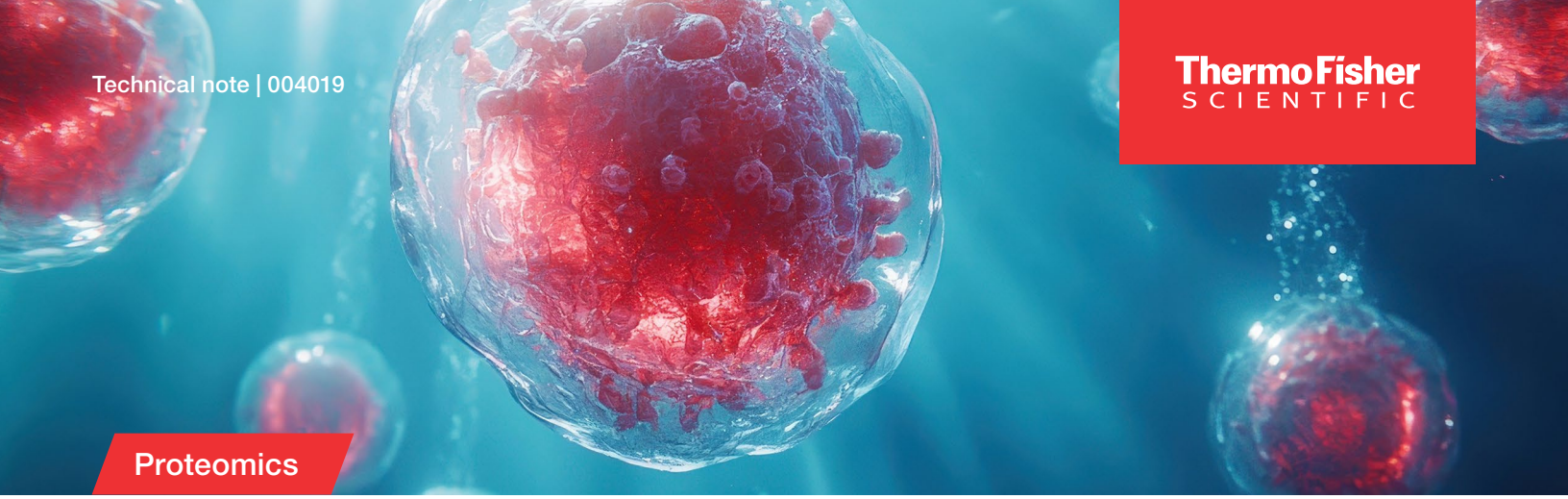

Technical note | 004019

Proteomics

Enhanced sensitivity of the Orbitrap Astral Zoom mass spectrometer for deeper proteome coverage in single-cell proteomics

Authors

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Keywords

Orbitrap Astral Zoom MS, Vanquish Neo UHPLC, FAIMS Pro Duo Interface, data-independent acquisition, DIA, single-cell proteomics low input mode

Goal

To assess proteome coverage, precision, quantitation accuracy, and sample throughput for low-input samples using a library-free data-independent acquisition (DIA) method on the new Thermo Scientific™ Orbitrap™ Astral™ Zoom Mass Spectrometer.

Introduction

Unlike traditional proteomics applications using LC-MS/MS, which analyze thousands of cells simultaneously and present changes as a cumulative response, single-cell proteomics analysis enables a more detailed investigation of protein composition and function at the single-cell level. However, confidently characterizing these changes requires analyzing many individual cells. Working with individual cells poses challenges due to limited sample amounts, sample preparation, throughput, and depth of coverage. Therefore, workflows that minimize human interaction (automated sample preparation), reduce sample losses (by minimizing pipetting steps and sample transfers), and maximize proteome coverage (instrument sensitivity) and throughput (data acquisition and processing speed) are crucial for SCP applications.

Recent advances in LC-MS have enabled unprecedented depth of proteome coverage and measurement accuracy, propelling the field forward. The Orbitrap Astral Zoom mass spectrometer is a next-generation instrument designed for high-throughput, high-sensitivity, and high-resolution applications with robustness and reproducibility at its core. Compared to the Thermo Scientific™ Orbitrap™ Astral™ Mass Spectrometer, the Orbitrap Astral Zoom MS achieves acquisition speeds of up to 270 Hz (35% faster) due to improved ion optics settling times and faster ion transfer. Additionally, it features enhanced spectral processing capabilities, higher sensitivity through ion pre-accumulation in the bent trap and “Low Input” application mode that increases single ion detection probability by 10%.

Experimental

Consumables and chemicals

- Fisher Chemical™ Optima™ LC-MS grade water with 0.1% formic acid (FA) (Cat. No. LS118-500)
- Fisher Chemical™ Optima™ LC-MS 80% acetonitrile (ACN) with 0.1% formic acid (Cat. No. LS122500)
- Fisher Chemical™ Optima™ LC-MS grade formic acid (Cat. No. A117-50)
- Fisher Chemical™ Optima™ LC-MS grade water (Cat. No. 10505904)
- Fisher Chemical™ Optima™ LC-MS grade acetonitrile (Cat. No. A955-1)
- Fisher Chemical™ Optima™ LC-MS grade isopropanol (Cat. No. A461-212)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), sequencing grade (Cat. No. 28904)
- Thermo Scientific™ n-Dodecyl-β-D-maltoside DDM (Cat. No. 89902)
- Eppendorf™ twin.tec™ 96 Well LoBind™ PCR Plates, Skirted (Cat. No. E0030129512)
- Axygen™ AxyMats™ 96 Round Well Sealing Mat for PCR Microplates (Cat. No. AM-96-PCR-RD)

