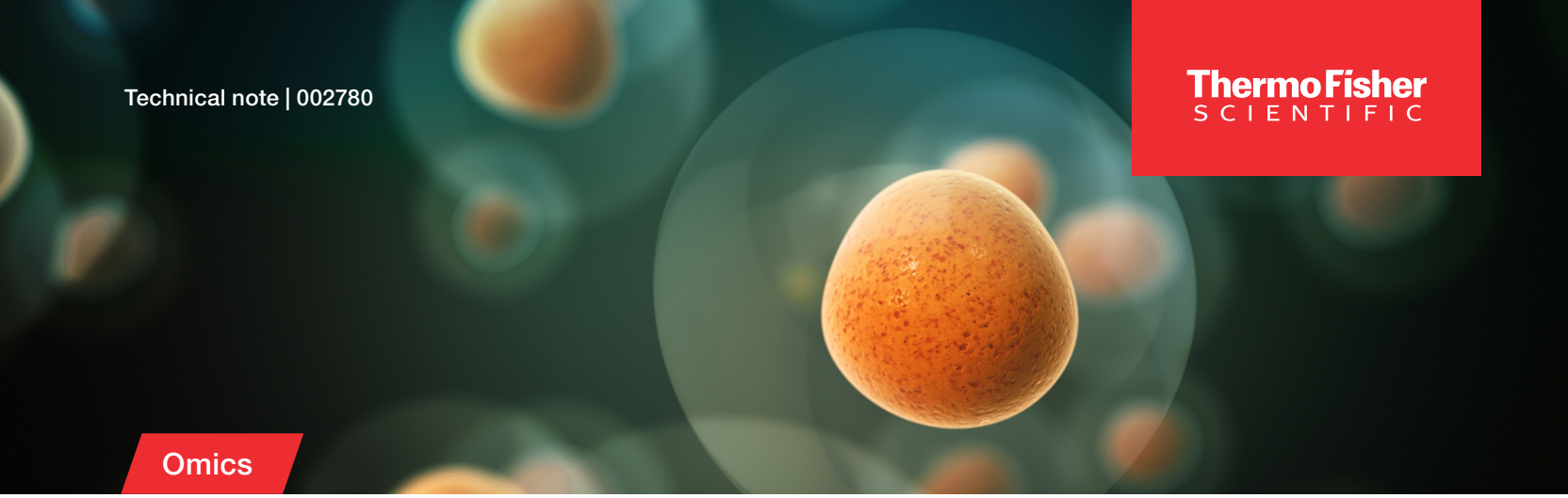

Technical note | 002780

Omics

Deeper proteome coverage and faster throughput for single-cell samples on the Orbitrap Astral mass spectrometer

Authors

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Keywords

Orbitrap Astral mass spectrometer, Vanquish Neo UHPLC, column, FAIMS Pro interface, data-independent acquisition, proteomics

Goal

To assess proteome coverage and sample throughput performance for single-cell samples using a library-free and library-based data-independent acquisition (DIA) method on the new Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer

Introduction

In recent years, there has been a shift to analyzing smaller sample quantities, driven by the interest in profiling the proteome of individual cells. This increased focus on LC-MS analysis of limited sample amounts (e.g., single cells) requires the highest possible sensitivity. At the same time, throughput and chromatographic performance must be preserved to generate sufficiently high-quality data from large data sets to draw meaningful conclusions. This trend toward analyzing smaller sample quantities reflects the need to understand the heterogenous nature of biology through the dissection of complex systems into their individual parts or specifically individual cells. Traditional proteomics approaches read out the average of a sample, while single-cell proteomics provides insights into the exact nature of each of the components in such samples.

The Orbitrap Astral mass spectrometer delivers a new standard for single-cell samples, with both deeper proteome coverage and faster throughput due to its high sensitivity. The Orbitrap Astral mass spectrometer enables this deeper coverage and high throughput through the synchronized acquisition of high-resolution and high-dynamic range full scan spectra with the Thermo Scientific™ Orbitrap™ mass analyzer and high speed and sensitivity MS/MS spectra with the Thermo Scientific™ Astral™ mass analyzer. The Thermo Scientific™ Vanquish™ Neo UHPLC system delivers maximum performance in terms of precise low flow rates and gradient formation, as well as low injection volumes, an important benefit for single-cell proteomics and limited sample amounts. The Thermo Scientific™ µPAC™ Neo HPLC columns with novel micro-pillar array deliver highly reproducible chromatographic separation and increased sensitivity with maximum resolution. The Thermo Scientific™ FAIMS Pro interface enhances sensitivity by removing background signals.

In this technical note, we present a single-cell proteomics workflow (Figure 1). To first evaluate sensitivity and quantitative precision, accuracy, and linearity of the workflow, a dilution series from 5 ng to 50 pg of the Thermo Scientific™ Pierce™ HeLa protein digest standard was analyzed.

