Performance Evaluation of a Modified Quadrupole Orbitrap Mass Spectrometer

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ABSTRACT

Purpose: Evaluate the performance of the new Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer in bottom-up proteomics and Biopharma applications.

Results: The novel Orbitrap Exploris 480 mass spectrometer has a smaller footprint than its predecessors, while providing equivalent performance for DDA and DIA applications and delivering consistent results. In addition, with the TurboTMT option and the capability of coupling it with the Thermo Scientific™ FAIMS Pro™, more peptides\protein groups can be identified\quantified.

INTRODUCTION

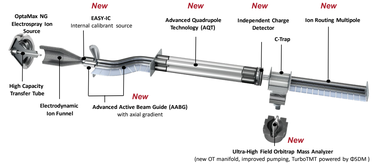
The increasing importance of understanding how, when and where proteins are expressed, together with the interaction with other proteins and what functions they perform, is driving advancements in mass spectrometric instrumentation. Since its introduction, Orbitrap-based instruments have and are still playing a pivotal role in many different research areas such as proteomics, metabolomics, biopharma, etc. Each of these applications come with different challenges to mass spectrometric instrumentation. To address some of these challenges, new technological developments as well as improvements for existing mass spectrometers is a necessity. Here we evaluated the Orbitrap Exploris 480 mass spectrometer for proteomics applications with focus on data dependent acquisition and data independent acquisition. Additionally, we assessed the use of the ΦSDM processing algorithm on TMT11plex labeled samples.

MATERIALS AND METHODS

For DDA and DIA experiments, HeLa protein digest was used as standard. For the LFQ experiments different amount of Yeast protein digest (Promega Corporation) was mixed in a constant background of the Thermo Scientific™ Pierce™ HeLa Protein Digest (200ng) to obtain a ratio of 2, 5 and 10.
The TMT experiments were done using Thermo Scientific Pierce™ HeLa Protein Digest labeled with TMT11plex mixed in a ratio of 1:1.
The samples were loaded onto Thermo Scientific™ Acclaim™ PepMap™ RSLC C18, 25 cm or 15 cm × 150 µm C18 columns and separated using different gradients. The peptides were separated using either the Thermo Scientific™ EASY-nLC™ 1200 System or Thermo Scientific™ Ultimate™ 3000 RSLCnano. The eluting peptides were analyzed on the new Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer. The mass spectrometer was operated in a data dependent mode. In addition, the acquisition of the TMT samples was also acquired with the TurboTMT option.
The novel Orbitrap Exploris 480 mass spectrometer (Figure 1) includes:

- A high capacity transfer tube (HCTT) and an RF-only electrodynamic ion funnel for increased ion flux.
- An Advanced Active Beam Guide (AABG) to prevent neutrals from entering the quadrupole and improve robustness by eliminating the effects of local charging.
- Advanced quadrupole technology (AQT): It comprises a hyperbolic segmented quadrupole, for higher transmission efficiency and optimized isolation window shape.
- An Independent Charge Detector(ICD) for ion control which is located after the segmented quadrupole.
- A Curved Linear Trap for ion trapping, cooling and injection into the Orbitrap
- A novel ion routing multiple for dynamic trapping and HCD fragmentation
- An ultra-high vacuum on the Orbitrap Exploris which is provided by a single 6 stage turbomolecular pump.
- An Ultra-high-field Orbitrap mass analyzer which offers resolutions up to 480,000 and scan speeds up to 40 Hz.

Figure 2. Schematic representation of the Orbitrap Exploris



Data Analysis

Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.4 software. The raw files were searched against the Homo sapiens (SwissProt TaxID=9606) fasta and or Saccharomyces cerevisiae (SwissProt TaxID=4932_and_subtaxonomies) (v2017-07-05) database using the SEQUEST HT algorithm with a Chimeric search node. The following settings were applied: precursor mass tolerance of 10 ppm, fragment mass tolerance of 20 mmu. Trypsin was specified as digesting enzyme and 2 missed cleavages were allowed. Cysteine carbamidomethylation was defined as fixed modifications and methionine oxidation and deamidation were variable modifications. Only high confident peptides with FDR < 1% and first ranked peptides were included in the results. For the quantitation, TMT reporter ion quantification method within Proteome Discoverer software was used to calculate the reporter ratios with mass tolerance ±20 ppm. Isotopic correction factors were applied according to the pertaining CoA. Only confidently identified peptides containing all reporter ions were designated as "quantifiable spectra". Protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the unique peptides pertaining to that protein group.