Samples

- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (Cat. No. 88328)
- HEK293F single cells (gift from Erwin Schoof Lab)
- Promega™ MS-Compatible Yeast Protein Extract, Digest, 100 µg (Cat. No. V7461)
- Waters™ MassPREP™ *E. coli* Digest Standard (Cat. No. 186003196)

LC columns

- IonOpticks Aurora® Ultimate™ 25×75 XT C18 UHPLC column (Cat. No. AUR3-25075C18-XT)
- IonOpticks HeatSync™ Column Heater (Cat. No. COLHTR01)

HPLC system

- Thermo Scientific™ Vanquish™ Neo™ UHPLC system
- Thermo Scientific™ Vanquish™ Neo™ Pump/Autosampler (Cat. No. VN-S10-A-01)
- Thermo Scientific™ Vanquish™ Column Compartment (Cat. No. VN-C10-A-01)

Mass spectrometer

- Orbitrap Astral Zoom mass spectrometer
- Thermo Scientific™ FAIMS Pro Duo Interface (Cat. No. FMS03-10001)
- Thermo Scientific™ EASY-Spray™ NG Source (Cat. No. ES082)

Data analysis

- Biognosys Spectronaut® 19.5 Software

HeLa digest standard preparation

200 µL of aqueous 0.1% TFA with 0.015% DDM was added to a vial containing 20 µg of lyophilized Pierce HeLa Protein Digest Standard. The resulting mixture was then sonicated at room temperature for 5 minutes to obtain a stock solution of 100 ng/µL. Then, 5 µL of the stock solution was diluted to 5 ng/µL by adding 95 µL of 0.015% DDM solution in an Eppendorf 96-well low-binding autosampler well plate and vortexed at 2,000 rpm for a few seconds.

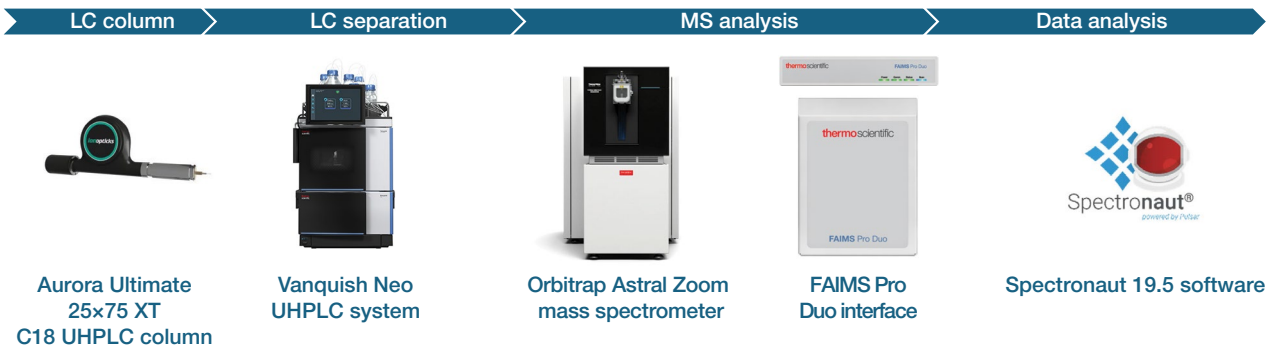

Figure 1. Single-cell proteomics workflow.

Single cell sample preparation

HEK293F cells were sorted on a Sony™ MA900 cell sorter by a fluorescence-activated cell sorting method into a 384-well Eppendorf LoBind PCR plate with 1 µL of lysis buffer consisting of 80 mM triethylammonium bicarbonate (TEAB) at pH 8.5 and 20% of 2,2,2-trifluoroethanol (TFE). After sorting, the plate was placed on dry ice for 5 minutes and stored at -80°C until further analysis. Before digestion, the plate was thawed and heated to 95°C on a PCR machine for 5 minutes followed by another freezing cycle. 2 ng of Promega™ Trypsin Platinum was added to each well. The samples were incubated at 37°C overnight and quenched with 1 µL of 1% TFA.

Three-proteome mix sample preparation (HeLa, yeast, *E. coli*)

Three-proteome mix samples of HeLa, yeast, and *E. coli* protein digests were prepared from lyophilized peptides from Pierce HeLa Protein Digest Standard, Promega MS-Compatible Yeast Protein Digest, and Waters MassPREP *E. coli* Digest Standard by re-suspension in aqueous 0.1% FA with 0.015% DDM to obtain a 100 ng/µL stock solution. Two mixtures, A and B, were prepared from the stock solution and diluted to 5 ng/µL before injection. Mix A contained 65% of HeLa digest, 15% of yeast digest, and 20% of *E. coli* digest. Mix B contained 65% of HeLa digest, 30% of yeast digest, and 5% of *E. coli* digest.

HPLC conditions

The LC separation was carried out using a Vanquish Neo UHPLC system configured in a direct injection mode and an Aurora Ultimate 25×75 XT C18 UHPLC column. The column was connected online to an EASY-Spray ion source. To enable efficient analysis of a large number of samples, an LC method with a throughput of 50 samples per day (SPD) was employed, with a total runtime of 28.8 minutes from injection to injection. The LC conditions and gradient method used for the separation are detailed in Tables 1 and 2.

