RESEARCH ARTICLE

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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One of the major additions in MS 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-hibrid mass spectrometers for (phospho-) peptide identification and quantitation using different gradient lengths, sample amounts, and combinations of different peptide fragmentation types and mass analyzers. 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:
Mass spectrometry / Phosphorylation / Protein identification / Protein quantitation

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1 Introduction

MS 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 LC and analyzed by MS [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 posttranslational modifications, have made the analysis of proteomes a challenge for the current proteomics technology [7].

In the last decade, the field of MS-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 [8–13]. A recent major addition in MS technology has been the introduction of a new type of tri-hybrid mass spectrometers that combine a quadrupole mass filter with a high-field

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Abbreviations: AGC, auto gain control; **IT**, ion trap; **PSM**, peptide-spectrum matches

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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.

Orbitrap mass analyzer and a dual cell linear ion trap (IT) [14], which resulted in exceptional capabilities in peptide and protein identification [15]. These new mass spectrometers enable several peptide fragmentation techniques including collision-induced dissociation (CID), high-energy collisional dissociation (HCD), electron-transfer dissociation (ETD), and their combinations, as well as the possibility of routing ions to the different types of mass analyzers 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 analyzers in the identification and MS1 label-free quantitation of peptides and phosphopeptides using data-dependent methods.

2 Materials and methods

2.1 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% CO_2 waterlogged atmosphere using DMEM (GIBCO, Invitrogen), supplemented with 10% 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, 13 000 × *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 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 twofold again and digested overnight with trypsin at 37°C. Tryptic peptides were desalted using a C18 column, evaporated to dryness.

Two hundred fifty micrograms of dried HeLa protein digest were dissolved with 10 μL of 3% ACN, 0.1% TFA), and further diluted with 90 μL of TiO_2 loading buffer (80% ACN, 6% TFA). Phosphopeptides were enriched with TiO_2-beads as previously described [17]. Briefly 10 mg of "Titansphere TiO_2 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. Fifty microliters 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 μL of washing buffer (0.1% TFA, 80% ACN). Phosphopeptides were eluted from the beads with 25 μL of 25% ACN v/v containing 25% NH_4OH m/v, acidified with 10 μL of 10% TFA and vacuum concentrated to dryness. Samples were finally diluted to 10 μL with H_2O + 0.1% formic acid prior and 4 μL was loaded into the chromatographic column for mass spectrometric analysis.

2.2 MS 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* 350–1500 with detection in the Orbitrap (120 K 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 35% (CID). The number of selected precursor ions for fragmentation was determined by the *"Top Speed"* acquisition algorithm and a dynamic exclusion of 60 s. Fragment ion spectra were acquired in the linear IT or the Orbitrap (OT, 30 K resolution) depending on the method, with an AGC of 4000 and a maximum injection time of 300 ms for IT 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 (Tune v2.0 1258).

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 μm id, PepMap RSLC C18, 2 μm particles, 45°C) and hyphenated to an Orbitrap Fusion LumosTribrid mass spectrometer. Different chromatographic gradient lengths from 60 to 240 min were tested for peptide separation. All gradient started at 5% ACN (ACN:H_2O (5:95), 0.1% FA) and went up to 32% ACN (ACN:H_2O (32:68), 0.1% FA).

Table 1. Orbitrap Fusion Lumos MS1 and MS2 acquisition parameters

	MS1			
Detection type	Orbitrap			
Resolution	120 000			
Scan range (*m/z*)	350–1500			
RF lens (%)	30			
AGC target	1.00×10^5			
Maximum injection time (ms)	50			
Change state	2–5			
Exclusion duration (s)	60			
Mass tolerance (ppm)	±5			
Intensity threshold	3.00×10^4			
Data-dependent mode	Top speed			
Precursor priority	Most intense			
	MS2			
	HCD-IT	HCD-FT	CID-IT[a)]	CID-FT[a)]
Isolation mode	Quadrupole			
Isolation window (*m/z*)	1.6			
Activation type	HCD	HCD	CID	CID
Collision energy (%)	28	28	35	35
Detection type	Ion trap	Orbitrap	Ion Trap	Orbitrap
Orbitrap resolution	NA	30 000	NA	30 000
AGC target	4.00×10^3	3.00×10^4	4.00×10^3	3.00×10^4
All available parallelizable time	Yes	No	Yes	No
Maximum injection time (ms)	300	80	300	80

a) In phosphoproteome methods CID with multistage activation (MSA) and a neutral loss of 98 Da were used.
Note that the paramenters 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 (PN-49014 from Thermo).

