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Multiplexed data independent acquisition (MSX-DIA) applied by high resolution mass spectrometry improves quantification quality for the analysis of histone peptides

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Abstract

We present the mass spectrometry (MS) based application of the innovative, although scarcely exploited, multiplexed data-independent acquisition (MSX-DIA) for the analysis of histone post-translational modifications (PTMs). Histones are golden standard for complexity in MS based proteomics, due to their large number of combinatorial modifications, leading to isobaric peptides after proteolytic digestion. DIA has thus gained popularity for the purpose as it allows for MS/MS-based quantification without upfront assay development. In this work, we evaluated the performance of traditional DIA versus MSX-DIA in terms of MS/MS spectra quality, instrument scan rate and quantification precision using histones from HeLa cells. We used an MS/MS isolation window of 10 and 6 m/z for DIA and MSX-DIA, respectively. Four MS/MS scans were multiplexed for MSX-DIA. Despite MSX-DIA was programmed to perform 2-fold more MS/MS events than traditional DIA, it acquired on average ~5% more full MS scans, indicating even faster scan rate. Results highlighted an overall decrease of background ion signals using MSX-DIA, and we illustrated specific examples where peptides of different precursor masses were co-fragmented by DIA but not MSX-DIA. Taken together, MSX-DIA proved thus to be a more favorable method for histone analysis in data independent mode.

Introduction

Histones are crucial components of chromatin, as they contribute to its 3D structure in the cell nucleus. Chromatin structure directly affects fundamental biological events including regulation of gene expression, DNA repair and DNA hypercondensation into chromosomes during mitosis and meiosis [1]. Histones are assembled in octamers named nucleosomes, each of them composed by two copies of four canonical histone isotypes, which can be replaced by specific variants in different genomic regions for functions not always unraveled [2]. Nucleosomes are wrapped by DNA every ~200 base pairs and heavily modified by dynamic post-translational modifications (PTMs), which affect chromatin condensation due to their chemical properties and their capability in recruiting histone writers/readers/erasers [3]. These characteristics, combined with the potential of being epigenetically inherited

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through cell division [4], make histone PTMs a landmark in biology that justifies the immense scientific literature of about ~80,000 research articles (PubMed index) spanning over more than 100 years (the first indexed article is [5]). Moreover, several links between aberrations of histone PTM levels and development of diseases have been found in the last 15–20 years [6, 7], demonstrating the importance of histone PTMs in the homeostasis of cell phenotype.

Technologies have evolved to improve throughput and confidence in the analysis of histone PTMs. Mass spectrometry (MS) has become the method of choice for histone PTM characterization, especially if in large scale and combined with liquid chromatography (LC). This is also because it allows for identification and quantification of PTM combinatorial patterns using strategies named middle-down and top-down proteomics [8, 9], opposing to the more traditional peptide-centric bottom-up strategy. Nevertheless, bottom-up remains the most widely adopted strategy, as it is less technically challenging in terms of chromatographic separation of analytes, MS detection and bioinformatics analysis of spectra. Trypsin, the most common proteolytic enzyme in bottom-up proteomics, is not suitable per se for histone digestion, as they are highly enriched in basic amino acid residues, which are targets of trypsin for cleavage. Thus, histones are often derivatized at the lysine residue side chains with either acetic or propionic anhydride before and after proteolytic digestion with trypsin [10, 11]. Both protocols lead to (i) generation of peptides only cleaved after arginine residues, thus with suitable size for MS detection, and (ii) increased hydrophobicity also thanks to peptide N-termini derivatization, enhancing peptide LC retention which can be crucial for short hydrophilic ones.