MS acquisition

Data acquisition was performed on the Orbitrap Astral Zoom MS. The FAIMS Pro Duo interface was utilized to reduce background interference that affects peptide signals, especially at low sample amounts, thus improving the signal-to-noise ratio. MS acquisition parameters, DIA isolation window, and maximum injection time were adjusted accordingly to accommodate different loading amounts or numbers of cells on the column. All other parameters were kept constant. A detailed summary of the method parameters is shown in Tables 3 and 4.

Table 1. LC conditions.

HPLC method parameters	
Mobile phase A	0.1% formic acid (FA) in water
Mobile phase B	0.1% FA in 80% acetonitrile (ACN)
Flow rate	0.2 µL/min
Column	IonOpticks Aurora Ultimate 25 cm × 75 µm ID, 1.7 µm C18 (Cat. No. AUR3-25075C18-XT)
Column temperature	50°C
Autosampler temperature	7°C
Injection wash solvents	Strong wash: 0.1% FA in 80% ACN Weak wash: 0.1% TFA in water

Table 2. Gradient method.

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Run			
0	0	0.45	4
0.1	0.1	0.45	6
1.9	1.8	0.45	12
2	0.1	0.2	12.1
15	13	0.2	25.5
20.5	5.5	0.2	45
Column wash			
22	1.5	0.3	99
24	2	0.3	99
Stop run			
Column equilibration			

Data processing parameters

DIA raw data files from either triplicate runs of each HeLa sample amount or the entire dilution series were processed with Spectronaut 19.5 software using the directDIA+™ workflow with the Spectronaut® Pulsar search engine against the Human UniProt protein database (20,607 FASTA entries). For the Pulse search, the following FDR settings were used: PSM FDR = 0.01, Peptides FDR = 0.01, and Protein Group FDR = 0.0.1. For the DIA analysis, the default Qvalues were used for precursor (Precursor Qvalue Cutoff = 0.01, Precursor PEP Cutoff = 0.2) and proteins (Protein Qvalue Cutoff [experiment] = 0.01, Protein Qvalue Cutoff [run] = 0.05, and Protein PEP Cutoff = 0.75). For HEK293F cells, all raw files were processed together without a library or booster channel using the same Spectronaut parameters as described above for the dilution series.

Table 3. MS conditions.

Source properties	
Spray voltage (V)	2,000
Ion transfer tube temperature (°C)	275
FAIMS carrier gas (L/min)	3.5
Orbitrap full scan properties	
Orbitrap resolution	240,000
RF lens (%)	45
Scan range (*m/z*)	400–800
FAIMS CV	-48
Normalized AGC target (%)	500
Max injection time (ms)	100
Data-independent acquisition properties	
Precursor mass range (*m/z*)	400–800
Isolation width	See Table 4
Window placement optimization	On
HCD collision energy (%)	25
Detector type	Astral
Scan range (*m/z*)	150–2,000
Injection time (ms)	See Table 4
AGC target (%)	800
Loop control	Time
Time (s)	0.6

Table 4. Optimized DIA isolation window and maximum injection time for different sample loading amounts. (A) HeLa digest standard and (B) three-proteome mixture.

A

Isolation width, [Th]	Injection time, [ms]	Sample amount [pg]
20	60	HEK293F single cells
20	60	50
20	60	100
20	40	250
20	40	500
10	20	1,000
8	16	2,000

B

Isolation width, [Th]	Injection time, [ms]	Sample amount [pg]
20	60	100
20	40	250
15	40	500
12	30	1,000
10	20	2,000
5	10	5,000

For the three-proteome mix, raw files were processed with Spectronaut 19.5 software using the directDIA workflow. The search was performed against the Human UniProt protein database (20,607 FASTA entries), yeast (*Saccharomyces cerevisiae,* 6,059 entries), and *E. coli* (4,402 entries) databases with default settings and the same modifications as above. Normalization in Spectronaut software was performed after identification using the human FASTA. For each load, two sets of triplicates (three from mix A and three from mix B) were processed together. MS^1 quantitation was used for 100 pg to 1 ng load range, while MS^2 quantitation was used for 2 and 5 ng loads.

Results and discussion

Enhanced sensitivity with Orbitrap Astral Zoom MS Low Input mode

To evaluate the performance differences between the new Orbitrap Astral Zoom MS and the Orbitrap Astral MS, 250 pg from bulk HeLa digest was analyzed on five Orbitrap Astral Zoom MS and five Orbitrap Astral MS systems. The Orbitrap Astral Zoom MS systems were operated in the Low Input application mode. Triplicate raw data files from individual instruments were processed in Spectronaut software without using a library. Compared to the Orbitrap Astral MS results, the Orbitrap Astral Zoom MS results showed an average increase in protein groups of 6.8%, from 6,247 to 6,674 (Figure 2A) and a 20.8% increase in peptides, from 49,014 to 59,231 (Figure 2B).

High level of identifications and precision of quantitation

From bulk HeLa digest standard, we created a dilution series in the range of 50–2,000 pg and separated each sample amount using the same LC methods (Table 2) at a throughput of 50 SPD. The injection times and the DIA isolation width (Tables 3 and 4) were adjusted based on the sample amount.

Triplicate raw files from each sample amount were analyzed with Spectronaut software using a library-free approach. From 50 pg HeLa digest, we identified 5,681 protein groups and 41,724 peptides. Increasing the sample load to 250 pg resulted in higher numbers of identified protein groups (6,807) and peptides (57,362). At 2,000 pg, we identified 7,992 protein groups and 80,916 peptides (Figure 3). When all files were processed together using a library-free approach (match-between-runs (MBR)), we identified 7,371 and 7,848 protein groups and 61,700 and 74,752 peptides for 50 pg and 250 pg, respectively. As expected, this approach yielded higher numbers of identified protein groups compared to processing individual sample amounts.

