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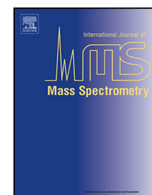

High resolution data-independent acquisition with electron transfer dissociation mass spectrometry: Multiplexed analysis of post-translationally modified proteins

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A B S T R A C T

Data-dependent acquisition (DDA) mode is the most commonly used method in bottom-up proteomics. Recently, data-independent acquisition (DIA) modes have become popular alternatives because of their unbiased analysis, leading in general to more comprehensive, global qualitative profiling of proteome systems and also higher quantitative reproducibility in such profiling. Most of the previously established DIA methods are based on collision-induced dissociation (CID). However, when it comes to the analysis of labile post-translational modifications (PTMs), electron capture/transfer dissociation (ECD/ETD) may be better suited. In addition to the bottom-up approach, the middle-down approach, which analyzes peptides in the range of 3,000–10,000 Da has emerged as an attractive alternative, including the analysis of highly modified and highly variable protein variants that exist in key system functions, such as histone signaling cascades. Here, we establish that a data-independent (DIA) middle-down ETD approach is a superior strategy in the differential characterization of PTM changes in histone H2B. We suggest that this strategy can further be used for other approaches where dynamic PTM characterization or changes due to different conditions are fundamental to accurate understanding of biological systems and function.
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1. Introduction

The method of choice for global proteomic analysis is the bottom-up approach in data-dependent acquisition (DDA) mode. Peptides and their post-translational modifications are analyzed after trypsin digestion using liquid chromatography–mass spectrometry, where the top N eluting precursor ions are subsequently analyzed by MS/MS. A principal disadvantage of any data-dependent analysis is the under-sampling that occurs, when only the top N peaks in a given MS spectrum are being analyzed [1]. This is mostly due to the irreproducible precursor ion selection [2] and has led to the development of data-independent acquisition (DIA) approaches [3–7]. Instead of generating MS/MS for every precursor, wide precursor windows (e.g. 10 m/z) or 'swaths' are co-isolated and fragmented together, generating spectra containing all fragment ions. The DIA approach also permits co-isolation, and thus multiplexed analysis, of variably modified protein forms concurrently isolated in the same mass window. Most of the previously established DIA methods are based on collision-induced dissociation (CID). However, when it comes to the analysis of labile post-translational modifications (PTMs), non-ergodic electron based dissociation techniques, such as electron capture dissociation (ECD) [8] and electron transfer dissociation (ETD) [9], may be better suited [10–24] due to a proposed fragmentation mechanism that is induced by random, free radical (electron)-driven bond destabilization, which can result in more comprehensive breakage across the peptide backbone versus preferential cleavage at energetically weaker PTM bonds in energetically driven CID fragmentation mechanisms.

In addition to the bottom-up approach, the middle-down approach, which analyzes peptides in the range of 3,000–10,000 Da, has emerged as an attractive alternative [25–35]. The basic advantage of this approach is that larger peptides retain more information

Abbreviations: DDA, data-dependent acquisition; DIA, data-independent acquisition; ECD, electron capture dissociation; ETD, electron transfer dissociation; LC, liquid chromatography; PTM, post-translational modifications.

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about protein isoforms and variants and their combinatorial PTMs [26,36–38]. It is, therefore, not surprising that middle-down approaches have successfully been used as an alternative strategy for several applications, including comprehensive histone analysis [25,28,29,36,39–42].

Here, we explore the feasibility of the DIA middle-down ETD strategy for the comprehensive analysis of post-translationally modified proteins on an Orbitrap Fusion Tribrid high resolution accurate mass spectrometer. The Orbitrap Fusion Tribrid is a newly designed tribrid quadrupole-orbitrap-linear ion trap mass spectrometer equipped with a novel Townsend discharge ETD ion source in the front, a quadrupole mass filter and an Ultra-High Field Orbitrap mass analyzer offering resolving power in excess of 400,000. Thus, this represents the first commercial mass spectrometer to offer the capability to execute DIA–ETD experiments with high resolution MS/MS necessary for the identification of larger peptides such as highly modified histone tails. It should be noted that Carvalho et al. [43] were the first to use middle-down ETD, albeit with low resolution MS/MS acquisition. Exemplary of highly modified proteins, we investigated the approach here using GluC-digested histone H2B fraction in the presence or absence of propionic acid. We focused on 10 *m*/*z* windows in the range between *m*/*z* 475 and *m*/*z* 540 in a similar fashion to that which was earlier described for data-independent proteomics experiments using CID [3–7]. We also employed a 'duplexed' strategy, using five 10 *m*/*z* precursor isolation windows, where the second set of precursor isolation windows was offset by 5 *m*/*z* versus the first to encourage preservation of signal intensity for all peaks across the target mass ranges, including those ions close to the edge of the DIA *m*/*z* window. Because the peptides in middle-down analysis are larger and highly charged, a resolving power of 60,000 was used for MS/MS analysis to enable the accurate determination of the charge states and masses of the product ions. The resulting high mass accuracy also increased confidence in PTM localization. In comparison to DIA–CID and DDA–ETD, we show that our DIA middle-down ETD approach is a superior strategy in the differential characterization of PTM changes in histone H2B. We suggest that this strategy can be used for other proteins where PTM characterization or changes in different conditions are important.

