High-throughput proteomics using narrow window DIA on the Orbitrap Astral Zoom MS

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Abstract

Purpose: Demonstrate the performance of the new Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer for high-throughput proteomics using narrow window data-independent acquisition (nDIA) methods.

Methods: HeLa digest standard measured on Orbitrap Astral Zoom MS and Thermo Scientific™ Orbitrap™ Astral™ MS coupled to Thermo Scientific™ Vanquish™ Neo UHPLC utilizing 30-300 samples-per-day (SPD) LC-MS methods.

Results: Fast LC-MS methods with up to 300 SPD throughput on the Orbitrap Astral Zoom mass spectrometer generate deep coverage and precise quantitation of HeLa samples.

Introduction

The recently introduced Orbitrap Astral mass spectrometer has significantly expanded the scale and scope of proteomics experiments, advancing discovery and translational research. Building on this innovation, we demonstrate the performance of the next evolution of the platform, the Orbitrap Astral Zoom mass spectrometer, at throughputs ranging from 30 to 300 samples-per-day (SPD) analysing a HeLa digest using narrow window data-independent (nDIA) acquisition. The novel instrument features a higher acquisition rate (270 Hz vs. 200 Hz) achieved through improved ion optics settling times and faster ion transfers, increased sensitivity via pre-accumulation, and deeper coverage through enhanced spectral processing, among other improvements. Comparative analysis with the Orbitrap Astral MS reveals further improvements in peptide and protein group identifications enabling even deeper coverage at high throughput whilst maintaining precise quantitation.

Materials and methods

Sample Preparation

Thermo Scientific™ Pierce™ HeLa Digest Standard (20 µg/vial) was reconstituted in H_2O + 0.1 % FA to reach a final peptide concentration of 100 or 200 ng/µL and transferred to 96-well plates for LC-MS measurement.

LC-MS Methods

For the throughputs of 60, 100, 180 and 300 SPD, samples were separated on a Thermo Scientific™ EASY-Spray™ 150 µm × 150 mm C18 column (ES906) using a Vanquish Neo UHPLC system operated in NanoCap Trap&Elute configuration with the Thermo Scientific™ PepMap™ Neo 5 µm C18 300 µm × 5 mm cartridge as a trap column. For the throughput of 30 SPD, the samples were separated on an IonOpticks Aurora™ Ultimate XT 75 µm × 25 cm C18 column using a Vanquish Neo UHPLC system operated in NanoCap Direct Injection configuration. Flow rates and gradients were varied depending on the throughput and are shown in Table 1. The run-to-run throughputs (SPD) are defined for 1 µL injections, column re-equilibration was performed in parallel to sample loading. The columns were connected via an EASY-Spray source to an Orbitrap Astral MS or Orbitrap Astral Zoom MS operated in a data-independent acquisition mode, MS method parameters are given in Table 2.

Data Analysis

The raw data files were processed with Spectronaut® 19.5 software (Biognosys AG). The data was searched against the Homo sapiens SwissProt database (TaxID=9606: 42,252 sequences) and contaminants FASTA file. The results were filtered to 1% false-discovery rate (FDR).

Orbitrap Astral Zoom MS

Figure 1. Schematic of the Orbitrap Astral Zoom mass spectrometer

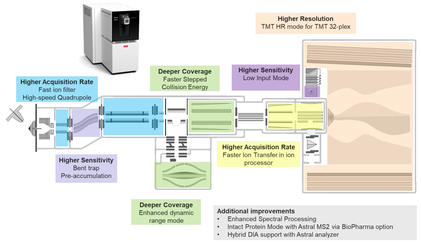


Table 1. LC gradients.