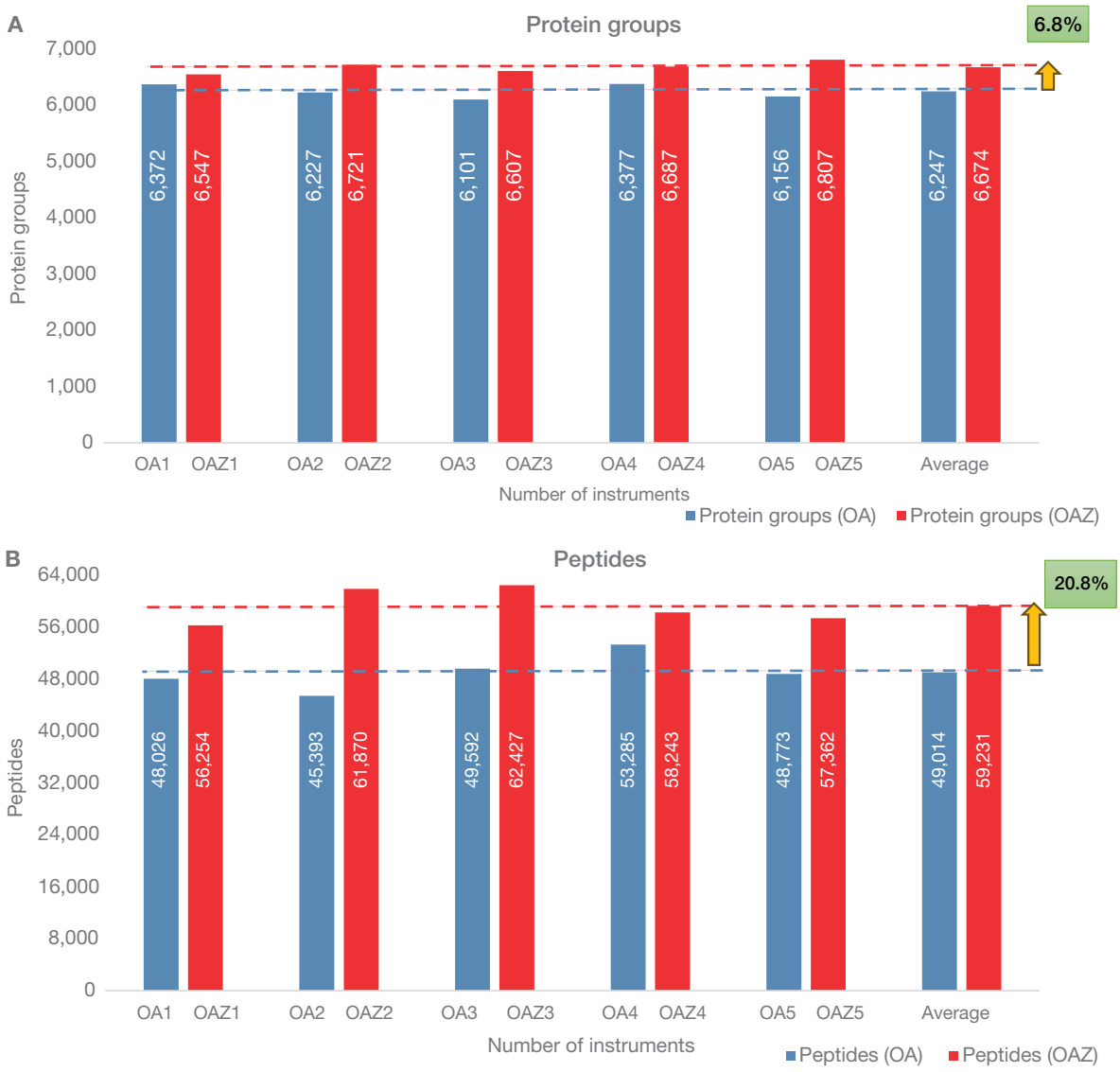

Figure 2. Histogram showing protein groups (A) and peptides (B) identified from five Orbitrap Astral Zoom instruments (red columns) and five Orbitrap Astral MS instruments (blue columns) and the average percentage gain in protein groups and peptides identifications for the Orbitrap Astral Zoom MS instruments relative to the Orbitrap Astral MS instruments.