To demonstrate the new standard in qualitative and quantitative performance, HeLa cells prepared on a Cellenion CellenONE™ Sample Prep Platform were analyzed on the Orbitrap Astral mass spectrometer using the data-independent acquisition (DIA) method. The data was analyzed using Spectronaut™ 18 software with library-free and library-based approaches and using Thermo Scientific™ Proteome Discoverer™ 3.1 software.

Experimental

Recommended consumables

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (FA) (P/N LS118-500)
- Fisher Scientific™ LC-MS grade 80% acetonitrile (ACN) with 0.1% formic acid (P/N LS122500)
- Fisher Scientific™ LC-MS grade formic acid (P/N A117-50)
- Fisher Scientific™ Optima™ LC-MS grade water (P/N 10505904)
- Fisher Scientific™ Optima™ LC-MS acetonitrile (P/N A955-1)
- Fisher Scientific™ Optima™ LC-MS isopropanol (P/N A461-212)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), sequencing grade (P/N 28904)

Samples

- Pierce HeLa protein digest standard (P/N 88328)

LC columns

- Thermo Scientific™ µPAC™ Neo HPLC 50 cm column (P/N COL-NANO050NEOB)
- Fused silica emitters, 10 µm i.d. (EvoSep Biosystems, P/N EV1111)
- IonOpticks Aurora Ultimate TS 25 cm column (AUR3-25075C18-TS)

HPLC system

- Vanquish Neo UHPLC system (P/N VN-S10-A-01) that includes a Vanquish Column Compartment (P/N VN-C10-A-01)

Mass spectrometer

- Orbitrap Astral mass spectrometer
- FAIMS Pro Duo interface
- Thermo Scientific™ EasySpray™ ion source
- Column oven for µPAC columns (P/N PRSO-V2-PF)

Data analysis software

- Spectronaut 18 software (Biognosys AG)
- Proteome Discoverer 3.1 software

HeLa digest standard

All proteomics experiments were performed using the Pierce HeLa protein digest standard. 100 µL of 10% ACN in 0.1% TFA was added to the vial containing 20 µg of protein digest. The vial was then sonicated at room temperature for 5 minutes, followed by adding another 100 µL of 0.1% TFA to make a final concentration of 100 ng/µL.

To the autosampler vial, 95 µL of 0.1% TFA and 5 µL of 100 ng/µL HeLa digest were added to make the final concentration to 5 ng/µL. This solution was pipetted up and down several times to mix and centrifuged at 8,000 × g for 2 minutes.

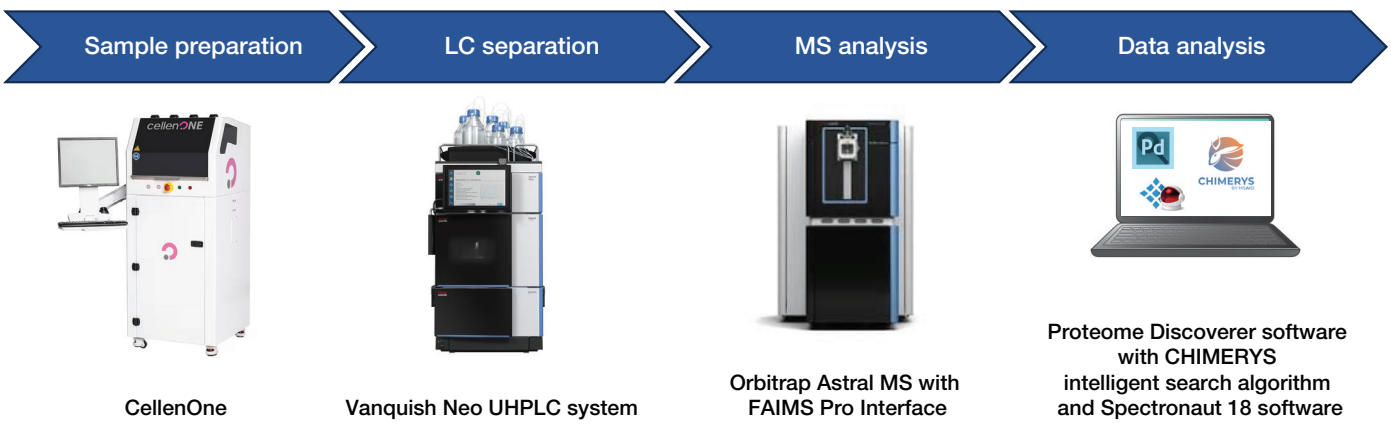


Figure 1. Single-cell proteomics workflow with the Orbitrap Astral mass spectrometer for a new standard in sensitivity, depth of coverage, and quantitative precision and accuracy. Library-based searches were processed with Spectronaut 18 software. Spectral libraries were generated using DIA data with the Pulsar search engine in Spectronaut 18 software against the Human UniProt Protein database containing 20,607 sequences.