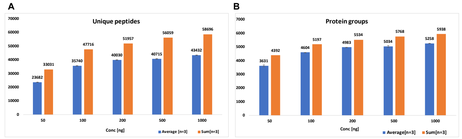
RESULTS

Applications

Bottom-up Proteomics (Data Dependent Analysis)

To evaluate the performance of the Orbitrap Exploris, different amounts of HeLa protein digest were loaded on column and separated on the EasynLC 1200. Figure 2 below shows the average (blue bars) as well as the sum (orange bars) of number of proteins and peptides identified in triplicate injections. The average number of IDs increased with increasing concentration, from approx. 3600, for 50 ng sample on column, to 5200 (1000ng on column) protein groups in triplicate experiments.

Figure 2. Number of unique peptides\protein groups identified from MPI HeLa digest in triplicate experiments using 60min gradient (8-60% B (80% ACN in 0.1% FA) in 60 min, Flow rate:300nl\min).
FS resolution setting: 60k (120k for 50 ng), AGC target: 300%, inject time: 20ms, ddMS2 resolution setting: 15k (30k for 50 ng), AGC target: 75%, inject time: 22ms (80ms for 50 ng), TopN: 20, intensity threshold: 5e3. Sample 50ng-1000ng, 50 cm column.

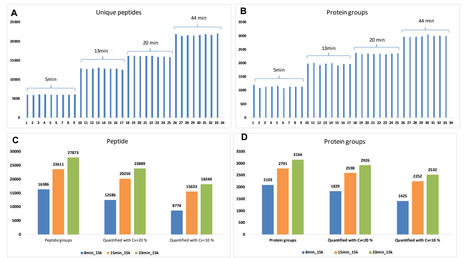


Furthermore, the performance of the Orbitrap Exploris was evaluated with variable gradients both in DDA and DIA experiments. For these experiments 200 ng HeLa digest was injected on column and separate with 5min (8min in total), 13 (15min in total), 20min (23min in total) and 44min (46min in total) gradients.
Figure 3 A and B shows consistence identification even at short gradients of 5min (>6000 peptides and 1000 protein groups).
For the DIA experiments, using only 3 (8, 15, 23min) of the 4 gradients, 87 % of the identified proteins were quantified with CVs below 20 % for the 5min gradients. The percentage of quantification increased to 93 % with gradient length. Also, the number of quantified peptides increased from 77 % to 85 % going from the 5 to 23min gradient (Figure C&D). The median coefficient of variation (CoV) for quantified peptides in all 3 gradients were less than 10 %.

Table 1. Short LC gradients on the Ultimate3000 RSLC

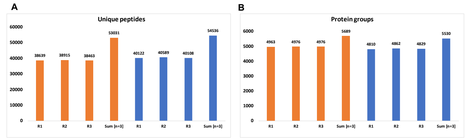
Time [min]	Flow rate [ul\min]	% B	Time [min	Flow rate [ul\min]	% B	Time [min]	Flow rate [ul\min]	% B	Time [min]	Flow rate [ul\min]	% B
0.2	1.5	10	0.15	1.5	8	0.15	1.5	8	0.15	1.3	8
5	1.5	35	1	1.5		1	1.5		1	1.3	
5.9	1.5	99	1.1	1		1.5	0.8		1.5	0.6	
6.4	1.5	99	10	1	30	17	0.8	30	40	0.6	30
6.9	1.5	8	12.8	1	50	20.8	0.8	50	44.8	0.6	50
			13	1.5	99	21.2	1.3	99	45.2	1.3	99
			13.2	1.5	99	22.7	1.3	99	46.7	1.3	99
			13.3	1.5	8	22.8	1.3	4	46.8	1.3	4

Figure 3. Protein groups\peptides identified using short gradients. A&B) Reproducible identification in multiple experiments using different gradients. C&D) Peptides\Proteins groups quantified using short gradients in DIA experiments, with median CoVs <10 %.
FS resolution setting: 60k, AGC target: 300%, inject time: 20ms, ddMS2 resolution setting: 7.5k, AGC target: 75%, inject time: 16ms, Iso Width: 2, TopN:40, 200ng sample load.



