Evaluation of different peptide fragmentation types and mass analyzers in data-dependent methods using an Orbitrap Fusion Lumos Tribrid mass spectrometer

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ABSTRACT

One of the major additions in mass spectrometry technology has been the irruption of the Orbitrap mass analyzer, which has boosted the proteomics analyses of biological complex samples since its introduction. Here, we took advantage of the capabilities of the new Orbitrap Fusion Lumos Tribrid mass spectrometer to assess the performance of different data-dependent acquisition methods for the identification and quantitation of peptides and phosphopeptides in single-shot analysis of human whole cell lysates. Our study explored the capabilities of tri-hybrid mass spectrometers for (phospho-)peptide identification and quantitation using different gradient lengths, sample amounts, and combinations of different peptide fragmentation types and mass analysers. Moreover, the acquisition of the same complex sample with different acquisition methods resulted in the generation of a dataset to be used as a reference for further analyses, and a starting point for future optimizations in particular applications.

Keywords

Protein quantitation, protein identification, phosphorylation, mass spectrometry

Statement of significance of the study

In this work we used a new generation tri-hybrid mass spectrometer to systematically compare the performance of different combinations of peptide fragmentation and mass analyzers acquisition methods for single-shot analysis of the proteome and phosphoproteome of human whole cell lysates.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict of interest statement

The authors have declared no conflict of interest.

INTRODUCTION

Mass spectrometry has become a robust technology for the identification and quantitation of a myriad of proteomes [1, 2]. In most high-throughput proteomic experiments, proteins are digested by site-specific proteases, and the resulting peptide mixes are separated by liquid chromatography and analysed by mass spectrometry [3]. Peptide ions eluting from the chromatographic column are often detected in a survey scan (MS1) and most intense peptide precursor ions are then further selected for peptide fragmentation and mass analysis (MS2). The comparison of the acquired MS2 spectra to theoretical fragmentation spectra or to spectral libraries retrieves the corresponding identification of peptide sequences, and protein inference algorithms assign them to protein sequences [4]. This bottom-up acquisition strategy, known as shotgun data-dependent proteomics, has become the method of choice in biological studies that demand deep proteome coverage [5, 6]. However, the complexity of proteomes due to the large dynamic range of protein abundances, the existing multiple protein variants, and the presence of a vast number of dynamic post-translational modifications, have made the analysis of proteomes a challenge for the current proteomics technology [7].

In the last decade, the field of mass spectrometry-based proteomics has benefited from the advances in instrument sensitivity, mass resolution and scanning speed, which have facilitated the analysis of complete proteomes with minimal sample fractionation and reduced chromatographic separation times [8, 9, 10, 11, 12, 13]. A recent major addition in mass spectrometry technology has been the introduction of a new type of tri-hybrid mass spectrometers that combine a quadrupole mass filter with a high-field Orbitrap mass analyser and a dual cell linear ion trap [14], which resulted in exceptional capabilities in peptide and protein identification [15]. These new mass

spectrometers enable several peptide fragmentation techniques including CID, HCD, ETD and their combinations, as well as the possibility of routing ions to the different types of mass analysers depending on the desired acquisition application. Although, previous works have explored the use of different peptide fragmentations [15] or acquisition methods [16] in the first generation of tri-hybrid mass spectrometers, here we took advantage of the capabilities of the new Orbitrap Fusion Lumos Tribrid mass spectrometer to perform an extensive evaluation of combining different peptide fragmentations and mass analysers in the identification and MS1 label-free quantitation of peptides and phosphopeptides using data-dependent methods.

MATERIALS AND METHODS

Sample preparation

HeLa cells were purchased at the America Type Culture Collection (<http://www.atcc.org>). Cells were cultured in a 100-mm culture plate at 37°C in a 5% CO₂ waterlogged atmosphere using Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Invitrogen), supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Confluent cells were harvested by scraping with 600 µl of 6 M Urea and 100 mM ammonium bicarbonate. Cell lysates were centrifuged (4°C, 30 min, 13000 g), the supernatant was collected and its protein content was determined using the BCA Protein Quantification Kit (Thermo Fisher Scientific).