	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
300 SPD	0.000			Run		
	0.000	0.000	3.800	8.0	0.00	0.00
	0.100	0.100	3.800	10.0	0.38	0.21
	0.150	0.050	3.500	10.5	0.18	0.10
	2.150	2.000	3.500	28.0	7.00	3.94
	3.000	0.850	3.500	50.0	2.98	1.68
	3.000			Column Wash		
	3.100	0.100	3.800	99.0	0.37	0.21
	3.700	0.600	3.800	99.0	2.28	1.28
	3.700			Stop Run		
	3.700			Column Equilibration		
180 SPD	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
	0.000			Run		
	0.000	0.000	2.500	4.0	0.00	0.00
	0.200	0.200	2.500	8.0	0.50	0.28
	3.800	3.600	2.500	20.0	9.00	5.07
	5.600	1.800	2.500	35.0	4.50	2.53
	5.600			Column Wash		
	6.000	0.400	3.000	99.0	1.10	0.62
	6.700	0.700	3.000	99.0	2.10	1.18
	6.700			Stop Run		
	6.700			Column Equilibration		
100 SPD	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
	0.000			Run		
	0.000	0.000	1.800	3.0	0.00	0.00
	0.500	0.500	1.800	6.0	0.90	0.51
	6.600	6.100	1.800	21.0	10.98	6.18
	10.600	4.000	1.800	35.0	7.20	4.05
	11.400	0.800	2.500	55.0	1.72	0.97
	11.400			Column Wash		
	11.900	0.500	2.500	99.0	1.25	0.70
	12.800	0.900	2.500	99.0	2.25	1.27
	12.800			Stop Run		
	12.800			Column Equilibration		
60 SPD	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
	0.000			Run		
	0.000	0.000	0.800	3.0	0.00	0.00
	11.000	11.000	0.800	21.0	8.80	4.95
	16.500	5.500	0.800	31.0	4.40	2.48
	19.500	3.000	0.800	41.0	2.40	1.35
	19.500			Column Wash		
	20.000	0.500	3.000	99.0	0.95	0.53
	22.000	2.000	3.000	99.0	3.80	3.38
	22.000			Stop Run		
	22.000			Column Equilibration		
30 SPD	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
	0.000			Run		
	0.000	0.000	0.300	4.0	0.00	0.00
	0.400	0.400	0.300	6.0	0.12	0.16
	0.900	0.500	0.300	8.0	0.15	0.20
	31.800	30.900	0.300	28.0	9.27	12.53
	37.800	6.000	0.300	45.0	1.80	2.43
	37.800			Column Wash		
	39.000	1.200	0.300	95.0	0.36	0.49
	42.000	3.000	0.300	95.0	0.90	1.22
	42.000			Stop Run		
	42.000			Column Equilibration		

Table 2. Mass spectrometer method parameters.

Full Scan (OT)	
Orbitrap Resolution	240k
Scan Range (m/z)	380-980*
RF Lens (%)	40
AGC Target (%)	500
Max. Injection Time (ms)	3-5*
DIA (Astral)	
Precursor Mass Range	380-980*
Isolation Window (m/z)	2-5*
Window Overlap (m/z)	0
Window Placement Opt.	(On)
NCE	25
Scan Range (m/z)	150-2000
RF Lens (%)	40
AGC Target (%)	500
Pre-Accumulation	(On)
Maximum Injection Time (ms)	2-10*
Loop Control (s)	0.6

* Depending on the gradient length, sample load and desired MS2 data points per peak, the Scan Range / Precursor Mass Range, Isolation Windows width and Maximum Injection Times (MS1 and MS2) can be adjusted and optimized.

Narrow-window DIA (nDIA)

For many applications, especially single-shot label-free analyses utilizing short gradients, data-independent acquisition (DIA) has emerged as the preferred method for proteome profiling due to its superior reproducibility, coverage, and quantitative performance compared to data-dependent acquisition (DDA). Narrow window DIA (nDIA) addresses limitations, such as insufficient precursor selectivity and ambiguous precursor to fragment assignments, by using small isolation windows, similar to those used in DDA, to improve specificity and reduce chimeric spectra of many co-eluting peptides, that are especially prominent in complex samples eluting over very short gradients.

The Orbitrap Astral MS platform uniquely meets the needs of high acquisition speed, sensitivity and dynamic range to employ nDIA with high-throughput gradients.

