also provide quantitative information.

In this review, a number of selected DIA and HRM methods reported, to date, are introduced and discussed with respect to how these new MS approaches could be used in to advanced protein biomarker discovery and validation. Their advantages, limitations and potential impacts on proteomics are being considered.

An overview of the data independent & targeted acquisition

DIA and HRM experiments are conducted on a hybrid system with full scan data being recorded in a time-of-flight (ToF), Orbitrap or other high resolution mass analyzers. A major difference in these methods lies on the width (or the absent) of the precursor windows at the first stage of ion isolation. This in turn determines the range of ions being transmitted, fragmented and sequenced. The precursor isolation window ranges from full mass range (normally, 400–2000 Th), to wide (10–100 Th) or relatively narrowed (0.4–4 Th) width. A consequence of having a wide precursor isolation window in DIA approaches is that it produces a very complex data structure and requires coherent and intricate data processing. Conversely, narrow isolation windows leads to high precursor selectivity, such as those in the HRM methods, and the data can be reconstructed subsequently in a manner as if it was acquired by SRM in a triple quadrupole mass spectrometer. This allows one to refine and re-mine the SRM transitions post-analytically. A summary of the selected approaches supported by the various instrument manufacturers and their advantages, limitations and potential impact to proteomics are given in Table 1. An overview of these approaches is given below in accordance to the bandwidth of the precursor isolation windows.

MS^E

MS^E is a DIA approach that acquires MS1 and MS2 mass spectra in an unbiased and parallel manner. As such, it increases both the number of peptides detected and the reproducibility of the peptides sampling during an LC-MS experiment. The MS^E method has been made commercially available since the version B of Q-TOF Premier mass spectrometer, although techniques similar to MS^E, had been implemented in the earlier generations of Q-TOF systems by the manufacturers [38–40] and independently by other research groups [41,42]. During data acquisition, the energy of the gas-filled travelling-wave collision cell is dynamically switched between a low-energy and an elevated-energy status. This produces alternating composite mass spectra of all intact molecular ions, followed by chimeric mass spectra of all precursors. The MS^E raw data files are then processed by three different algorithms in ProteinLynx Global SERVER (PLGS). Ion detection and charge/isotopic deconvolution are performed in data pre-processing. The 'reconstructed' DDA-like data is then evaluated by database searching algorithm prior to the peptide identification.

The first algorithm, Apex3D, filters out all the signals below the intensity threshold specified by the user (consider as noise) and integrates ion current signals across their chromatographic profile. A second algorithm, Pep3D, performs charge-state reduction and deisotoping to further reduce the volume of the data. Despite multiple precursor ions are being fragmented simultaneously during the data acquisition, on the principle of time-alignment correlation, a particular set of product ions can be grouped or deconvoluted to the specific subset of precursors based on their chromatographic elution (and mobility) profiles, but not to all the intact ions in the composite mass spectra (Figure 1A) [43]. The correlation efficiency is determined with a number of chromatographic peak characteristics such as start time, end time, apex retention time, width at half maximum and the chromatographic peak asymmetry. The outputs of data pre-processing are lists of time-aligned precursors and product ions annotated with their respective accurate mass, retention time, intensity, charge state and other physicochemical properties (e.g., arrival time in case of mobility measurement). However, it is worth noting that at this initial stage of precursor/product ion assignation, the same product ions can be associated with numerous precursors. The database search is then performed by Ion Accounting algorithm ($IDENTITY^E$).

Table 1. A summary of selected emerging data-independent analysis/HSM approaches and their respective pros and cons.

Method	Manufacturer	Instrument type	Brief description	MS1 isolation window	Duty cycle, *m/z* range	Advantages	Disadvantages	Potential impacts on proteomics
MS^E	Waters	Q-TOF	The collision cell switches rapidly and continuously between low and elevated collision energy states. At low energy state, no fragmentation occurs and precursor ion spectra are recorded. At high energy state, ramped collision energy is used to generate fragment ions. Product ions spectra of all precursors are recorded. Complex sample requires efficient separation using UPLC or further with ion-mobility separation.	Typically, 300–2000 Th (filter low mass ions)	1 s (0.5 s for intact/ precursor ions scan and 0.5 s for fragment ions scan), *m/z* 300–1990	Requires a minimum method development. Need no prior knowledge of the sample components. Unbiased data acquisition, regardless of the ion intensity. Closes to 100% duty cycle and potentially allow a higher number of proteins to be identified. Label-free quantitation.	The method is not a replacement of DDA and is limited by the extent to which correct assignation of fragment ions to precursors is achieved. Identity of the peptides may require further validation with directed DDA. US FDA determination can only achieved with third party package.	Circumvent the issues surrounding the stochastic sampling of precursors in DDA approach. Significantly enhance sequence coverage and hold promises to locate the sites of post-translation modifications.
AIF	Thermo scientific	Benchtop Orbitrap and quadrupole-Orbitrap	The system is set to perform alternating MS scans of the precursor ions and AIF scans in which all the precursors are transferred to the HCD cell and fragmented.	None	2 s (1 s for intact/ precursor ions scan and 1 s for fragment ions scan), *m/z* 100 –1600	Same as MS^E but with wider dynamic range and higher mass accuracy due to the use of Orbitrap mass analyzer. MaxQuant allows FDR estimation.	MaxQuant's peak recognition algorithm is not as sophisticated as IDENTITY.	Same as above
SWATH-MS	AB Sciex	Q-ToF	The system performs MS2 scan in a sequential 25 Da MS1 windows. Fragmented ions are measured with high resolution TOF mass analyzer. During data post-processing, SWATH-MS spectra are extracted to SRM chromatographs.	25 Th	3.2 s, *m/z* 400 –1200	Allow quantitative assay of complex samples in the same manner as SRM but enjoy the potential to monitor a higher number SRM transitions than conventional SRM and able to refine the SRM transitions retrospectively.	Incompatible with database search, and thus does not confirm the identity of the peptide detected. Require validation.	Fill the gap between discovery-based and hypothesis driven investigation. To test new hypotheses without the need to repeat the entire experiment and expedite biological discovery. Further targeted or routine analysis can be performed by SRM or MRM^{HR}

AIF: All-ion fragmentation; DDA: Data-dependent acquisition; DIA: Data-independent analysis; HCD: Higher energy collisional dissociation; IDENTITY: Ion Accounting algorithm; MRM: Multiple reaction monitoring; MS: Mass spectroscopy; PRM: Parallel reaction monitoring; pSRM: Pseudo-SRM; Q-TOF; Quadrupole orthogonal acceleration time-of-flight; UPLC: Ultra-performance liquid chromatography.