LC conditions

To evaluate the performance of our workflow, an 80 sample per day (SPD) method was employed, consisting of an 8-minute gradient and 10-minute washing, equilibration, and injection steps for a total injection-to-injection cycle time of 18 minutes. HPLC conditions are described in Table 1, with the gradient details for the 80 SPD method in Table 2 and the 50 SPD method in Table 3.

Table 1. HPLC conditions

HPLC method parameters	
Mobile phase A	0.1% FA in water
Mobile phase B	0.1% FA in 80% ACN
Flow rate	0.2 µL/min
Column	50 cm µPAC Neo HPLC column, C_{18} silicon chip stationary phase
Column temperature	50 °C
Autosampler temperature	7 °C
Injection wash solvents	• Strong wash: 0.1% FA in 80% ACN • Weak wash: 0.1% FA in water
Needle wash	Enabled after-draw

Table 2. 80 SPD method (µPAC Neo HPLC 50 cm column)

	Time (min)	Duration (min)	%B	Flow rate (µL/min)
	Run			
Active gradient	0.0	0.0	4.0	0.75
	0.2	0.2	8.0	0.75
	2	1.8	18.0	0.75
	2.1	0.1	18.1	0.2
	5.1	3.0	28.0	0.2
	7.6	2.5	48.0	0.18
	Column wash			
	8	0.4	99.0	0.18
	16	8.0	99.0	0.18
	Stop run			
	Column equilibration			

Table 3. 50 SPD method (Aurora Ultimate TS 25 cm column)

	Time (min)	Duration (min)	%B	Flow rate (µL/min)
	Run			
Active gradient	0.0	0.0	1.0	0.45
	0.1	0.1	4	0.45
	1.9	1.8	12	0.45
	2.0	0.1	12	0.2
	12	10.0	22.5	0.2
	19.5	6.5	40.0	0.2
	Column wash			
	22	2.5	99.0	0.3
	25	3	99.0	0.3
	Stop run			
	Column equilibration			

MS parameters

The Orbitrap Astral mass spectrometer was operated with the parameters shown in Table 4.

Table 4. MS parameters

Source parameters	
Spray voltage	1.9 kV
Capillary temperature	275 °C
FAIMS CV	–45 V
Orbitrap MS full scan parameters	
Resolution	240 k
Normalized AGC target	500%
Maximum IT	100 ms
RF lens	45%
Scan range	400–800 *m/z*
Astral DIA MS^2 scan parameters	
Precursor mass range	400–800 *m/z*
DIA window type	Auto
Window placement optimization	On
DIA window mode	*m/z* Range
HCD collision energy	25%
Scan range	150–2,000 *m/z*
RF lens	45%
Normalized AGC target	800%

The DIA isolation window and maximum injection time used in these experiments were varied based on the sample load as shown in Table 5.

Table 5. DIA isolation windows and maximum injection times for the dilution series

	Sample amount			
	≤500 pg	750 pg–1 ng	2 ng	5 ng
DIA isolation window (*m/z*)	20	10	8	5
Maximum injection time (ms)	40	20	14	10

Data processing parameters

The HeLa protein digest standard dilution data containing triplicates of 50 pg, 100 pg, 250 pg, 500 pg, 750 pg, 1 ng, 2 ng, and 5 ng DIA runs were processed using individual triplicate runs with Spectronaut 18 software using the directDIA™ workflow with the Spectronaut™ Pulsar search engine, against the Human UniProt protein database (20,607 reviewed, canonical entries). A false-discovery rate (FDR) of 1% was applied at the precursor, peptide, and protein levels. Default parameters were used.

Table 6. DIA isolation windows and maximum injection times for the single cells

Number of cells	1 cell	20 cells
DIA isolation window (*m/z*)	20	5
Maximum injection time (ms)	40	10

Results and discussion

Workflow check before running single cells

Before running single cells, it is advised to perform a system check using a dilution series or at a minimum, a diluted standard. In addition to a list of protein identifications, quantitative data is necessary to study biomarkers and gain insights into biological pathways. The quantitative data must be highly precise, accurate, and linear to reflect subtle changes in biological systems.

To assess the quantitative performance, we injected three replicates of a dilution series consisting of 50 pg, 100 pg, 250 pg, 500 pg, 750 pg, 1 ng, 2 ng, and 5 ng. This covers most of the range of single-cell protein content. Processing the replicates together in library-free mode resulted in more than 2,700 protein groups quantified for 50 pg to more than 6,650 protein groups software quantified for 5 ng (Figure 3). Processing all files together (Figure 4) resulted in an increased number of quantified protein groups ranging from almost a 70% increase in numbers for 50 pg to no increase for 5 ng. On average, this method produced more than 6,100 quantified protein groups for 250 pg.