HeLa protein extracts were sequentially digested in-solution with endoproteinase Lys-C and trypsin. Briefly, samples were initially reduced with dithiothreitol (DTT, 10 mM, 37 °C, 60 min), alkylated with iodoacetamide (IAM, 20 mM, 25 °C, 30 min), and they were diluted to 2 M urea for overnight digestion with Lys-C at 37 °C. Samples were then diluted 2-fold again and digested overnight with trypsin at 37 °C. Tryptic peptides were desalted using a C18 column, evaporated to dryness.

250 µg of dried HeLa protein digest were dissolved with 10 µl of 3 % ACN, 0.1 % TFA), and further diluted with 90 µl of TiO₂ loading buffer (80% ACN, 6%TFA). Phosphopeptides were enriched with TiO₂-beads as previously described [17]. Briefly 10 mg of "*Titansphere TiO₂ 5 µm*" (GL Sciences Inc. Japan) were washed with 1 ml of 80% ACN, 0.1% TFA, and resuspended in 1 ml of loading buffer. 50 µl of the slurry were transferred and immobilized in a pipette-column and washed with 50 µl of loading buffer. The sample was loaded in the pipette-column and it was washed with

50 μ L of washing buffer (0.1 % TFA, 80 % ACN). Phosphopeptides were eluted from the beads with 25 μ L of 25 % ACN (v/v) containing 25 % NH_4OH (m/v), acidified with 10 μ L of 10 % TFA and vacuum-concentrated to dryness. Samples were finally diluted to 10 μ l with H_2O + 0.1% formic acid prior and 4 μ l were loaded into the chromatographic column for mass spectrometric analysis.

Mass spectrometry acquisition

The mass spectrometer was operated in positive ionization mode with an EASY-Spray nanosource with spray voltage set at 1.4 kV and source temperature at 275 °C. The EASY-IC mode was used to perform internal calibration (lock-mass) using the fluoranthene radical cation signal at m/z 202.0777 [18]. The instrument was operated in data-dependent acquisition mode, with full MS scans over a mass range of m/z 350–1,500 with detection in the Orbitrap (120K resolution) and with auto gain control (AGC) set to 100,000. In each cycle of data-dependent acquisition analysis, following each survey scan, the most intense ions above a threshold ion count of 30,000 were selected for fragmentation at normalized collision energy of 28% (HCD) or 30% (CID). The number of selected precursor ions for fragmentation was determined by the “*Top Speed*” acquisition algorithm and a dynamic exclusion of 60 seconds. Fragment ion spectra were acquired in the linear ion trap (IT) or the Orbitrap (OT, 30K resolution) depending on the method, with an AGC of 4,000 and a maximum injection time of 300 ms for ion trap MS2 detection, and an AGC of 30,000 and a maximum injection time of 80 ms for Orbitrap MS2 detection. All data were acquired with Xcalibur software v3.0.63.

Different amounts of peptide mixtures derived from digested HeLa cell extract were injected into a nano-UPLC system (EASY-nanoLC 1000 liquid chromatograph) equipped with a 50-cm C18 column (EASY-Spray; 75- μ m ID, PepMap RSLC C18, 2-

µm particles, 45 °C) and hyphenated to an Orbitrap Fusion Lumos Tribrid mass spectrometer. Different chromatographic gradient lengths from 60 to 240 minutes were tested for peptide separation. All gradient started at 5% ACN (ACN:H₂O (5:95), 0.1% FA) and went up to 32% ACN (ACN:H₂O (32:68), 0.1% FA).

Data analysis

Proteome Discoverer software suite (v2.0, Thermo Fisher Scientific) and the Mascot search engine (v2.4, Matrix Science) were used for peptide identification and quantitation. The data were searched against the Swiss-Prot human database (version Nov 2015). At the MS1 level, a precursor ion mass tolerance of 7 ppm was used, and up to three missed cleavages were allowed. The fragment ion mass tolerance was set to 20 mmu for the Orbitrap MS2 detection methods and to 0.5 Da for the linear ion trap MS2 detection methods. Oxidation of methionine, and N-terminal protein acetylation were defined as variable modifications whereas carbamidomethylation on cysteines was set as a fixed modification. Phosphorylation in serine, threonine and tyrosine was also set as variable modification in the analysis of the phospho-enriched samples. In all cases, false discovery rate (FDR) in peptide identification was limited to a maximum of 0.01 by using a decoy database and the Percolator algorithm [19]. Phosphorylation site localization was calculated using the phosphoRS algorithm [20]. Quantitation data were retrieved from the “Precursor ion area detector” node from Proteome Discoverer (v2.0) using 2 ppm mass tolerance for the peptide extracted ion current (XIC).