Table 1. A summary of selected emerging data-independent analysis/HSM approaches and their respective pros and cons (cont.).

Method	Manufacturer	Instrument type	Brief description	MS1 isolation window	Duty cycle, *m/z* range	Advantages	Disadvantages	Potential impacts on proteomics
MSX	Thermo scientific	Benchtop quadrupole-Orbitrap	An inclusion list containing a sequence of ~5000 isolation windows is defined in Skyline before an experiment. During an analysis, the instrument loops through the inclusion list sequentially, choosing five isolation windows for each MSX scan. When an MSX scan is acquired, each isolation window is isolated, fragmented and trapped serially in time before mass analysis in the Orbitrap. The MSX spectra are demultiplexed during data post-processing and are extracted to SRM chromatographs.	5 × 4 Th	3.5 s (with 2 MS1 scans), *m/z* 500—900; max. fill time for each isolation window 20 ms, Orbitrap resolving power: 17,500	Same as above An improved DIA/SWATH-MS method that has a reduced chemical background and fragment-ion interference in the extracted SRM chromatography bought by increasing the precursor selectivity. MS1 scan can be acquired at the same experiment and this produces complementary quantitative data.	Same as above The method is a compromise between the data quality and the scanning rate of system.	Same as above. The method potentially improves the precision and accuracy of the measurement relative to the SWATH-MS.
PRM	Thermo Scientific	Benchtop quadrupole-Orbitrap	A quadrupole mass filter isolated narrow mass windows. Fragmented ions are measured with ultra-high resolution Orbitrap mass analyzer.	2 Th	Maximum fill times of 3s, at resolution of 70 000 (at *m/z* 200), and target AGC values of 3e6	Increase the number of peptides to be monitored in one experiment. Taking the advantages of the high-resolution and accurate mass measurement to increase the degree of selectivity such that the targeted peptides can be discriminated against background interferences. High selectivity, dynamic range, and sensitivity. The MS2 spectra are compatible with database search.	The number of precursor ions that can be monitored is dependent on the duty cycle or transient length of the mass analyzer. Although a higher number of precursor ions can be monitored than SRM, the number precursor ions are still limited in an experiment. The same argument applies to pSRM/ MRM^{HR}	Advancing targeted proteomics.

AIF: All-ion fragmentation; DDA: Data-dependent acquisition; DIA: Data-independent analysis; HCD: Higher energy collisional dissociation; IDENTITY: Ion Accounting algorithm; MRM: Multiple reaction monitoring; MS: Mass spectroscopy; PRM: Parallel reaction monitoring; pSRM: Pseudo-SRM; Q-TOF; Quadrupole orthogonal acceleration time-of-flight; UPLC: Ultra-performance liquid chromatography.

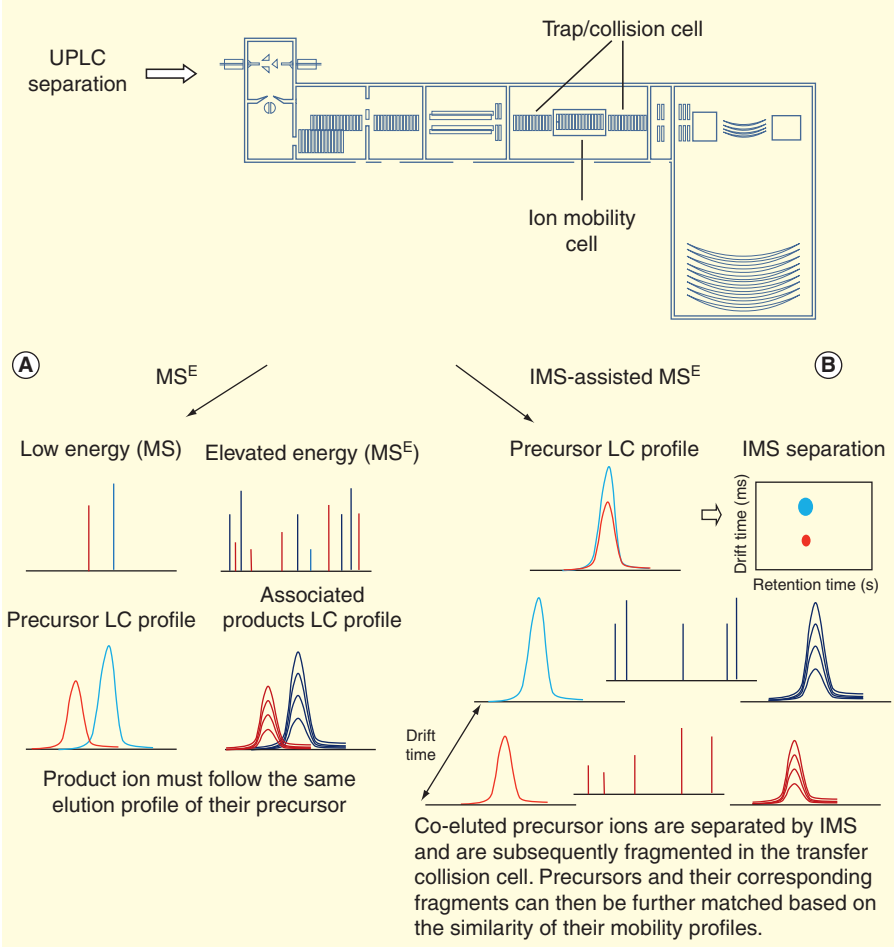

Figure 1. The principle of ion mobility spectrometry (IMS) assisted MS^E (HDMS^E). **(A)** In standard MS^E, the collision energy of the collision cell is dynamically switching between low-energy and elevated-energy status, producing alternated scan of intact molecular ions at low-energy status and their corresponding fragment ions at elevated-energy status. An algorithm dictates the relationship of precursors and fragments based on time-resolved mass measurement obtained from an LC-MS^E experiment. **(B)** In the analysis of complex biological mixture, co-elution of peptides is common. Gas-phase ion mobility separation allows an additional orthogonal separation to LC. Fragmentation occurs after the IMS separation. The alignment the precursors and fragments is not just based on the LC retention time but also on the drift time of the precursors resulting in more accurate assignment of fragment ions to precursors. HDMS^E allows up to 60% higher proteome coverage and higher confidence of protein and peptide identifications than standard MS^E. IMS: Ion mobility spectrometry; LC: Liquid chromatography; LC-MS^E: Liquid chromatography-mass spectroscopy; MS: Mass spectroscopy.