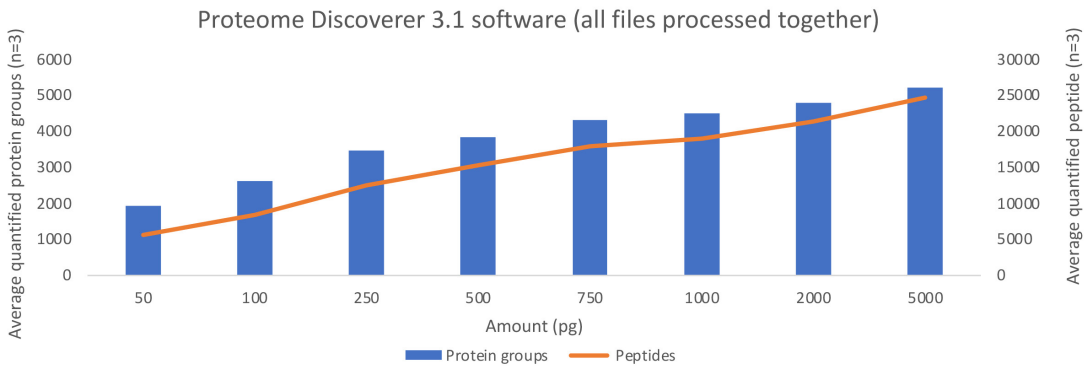


Figure 2. Quantified protein groups and peptides for the 80 SPD method, processing all files together using Proteome Discoverer 3.1 software

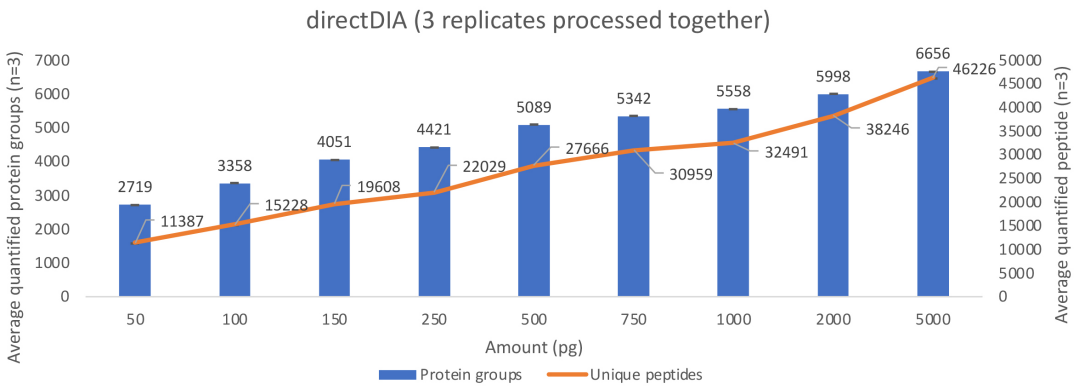
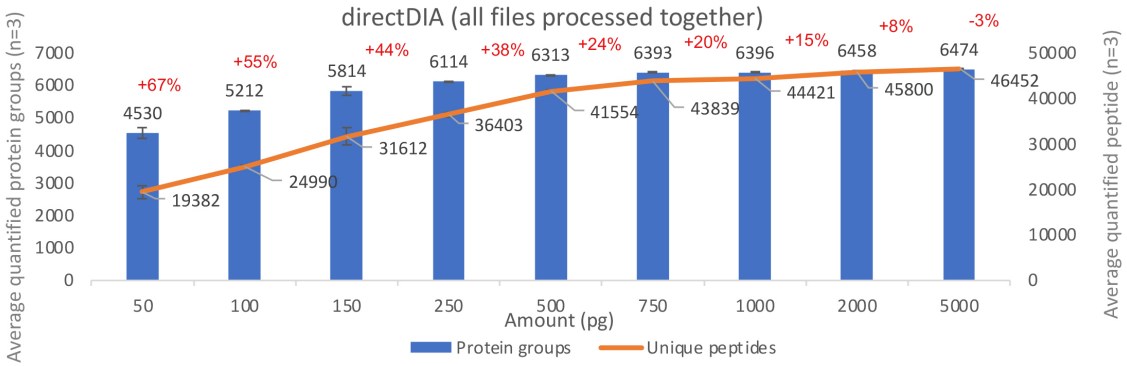


Figure 3. Quantified protein groups and peptides for the 80 SPD method; number method, processing the replicates together using Spectronaut 18 software



Red numbers indicate the increase in percentage compared to the results obtained by processing the replicates together (Figure 3).

Figure 4. Quantified protein groups and peptides for the 80 SPD method; number, processing all files together using Spectronaut 18 software

Precision, accuracy, and linearity of the workflow

The median CVs of all the dilution points are below 10%; for 250 pg it is 7% (Figure 5). Processing all the files together slightly increases the median CV for most of the dilution points but is still below 10%, except for 50 pg. The increase for the lowest dilution point can be explained by the fact that approximately 70% more (very low abundant) proteins are quantified.

The accuracy of the ratios is shown in a box and whisker plot (Figure 6). The measured values are very close to the expected ratios for all the proteins. By processing all the files together, the accuracy is improved, especially for the larger ratios (1/10 to 1/100).