MS based proteomics is a highly dynamic field of study, and several MS acquisition methods have been developed for peptide identification and quantification [12]. The most widely adopted acquisition method for histone analysis is still to combine data dependent acquisition (DDA) and targeted MS/MS scans of isobaric peptide masses within the same method [13]; in this way isobaric and co-eluting peptides can be differentially quantified by extracting the MS/MS chromatogram of the unique fragment ions belonging to each of the given species. This method, however, is ideal only if the retention time and the mass of the isobaric forms are known prior LC-MS analysis, as targeting several species through the entire run would drastically slow down the instrument duty cycle. Furthermore, such data become unusable for further data mining, in case the user aims to investigate previously unpredicted isobaric species. Data independent acquisition (DIA) is an effective alternative if just quantification and not identification is required, which is the case of typical histone analysis where the peptide list is known. DIA methods are programmed as cycles alternating a full MS scan followed by a series of sequential MS/MS scans spanning over the same m/z window as the full scan, each of them of 5–50 m/z . By using such windows, instead of generating an MS/MS of the entire mass range, the sensitivity of the analysis increases and the complexity of each individual MS/MS reduces, facilitating confident MS/MS extracted ion chromatography [14]. DIA proved to be highly effective for histone analysis [15–17], since histone peptides have numerous isobaric forms. Importantly, the MS/MS extracted ion chromatogram is used only to discriminate the relative abundance of isobaric and co-eluting peptides; the actual abundance of the peptides is achieved by extracting the precursor MS ion chromatogram in the same manner as DDA.

Based on an idea of Wilson and Vachet [18] Thermo Fisher Scientific^{TM} implemented in their Q-Exactive^{TM} and subsequent MS instrumentation an option that allows co-isolation of different precursor masses prior their simultaneous fragmentation, namely multiplexed MS (MSX). This approach turns advantageous to reduce duty cycle time in elaborate methods that target long lists of peptides using either single ion monitoring (SIM) or parallel reaction monitoring (PRM) [19]. This idea has been recently transferred to DIA as well [20], using an approach analogous to the Hadamard multiplexing [21]; i.e. at each scan cycle the instrument chooses randomly *N* MS/MS fragmentation windows for co-isolation followed by fragmentation. Each mixed MSX spectrum contains in the header the *m/z* windows selected for fragmentation, which can be used by software like Skyline [22] to demultiplex and perform MS/MS extracted ion chromatography. Despite the apparent advantage over traditional DIA only the publication of MacCoss and colleagues [20] described and exploited MSX-DIA for proteomics so far, suggesting that this acquisition method still has ample room for exploration.

In this work we applied MSX-DIA on an Orbitrap Fusion^{TM} Lumos^{TM} Tribrid^{TM} to investigate histones extracted from HeLa cells and prepared for bottom-up proteomics using our in-house developed protocol [10, 11]. MSX-DIA was compared with traditional DIA using the same instrument. Results highlighted that, upon accurate planning of fragmentation windows, MSX-DIA both decreases instrument duty cycle and reduces the complexity of extracted ion chromatograms, both aspects leading to more accurate and confident peptide quantification. Moreover, we compared the performance of traditional DIA in an Orbitrap Fusion^{TM} Lumos^{TM} Tribrid^{TM} with the Orbitrap Fusion^{TM} Tribrid^{TM}, confirming that the newer Lumos^{TM} is ~2-fold more sensitive overall (data not shown). In summary, we confirmed that such acquisition method should be preferred over traditional DIA, at least for proteomics samples of medium complexity (e.g. histone peptides).