The database search is an iterative process whereby each pass incrementally increases the selectivity, specificity and sensitivity of the overall strategy. Prior to database query, a reverse or random decoy database is generated for monitoring the false positive identification rate of a particular search. A pre-assessment survey is conducted to construct an optimized fragmentation model [44]. This is achieved by assessing the experimental dataset and a database search encompressing the physicochemical properties of peptides, hypothetically produced by the enzymes (and other reagents) used. During the first pass, the algorithm considers each parent/product ion lists and matches them against protein database, but only to assign the peptides that are completely cleaved by the enzymes used. The tentative identified peptides are scored, based upon how well they correlate to 14 different models of specific physicochemical attributes (see ref. [44] for further detail). This process is followed by a peptide ranking process and collapsing the identified peptides into proteins. The process terminates when the rate of decoy protein identifications exceeds the false positive rate threshold specified by the user (default setting 4%). During the pass, two of the database search, only the depleted data from the pass one is used for the search. Identifying peptides can be subjected to modifications (including chemical and post translational modifications, post-translationally modifications [PTMs]), non-specific cleavage, neutral losses and in-source fragmentation, but are only assigned to proteins positively identified in the first pass. During the third pass, a fragment is allowed to have higher intensity than its precursor, a situation characteristic of in-source fragmentation of highly labile peptides. The proteins are scored and ranked and the depletion process is repeated until a minimum protein score can no longer be maintained or the specified false positive rate of identification is breached [44]. The results of various search iterations are aligned and a protein ranking process is initiated.

Compared to DDA, MS^E has a duty cycle close to 100% such that the method enhances reproducibility and widens sequence coverage for each of the identified protein. The improved sequence coverage holds promises for the characterization of the sites of PTMs. The algorithm used to deconvolute the multiplexed spectra is also capable of correcting the issues associated with co-eluting peptides [45]. The reconstructed data can also be extracted as if the data was acquired using precursor ion scan or neutral loss scan for further inspection [38]. Additionally, MS^E can be used for label-free differential quantitative assay across the series of samples [46]. It has been shown that not only the method produces good quantitative agreement with the iTRAQ approach [47], but also has a linear range of 3 orders in magnitude [46]. It has also been reported that based on a comparison of a mixture of known proteins, universal proteomics standard 1, commonly used for instrumental testing, the method is able to identify more unique peptides and proteins [46].

However MS^E is not a substitution of DDA, it is not suited for quantitative proteomic methods that involve isobaric labeling or tagging, for example. IDENTITY^E also lacks an estimation of false discovery rate (FDR) and the data generated may have to be cross validated by (directed) DDA [48]. Since all ions are being fragmented simultaneously, the data produced is highly convoluted. Hence, the method is limited by the post-processing software to accurately correlate the fragments to the precursors. To circumvent this issue, it is crucial to increase the resolving power to separate the peptides or the ions before the mass analysis such that high-quality data is generated for data processing. Ultra-performance liquid chromatography (UPLC) is normally employed for enhancing the peptide separation. The performance can be further enhanced with the use of ion-mobility gas-phase separation conducted on Synapt class systems. This is referred as ion mobility-assisted DIA or HDMS^E (Figure 1B). Ion mobility enhances the peak capacity and doing so it increases the proteome coverage of MS^E. It has been reported that in comparison of MS^E and HDMS^E, up to 60% higher proteome coverage and higher confidence of protein and peptide identifications can be achieved [49]. The same research group has also developed Synapter software package for post-PLGS data analysis [50]. This allows a deeper proteome coverage delivered by HDMS^E. Furthermore, the post-PLSG package permits FDR estimation which is currently unavailable function in PLGS.

MS^E has been a popular choice among authors and has found applications both in proteomics and metabolomics. The method is compatible with multivariate data analysis, such as principal component analysis (PCA) [51–53]. A number of label-free quantitative studies have successfully applied MS^E, including identification differentially expressed proteins in rat frontal cortex following subchronic treatment with haloperidol or olanzapine [52], profiling the serum [54] and post-mortem brain tissues of schizophrenia patients [53] and analyzing the human pituitary proteome [55]. MS^E has also been employed in the studies of the proteins from plants [56,57] and microorganisms [40,48,58,59].