Using all dilution points, calibration curves are drawn for each protein, divided into five abundance quantiles (Figure 7, left). Even for the lowest abundant proteins, the vast majority of the calibration curves have a linear correlation of >0.9. This is further improved by processing all files together (Figure 7, right).

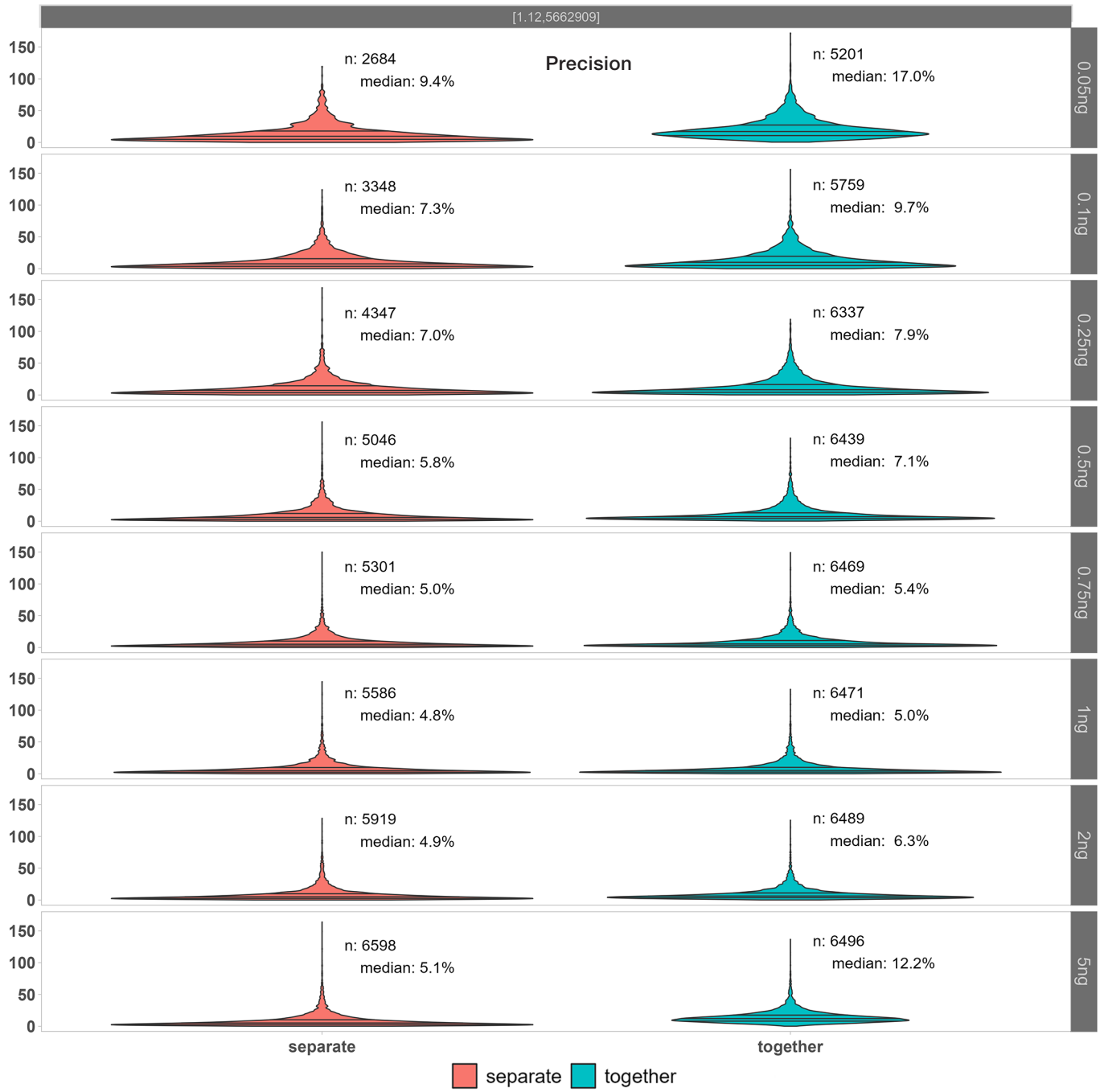


Figure 5. Precision of the two processing methods using Spectronaut 18 software

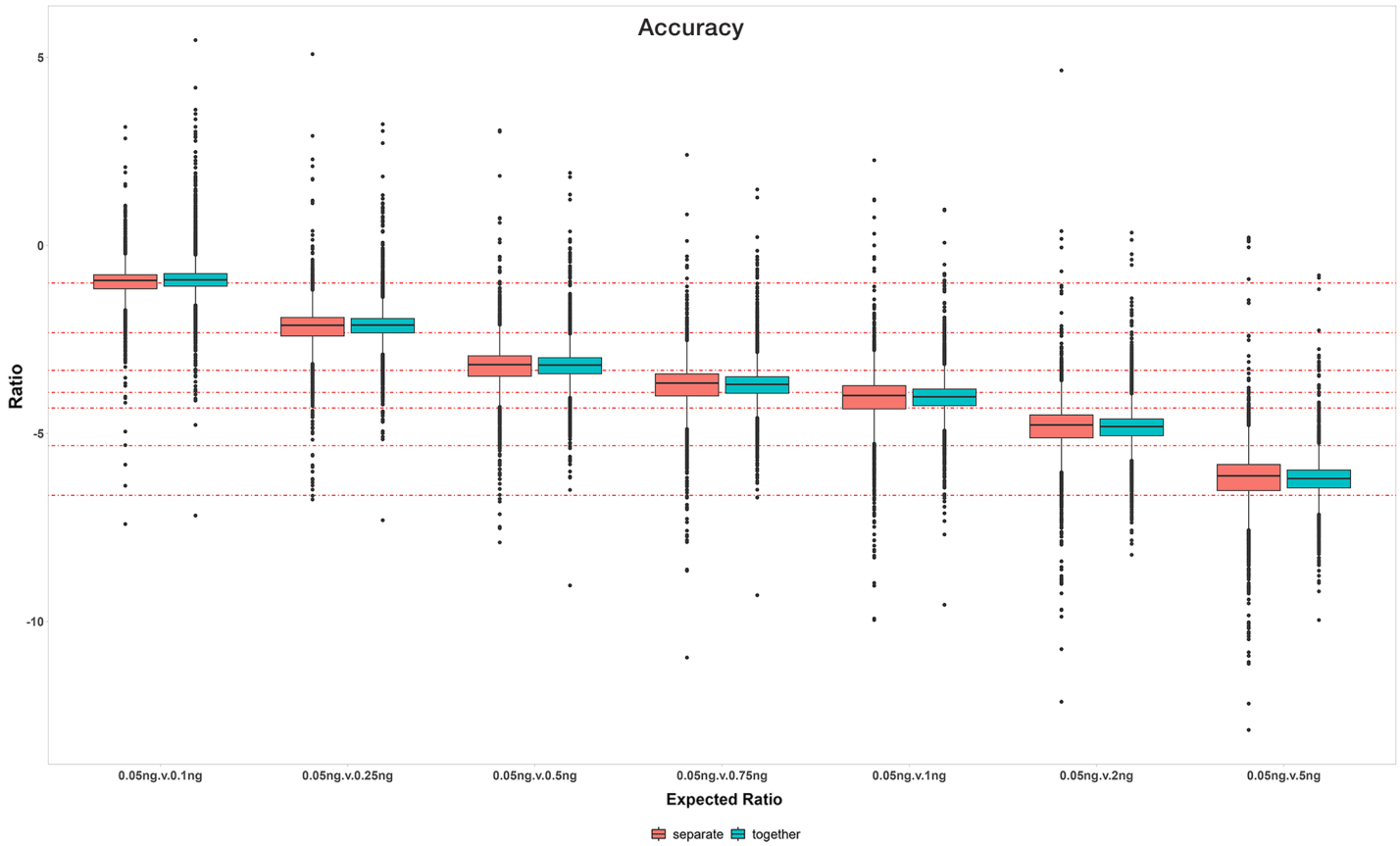


Figure 6. Accuracy of the protein ratios for both processing methods using Spectronaut 18 software