2.3 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 IT 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, 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.

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

3 Results and discussion

3.1 Chromatographic gradients and cell lysate amounts

Initially, we evaluated the performance of the LC–MS system on single-shot analyses of 1 μg 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 IT (Table 1 and Supporting Information Table 1). Samples analyzed with this acquisition method rendered a minimum of 4761 protein groups and 32 861 distinct peptides (FDR < 1%) when merging three single-shot technical replicates with 1 μg of HeLa digest loaded on column, and a maximum of 5979 protein groups and 50 476 distinct peptides with the longest gradient (Fig. 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 (Fig. 1D). This analysis showed that an increase of either 30 or 60 min (90- 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

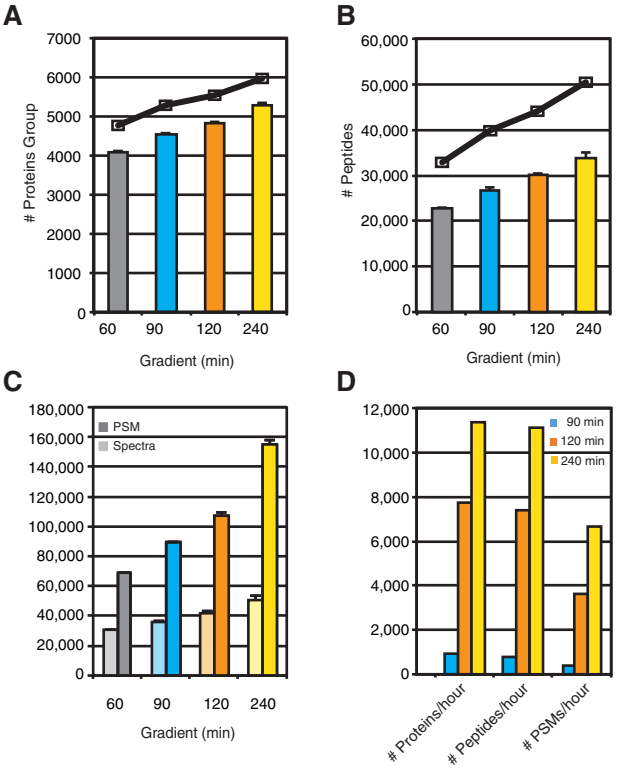

Figure 1. Number of protein groups (A), peptides (B), PSM and triggered MS2 spectra (C) identified from 1μg of total protein HeLa extract with 60, 90, 120 and 240 min gradient. Samples were injected in triplicate in an Orbitrap Fusion Lumos using HCD peptide fragmentation, and fragment ion detection in the IT. All reported identifications are filtered by a peptide FDR ≤ 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 compared to the 60-min gradient.

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 analyzed different amounts of digested cell extract ranging from 4 μg to 100 ng on column in a 90-min chromatographic gradients with HCD peptide fragmentation and MS2 detection in the linear IT (Table 1 and Supporting Information Table 2). Our analyses resulted on average in ~38 500 PSMs per replicate, in ~27 500 identified peptides, and ~4700 protein groups with 2 μg of HeLa digest, with no improvement over 2 μg of digest (Fig. 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 4100 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 five to six peptides per protein were identified (Supporting Information Fig. 1).

3.2 Combination of fragmentation techniques and mass analyzers

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 analyzer to use for fragment detection that balance analytical speed and spectra quality and thus, maximize 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 IT mass analyzer using 1 μg of HeLa digested proteome in a 90-min gradient (Table 1, Supporting Information Figs. 2–5, and Supporting Information Table 3). 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 IT-based MS2 methods in terms of the number of protein groups and peptide identifications compared to the methods that use the Orbitrap mass analyzer for fragment ion detection (Fig. 3, Table 2, and Supporting Information Table 4). The observed differences in performance between IT 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 IT-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 5291 protein groups merging all three replicates (90 min, MS2 HCD-IT), 3807 protein groups were consistently identified in every technical replicate and

Table 2. Summary of all identified peptides and phosphopeptides in the different acquisition methods assessed in this study[a)]