Materials and Methods

HeLa cells histones extraction and digestion

HeLa S3 cells were grown in flasks using a suspension type of culture, as previously described [23]. Cells were harvested using a standard protocol of centrifugation and PBS washes [13]. Histone purification was performed according to standard protocols [13]. Briefly, nuclei were isolated by suspending cells into nuclei isolation buffer (15 mM Tris–HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 250 mM sucrose, 0.2% NP-40) including the following inhibitors: 1 mM DTT, 0.5 mM AEBSF, and 10 mM sodium butyrate. Nuclei were separated by centrifugation (1000 g for 10 min). 2 mL of cold 0.4 N H₂SO₄ was added on the nuclei pellet and sample was incubated at 4°C with shaking for 2 h. The nuclei were pelleted at 3400 g for 5 min. The supernatant was collected, trichloroacetic acid (TCA) was added at a final concentration of 33% TCA (w/v), and the sample was incubated overnight at 4°C. Afterwards, precipitated histones were pelleted by centrifugation (3400 g) for 10 min. Pellet was washed from leftover acid using ice cold acetone. Purified histones were processed with two cycles of chemical derivatization, trypsin digestion, and desalting as previously described [13]. Sample was dissolved in 30 µl of 50 mM NH₄HCO₃, pH 8.0. Derivatization reagent was prepared by mixing propionic anhydride

with acetonitrile in the ratio 1:3 (v/v) and added to the histone sample in the ratio of 1:2 (v/v) for 20 min at room temperature. The reaction was performed twice to obtain complete labeling. Histones were then digested with trypsin with an enzyme:sample ratio of 1:20 (w:w) overnight at room temperature in 50 mM NH_4HCO_3 , pH 8.0. After digestion, the derivatization reaction was performed again twice to derivatize peptide N-termini. Samples were then desalted by using C_{18} Stage-tips [24].

Nano liquid chromatography – tandem mass spectrometry (nLC-MS/MS)

Sample separation and introduction into the mass spectrometer was performed using a reversed phase high performance nLC with a two component mobile phase system: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A Thermo Scientific™ Easy nLC™ 1000 nano-uHPLC system and 75 μm x 50 cm Thermo Scientific™ PepMap™ Easy-Spray™ column were employed at a flow rate of 300 nL/min. Peptides were eluted using a gradient of 5% to 22% B over 105 minutes followed by 22% to 32% B over a subsequent 10 minutes. A second blank comprised of 5 μL injection of 10% isopropyl alcohol and 5% formic acid was injected between each replicate to regenerate the column. Using a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ and Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometers to combat the challenging complexity of histone modifications, a data independent acquisition method was constructed using small 10 m/z quadrupole isolation windows. A full scan at 120,000 FWHM resolving power (at 200 m/z) was performed followed by sequential HCD MS/MS scans at normalized collision energy of 30 and 15,000 FWHM resolution. The range between 400 and 900 m/z was surveyed by these experiments with maximum injection times of 50 ms and 45 ms for MS and MS/MS, respectively. AGC values were set to 4×10^5 for MS and 10^6 for MS/MS. MS/MS scan range was set to 200–2000 m/z . As a follow-up, a second method using MSX-DIA acquisition was performed on the Orbitrap Fusion™ Lumos™ Tribrid™. The method surveyed a range from 300 to 900 m/z using 6 m/z quadrupole isolation windows, multiplexing these windows together randomly at the rate of 4 per scan. Maximum injection times of 100 ms were defined for both MS and MS/MS scans. AGC values were set to 4×10^5 for MS and 10^6 for MS/MS. HCD fragmentation was performed using NCE of 30 and MS/MS scan range was set to 200–2000 m/z . All raw files are available at the Chorus database (<https://chorusproject.org/>, project ID: 1005).

Data analysis

The peptide list and consequent theoretical spectral library was manually generated using Skyline [22], considering mostly peptides commonly detected during a traditional histone analysis [16]. The selection of the chromatographic peaks was performed by Skyline [22] and manually assisted based on previous knowledge of peptide order of elution. High MS/MS resolution of 30,000 FWHM and precursor mass tolerance lower than 5 ppm were considered during peak integration, in order to discriminate false chromatographic peaks, including discrimination of nearly isobaric modifications like acetylation (42.011 Da) and trimethylation (42.047 Da). To assess quantification reproducibility we extracted the precursor peak areas of selected peptides and estimated the coefficient of variation between replicates (standard deviation / average).