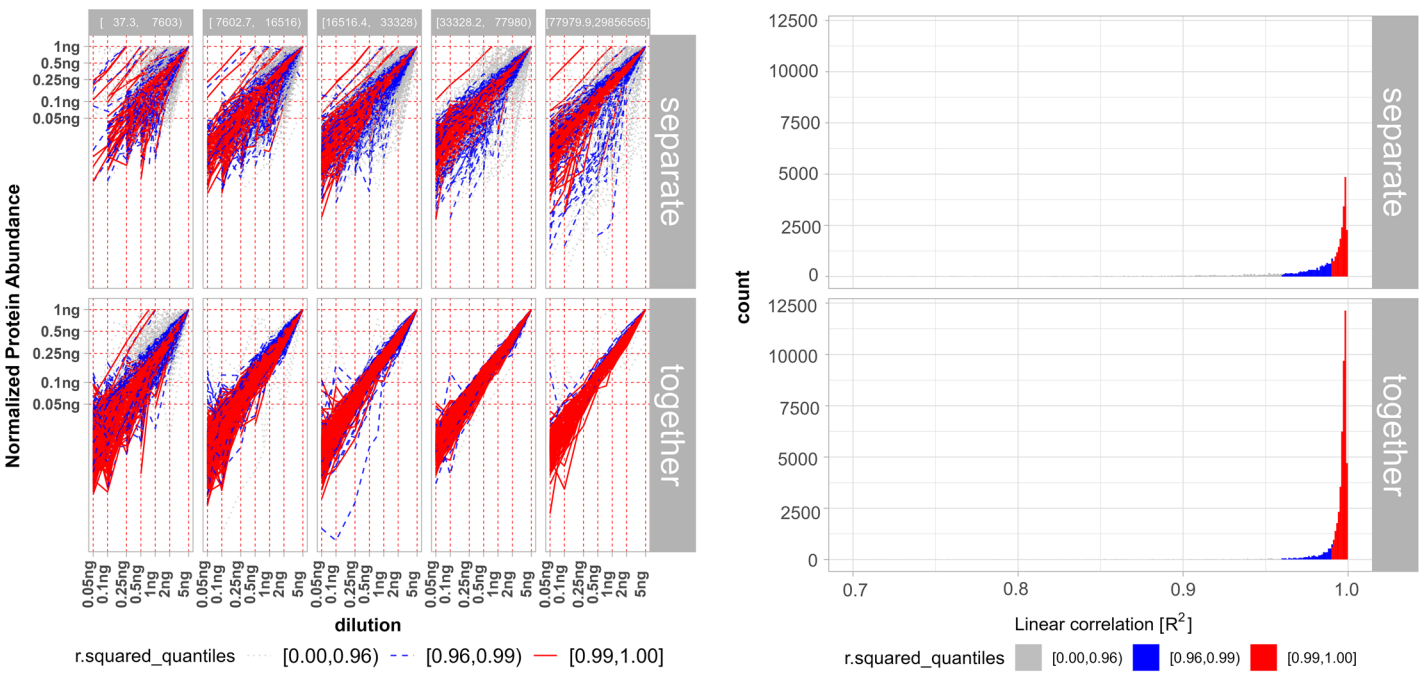


Figure 7. Linear correlation of the calibration curves of the proteins (left) and density plots of the linear correlation coefficient (right) using Spectronaut 18 software

Maximizing depth

When the highest number of identified and quantified protein groups and peptides is required, a 50 SPD method can be used (Figures 8, 9, and 10).

Almost 7,700 protein groups can be identified, on average, for 250 pg of a HeLa dilution (Figure 10).

Reproducibility and inter-lab comparison

To test the reproducibility of the methods, a 250 pg HeLa dilution was analyzed in triplicate on three different Orbitrap Astral mass spectrometers, in three different locations, and by three different operators (Figure 11). The relative standard deviation (RSD) of the quantified protein groups and quantified peptides was 2%. On average, >6,000 protein groups and more than 42,000 peptides were quantified using the 50 SPD method and processed with directDIA (Spectronaut 18 software).

Using a library of 10 ng, the number of quantified protein groups is increased by 27% to more than 7,700, and the number of quantified peptides is increased by 61% to more than 66,000 (Figure 12).

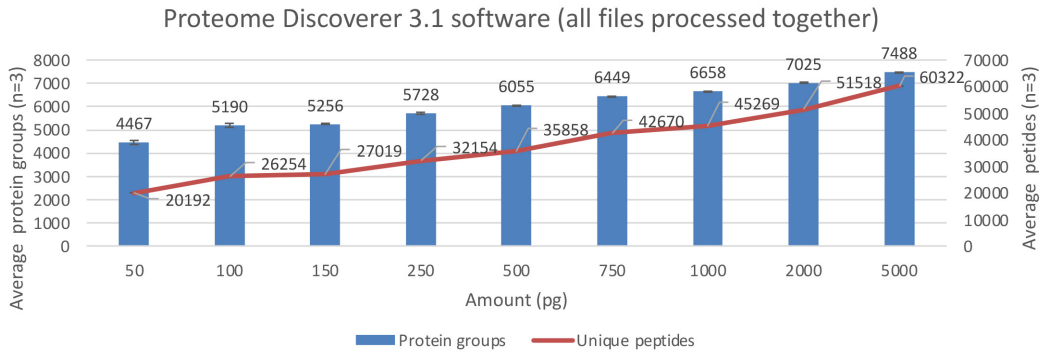


Figure 8. Identified protein groups and peptides for the 50 SPD method processing all files together using Proteome Discoverer 3.1 software

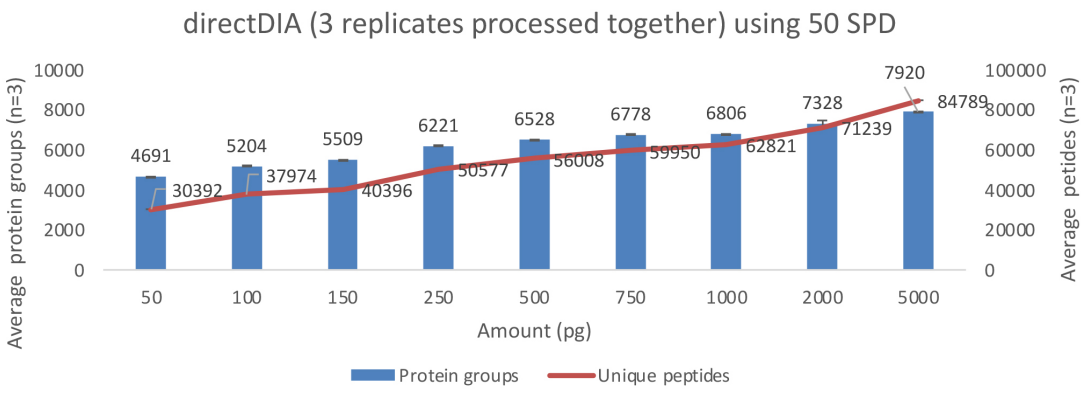


Figure 9. Identified protein groups and peptides for the 50 SPD method processing the replicates together using Spectronaut 18 software