All-ion fragmentation

All-ion fragmentation (AIF) was first introduced in bench-top Exactive class Orbitrap mass spectrometer that was initially developed for small molecule applications. AIF, despite being named differently by the manufacturer, is a similar acquisition mode to MS^E in which all precursor ions are fragmented without a pre-selection by the quadrupole [60]. Fragmentation, however, is obtained with a higher energy collisional dissociation (HCD) cell, located at the far side of the C-trap. During filling of the HCD collision cells, the energy can be set to step between values at specified percent values around the chosen middle energy regardless of the ion's characteristics. This is similar to the energy ramping normally employed in MS^E. Data processing and database searching can be performed in MaxQuant software. From the information presented in the ref. [60], the MaxQuant's peak recognition algorithm is also based on chromatographic elution profile correlation, similar to the deconvolution algorithms used in PLGS, to assign the AIF peaks to their potential precursors. A cosine correlation value of at least 0.7 was considered an acceptable value in the study [60]. The reconstructed pseudo MS/MS spectra are submitted to the database search engine, Andromeda [61]. Comparing to PLGS, MaxQuant's uses a less sophisticated approach in performing AIF database searching. The search engine treats the pseudo MS/MS spectra as if it was acquired with DDA. The ultra-high mass resolution and mass accuracy of the Orbitrap mass analyzer becomes critically important in ensuring the success of the method. In comparison to MS^E, based on the results reported in [46], 43 out of the total 48 proteins in the Universal Proteomics standard 1 (UPS 1) were identified with MS^E when using the complete human database, whereas 45 of the UPS 1 proteins were identified with AIF (with 1% FDR specified in MaxQuant). This may be contributed to the mass resolution and accuracy of the Orbitrap. However, the study also showed that the MS^E platform produced an average of 12.2 9.8 peptides per protein, whereas with the AIF mode only 8 7.2 was achieved [46]. The scanning speed of the system and the database search algorithm therefore play important roles in terms of sequence coverage. Linearity and dynamic range of the method were reported to be 3 and 4 orders in magnitude, respectively [60].

Fourier transform-all reaction monitoring

Fourier transform-all reaction monitoring (FT-ARM) is a newly proposed DIA method that does not depend on the precursor mass measurements. Instead, it relies on the specificity of peptide fragmentation patterns and high mass accuracy measurement [62]. In a typically FT-ARM experiment, which is conducted either on a LTQ-FT or a LTQ-Orbitrap instrument, all the ions in a continuous defined mass window (e.g., *m/z:* 700–800) are first isolated and fragmented in a linear ion trap. The isolation window remains throughout the entire chromatographic run. All fragment ions spanning across the whole mass range are then being transferred to a Penning trap or an Orbitrap, where high-resolution measurements are made. The data processing is accomplished in the software package developed by the authors (available free at Bruce lab web site). The multiplexed fragmentation spectra are searched against hypothetical spectra calculated by in silico digestion. The result is a score chromatogram for each peptide contained in the database. A reverse sequence decoy database is searched to estimate FDR. Typically, 1% FDR is used. In the work of Weisbrod *et al.* [62], the FT-ARM data was acquired at resolution of 25,000 (at *m/z:* 400) for the LTQ-FT, and 30,000 (at *m/z:* 400) for the LTQ-Orbitrap. In addition to identification, peptides from BSA were also quantified against the yeast whole cell lysate background. Linearity and limit of detection were found about 4 orders of magnitude and 1.5 fmol, respectively, in terms of total BSA protein loaded (i.e., not the actual concentration of the peptides).

The FT-ARM analysis differs from DDA and conventional database searching algorithms in that all possible peptides in a

given database are scored against every acquired spectrum [62]. It also differs from MS^E and AIF that MS1 data is not required or acquired. Identifications rely on accurate fragment ion matches only and do not take into account of chromatographic elution profile characteristics to determine the precursor-fragment ions relationships. Furthermore, quantification is performed on a large scale without the need for assay development. However, the authors also pointed out that the constraint of the method is upon that the dynamic range and sensitivity is somewhat limited by that of the linear ion trap and automatic gain control (AGC).

MS/MS^{ALL} with SWATH Acquisition & MRM^{HR}

SWATH (Sequential Window Acquisition of all THeoretical Mass Spectra) Acquisition was first introduced in TripleTOF system [63]. The method has been used to profile *N*-linked glycoproteins in human plasma [64] and to examine the sites of protein phosphorylation and acetylation at the receptor tyrosine kinase ErbB2 extracted from SK-BR-3 cells [65]. In SWATH Acquisition, the first quadrupole sequentially steps in a 25 Th precursor window across the mass range of interest and passing the ions into the collision cell recursively during the entire liquid chromatography (LC) separation. The transmitted ions are fragmented and the resulting fragment ions are then analyzed by the ToF mass analyzer [66]. This method of data acquisition generates a 3D retention time-fragment ion m/z-intensity map for each precursor ion selection window, called a SAWATH (Figure 2A): a term derived by making an analogy of the SWATH Acquisitions in Earth satellite scans. A compilation of all SAWATHs is complicated but complete record of the fragment ion spectra of all analytes is detectable by the system in a sample. Similar to other DIA methods, data interpretation is a challenge. However, it has been demonstrated that using targeted data extraction, the complex data acquired can be interrogated in the same manner as if they were acquired using SRM [66], but with the capability and flexibility to refine, expand and re-mine the SRM transitions post-analytically. Thus, one of the most significant advantages of SWATH Acquisition (and similar DIA approach described in the later section using ion trap or Orbitrap systems) in comparison to SRM is its potential to perform a significant larger number of SRM-like experiments concurrently.

To empower the SWATH-MS approach, the instrumental scanning speed has to be fast enough to allow acquiring an adequate number of data points across the typical chromatographic peak such that ion chromatography can be reconstructed with satisfactory signal-to-noise ratio (S/N). It has been estimated that at an accumulation/dwell time of 100 ms for each 25 Th window scan with 1 Th window overlap isolated by the quadrupole, it is able to scan *m/z* 400–1200 range in a total of 3.2 s. This cycling time is sufficient to construct the approximately 30 s wide chromatographic peak of each analyte for accurate quantification [66]. Relative to the typical SRM approaches, the selectivity of the method is ensured by taking the advantage of high mass accuracy measurement of the fragment ions. Linearity was found almost 4 orders in magnitude. The limit of detection (LOD) and limit of quantitation (LOQ) was found in the amol range. This sensitivity is generally much better than the typical DDA or MS1 (lower fmol), although not as much as sensitive as conventional SRM (lower amol). Precision was estimated to be 13.7–14.9% [64,66]. In addition to the official software package (PeakView) to process the SWATH-MS data, the data can be also processed with OpenSWATH (implemented in OpenMS [67,68]), Spectronaut [69,70] and Skyline [71,72] software packages. All of these packages are free for academic uses.