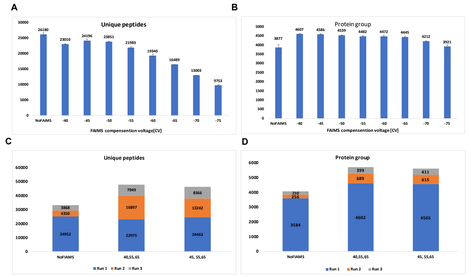
In addition, a direct comparison with the Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer was performed using 1 ug of HeLa digest on a 60 min gradient. The MS was operated in a DDA mode, either selecting the 20 most intense peaks from each survey scan. On average 39000 unique peptides were mapped to an average of 4800 protein groups (Figure 2 E and F). In total approximately 55000 unique peptides were matched to 5500 protein groups in the triplicate runs on the Orbitrap Exploris 480. Similar numbers were also identified on the Q Exactive HF-X mass spectrometer. These results show that both systems perform the same in DDA experiments.

Figure 4. Comparison of the Orbitrap Exploris 480 to the Q Exactive HF-X. The same number of protein and peptides are identified for a 60 min gradient (8-60% B (80% ACN in 0.1% FA) in 60 min, Flow rate:300nl\min.
FS resolution setting: 60k, AGC target: 300%, inject time: 20ms ddMS2 resolution setting: 15k, AGC target: 75%, inject time: 22ms, TopN: 20, intensity threshold: 5e3. Sample 50ng-1000ng Pierce HeLa digest.



Recently it was shown that interfacing FAIMS with the Orbitrap Fusion Lumos, boosted the total number of peptides\peptides identified and quantified. By looping CVs, single FAIMS compensation voltages (CV) or multiple CV looping, a set of ion populations will reach the detector while others will not. To evaluate the performance of FAIMS and the Orbitrap Exploris 480, 1ug HeLa was loaded on column and separated on a PepMap 15 cm x 150 µm CapLC column and analyzed with (single CVs) and without FAIMS. The number of protein groups increased with each FAIMS CV used as compared to without FAIMS. The percentage gain in IDs is different for different CV, the highest gain, 24%, being at -40. As expected for single CVs, the number of peptides identified with FAIMS is slightly less than without FAIMS. This is because only a fraction of the ion population is injected into the instrument. However, if IDs per given instrument time are compared as shown on figure 5 C&D by in-silico combining multiple CV (-40,-55, -65 and -40, -55, -70), the total number of peptide\protein IDs increases by at least 35 %.

Figure 5. Peptides and proteins identified from different CV in a 30 min gradient (8-30 % B in 30 min. 5 min to 50 B, 5 min to 90 % B, 1 min back to 8 % B and 5 min at 8% B, Flow rate: 1ul\min. C&D) In-silico combination of multiple CV compared to 3 injections without FAIMS.
FS resolution setting: 60k, AGC target: 300 %, inject time: 20ms, ddMS2 resolution setting: 7.5k, AGC target: 100%, inject time: 22ms,TopN:20, 1ug Pierce HeLa on column.



Relative Quantitation

Stable isotope-labeling approaches

The Orbitrap Exploris is equipped with the TurboTMT option for TMT analysis. Using this option, the instrument processes frequency spectra with the Phase-Constrained Spectrum Deconvolution Method (ΦSDM)[1,2,3] during acquisition. The advantage of (ΦSDM) is that it requires substantially shorter transients to achieve the same mass resolution relative to standard FT based approaches. However, due to the huge computational power requirement of the ΦSDM, only sections of the spectra can be processed in real time on the embedded PC. The TurboTMT takes advantage of ΦSDM by using a predefined section (TMT Tags, 126-131 Da) of the spectra, while the rest of the fragment ion will be processed with the standard processing method, eFT (see figure 6).

Figure 6. Randomly selected MS/MS spectra of a HeLa peptide, labeled with TMT11plex. A: Measured with 96ms transient length (45k).B: Measured with 32ms transient length (15k). C: Zoom in to the reporter region for both spectra, the resolution increased by approximately 3-fold in the reporter ion region.

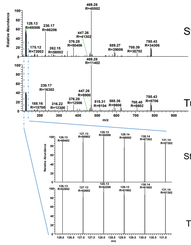
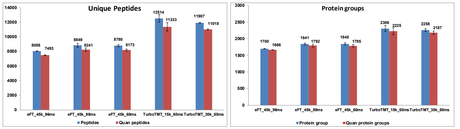


Figure 7. Average of triplicate injection of 500 ng on column HeLa digest labeled with TMT11plex and mixed in a 1:1 ratio and separated with a 50 min gradient.