	Replicate 1			Replicate 2			Replicate 3			Total		
	Number of protein groups	Number of peptides	Number of PSM	Number of protein groups	Number of peptides	Number of PSM	Number of protein groups	Number of peptides	Number of PSM	Number of protein groups	Number of peptides	Number of PSM
60 min	4050	22 819	30 606	4090	22 653	30 631	4096	22 904	30 538	4761	32 861	91 775
90 min	4545	27 115	36 753	4549	25 998	35 652	4566	26 868	36 436	5291	39 801	10 8841
120 min	4847	30 613	42 318	4850	30 000	41 403	4818	29 928	41 387	5541	43 991	12 5108
240 min	5321	34 203	50 740	5316	34 524	51 763	5183	32 366	49 023	5979	50 476	15 1526
100 ng	3612	19 057	24 607	3570	18 332	23 654	3642	18 832	24 250	4242	27 884	72 511
250 ng	4174	23 341	30 543	4185	22 871	30 071	4155	23 367	30 481	4871	34 582	91 095
500 ng	4359	25 165	33 393	4431	24 556	32 852	4431	24 884	32 925	5118	36 938	99 170
1000 ng	4540	27 166	36 670	4544	26 112	35 691	4570	26 899	36 346	5277	39 744	10 8707
2000 ng	4627	27 907	38 345	4646	27 411	38 236	4657	27 993	38 403	5343	40 626	11 4984
4000 ng	4705	28 451	39 447	4710	27 009	38 247	4714	27 737	38 790	5395	40 245	11 6484
HCD-IT	4594	27 895	37 575	4605	26 766	36 477	4620	27 571	37 177	5365	41 025	11 1229
HCD-FT	3511	18 665	23 125	3540	19 773	24 446	3536	19 134	23 711	4192	27 955	71 282
CID-IT	4134	21 077	27 648	4167	20 841	27 532	4142	21 093	27 781	4899	31 118	82 961
CID-FT	3628	19 401	24 166	3611	19 723	24 620	3530	18 486	23 170	4281	28 515	71 956

	Replicate 1	Replicate 2	Replicate 3	Total									
	Number of Phos-peptides	Number of Phos-peptides	Number of Phos-peptides	Number of Phos-peptides	1× Phos-peptides	2× Phos-peptides	3× Phos-peptides	Number of Phos-Ser	Number of Phos-Thr	Number of Phos-Tyr	Phospho RS >90	Phospho RS 50–90	Phospho RS <50%
HCD-IT	5734	5293	4848	7653	6957	715	33	5927	955	75	5530	869	557
HCD-FT	4876	4828	4175	6784	6314	488	15	5460	797	56	5318	580	416
CIDMSA-IT	5110	4707	4183	6919	6319	623	27	5476	787	56	4961	846	512
CIDMSA-FT	5060	4510	4337	6349	5837	531	17	5044	748	45	4983	495	359

a) Note that the numbers represent identified peptides that have at least one valid extracted area value (XIC) and are not matching to any potential contaminant.

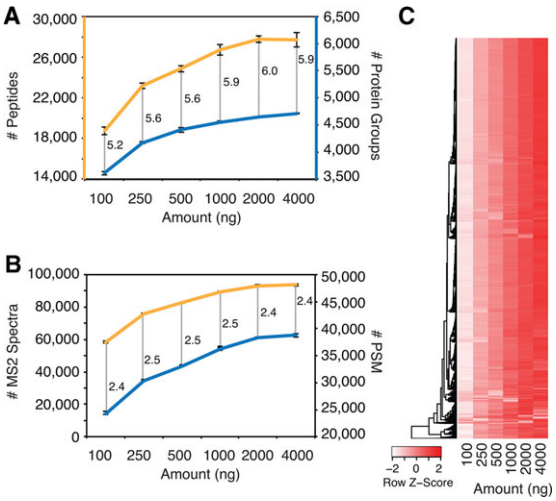

Figure 2. Average number of protein groups and peptides (A) and PSM and triggered MS2 spectra (B) identified per replicate from different amounts of total protein HeLa extract ranging from 100 ng to 4 μg. Samples were injected in triplicate with a 90-min gradient in an Orbitrap Fusion Lumos using HCD peptide fragmentation, and fragment ion detection in the IT. All reported identifications are filtered by a peptide FDR ≤ 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.