Nevertheless, SWATH Acquisition has a major drawback: the SWATH-MS data is incompatible with conventional database searching (or the development of a deconvolution algorithm to process the SWATH-MS data for database searching has not been achieved). There are number of challenges in designing a deconvolution algorithm to process such complex data. Firstly, as shown in Figure 2B, co-elution and co-fragmentation of a mixture of precursor ions produces a chimeric spectrum (superimposed with residual precursors). However, time-alignment correlation used in MS^E and AIF data processing breaks down. Furthermore, the mass resolution and accuracy of the TripleTOF system is not as high as Orbitrap or FT-ICR to give adequate selectivity. Several algorithms have been published previously to process chimeric spectra, such as M-SPLIT and MixDB from Wang *et al.* [73,74]. The Aebersold lab has also previously developed ProbIDtree to automatically assign several peptide precursors in MALDI TOF-TOF chimeric spectra [75]. Despite these computation approaches were being designed to assign low-resolution MS/MS data, their algorithms or principles may be modified or adapted to aid the data processing of the SWATH-MS data.

Another potential problem of SWATH Acquisition is the interferences that arise as a result of the 25 Th width of the precursor isolation widow used. The presence of interferences may affect the precision of quantification (see more on MSX in the later section). An alternative approach is termed MRM^{HR} [76], in which the Q1 is set to scan a set of predefined precursors and the precursors are fragmented in the collision cell. This produces a series of product ion scans. Typically, 30–40 precursors can be targeted per run without time scheduling MRM. As with SWATH Acquisition, SRM-like extracted ion chromatograms on sequence specific ions can be generated after the data acquisition, excepting that there is no flexibility to refine the precursor windows post-acquisition retrospectively. However, since the full high-resolution product ion spectra acquired are compatible with the conventional database search engines, the spectra can potentially provide the sequence information of the peptide.

Data-independent acquisition

The DIA method, or the origin of the SWATH-MS approach, was initially developed in a fast scanning LTQ linear ion trap mass spectrometer [77,78]. However, this method has also been

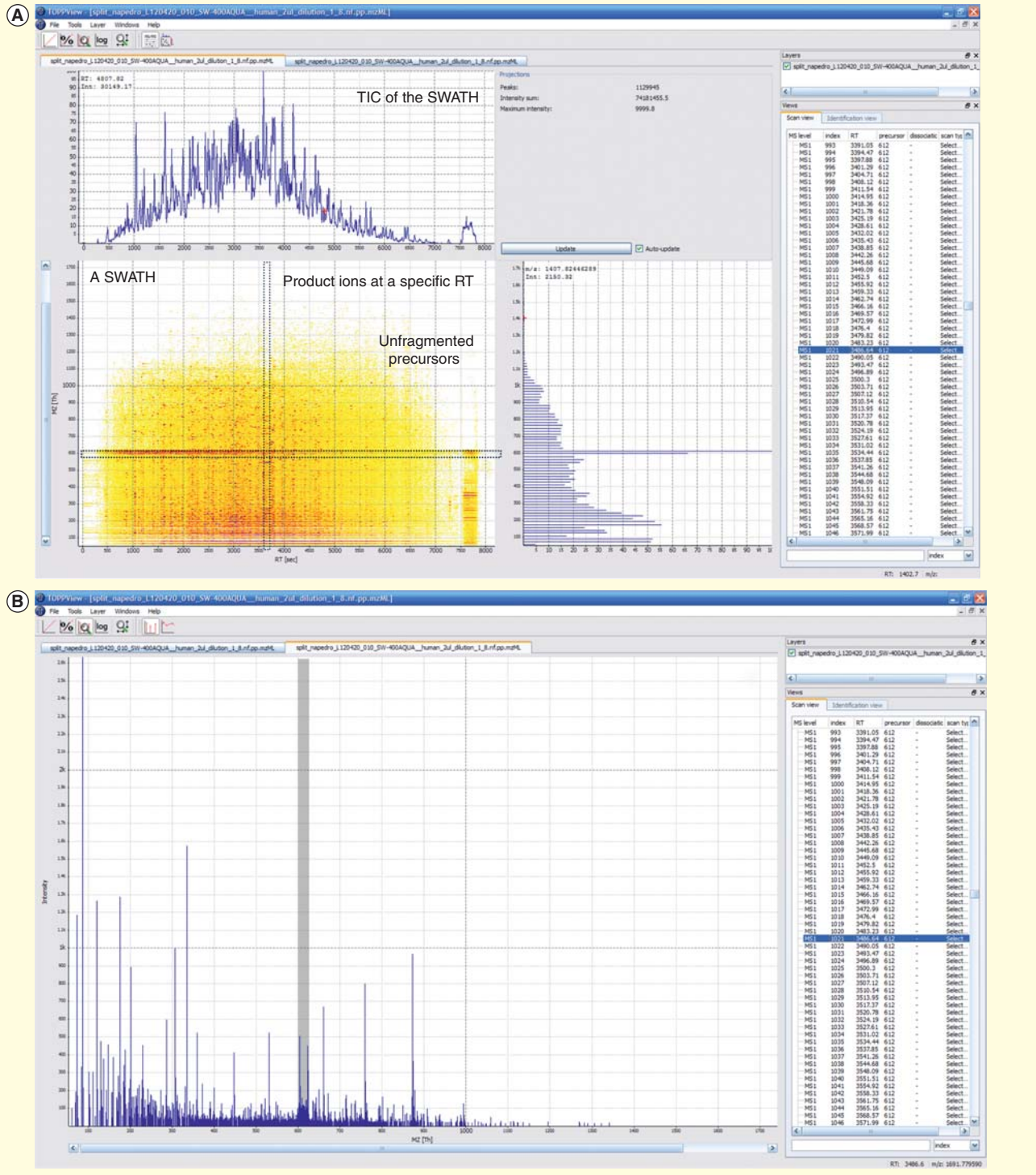

Figure 2. The data structure of SWATH-MS data. During SWATH-MS data acquisition, fragment ion spectra of a series of precursor isolation windows, typical 25 Th width, were sequentially acquired during the entire LC run. A compilation of all the fragment ion spectra acquired within a particular precursor isolation window are referred as a SWATH. **(A)** A contour plot of a single SWATH between 600–625 Th, with retention time as the abscissa, fragment ion *m/z* as the ordinate, and ion intensity represented by color intensity. The darker horizontal band visible between 600–625 Th (horizontal box) corresponds to un-fragmented precursor ions that have been transferred through Q1. Each data point in a SWATH (vertical box) consists of a fragment ion spectrum at that retention time. **(B)** The fragment ion spectrum at RT 3486.34s is shown in, which is a chimeric spectrum. The precursor isolation window is shaded. At least, two major and three minor peptide ions are co-fragmented within the isolation window. Screenshots were taken using OpenSWATH demo dataset on OpenMS ver. 1.10.0.