Results

We used two Thermo Fisher Scientific™ instruments, namely Orbitrap Fusion™ Tribrid™ and Orbitrap Fusion™ Lumos™ Tribrid™, to test the performance of data independent acquisition (DIA) in quantifying hypermodified histone peptides. Since histone peptides might be present in several isobaric forms, we used both MS and MS/MS extracted ion chromatography for accurate quantification. Moreover, we applied multiplexing to DIA (MSX-DIA) to assess the improvements in spectra quality and instrument speed as compared to traditional DIA. We performed at least 3 runs with each of the three setups, achieving high reproducibility confirmed by the low coefficient of variation of the quantification of precursor ions, i.e. 11.9%, 20.5% and 13.5% for the Orbitrap Fusion™ Tribrid™, the Orbitrap Fusion™ Lumos™ Tribrid™ and this last instrument using MSX-DIA, respectively. All raw files are available at the Chorus database (Project No. 1005). Skyline [22] result files are available upon request (*for review, please download Skyline file from <https://www.dropbox.com/sh/rdyx0n67pkilzp8/AADH49KrMr7QfwSk2vN4pxWGa?dl=0>*).

Histone peptides were LC separated adopting an unusually shallow gradient for histone peptide analysis, programmed to increase the concentration of acetonitrile of 0.16% per minute over 105 minutes. The column adopted was a 75 µm x 50 cm Thermo Scientific™ PepMap™ Easy-Spray™, aiming to achieve the highest separation, resolution and reproducibility for our LC system. Separation was outstanding, as we could accurately resolve isobaric peptides such as acetylated forms of histone H3 peptides, including H3K9/K14ac and H3K18/K23ac, and forms of the histone H4 peptide GKGKGLGKGGAKR (aa 4–17) which contains four lysine residues that can be acetylated (Fig. 1A). Peptide chromatographic peaks had an average base width of about 40 seconds, implying that an instrument duty cycle of ~4 seconds or less would be sufficient to have enough data point for accurate definition of its peak area. Both instruments, the Orbitrap Fusion™ Tribrid™ and Orbitrap Fusion™ Lumos™ Tribrid™, have the same scan frequency in DIA; specifically, in a combined run alternating one full MS scan (resolution 120,000) followed by 50 MS/MS scans (resolution 15,000) they achieved a scan rate of about 3.6–3.7 seconds per cycle. The Orbitrap Fusion™ Lumos™ Tribrid™ was used in MSX-DIA mode alternating one full MS scan followed by 100 MS/MS scans multiplexed four by four using the same resolutions, achieving a scan rate of about 3.4–3.5 seconds per cycle. Results were thus confirming that the instrument speed was compatible with chromatographic performance.

First, we manually assessed the quality of few MS/MS acquired using MSX-DIA as compared to traditional DIA, focusing on the most abundant species. As illustrated in Figure 1B both methods provided highly similar spectra. This indicated that multiplexing m/z fragmentation windows does not generate drawbacks on any crucial feature of an MS/MS spectrum, including fragment relative abundance, signal resolution (highlighted by accurate isotopic definition of the fragments) and general background noise. This was not obvious as, even though we show later in the manuscript that MS/MS extracted ion chromatography improves upon use of MSX-DIA (Fig. 4), individual MS/MS spectra should be naturally noisier by multiplexing.

Extracted ion chromatography was performed by using Skyline [22]. In order to achieve the highest automation in chromatographic peak picking the software was instructed to select the signals according to the smallest mass error, the ones displaying the same elution profile for precursor and product ions and between the three main isotopes of the precursor mass. As displayed in Figure 2, high mass accuracy and accurate Gaussian elution profile was achieved also for peptides normally known as low abundance ones [11], and assisted accurate selection of the proper LC-MS signal. The peptide H3K9me3S10ph was co-isolated for fragmentation using traditional DIA with the species H3K9me2S10ph (Fig. 2A); their mass difference is one methyl group (14.01 Da), corresponding for 7 m/z units in case of doubly charged species. The use of MSX-DIA with an m/z fragmentation window of 6 m/z avoided the generation of the two MS/MS chromatograms while extracting the signal of H3K9me3S10ph (data not shown). The importance of the high mass accuracy was crucial in presence of deamidated species; the extracted ion chromatogram of the m/z of the histone H3 peptide EIAQDFKTDLR (aa 73–83) generated three peaks using the full MS signal (Fig. 2B). However, the use of mass accuracy together with the MS/MS extracted ion chromatogram assured which the correct signal was.