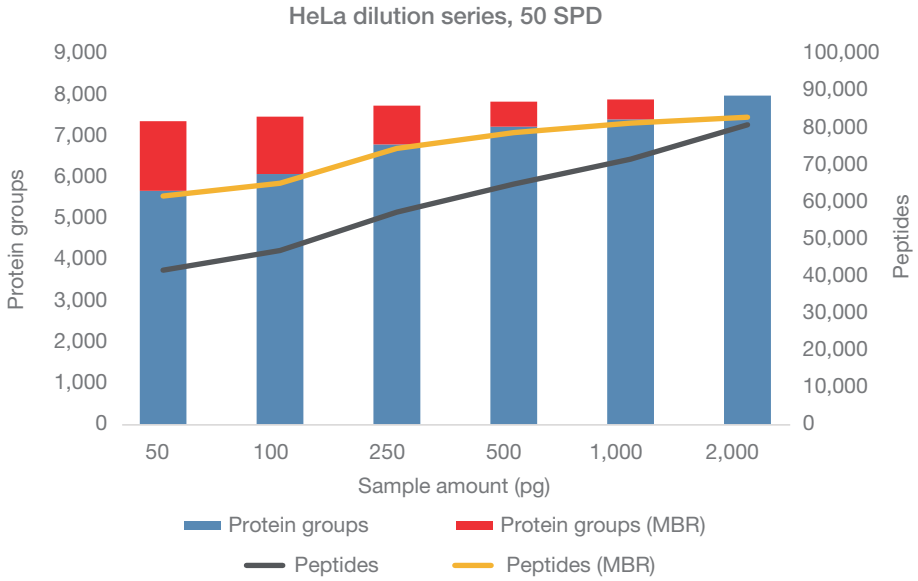

Figure 3. Peptides and protein groups identified from a dilution series of bulk HeLa digest acquired on the Orbitrap Astral Zoom MS using Low Input mode. The data was searched with Spectronaut 19.5 software using a library-free approach. The blue section of the stacked columns represents results obtained when triplicate raw files from each sample amount were searched together, and the whole column represents results obtained when raw files from the entire dilution series were processed together.

Next, we evaluated the reproducibility of protein quantification. When processing triplicate raw files for each sample amount, 86–88% of quantified proteins had CVs below 20%, and 67–71% had CVs below 10% (Figure 4). When processing raw files from the entire dilution series together, 70% of identified proteins had CVs <20%, and 58% had CVs <10%. The median coefficients of variation (CVs) of protein abundances across triplicate runs for each sample amount in the dilution series were less than 6.1%, and less than 7.5% when files from the entire dilution series were processed together. These results demonstrate the excellent reproducibility of the Orbitrap Astral Zoom MS.

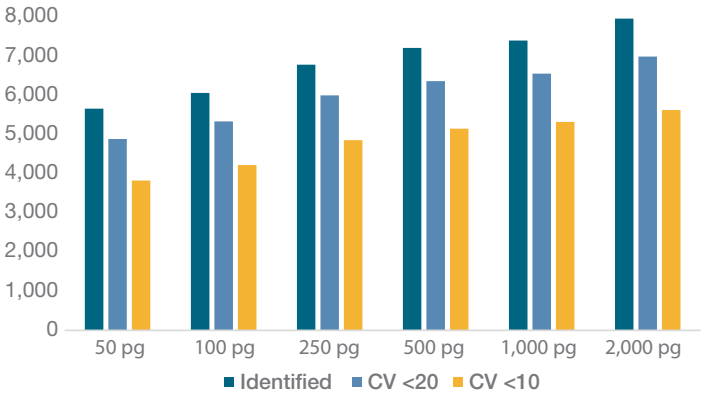

Figure 4. Total protein groups identified in triplicate runs (n=3) with protein groups CVs <20% (light blue) and <10% (gold) analyzed using a library-free search.

Quantitative performance

While CVs provide a good indication of quantitative precision across technical replicates, accuracy and precision of protein quantification are best demonstrated by comparing mixtures of different samples with known concentrations. For the evaluation of accuracy and precision on the Orbitrap Astral Zoom MS, we used a three-proteome mix sample consisting of human, yeast, and *E. coli*. Two samples, A and B, were made from the three proteome digests. The concentrations of yeast and *E. coli* were varied to mimic fold changes in biological systems, while human was kept constant. We then analyzed 250 pg of sample A and B on the Orbitrap Astral Zoom MS and Orbitrap Astral MS. For each sample amount, two sets of triplicates (three from mix A and three from mix B) were processed together using a directDIA+ workflow in Spectronaut 19.5 software (Figure 5).

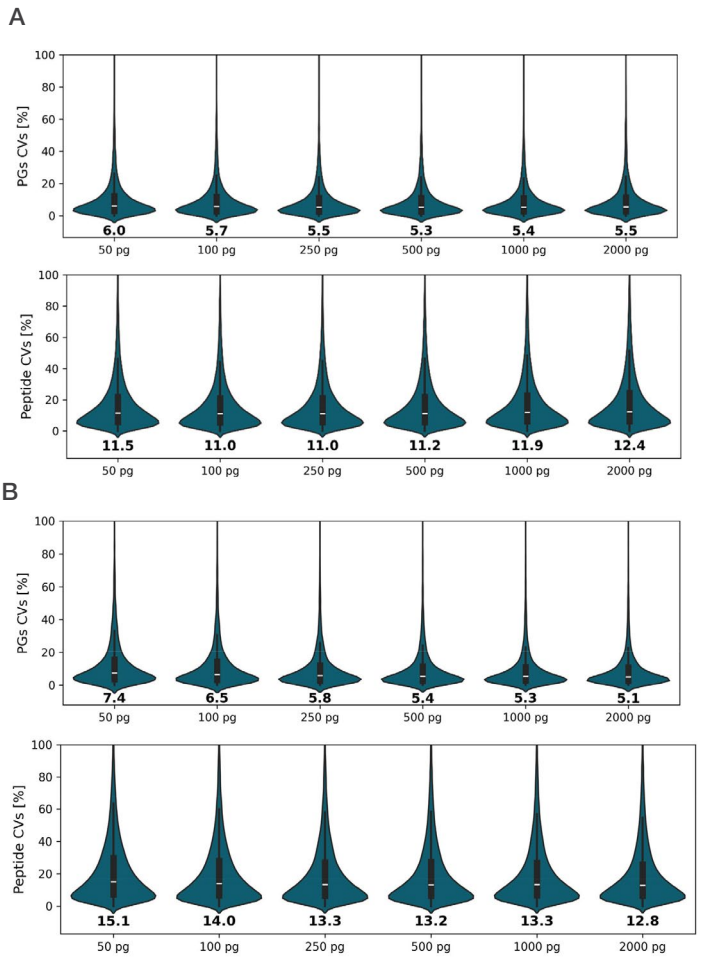

Figure 5. Median coefficients of variation for protein groups and peptides of the HeLa digest dilution series acquired with the Orbitrap Astral Zoom MS using Low Input mode. (A) Triplicates of each sample amount were searched together using the directDIA+ approach in Spectronaut software. (B) The raw files of the entire dilution series were searched together using the directDIA+ approach in Spectronaut software.