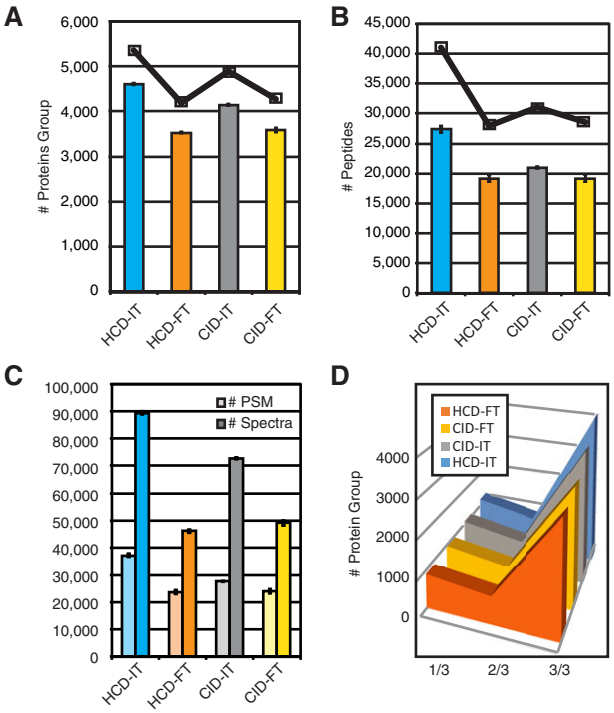

Figure 3. Number of protein groups (A), peptides (B), PSM and triggered MS2 spectra (C) identified from 1 μg of total protein HeLa extract with 90-min gradient with different peptide fragmentation and fragment ion detection methods. All reported identifications are filtered by a peptide FDR ≤ 1%. Error 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.

over 90% of the quantified distinct peptides exhibited a CV of peptide MS1 log2-areas below 4% (three technical replicates). Similar values of CV were obtained for the other methods under consideration (Fig. 4).

3.3 Instrument performance on phospho-enriched human samples

The performance of the new tri-hybrid mass analyzer with the different combinations of peptide fragmentations and mass analyzers 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 IT mass analyzer (Table 1, Supporting Information Fig. 6–9, and Supporting Information Table 3).

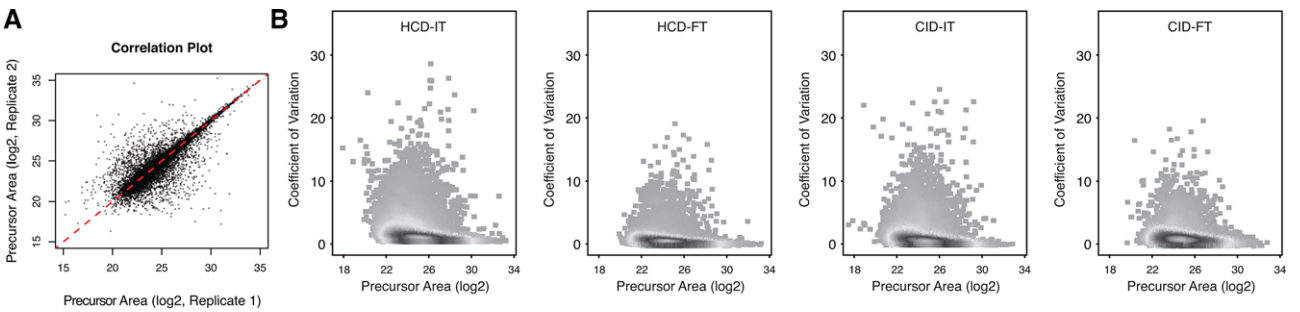

Figure 4. Correlation plot (A) and CV distribution (B) of the peptide log2-areas corresponding to three technical replicates of 1 μg of total protein HeLa extract analysed in a 90-min gradient with different combinations of peptide fragmentations and mass analyzers.

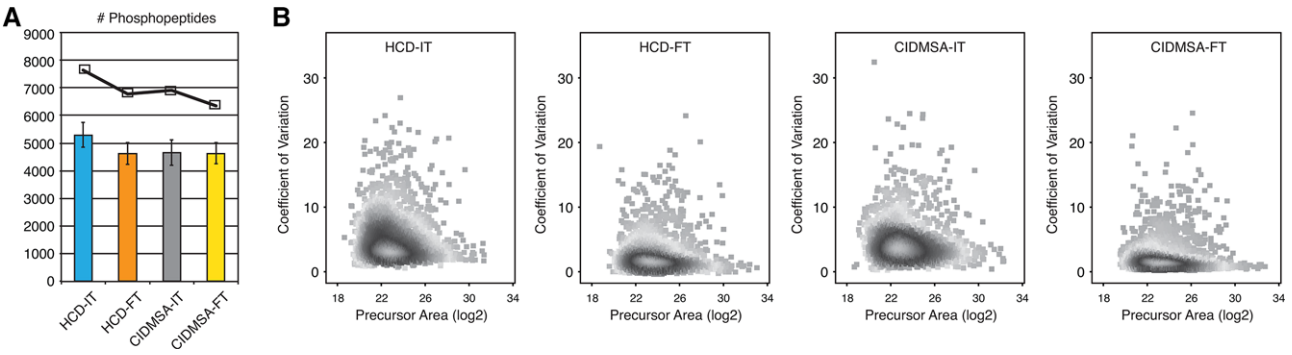

Figure 5. (A) Number of phosphorylated peptides identified from the phospho-enrichment protocol 90-min gradient with different peptide fragmentation and mass analyzers. All reported identifications are filtered by a peptide FDR ≤ 1%. Error bars correspond to standard deviation and solid lines indicate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