The presence of sequence variants includes a further layer of complexity in histone peptide analysis. The histone H3 peptide aa 27–40 is commonly present in mammalian samples in two variants, one representative of histone H3.2/H3.1 and one for H3.3. The mass difference (15.999 Da, one oxygen atom) is due to a switch between alanine (A) and serine (S) in position 31, which could be confused with a difference in degree of methylation plus inaccurate monoisotopic peak recognition (Fig. 3). For this reason, high mass accuracy and MS/MS extracted ion chromatogram play an essential role in defining the correct LC peak to integrate. Due to such minimal mass differences even MSX-DIA failed in avoiding peptide co-isolation for MS/MS. We thus display a typical example on how to discriminate the signals of the peptides (Fig. 3).

Next, we evaluated on selected candidates the improvements offered by MSX-DIA over DIA regarding noise reduction of MS/MS extracted ion chromatograms. Results highlighted that the use of smaller m/z fragmentation windows reduced, and in some cases completely eliminated, artifacts from selected MS/MS chromatograms (Fig. 4). The extracted ion chromatogram of the peptide carrying H3K36me2 (aa 27–40) showed two equally intense peaks generated each by two MS/MS ions (Fig. 4A). By using MSX-DIA one of the two signals completely disappeared, solving any doubt on which is the correct peak profile to integrate. An analogous example was the unmodified H3.3 peptide, where the noise was even higher in intensity than the correct one (Fig. 4B). The integration of the peptide containing H3K27me2 showed a different, but not rare, example of issues generated by MS/MS noise (Fig. 4C); the presence of a contamination which does not baseline resolve from the major signal leads to inaccurate peak area quantification, since software cannot easily define the borders of the two different peak profiles. In this specific example the artifact was depleted by using MSX-DIA, which isolated the correct peptide signal for MS/MS fragmentation from the contamination, i.e. H3K27me3. Together, the use of MSX-DIA proved to successfully reduce noise of selected MS/MS extracted ion chromatogram, leading to more confident and accurate peptide quantification.

Finally, we performed a quality control analysis of all our runs, including all replicates from each of the three experiments, in order to assess that our observations in representative peptides are reproducible overall. First, we verified that the retention time drift between replicates of the same experiment were constantly below 1.1 minute, proving that our nanoLC provided highly reproducible peptide elution (Fig. 5A). Next, we calculated the precursor mass errors of all 122 peptides in all replicates of the three experiments. Results showed that mass error was centered around 0 ppm, and never fell beyond ± 6 ppm window (Fig. 5B). This aspect highlighted that our analysis had high mass precision due to the narrow mass error, and high mass accuracy due to the mass error centered around 0 ppm. This increased overall the confidence that the correct signals were selected. Moreover, we calculated the variation of the precursor area integration for each peptide of each experiment between performed replicates to verify the reproducibility of the quantification within experiments. The observed variation, mostly below 20% (Fig. 5C), is considered as highly reproducible, as in line with SRM and SWATH^{TM} experiments [25].