The raw proteomics data have been deposited to the PRIDE repository with the dataset identifier PXD004940.

RESULTS AND DISCUSSION

Chromatographic gradients and cell lysate amounts

Initially, we evaluated the performance of the LC-MS system on single-shot analyses of 1 μ g of digested human cell lysates using a robust data-dependent acquisition method in different chromatographic gradients (60, 90, 120 and 240 min) with HCD peptide fragmentation and MS2 detection in the linear ion trap (Table 1 and Supplementary Table ST1). Samples analysed with this acquisition method rendered a minimum of 4,761 protein groups and 32,861 distinct peptides (FDR < 1%) when merging three single-shot technical replicates with 1 μ g of HeLa digest loaded on column, and a maximum of 5,979 protein groups and 50,476 distinct peptides with the longest gradient (Figure 1A-C, Table 2). To balance the sample analysis throughput and the number of identifications, we evaluated the gain in the number of identified protein groups, peptides and peptide-spectrum matches (PSM) with respect to the increase in time considering the 60-min gradient as baseline (Figure 1D). This analysis showed that an increase of either 30 or 60 minutes (90-min and 120-min gradients) resulted in a similar proportional increase of identifications, whereas increasing the analysis time to longer gradients (240-min) was much less efficient in terms of number of proteins and peptides identified per additional time. The 90 and 120-min gradients were the methods that best balanced the number of identified peptides with the instrument time allocated per sample in the analysis of complex proteomes, and therefore, the 90-min gradient for further experiments.

Next, we evaluated the effect that decreasing amounts of human cell digests had on the performance of the LC-MS system in terms of PSMs, peptide and protein identifications. Thus, we analysed different amounts of digested cell extract ranging from 4 μ g to 100 ng on column in a 90-min chromatographic gradients with HCD

peptide fragmentation and MS2 detection in the linear ion trap (Table 1 and Supplementary Table ST2). Our analyses resulted on average in ~38,500 PSMs per replicate, in ~27,500 identified peptides, and ~4,700 protein groups with 2 µg of HeLa digest, with no improvement over 2 µg of digest (Figure 2 and Table 2). However, the reduction of cell extract amounts did not result in drastic losses of identifications and, with the exception of 100 ng, the number of identified peptides and protein groups was only smoothly decreasing with over 30,000 PSM, 23,000 identified peptides and 4,100 protein groups with only 250 ng of HeLa digests on column. Within the range of 2 µg to 250 ng of HeLa digest the MS2 spectra assignment rate was around 40% and an average of around 5-6 peptides per protein were identified (Supplementary Figure S1).

Combination of fragmentation techniques and mass analysers

The ion routing capabilities and flexibility on peptide fragmentation techniques of tri-hybrid mass spectrometers often raise the question of which peptide fragmentation and mass analyser to use for fragment detection that balance analytical speed and spectra quality and thus, maximise peptide identification while maintaining good MS1 quantitation properties. A fair comparison of all combinations might be difficult due to the multiple variables involved that by definition cannot be equal in the compared methods. Similarly, given a set of parameters there exists always the possibility to further optimize them for a particular application and for a given sample type. Being aware of these myriad of possibilities, we set up to explore the performance of the different methods combining HCD or CID peptide fragmentation with fragment ion detection in the Orbitrap (FT) or the ion trap (IT) mass analyser using 1 µg of HeLa digested proteome in a 90-min gradient (Table 1, Supplementary Figures S2-5, and Supplementary Table ST3). The method parameters were chosen to balance the

number of identifications with the generation of high-quality data for MS1 label-free peptide quantitation, and the results obtained are meant to be a reference dataset that serves as starting point for further applications. Note that the parameters used in the IT-based MS2 acquisition methods conform what has been called the "Universal Method", which allows using the same instrument settings regardless of the sample amount to achieve excellent instrument performance [21].