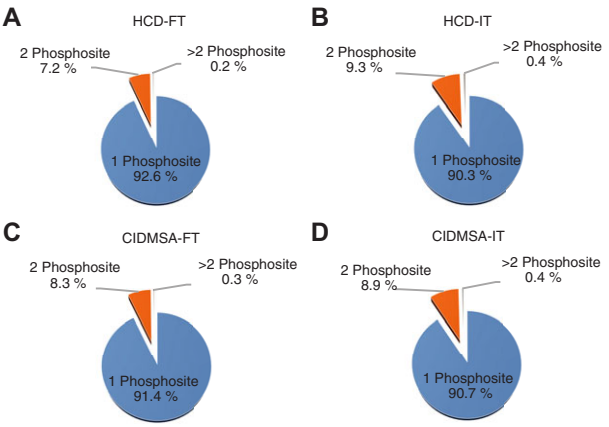

Figure 6. Percentage of identified peptides with one, two, or more phosphorylation sites with the HCD-FT (A), HCD-IT (B), CIDMSA-FT (C) and CIDMSA-IT methods.

The phosphoproteome analysis from three technical replicates provided an average identification of ca. 5300 distinct phosphopeptides per replicate with a 90-min gradient (MS2 HCD-IT), with more than 95% of the peptide log2 areas exhibiting a CV below 15% (Fig. 5, Table 2, and Supporting Information Table 5). Moreover, we observed that the linear IT 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 90% of the identified phosphorylated peptides were monophosphorylated regardless the acquisition method used (Fig. 6), and among them, most of the phosphorylation sites were identified on serine residues (Fig. 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 IT 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 (Fig. 7B).

4 Concluding remarks

In this study, we used the fragmentation and ion routing capabilities of the new tri-hibrid 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 analyzers were tested. Our results show that among the assessed methods, the HCD peptide fragmentation and IT-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

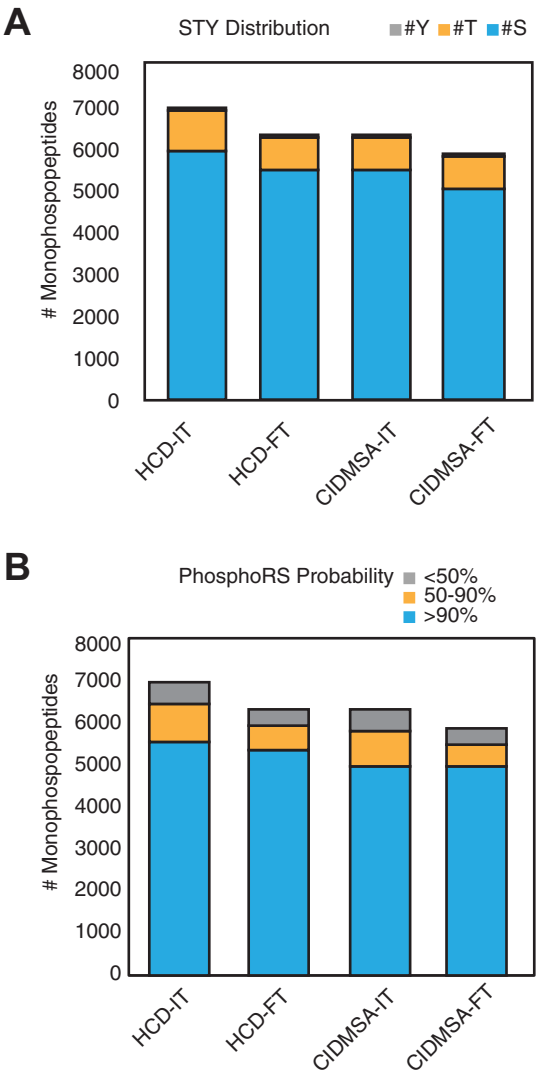

Figure 7. Distribution of phosphorylated residues (A) and PhosphoRS localization score confidence (B) among the identified mono-phosphorylated peptides with the different peptide fragmentation methods (HCD and CIDMSA) in combination with the different MS2 mass analyzers (FT and IT).

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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