The results in Figure 6A show that more than 8,390 protein groups were identified on the Orbitrap Astral Zoom MS; this is approximately 5% more than on the Orbitrap Astral MS (8,018 protein groups). Also, more than 8% additional peptide identifications were obtained with the Orbitrap Astral Zoom MS, 55,211 peptides, compared to 51,003 on the Orbitrap Astral MS (Figure 6A). The quantitative accuracy obtained with the Orbitrap Astral Zoom MS for the 250 pg load was within 0.6% for *E. coli* and 5.5% for yeast of the expected ratios (Figure 6B).

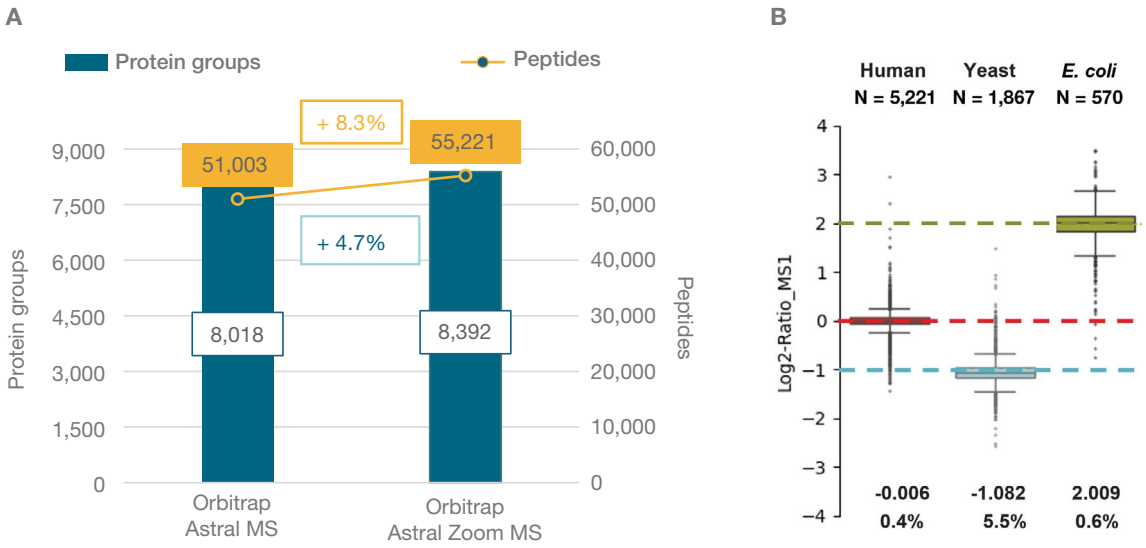

Figure 6. (A) Gain in peptide and protein identifications using the Orbitrap Astral Zoom MS with Low Input application mode for 250 pg three-proteome mixture and (B) boxplots depicting the protein abundance ratio measurement accuracy for the 250 pg three-proteome mixture. Six files (triplicates from mixA and mixB) were searched together using the directDIA approach in Spectronaut software. The numbers of identifications per proteome were obtained after filtering out missing values from triplicate runs. Absolute and relative (%) errors for each proteome are shown in the lower part of the figure.

Furthermore, a dilution series from 100 to 5,000 pg was analyzed with the Orbitrap Astral Zoom MS at a throughput of 50 SPD. The raw files from each sample amount were processed the same way as the 250 pg sample amount. A total of 7,283 protein groups and 44,374 modified peptides were identified in the 100 pg sample. This demonstrates the sensitivity of the Orbitrap Astral Zoom MS in the Low Input application mode. The depth of proteome coverage consistently increased with increasing sample amount. At 5,000 pg, we identified over 12,700 protein groups and over 121,000 peptides (Figure 7). The median protein group CVs for all sample amounts were below 8% (Figure 8), indicating low variation between measurements.

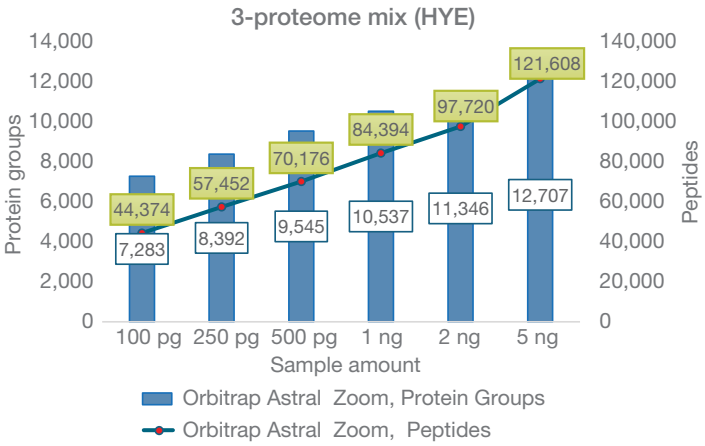

Figure 7. Dilution series of the three-proteome mix showing the total number of protein groups and modified peptides identified using the Orbitrap Astral Zoom MS. For each load, six files (triplicates from mixA and mixB) were searched together using the directDIA approach in Spectronaut software.