Figure 2. HeLa dilution series results across various throughputs using Orbitrap Astral Zoom MS. Protein Groups (**A**) and Peptides (**B**) as identified by Spectronaut 19 software.

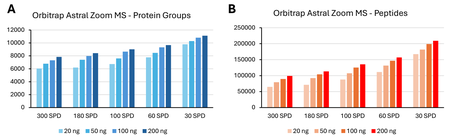


Figure 3. Comparative HeLa results of 200 ng load at various throughputs. (**A**) Run-to-run times and effective separation times in minutes of the used throughputs. (**B**) Protein group and peptide identifications. Consistent method parameters, available both on Orbitrap Astral Zoom MS and Orbitrap Astral MS, were used; pre-accumulation was additionally enabled on Orbitrap Astral Zoom MS. Data analyzed with Spectronaut 19 software.

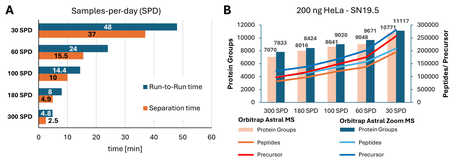
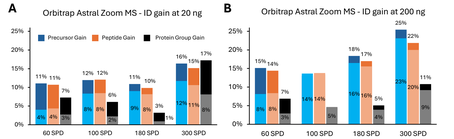


Figure 4. Gain of IDs of Orbitrap Astral Zoom MS vs. Orbitrap Astral MS. Precursor, peptide and protein group gain of Orbitrap Astral Zoom MS over Orbitrap Astral MS across various throughputs at 20 ng sample load (**A**) and 200 ng load (**B**). Identification gains using the same effective MS2 max. injection time (IT, Orbitrap Astral Zoom MS: 2 ms + approx. 1 ms pre-accumulation, Orbitrap Astral MS: 3 ms) are depicted in light color. Additional gains using pre-accumulation on top of 3 ms max. IT on Orbitrap Astral Zoom MS are depicted in deep colors.



Results

Orbitrap Astral Zoom MS excels at high-throughput DIA

We evaluated HeLa sample loads ranging from 20 ng to 200 ng on high-throughput methods ranging from 30 to 300 SPD. As expected, identification counts increased with sample load and longer gradients (Figure 2-3). Importantly, 200 ng HeLa at 300 SPD already identified approx. 8,000 PGs (approx. 100,000 peptides) with Spectronaut 19 software. Longer gradient times of 30 SPD provided a comprehensive and deep coverage of more than 11,000 PGs (>200,000 peptides).

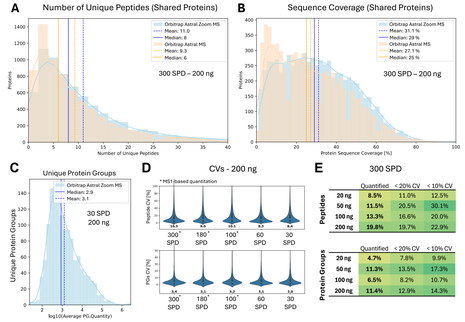
Orbitrap Astral Zoom MS generates a consistent gain in identifications

Comparative analysis of the novel Orbitrap Astral Zoom MS versus the Orbitrap Astral MS revealed significantly higher identifications by the instrument evolution. Exact gains are dependent on throughput and load. However, the Orbitrap Astral Zoom MS consistently identifies 10-15% more peptides and precursors and approx. 5 % more protein groups. Notably, the highest gains were observed at very short gradients with 16-25 % on peptides and precursors and 8-17% on protein groups (Figure 4).