2. Materials and methods

2.1. Sample preparation

Histones from MEL cells treated with either 5 mM propionic acid or DMSO were acid extracted with 0.4 N H_2SO_4 and precipitated with 27% (v/v) trichloroacetic acid as previously described [44]. The histone pellet was dried, resuspended in water, and the protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.2. Intact histone separation by liquid chromatography and GluC digestion

An Agilent Zorbax 300 SB-C3 column (150 mm × 4.6 mm, 5 μm) was used to separate 350 μg of extracted histones. Eluent A was 5% acetonitrile and 0.05% (v/v) TFA in water and eluent B was composed of 5% water and 0.05% (v/v) TFA % (v/v) in acetonitrile. The gradient was 0–30% B in 3 min, 30–38% B in 30 min and 38–60% B in 8 min. The flow rate was 1 ml/min and it was split right before entering into the mass spectrometer. Approximately 85% of the flow was collected and 15% was directed to MSD for fraction identification. The histone 2B fractions were selected and digested with Glu-C (Thermo Scientific) at an enzyme to protein ratio of 1:10 in 100 mM NH_4HCO_3 (pH = 4.0) at 25 °C for 5 h. All reactions were quenched by freezing at −80 °C. Prior to mass spectrometry analyses, digested histone peptides were resuspended in 0.2% formic acid to a concentration of 100 ng/μl.

2.3. Nanoflow liquid chromatography–tandem mass spectrometry (nanoLC–MS/MS)

All experiments were performed on a nanoflow liquid chromatography system, EASY-nLC 1000, (Thermo Scientific) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA), equipped with an EASY spray source (Thermo Scientific).

For the DIA experiments, 0.3 μg (3 μL) of Glu-C-digested H2B peptides were directly loaded at maximum flow rate to a pressure of 800 bar (∼800 nl/min) onto an analytical HPLC column (Easy Spray Pepmap C18 column, 75 μm ID × 15 cm, 3 μm particle size). The column was operated at 35 °C. Peptides were separated with a 55 min gradient from 2% to 34% acetonitrile/0.2% formic acid at a flow rate of 350 nl/min.

The Orbitrap Fusion was operated in DIA mode. The quadrupole isolated the following *m*/*z* ranges for data-independent MS/MS using ETD: 475–485, 485–495, 495–505, 505–515, 515–525, 525–535, 480–490, 490–500, 500–510, 510–520, 520–530, 530–540. ETD reaction time was set to 30 ms, ETD reagent target was 2×10^5 ions and maximum ETD reagent injection time was 200 ms. All fragment ions were acquired in the Orbitrap with resolving power set to 60,000. Scan range was *m*/*z* 200–2000. The automatic gain control (AGC) target value for the Orbitrap was set to 2×10^5 ions and maximum ion injection time of 150 ms. One microscan was used. MS/MS spectra were acquired in centroid mode. Data acquisition was controlled by Xcalibur 3.0.2 software (Thermo Scientific).

2.4. Data analysis

Individual precursor windows were summed across the relevant retention time period where the H2B N-terminal peptides elute and then deconvoluted using Xtract within XCalibur Qual Browser. From the deconvoluted spectra, all potential combinations of H2B sequence and methyl equivalents (14.0096 Da) were deduced from the upper mass range corresponding to an 8+ charge state for the given precursor window. Similar to DiMaggio et al. [45] a mixed integer linear program was used to identify a minimal set of proteoforms that would explain all of the observed fragment ions in the precursor window for both the treated and untreated samples.

3. Results and discussion

3.1. Data-independent middle-down nano-LC-ETD MS/MS strategy

The principle strategy for our data-independent middle-down nano-LC-ETD MS/MS strategy is shown in Fig. 1. To test this strategy, treated and untreated histones were used as an example of heavily post-translationally modified proteins. After separation of the intact histones on a reversed phase HPLC column, the H2B fraction was used for further analysis. The fraction was digested with Glu-C and the resulting N-terminal peptides were analyzed using an nLC coupled online to an Orbitrap Fusion, operated in DIA mode with all ion ETD fragmentation. Instead of performing a DDA where the full *m*/*z* range from *m*/*z* 300 to 1700 is surveyed, we focused on five 10 *m*/*z* windows in the range of *m*/*z* 475–535 covering the +8 charge state of the histone H2B N-terminal tails. A second set of DIA windows offset by 5 *m*/*z* was also executed in the scan sequence, resulting in the range of *m*/*z* 480–540 to cover overlapping peaks. These ten precursor isolation windows were continuously analyzed during the entire chromatogram regardless of the precursor. Executing a resolving power setting of 60,000, this resulted in a duty

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