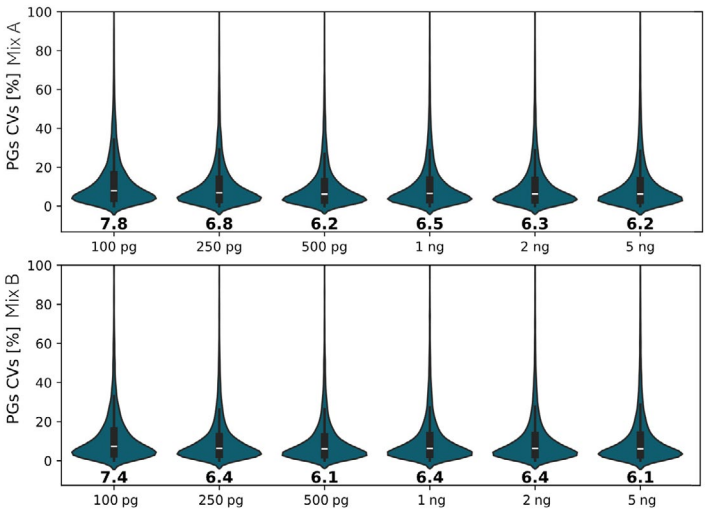

Figure 8. Median coefficients of variation for 100 pg to 5 ng of the three-proteome mix, using the Orbitrap Astral Zoom MS with Low Input application mode. Data was analyzed with a library-free approach using directDIA+ in Spectronaut software.

Since the ratio of proteomes in mixtures A and B is known, quantitation accuracy can be determined from the distribution of ratios obtained for individual proteins around the expected fold change. A relative error of experimental median log_2-ratio was within 7.3% for *E. coli* and 6.1% for yeast across the entire dilution series (Figure 9 and Table 5).

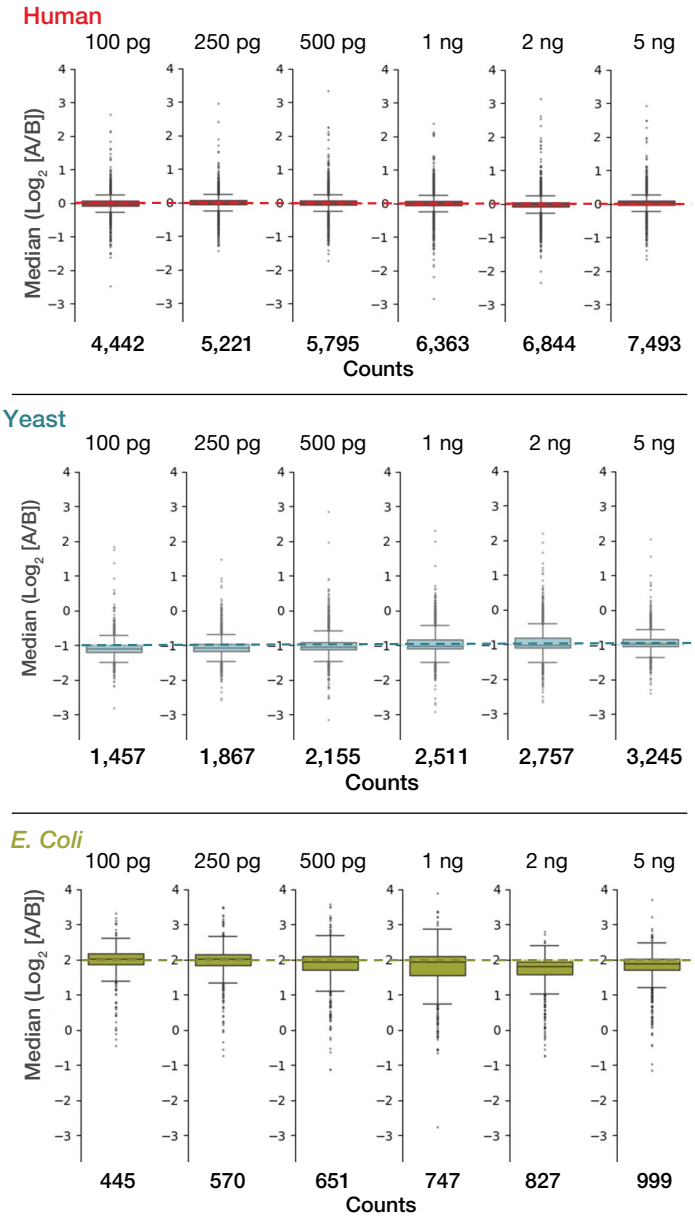

Figure 9. Boxplots depicting the median log_2 of the protein abundance ratio measurement accuracy for human, yeast, and *E. coli* proteomes for loads from 100 pg to 5 ng of the three-proteome mix, using the Orbitrap Astral Zoom MS with Low Input application mode. For each load, six files (triplicates from mixA and mixB) were searched together using the directDIA approach in Spectronaut software. Number of counts represents the number of quantified proteins per proteome after filtering out missing values from triplicate runs. Median log_2 values were calculated based on the abundance data for these counts.

Table 5. Quantitation accuracy for the three-proteome mix samples from 100 pg to 5 ng loads, using the Orbitrap Astral Zoom MS. Six files (triplicates from mixA and mixB) were searched together using the directDIA approach in Spectronaut software. Quantitation accuracy was within 7.5% of the expected across the entire low-input load range analyzed.