In MSX-DIA we expected to observe a less complex MS/MS extracted ion chromatogram for our peptide list as compared to traditional DIA, due to the shuffling nature of the acquisition method. This is because interfering ions generated by peptides with different masses co-isolated during MS/MS are less likely to provide a peak profile. This should result in an overall reduced total MS/MS area of the extracted ion chromatogram, as interfering ions should be overall less present. We indeed observed that the total areas of the MS/MS extracted ion chromatograms were lower in MSX runs as compared to traditional DIA in both Fusion^{TM} and Fusion^{TM} Lumos^{TM}, considering the area of the precursor mass as normalization factor (Fig. 5D). This suggests that the MS/MS extracted ion chromatogram in MSX-DIA was globally “cleaner” than traditional DIA. This phenomenon can be visualized for instance in Figure 4C; by using MSX-DIA we avoid co-fragmentation of species which would inevitably produce fragments integrated together with the main peptide signal by Skyline [22], or any other software. However, this dramatic reduction in the ratio between fragment and precursor extracted ion chromatogram from DIA to MSX-DIA includes as well a general lower abundance of the fragments of MSX acquisition (data not shown). This is easily explained due to the nature of the fragmentation window, which for MS/MS events of MSX acquisition are indeed more complex (4 mixed windows of 6 m/z each) than traditional DIA (1 window of 10 m/z). Notably, this affected also the reproducibility of the extracted MS/MS ion chromatograms, as the peak areas of the fragment ions were on average more variable in MSX-DIA than traditional DIA (Fig. 5E). However, even though this is relevant to highlight, this effect should not be considered as major drawback of MSX-DIA since (i) this effect is pronounced mostly for b-type fragments, which are commonly not used in histone analysis due to their low intensity, which is a consequence of N-terminal peptide propionylation [26]; (ii) the calculated coefficient of variation in Figure 5E inevitably considers also extracted chromatograms of interfering ions, as they are not always possible to discriminate from correct fragments. In summary, this section highlights the high quality and reproducibility of our analysis, and how MSX-DIA facilitates MS/MS extracted ion chromatograms with lower complexity.

Discussion

We here presented the use of MSX-DIA for the analysis of hypermodified histone peptides. For the purpose, we used state-of-the-art instrumentation such as ultra high pressure LC (~1000 bar limit) and the Orbitrap Fusion™ Lumos™ Tribrid™ for MS detection. High performing chromatography is undoubtedly an essential aspect for result quality. In this work we pushed the limit of LC by using a long (50 cm) nano column and a very shallow gradient for peptide elution. With this, we achieved separation even for a number of isobaric peptides (Fig. 1A), which are commonly very difficult to separate due to their nearly identical chemical properties. Although these long runs are not convenient for large scale analyses of large numbers of samples, we made this choice to demonstrate that isobaric species can be chromatographically resolved, simplifying data interpretation post LC-MS run.

Scientific publications where DIA was adopted as acquisition method have risen in the last three years, demonstrating the convenience of producing MS data that can be fully re-mined, and where quantification confidence is enhanced by the possibility of extracting both MS and MS/MS ion chromatograms. It is clear that the introduction of SWATH™-MS by ABSciex™ paved the way to the so-called “*in silico* selected reaction monitoring (SRM)”. However, such an approach is a constant compromise between the width of fragmentation window, i.e. if larger more noisy MS/MS spectra are generated, and instrument duty cycle, i.e. the more MS/MS events the slower the cycle becomes. Multiplexing data independent MS/MS scans was introduced by Thermo Fisher Scientific™ as promising solution, but after the proof of concept [20] no scientific articles highlighted the advantages of this method. Here, we show that MSX-DIA provides MS/MS spectra with comparable signal-to-noise levels (Fig. 1B) and assists confident quantification if smaller MS/MS fragmentation windows are planned (Fig. 4). The use of smaller fragmentation windows does not become a duty cycle issue, as by multiplexing MS/MS events the instrument saves scan time becoming comparable to traditional DIA. Moreover, label-free quantification is prone to inaccurate quantification due to imprecise LC peak definition. In a recent publication, Coon and co-workers introduced metabolic labeling using neutron-encoded (NeuCode) SILAC to DIA (NeuCoDIA), aiming to reduce technical variability allowing the mixing of multiple conditions in the same MS run and DIA based quantification [27]. In our work we showed how MSX-DIA can improve the accuracy of the quantification in label-free experiments as well; the presence of a “shoulder” in the extracted ion chromatogram in Figure 4C is due to co-fragmentation of undesired peptides. During peak area integration any software, even if manually assisted, would struggle in defining the real shape of the peak of interest, ending up integrating part of the erroneous signal. In this specific case MSX-DIA solved the issue by avoiding co-fragmentation of the undesired peptide that shared some fragment ion masses.