Orbitrap Astral Zoom MS generates better IDs and precise quantitation

In addition to the deeper proteome coverage by the Orbitrap Astral Zoom MS, the shared identifications (IDed with Orbitrap Astral Zoom MS and Orbitrap Astral MS) are based on additional unique peptides (at 300 SPD, 200 ng: +2 unique peptides in median) and consequently feature higher sequence coverage (Figure 5A-B). Investigation of the protein groups uniquely identified by the Orbitrap Astral Zoom MS at 30 SPD and 200 ng load shows a bias towards low abundant proteins, showcasing the increased sensitivity of the novel instrument (Figure 5C). Importantly, the boost in identifications is achieved while maintaining the good quantitative precision of the Orbitrap Astral MS platform (Figure 5D). In fact, the percentage gains on protein groups and peptides below 10% CV significantly exceed the gain in total quantified protein groups and peptides (Figure 5E).

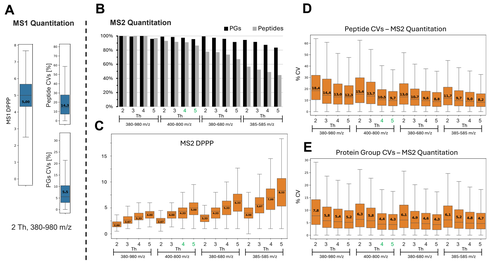
Figure 5. Orbitrap Astral Zoom MS generates high quality IDs and precise quantitation. (**A**) Distributions of unique peptides per protein. (**B**) Protein sequence coverage of identifications generated both by Orbitrap Astral Zoom MS and Orbitrap Astral MS. (**C**) Distribution of protein groups uniquely identified by Orbitrap Astral Zoom MS at 30 SPD with 200 ng load. (**D**) Peptide and protein group CVs of 200 ng HeLa measured on Orbitrap Astral Zoom MS (300-30 SPD). (**E**) Increase in total quantified, quantified <20% and <10% CV peptides and protein groups at 300 SPD with Orbitrap Astral Zoom MS.



Optimizing MS parameters of high-throughput methods for MS2 quantitation

For high-load HeLa samples, the standard 2 Th 380-980 m/z DIA isolation window scheme generates the maximum of identifications. Using this in combination with very short gradients of 300, 180 and 100 SPD MS1 quantitation generates good quantitative precision, as the detector parallelization of the Orbitrap Astral Zoom MS generates MS1 datapoints at 240k resolution every 0.6 seconds (Figure 5 D-E, Figure 6 A). However, the platform offers the full flexibility to balance identifications with precise MS2-based quantitation by simply widening the isolation windows slightly to 3-5 Th and potentially limiting the precursor selection range to further decrease the MS2 cycle time. An exemplary study showcasing this flexibility for 300 SPD is shown in Figure 6, generating outstanding precision for this 4.8 min run-to-run method.

Figure 6. DIA window schemes offer full flexibility to focus on MS2 quantitation. (**A**) MS1 data-points-per-peak (DPPP), peptide and protein groups CVs of the maximum ID method (2 Th, 380-980 *m/z*) using MS1 quantitation. (**B**) Percentage drop in IDs versus the maximum ID method when using incrementally wider isolation windows and limiting the precursor range. MS2 DPPP (**C**), peptide CVs (**D**) and protein group CVs (**E**) of the various DIA window schemes using MS2 quantitation. Data extracted from Spectronaut 19 reports.



Importantly, this general strategy can be applied and optimized for all throughputs, if an even faster MS2 cycle time and consequently more MS2 DPPP for quantitation are desired. The longer the gradient (increased peak width), the smaller the adjustments need to be. Besides the excellent quantitative precision of the Orbitrap Astral Zoom MS, its quantitative accuracy is also outstanding. Three-proteome-ratio studies illustrating this are shown on a separate poster (ASMS 2025, TP 720, Pashkova et al.).

Conclusions

The novel Orbitrap Astral Zoom mass spectrometer is, among its broad and comprehensive range of applications, an excellent instrument for high-throughput proteomics.

- High-throughput nDIA on the Orbitrap Astral Zoom MS generates deep coverage of proteomes
- The novel Orbitrap Astral Zoom MS further pushes the boundaries in terms of quantity and quality of identifications
- The Orbitrap Astral Zoom MS enables precise quantitation with full flexibility to focus on MS2-based quantitation

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