used in LTQ-Orbitrap, for example, using electron transfer dissociation with supplemental activation (ETCaD) [79]. Both of the cited works were conducted independently from the manufacturer. In the study that used a LTQ linear ion trap mass spectrometer, the system was set to sequentially isolate and fragment precursor windows of 10 Th (in the ion trap) by collision-activated dissociation (CAD) until a desired range was covered. It was shown that there was a three to fivefold improvement in the signal-to-noise ratio of the ion chromatograms in comparison to DDA. Furthermore, the method provided time-consistent ion sampling and was able to identify peptides undetected in MS1. The increased sensitivity is due to the ability of the linear ion trap to accumulate selected precursor ions for MS2, thereby becoming less affected by chemical noise than MS1. Automatic gain control (AGC) on ion-trap mass spectrometers further improves the detection of low abundance molecular species. Although the precursor window is smaller than that in SWATH-MS approach, equally, the lack of precise knowledge of the peptide precursor ion and the overlapping of spectra owning to co-elution of peptides do cause problems in standard peptide identification programs, though effort has been made to develop software program to deconvolute complex DIA [78] or multiplexed spectra [80]. A program named XDIA Processor from Yates's laboratory [79] has been made available to pre-process the DIA spectra acquired on Orbitrap mass spectrometers prior to database searching.

Multiplexed MS/MS

In SWATH Acquisition and other similar DIA approaches, co-fragmentation of precursors is inevitable due to the width of the precursor isolated windows used. It has been estimated that when 25 Th wide isolation windows are used in DIA, approximately 85% of the windows isolate and fragment two or more peptide precursors from a sample of cell lyate. On an average 3.4 peptide precursors are co-fragmented in each window. This significantly increases the likelihood of fragment-ion interference in the extracted SRM chromatograph and decreases the specificity of the method [81]. Furthermore, with 25-Th isolation windows, a number of peptides and their modified forms (e.g., N-terminal acetylation, oxidized methionine) may be isolated in the same window. However, they are difficult to differentiate due to overlapping fragmentation patterns and this potentially hinders quantitation [81]. In strike contrast, when the width precursor isolation window is reduced to 4 Th, <10% of the isolation windows contain two or more peptides [81]. However, using narrow precursor isolation windows in DIA is hurdled by the sampling frequency of the instrument.

To increase the precursor selectivity and to overcome the constraint on the scanning speed of the instrument, the MacCoss lab has introduced an improved DIA framework, termed multiplexed MS/MS or MSX [81]. The MSX technique has been demonstrated in Q Exactive instrument by taking its ability to isolate, fragment and trap ions from multiple precursor windows prior to mass analysis (i.e., multiplexing). In the proposed workflow, an inclusion list is first calculated in Skyline. For example, using isolation windows width of 4 Th to monitor mass range of 500–900 Th and performing multiplexed acquisition with five isolation windows per scan, the software tool generates an inclusion list of 4935 entries (firmware inclusion limit is 5000 [entries]). The list contains 47 sets of 105 center/target masses. However, the sequence of the center masses in each set is randomized. The instrument is then set to perform a DIA experiment with 4 Th width precursor isolation windows around the center masses. During the data acquisition, the Q1 isolates the precursor windows on the sequence of the inclusion list, but the Orbitrap performs a MSX scan after five of these 4 Th isolation windows have been fragmented in the HCD cell and trapped in the C-trap. Therefore, each of the MSX scan is a chimeric spectrum of five distinct precursor isolated windows. Since the fragmentation and MSX scanning are conducted in parallel, the whole mass range can be covered in 3.5 s using the highest scanning rate of the system. The process continues until it reaches the end of the inclusion list and the whole process repeats again. During data post-processing, the MSX spectra are demultiplexed and reconstructed into pSRM chromatographs. The confidence of the assignment is evaluated by a score based system (the dot-product in Skyline).

The demultiplexed extracted SRM chromatographs have reduced chemical background and fragment-ion interferences, which resulted in a higher dot-product similarity (0.96 vs 0.94 for demultiplexed and non-demultiplexed fragments, respectively) to a DDA spectrum for the same peptide acquired with a 2-m/z-wide isolation window. Accordingly, an advantage of MSX is that the method has an improved selectivity than other DIA methods using 10 Th or larger precursor isolation windows. The report had only compared the level of interferences of MSX against a DIA method using 20 Th wide isolation windows, but had not quantified the improvement on precision and accuracy brought by enhancing the precursor selectivity of the proposed method. The authors, however, had compared the analytical performance of MSX against MS1 in a spike-in experiment with 36 peptides [81]. On an average, the MSX method was less sensitive than the MS1 method. They found that the lower limit of detection for MSX and MS1 was 8.66 and 4.98 fmol, respectively. However, the authors also noted that seven peptides suffered from interference in the MS1 signal, and consequently MSX gave an average 3.4-fold improvement in sensitivity in those cases. The CV of 18 replicate measurements of six peptides averaged 0.15 and 0.10 by MSX and MS1, respectively. The standard deviation in these measurements was 2.29×10^7 and 1.56×10^8 for MSX and MS1, respectively [81].

MSX also has some drawbacks. The improvement on selectivity is only appreciable on highly complex samples. Simple mixture may or may not gain benefit from the increase of precursor selectivity, although the method potentially enhances the ability to identify peptides (e.g., using XDIA). The proposed workflow used a maximum of 20 ms HCD fill time, in other words, 100 ms for five isolation windows, to scan a mass range of 400 Th. If a higher maximum fill time is being used, the

duty cycle of the method would be too slow. Accordingly, the majority of the scans would hit the maximum fill time, but has not yet hit the AGC target. That likely affects the ability to detect low abundance peptides from a complex mixture. Alternatively, one has to adjust the LC gradient such that a higher HCD fill time can be used to permit detection of low concentrated species. Thirdly, demultiplexing of the MSX data into pSRM chromatographs is computational intensive and may take several hours in a modern personal computer for a signal injection.