With the method parameters assessed in this study, we showed a higher performance of ion trap-based MS2 methods in terms of the number of protein groups and peptide identifications compared to the methods that use the Orbitrap mass analyser for fragment ion detection (Figure 3, Table 2, and Supplementary Table ST4). The observed differences in performance between ion trap and Orbitrap-based MS2 methods were larger when combined with HCD peptide fragmentation, and among all tested combinations, the method with HCD peptide fragmentation and ion trap-based MS2 acquisition (HCD-IT) was the method rendering the highest number of protein groups, peptides and PSMs. Noteworthy, all evaluated acquisition methods were highly reproducible within the acquired technical replicates both in terms of protein and peptide identification, and MS1-based peptide quantitation. For instance, from the identified 5,291 protein groups merging all three replicates (90 min, MS2 HCD-IT), 3,807 protein groups were consistently identified in every technical replicate and over 90 % of the quantified distinct peptides exhibited a coefficient of variation of peptide MS1 log2-areas below 4 % (three technical replicates). Similar values of coefficient of variation were obtained for the other methods under consideration (Figure 4).

Instrument performance on phospho-enriched human samples

The performance of the new tri-hybrid mass analyser with the different combinations of peptide fragmentations and mass analysers was further assessed with single-shot

analyses of phospho-enriched human samples using a data-dependent acquisition method. We injected 25% of a TiO₂-phosphoenriched HeLa sample (250 µg of starting material) in a 90-min gradient combining HCD or CID-MSA peptide fragmentation with fragment ion detection in the Orbitrap (FT) or the ion trap (IT) mass analyser (Table 1, Supplementary Figures S6-S9, and Supplementary Table ST3).

The phosphoproteome analysis from three technical replicates provided an average identification of *ca.* 5,300 distinct phosphopeptides per replicate with a 90-min gradient (MS2 HCD-IT), with more than 95% of the peptide log₂-areas exhibiting a coefficient of variation below 15% (Figure 5, Table 2, and Supplementary Table ST5). Moreover, we observed that the linear ion trap MS2 detection methods rendered higher number of identified phosphorylated peptides compared to Orbitrap-based MS2 methods. Similarly to what we had observed in the proteome analyses, here the HCD peptide fragmentation methods also outperformed CID-MSA fragmentation methods in terms of number of phosphopeptide identifications. Over ninety per cent of the identified phosphorylated peptides were mono-phosphorylated regardless the acquisition method used (Figure 6), and among them, most of the phosphorylation sites were identified on serine residues (Figure 7A). The Orbitrap-based MS2 detection method resulted in the highest percentages of confidently assigned phosphorylated sites, probably due to the high accuracy in the MS2 measurements, with around 90% of the identified phosphosites exhibiting a phosphoRS score >90%. Detection of phosphopeptide fragments in the linear ion trap resulted in lower percentages of high-confident phosphorylation sites, but at the same time rendered a higher absolute number of identified phosphopeptides with high-confident site localization (Figure 7B).

CONCLUDING REMARKS

In this study we used the fragmentation and ion routing capabilities of the new tri-hybrid mass spectrometers to evaluate the performance of different data-dependent acquisition methods in the identification and MS1 label-free quantitation of peptides and phosphopeptides in complex biological samples. Several gradients, sample quantities, peptide fragmentation methods and mass analysers were tested. Our results show that among the assessed methods, the HCD peptide fragmentation and ion trap-based MS2 acquisition (HCD-IT) was the acquisition method rendering the highest number of identified (phospho-)peptides in both the proteome and phosphoproteome data-dependent analyses, with good quantitative features and high-confident site localization of phosphorylated sites.

Moreover, our results reveal the capability of the new Orbitrap Fusion Lumos Tribrid mass spectrometer to identify and quantify thousands of peptides and phosphopeptides in single-shot analyses of human whole cell lysates with minimal sample fractionation and reduced chromatographic separation times. Indeed, the new Orbitrap Fusion Lumos Tribrid mass spectrometer includes a segmented quadrupole, and incorporates a new high-capacity transfer tube and an ion funnel that improve ion transmission and selectivity, thus reducing accumulation times and increasing scanning rates. These features, that improve ion transmission, facilitate the in-depth analysis of human proteome and phosphoproteome from whole cell lysates, which is of high relevance for the high-throughput analysis of *in-vivo* samples such as primary cells and biopsies, as well as, for systems biology applications that require protein and phosphopeptide quantitation in multiple samples and experimental conditions.