High resolution is also recommended for confident peak picking, although we previously demonstrated that it is not mandatory for both DDA [28] and DIA [17]. We did not test MSX-DIA using low resolution mass analyzer, e.g. ion trap, as the instrument duty cycle was sufficiently fast for our purpose. Moreover, there is no current need to prove its

applicability with low resolution mass analyzers like standalone ion traps, as this software feature is not available on this type of instrumentation.

Interestingly, MSX-DIA is still very scarcely exploited in the scientific community. The major reason is that this method is currently available only on Thermo Fisher Scientific™ instrumentation, and only for Q-Exactive™ or more recent MS. However, it is also important to consider that DIA is a relatively novel acquisition method per se, and thus innovations are received with more caution, e.g. some laboratories might have this method just established. Moreover, some resistance might be due to the fact that, being a novel acquisition method, software might not be able to deal with this kind of raw files yet. To our knowledge, any software that processes DIA data is equally suitable for MSX-DIA.

Together with DIA, in recent years also middle-down and top-down proteomics proved to be suitable for medium/large scale analyses [29, 30], especially for histone analysis due to the possibility to characterize coexisting PTMs. It is thus reasonable to speculate that DIA might be applicable for these proteomics strategies as well since, for instance, quantification in middle-down is mostly performed using the intensity of the fragment ions [31, 32]. However, it is harder to imagine an applicability of MSX-DIA for the purpose, as the limitations of these strategies are not instrument duty cycle, but the complexity of spectra analysis. Decreasing the fragmentation windows by multiplexing spectra has no apparent advantage to simplify extracted MS/MS ion chromatograms in middle-down and top-down analyses, as the biggest challenge of these strategies is discriminating species that are isobaric. We expect that further studies will be performed on these aspects. Currently, we are confident that MSX-DIA will increase popularity in bottom-up type of studies, as the advantages we described do not seem to be juxtaposed by evident drawbacks.

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Significance of the study

DIA has rapidly gained popularity in proteomics studies, as it combines the accuracy of quantification of targeted methods and the possibility of data re-mining typical of unbiased acquisitions. Alongside the success of a new technology it is required to apply efforts aiming to improve the technology itself. In this work we show a modification of the traditional DIA method; by performing MS/MS fragmentation of co-isolated multiple fragmentation windows it is possible to save precious instrument scan time, which can be exploited by reducing the size of these windows and increase quantification quality. These results will therefore challenge the most widely adopted DIA, as the proposed acquisition method demonstrates that reducing noise in MS/MS scans has no harm in instrument duty cycle.

Figure 1. Examples of high performance chromatographic separation and MS/MS spectra quality

(A) Three examples of MS extracted ion chromatograms of isobaric peptides. Baseline separation was observed for histone peptides with the same molecular mass, including (i) KSTGGKAPR (histone H3, aa 9–17) with 1 acetyl group, (ii) KQLATKAAR (histone H3, aa 18–26) with 1 acetyl group and (iii) GKGGKGLGKGGAKR (histone H4, aa 4–17) with 3, 2 and 1 acetyl groups. This last peptide has four lysine residues that can be all modified with acetylation. Only some of the forms could be nLC separated. (B) Zoom of an MS/MS spectrum of four multiplexed m/z windows including the mass of the peptide H3K23ac, achieved using the MSX-DIA method. The fragment ions of the peptide could be confidently annotated despite multiple isolation windows were co-selected for fragmentation. Below, the corresponding MS/MS spectrum of the m/z window including the mass of the peptide H3K23ac acquired with traditional DIA.