Pseudo-SRM & parallel reaction monitoring

Both pSRM and parallel reaction monitoring (PRM) experiments are targeted MS/MS analyses, in which full fragment ion spectrum of each precursors in a target list is recorded continuously throughout the entire LC separation. In contrast to conventional SRM, in which only the selected transitions are being measured, pSRM and PRM record all the products of the selected precursor's windows. However, the term pSRM appears in literature to refer to experiments conducted exclusively on LTQ linear ion trap or LTQ-Orbitrap mass spectrometers [82,83], whereas the term PRM refers to the experiment specifically carried out on Q Exactive instruments [84,85]. Hence, in pSRM, the first stage of ion isolation is performed in a linear ion trap rather than *via* a quadrupole mass filter. This isolation step is essential for quantitative measurements because the space charge effect is intrinsically associated with ion trapping devices, that limits the dynamic range of the measurements. However, the use of a quadrupole frontend in PRM provides some advantages, such as ion isolation speed and effectiveness, the availability of multiplexed single ion monitoring (SIM) and manufacturing cost.

Although precursor ion spectrum is not recorded in standard PRM mode, one can still use an alternative setting to acquire a full MS1 scan or SIM spectrum in an expense of duty cycle (termed targeted-DDA or directed-DDA [86] to conduct MS1 and MS2 based analyses, whereas pSRM experiments conducted on LTQ-Orbitrap allows concurrent MS1, MS2 and MS3-based analyses and quantifications [82]. SRM chromatographs can be extracted from the full MS2 or even MS3 spectra and peak areas of the transitions are summed for label-free quantification.

The main advantage of pSRM and PRM is the use of ultra-high resolution Orbitrap mass analyzer that is able to separate interferences from the true signals and thus significantly enhancing the selectivity of the method compared to the conventional SRM approach [87]. A drawback is that the number of precursor ions that can be monitored is dependent on the duty cycle or transient length of the (Orbitrap) mass analyzer and the chromatographic conditions, although the number can be increased with the use of time-scheduling, parallelization (up to 10 precursor windows at one time) and relaxation on the Orbitrap resolving power. For example, Galliem *et al.* used a multiplexing of four precursors, scheduled elution window in 1.5–2.5 min retention time and they were capable of monitoring 770 tryptic yeast peptides (corresponding to 436 proteins) grouped in subsets of four peptides according to their chromatographic elution order [85].

The pSRM method has been employed for quantification of six site-specific phosphorylations in the EGFR in epidermal growth factor-stimulated A431 cells using SID and internal reference peptides (IRP) methods. The precision of the method was reportedly ranging from 7.3 to 15.7% [82]. In other studies, the linearity was found over 5 orders in magnitude [83] and the method had higher sensitivity and selectivity than SRM [88]. At least two different scoring systems have also been proposed to assess the confidence of peptide identification of the method [88,89]. The score was determined either based on the absolute intensity and number of product ions in the MS2 spectrum matched with the reference spectrum, or on the mass accuracy and retention time accuracy with reference to predicted and measured values.

Similarly, the PRM method has also been used to quantify 10 different polyubiquitin chains from yeast cell lysates using custom-made isotopically labeled peptides as internal standards [90]. Consistent with the earlier study [84], the dynamic range of the method was reportedly at least 3 orders in magnitude [90]. The precision of PRM was found to be <10% in most cases, depending on the concentration of the peptide [84]. The authors have also noted that neither isolation width nor the presence of matrix resulted in statistically significant differences in precision. LOD was found to be as low as 0.1 amol [85]. The pSRM and PRM data processing can be performed in Pinpoint software as well as in Skyline.

Expert commentary

DDA has been widely employed in shotgun proteomic workflow, not only has it been used in identification and characterization of proteins in a complex biological matrix, the method is also used in quantitative proteomics, particularly with the use of isobaric labeling or tagging. While a large number of studies have been conducted using DDA, the method suffers from various limitations, including reproducibility issue, narrow dynamic range and under-sampling. For that reason, DDA is often incapable of detecting low abundant peptides and is less suited for quantitative analysis. Targeted proteomic method such as SRM has a great potential to validate the results produced by shotgun proteomics. Despite its potential in quantification, SRM lacks flexibility and ease of use. The method requires a prior knowledge of the surrogated peptides, and their respective SRM transitions, in the samples. The method also involves a time consuming and labor intensive procedure in optimizing of the instrumental parameters for each type of assay. Besides, the use of low-resolution triple quadrupole-type instruments in SRM may lead to false positive results, particularly to low abundant species. For these reasons, using SRM to validate the vast amount of information generated by shotgun proteomics is uneconomical or impractical. It is not surprising that targeted peptide analysis by SRM has only been adopted in a relatively small proportion of laboratories that are dedicated to the advance of MS-based proteomic techniques and

the development of associated bioinformatics. Indeed, neither of the two methods seems to be ideal and has restricted the proteomic researchers from examining complex biological systems with both depth and width.

With the advance of MS instrumentation and scanning speed, various alternatives strategies, collectively referred here as DIA and HRM, are being introduced. Their advantages, drawbacks and their potential impacts on proteomics have been considered.

The sequencing event in MS^E, AIF and FT-ARM is initiated indiscriminately and all peptides in a sample are sequenced. These methods potentially permit low abundant peptides to be detected and sequenced despite in the presence of high abundant peptides. PTMs of proteins are known to be biologically important but these modified proteins often exist at very low concentrations. Characterization of PTMs would benefit from selective enrichment and purification, which is not possible for all the PTMs. For this reason, the DIA methods could be an alternative to the conventional approach. A relatively higher number of MS laboratories have adopted the MS^E technique in their workflow. However, the data generated by these methods could be extremely complex and these methods are currently restricted by the accuracy of the associated bioinformatic tools. Furthermore, much of the results reported in the past have not been obtained with a control, nor have the FDR estimated. Thus, there are concerns about the confidence of the peptide or protein assignation, or whether the results obtained by different research laboratories or methods are comparable.