Finally, with the acquisition of the same complex sample with different methods assessed in this study, we provide a complete dataset to be used as a reference dataset

for further analyses, and as starting point for future optimizations in particular applications.

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FIGURES

Figure 1: Number of protein groups (A), peptides (B), peptide-spectrum matches and triggered MS2 spectra (C) identified from 1 µg of total protein HeLa extract with 60, 90, 120 and 240 minutes gradient. Samples were injected in triplicate in an Orbitrap Fusion Lumos using HCD peptide fragmentation, and fragment ion detection in the ion trap. All reported identifications are filtered by a peptide FDR $\leq 1\%$. Error bars correspond to standard deviation and solid lines indicate the number of proteins and peptides identified considering all three technical replicates as merged files. D) Additional number of proteins, peptides and PSM identifications obtained per time unit of gradient length increment.

Figure 2: Average number of protein groups and peptides (A) and peptide-spectrum matches and triggered MS2 spectra (B) identified per replicate from different amounts of total protein HeLa extract ranging from 100 ng to 4 µg. Samples were injected in triplicate with a 90-min gradient in an Orbitrap Fusion Lumos using HCD peptide fragmentation, and fragment ion detection in the ion trap. All reported identifications are filtered by a peptide FDR $\leq 1\%$. Error bars correspond to standard deviation, and the numbers between lines represent the average number of peptides per protein group, and the average number of triggered MS2 spectra per assigned spectra. C) Heatmap representing the areas for all quantified peptides after mass spectrometric acquisition of the different amounts of total HeLa protein extract.

Figure 3: Number of protein groups (A), peptides (B), peptide-spectrum matches and triggered MS2 spectra (C) identified from 1 µg of total protein HeLa extract with 90-

min gradient with different peptide fragmentation and fragment ion detection methods. All reported identifications are filtered by a peptide FDR $\leq 1\%$. Error bars correspond to standard deviation and solid lines indicate the number of proteins and peptides identified searching the three technical replicates as merged files. D) Number of protein groups confidentially identified in one, two or three out the three technical replicates.

Figure 4: Correlation plot (A) and coefficient of variation distribution (B) of identified peptides log₂-areas of three available technical replicates of 1 µg of total protein HeLa extract analysed with 90-min gradient with different combinations of peptide fragmentations and mass analysers.

Figure 5: A) Number of phosphorylated peptides identified from the phospho-enrichment protocol 1 µg of total protein HeLa extract with 90-min gradient with different peptide fragmentation and fragment ion detection methods. All reported identifications are filtered by a peptide FDR $\leq 1\%$. Error bars correspond to standard deviation and solid lines indicate the number of proteins and peptides identified searching the three technical replicates as merged files (A) and coefficient of variation distribution (B) of identified peptides log₂-areas of the three available technical replicates of 1 µg of total protein HeLa extract analysed with 90-min gradient with different combinations of peptide fragmentations and mass analysers.

Figure 6: Percentage of identified peptides with one, two, or more phosphorylation sites with the HCD-FT method (A), the HCD-IT method (B), the CIDMSA-FT method (C) and the CIDMSA-IT method.

Figure 7: Distribution of phosphorylated residues (A) and PhosphoRS localization score confidence (B) among the identified mono-phosphorylated peptides with the different peptide fragmentation methods (HCD and CIDMSA) in combination with the different MS2 detectors (FT and IT).

TABLES

Table 1: Orbitrap Fusion Lumos MS1 and MS2 acquisition parameters.

Table 2: Summary of all identified peptides and phosphopeptides in the different acquisition methods assessed in this study.

SUPPORTING INFORMATION

The supporting information includes the tables with the proteins, peptides and PSM identified in the different experiments reported in the manuscript, as well as additional figures that illustrate the raw data quality and complement the differences among methods described in the main text.

Figure 1

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