Figure 2. MS and MS/MS extracted ion chromatograms of low abundance peptides in traditional DIA

(A) On the top, MS extracted ion chromatogram of the peptide H3K9me3S10ph. The three colored lines correspond to the ion chromatogram of the monoisotopic peptide mass and the first two isotopes. Below, MS/MS extracted ion chromatogram of the same peptide. The peptide H3K9me2S10ph was co-isolated and co-fragmented, evidenced by the fact that only the y fragment series has the same mass as H3K9me3S10ph. This is confirmed by the MS chromatogram, as the extracted precursor mass did not generate a profile. (B) MS and MS/MS extracted ion chromatogram of the peptide H3K79me1. At the precursor mass level the lower intense signals correspond to isotopes of the deamidated form. The MS/MS extracted ion chromatogram presents some signals corresponding to the unmodified form of the peptide, since some fragment ions share the same mass.

Figure 3. Deconvolution of signals for the extracted ion chromatogram of the peptide H3.3K27me1

At the MS level (top) three major peaks are present upon extracting the ion mass of the histone H3.3 peptide KSAPSTGGVKKPHR (aa 27–40) modified with a methyl group in K27. This peptide is unique for the histone variant H3.3, due to the presence of a serine (S) instead of an alanine (A) in position 31. The MS chromatogram reveals one peak (on the right) with a larger mass error than expected (12 ppm), actually corresponding to the third isotope of the peptide K27me1K36me1 of canonical histone H3 (MS spectrum in the middle panel). The peptide sequences of the two histone H3 variants have a mass difference of ~16 Da (due to A/S switch), approximately corresponding to the same delta mass of a methyl group (14.01 Da) plus two isotopes ($2 \times {}^{13}\text{C} = 2 \text{ Da}$). The correct signal is the peak in the middle, as the one on the left is the peptide H3.3K36me1, evidenced by the lack of signals when extracting the MS/MS ion chromatogram of the species H3.3K27me1.

Figure 4. MS/MS noise reduction using MSX-DIA as compared to traditional DIA
 (A) MS/MS extracted ion chromatogram of the peptides KSAPATGGVKKPHR (histone H3, aa 27–40) dimethylated in K36, (B) KSAPSTGGVKKPHR (histone H3.3, aa 27–40) with no modifications, and (C) KSAPATGGVKKPHR (histone H3, aa 27–40) dimethylated in K27. All three show nearly identical chromatograms in runs performed using traditional DIA with the Orbitrap Fusion^{TM} Tribrid^{TM} and the Orbitrap Fusion^{TM} Lumos^{TM} Tribrid^{TM}, but with cleaner chromatograms when performing MSX-DIA with the Orbitrap Fusion^{TM} Lumos^{TM} Tribrid^{TM}. The y-axes evidence that the signal of the Orbitrap Fusion^{TM} Tribrid^{TM} is about half intense as compared to the Orbitrap Fusion^{TM} Lumos^{TM} Tribrid^{TM}.

Figure 5. Global quality control of the performed experiment and comparison of variability of DIA vs MSX-DIA

(A) Variance of retention time between replicates of the same experiment. As reference the average retention time of all replicates was used. The box plot includes all variances of all peptides from all three experiments. (B) Distribution of the precursor mass error (in ppm) of all peptides from all replicates of all experiments. (C) Coefficient of variation of the precursor MS extracted ion chromatogram between replicates of the same experiment. (D) Average ratio between total MS/MS extracted ion chromatography and total MS extracted ion chromatography for each of the three experiments. Error bars represent standard deviation between replicates. (E) Coefficient of variation of MS/MS extracted ion chromatograms for the experiments performed in the Fusion^{TM} Lumos^{TM} divided for each individual ion type.