In contrast to biomolecular discovery, the hurdle in MS-based validation lies in the complexity to conduct targeted proteomic assays. SWATH Acquisition, MSX and HRM approaches allow one to perform targeted proteomic studies without the restrictions associated with the conventional SRM approach. Established SRM methods can directly be transferred, while new assays would be easier to set up. SWATH-MS enjoys the advantages of targeted approach, such as high precision and quantitative accuracy, while having the strengths and flexibilities of shotgun proteomics. The technique could be used as a multiplexed label-free quantitative proteomic approach and it may well find applications in the metabolomics. A multiplexing DIA strategy, MSX, has also been introduced. The method aims to tickle the drawbacks in relation to the chromatographical nose and fragment-ion interferences in SAWATH Acquisition and similar DIA approaches. In MSX, the instrument is set to fragment and trap five separate 4-*m/z*-isolation windows before a MSX mass analysis. The strategy permits the sampling frequency of a DIA approach using 20-*m/z*-wide precursor isolation windows, while having the precursor selectivity of an approach using 4-*m/z*-wide isolation windows. The resulting extracted SRM chromatography has a quality approaching PRM. HRM approaches (MRM^{HR}/pSRM/PRM) are the targeted MS/MS analyses. These targeted methods provide the highest sensitivity and selectivity. At the time of writing, few reports on these emerging methods have been published. The applications and true impacts of this novel MS technique awaits to be revealed. It is foreseeable that they will continue to gain popularity and the methods such as MSX and PRM may be further benefited with the introduction of Orbitrap fusion instrument.

Five-year view

The rapid advances of systems biology, particularly proteomics, have driven by the methodological and technological advances of MS. MS instruments are becoming more and more powerful. The increased mass resolution has significantly enhanced the selectivity of the ions, while the increased scanning speed has allowed more peptides to be sequenced. The sensitivity of the systems has also increased considerably. Another noticeable direction of development includes the coupling or incorporation of ion mobility spectroscopy to the mass spectrometers that allows separation of ions in the gas-phase orthogonal to the separation of LC. The combination of these advances has already permitted researchers to identify and/or quantify proteins in complex biological systems several more times than what it used to be. The emerging methods of data acquisition and associated data minding or interrogation approaches are only possible with the improved performance of mass spectrometers. It can be foreseen that the number of publications employing these techniques in the next 5 year will not be just based on their merits, but also on the popularity and the cost of the platform associated with those techniques. The Orbitrap platform, for example, offers a great variety of approaches, from AIF, DIA, MSX, to pSRM/PRM. The success of these methods greatly relies on the ultra-high mass resolution and accuracy of the mass analyzer which resolves isobaric ions in multiplexed/chimeric spectra. Continual technological development of the platform will certainly increase the scanning speed of the mass analyzer. In contrast, the success of MS^E relies on efficient separation of the peptides and/or ions before mass analysis. Further improvement on chromatography and the next generation of travelling-wave ion mobility technology will greatly benefit the MS^E method. The introduction of SAWATH-MS has initiated interest in DIA/HRM approaches among proteomic community and the method has been adapted in a number of laboratories in a relatively short-time of introduction, although SWATH-MS may have the least technological advances among the all methods discussed herein. Another factor to determine the success or the failure of an individual technique is associated with the availability or the development of associated informatic tools. The most noticeable developments have been Skyline and Spectronaut software tools originally developed for conventional SRM. Their functionalities will undoubtedly be extended and newer HRM methods will emerge. For example, recently, the Skyline software tool has introduced an overlapping mode for SWATH-MS and other DIA approaches. In this method, two sets of precursor isolation windows, offset by 50% (or other amount defined by the user), are monitored during data acquisition. Such an approach is said to have increased precursor selectivity while maintaining the sampling frequency. Although the authors do not foresee that these new DIA/HRM techniques or strategies will result in a paradigm shift in MS-based proteomics in the next 5 years, there is no doubt that these methods

will become useful additional tools contributing toward biomedical discovery and the verification of biomarker candidates.

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Key issues

- Data dependent acquisition employed in shotgun proteomics and selected reaction monitoring (SRM) employed in targeted proteomics have a number of short-comings that limited researchers from examining complex biological systems in terms of depth and width.
- Emerging mass spectrometry approaches, known as data independent analysis and hyper reaction monitoring, riding on the advantages of high speed and high-resolution hybrid mass spectrometers (e.g., Q-ToF and Orbitrap), could address the short-comings of DDA and SRM.
- One such approach eliminates the ion selection stage and produces alternating scan of precursor ions and fragments of all the precursors. The resulting product ion spectra are highly convoluted, but an algorithm capable of correlating the products with the precursors based on the retention time, chromatographic peak shapes, charge state, etc., for the ions is available. Enhancements can be brought by the use of ultra-performance liquid chromatography and ion-mobility gas-phase separation.
- An alternative approach sequentially fragments all the ions in a relative wide isolation window, typically from 10–100 Th. The resulting product ion spectra are convoluted but can be searched against theoretical reference spectra or be mined with SRM-like targeted data extraction.
- There are also novel targeted methods that sequence the precursor ions in a relative narrow precursor isolation window in a way similar to SRM. However, these methods do not measure the SRM transitions but to record the whole product ion spectra. The spectra can either be searched against conventional database or extracted with SRM-like ion chromatograph.
- There is also a strategy, in which multiple but separated narrow precursor isolation windows are analyzed simultaneously. The multiplexed spectra are demultiplexed and extracted to SRM ion chromatographs. Such approach enjoys the sampling rate of the methods using wide isolation windows but has the selectivity of the methods using relative narrow precursor isolation windows.
- These new approaches are not faultless themselves and are not substitution to data dependent acquisition or SRM. However, they are useful additional tools or new opportunity for biomedical and bimolecular investigations and may produce an impact to the field in the next few years.

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