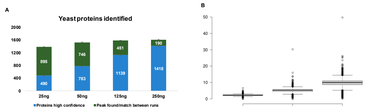
eFT_45k_96ms: (eFT processing using 96ms transients in MS2 and 96ms MS 2 injection time for the 50 min gradient.
eFT_45k_80ms: (eFT processing using 96ms transients in MS2 and 80ms MS 2 injection time for the 50 min gradient
eFT_45k_60ms: (eFT processing using 96ms transients in MS2 and 60ms MS 2 injection time for the 50 min gradient
TurboTMT_15k (32ms transients in MS2 and 60ms MS/MS injection time for 50 min gradient
TurboTMT_30k (64ms transients in MS2 and 60ms MS/MS injection time for 50 min gradient

Using the TurboTMT for TMT11plex analysis, approximately 30 % more peptides and proteins from 500 ng HeLa labeled with TMT11plex were identified and quantified. The variation between injections is higher in the 15k resolution setting method than for the 30k method. The Percentage gain will vary for different sample amounts and for different gradient lengths.

Label free quantitation

Unlike stable isotope-labeling approaches, protein samples in label-free approaches are measured individually and the relative protein expressions is determined either by means of spectral counting or mass spectral peak intensities. To evaluate the capability of the Orbitrap Exploris 480 in this application, different concentration of yeast protein digest were spiked into a constant HeLa protein digest background (200ng) to mimic expression level of 2, 5- and 10-fold changes. Only proteins identified with at least 3 peptides were used for quantitation (Figure 8B). The results below show that the absolute precision (width of box) is better for smaller ratios and the accuracy is better with higher ratios. Also, the table below show that the ratios (<10 %) were not under- nor overestimated (Figure 8C).

Figure 8. A. No of yeast protein identified with or without match between runs. B Precision of quantitation (width of boxes) for the different concentration spiked into HeLa C. Expected versus detected ratios as well as the high confident yeast proteins used for quantitation.



C

Expected ratio	2	5	10
Actual (median)	2.26	5.22	9.97
ng yeast	50ng	125ng	250ng
Yeast Proteins	783	1139	1418

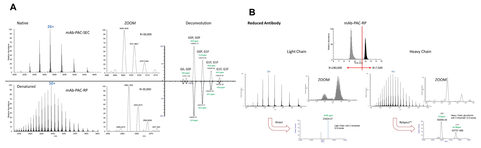
Biopharma applications

Applying intact protein mode on the Orbitrap Exploris 480 for the analysis mAbs enables increased scanning range up to 8000 m/z. Analysis can be conducted in either native or denaturing conditions.

- Native analysis were achieved by volatile salt-based buffers at physiological pH, separation was conducted by size exclusion chromatography. In these conditions, the mAb retain his native conformations resulting in lower charge states.
- In denaturing condition, the protein unfolds and increase protonation, due to mobile phases at acidic pH containing organic solvent. Separation was conducted by RP chromatography.

With the Orbitrap Exploris 480 we are able to determine the protein mass and confirm the glycoform ratios of intact monoclonal antibodies in both native and denatured conditions.

Figure 9. A) Analysis of monoclonal Antibody, Herceptin, in native and denatured condition. B) Herceptin reduced with TCEP, Separation by RP chromatography



CONCLUSIONS

- In shotgun proteomics using the DDA approach, the Orbitrap Exploris delivers consistent results with different concentrations of HeLa digest and different gradient lengths (5-44 min gradient), and performs equivalently to the Q Exactive HF-X.
- For DIA experiments, as many as 2000 proteins were identified using a 5 min gradient; 87 % percent of these were quantified with CoVs less than 20 %.
- The use of the TurboTMT option for TMT11 plex showed approximately 30 % increase, for 500ng HeLa digest labelled with TMT11plex, in the number of identified\quantified peptides and protein groups.
- Label free experiments using different concentration of yeast spiked to a constant background of HeLa on the Orbitrap Exploris shows accurate determination of the ratios (2, 5, 10). Accuracy was below 10 % for the different concentrations.
- With the Orbitrap Exploris, we are able to determine the mass and confirm the glycoform ratios of intact monoclonal antibodies in both native and denatured conditions.

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TRADEMARKS/LICENSING

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