Load	Human	Human	Human	Yeast	Yeast	Yeast	*E. coli*	*E. coli*	*E. coli*
	Median log_2-ratio	Calculated median ratio	Relative error, %	Median log_2-ratio	Calculated median ratio	Relative error, %	Median log_2-ratio	Calculated median ratio	Relative error, %
100 pg	-0.004	0.997	0.26	-1.091	0.469	6.1	2.021	4.058	1.4
250 pg	-0.006	0.996	0.44	-1.082	0.473	5.5	2.009	4.024	0.6
500 pg	-0.003	0.998	0.18	-1.043	0.485	2.9	1.939	3.835	4.1
1 ng	0.002	1.002	0.16	-1.014	0.495	0.9	1.932	3.815	4.6
2 ng	-0.010	0.993	0.69	-1.018	0.494	1.2	1.890	3.707	7.3
5 ng	-0.003	0.998	0.19	-0.965	0.512	2.5	1.893	3.714	7.2

Sensitive and reproducible measurements on true single cells

For the evaluation of our method with true single cells, we selected the HEK293 cell line. It is derived from embryonic kidney cells and has been widely used in research due to its reliable and fast growth and ease of transfection. Hence, HEK293 cells are often used to produce therapeutic proteins and viruses for gene therapy.[3] The HEK293F variant that was used for our experiments was grown, sorted, and digested as described in the Experimental section. The final volume of 2 µL from each individual digested cell was then injected, separated, and analyzed on the Orbitrap Astral Zoom MS using the 50 SPD method (see the LC-MS method details in Figures 1 and 2). All generated raw files were processed in Spectronaut 19.5 software without a library. From 24 individual single HEK293F cells, we identified an average of 5,842 protein groups and 34,538 peptides (Figure 10). To our knowledge, this is by far the highest number of protein groups identified from HEK293F cells.

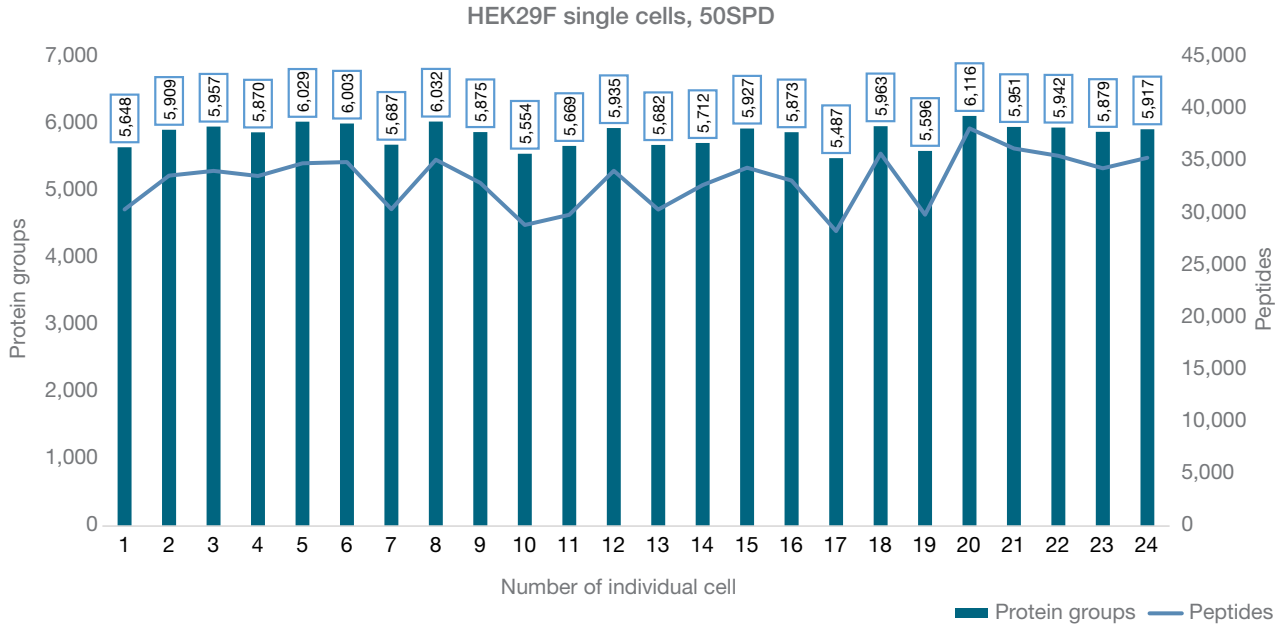

Figure 10. Number of protein groups, precursors, and peptides identified across 24 isolated HEK293F single cells using the 50 SPD method. Data were searched using Spectronaut 19.5 software with a library-free approach.

Conclusion

- The high-resolution DIA workflow shows sensitive and reproducible results on the Orbitrap Astral Zoom mass spectrometer for single-cell and low-input proteomics applications.
- Through the combination of advanced instrumentation, optimized DIA workflows, and complementary technologies such as the FAIMS Pro Duo interface and Vanquish Neo UHPLC system, we achieved deep, sensitive, and reproducible proteome coverage from sample inputs as low as 50 picograms.
- The Low Input application mode significantly improved identification rates, enabling detection of 6,674 protein groups on average from 250 pg HeLa standard digests and an average of 5,842 protein groups from individual HEK293F cells.
- The workflow also delivered high quantitative accuracy, as shown by the tight distribution of measured protein abundance ratios for the three-proteome mixtures, closely matching expected fold changes (relative error of quantitation within 7.5% across 100–5,000 pg load range). Together, these results confirm that the Orbitrap Astral Zoom MS is an excellent choice for high-throughput, high-sensitivity proteomic analysis and represents a transformative solution for routine single-cell proteomics across diverse biological and clinical research applications.

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