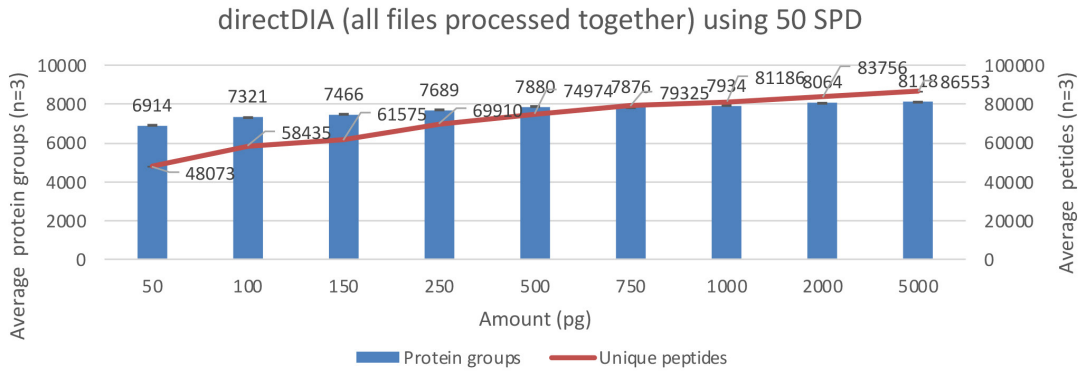


Figure 10. Identified protein groups and peptides for the 50 SPD method processing all files together using Spectronaut 18 software

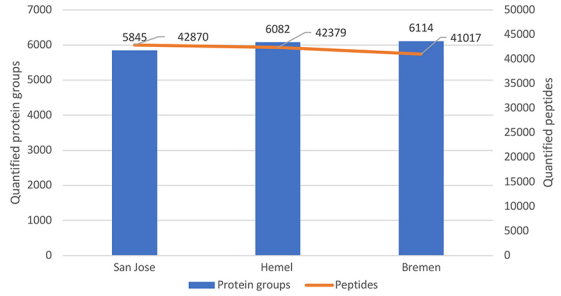


Figure 11. Inter-lab comparison of 250 pg HeLa dilution using the 50 SPD method

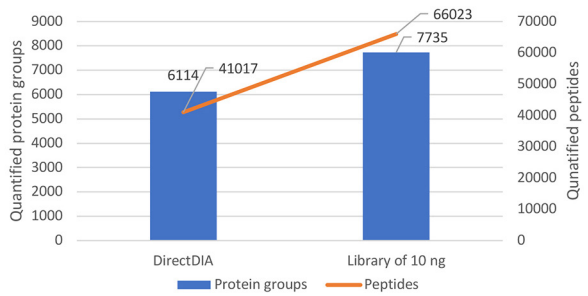


Figure 12. Overview of different search strategies for 250 pg HeLa dilution

Single-cell data

Using the 80 SPD method, 12 single cells were analyzed from HeLa. The result of the individually processed raw files can be found in Figure 13. On average, >3,400 protein groups and 15,300 peptides were quantified using directDIA (Spectronaut 18 software) from each cell (Figure 14). Processing the 12 single cells together on average resulted in the quantification of >4,250 protein groups and >23,550 peptides (Figure 15).

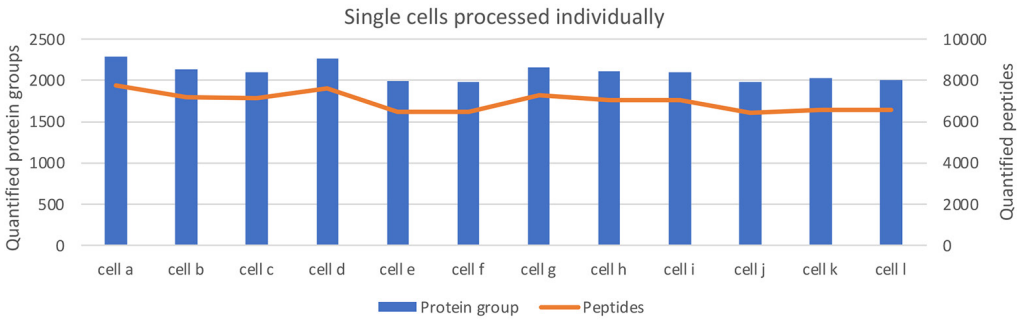


Figure 13. Number of quantified protein groups and peptides for individual single cells using Proteome Discoverer 3.1 software for the 80 SPD method

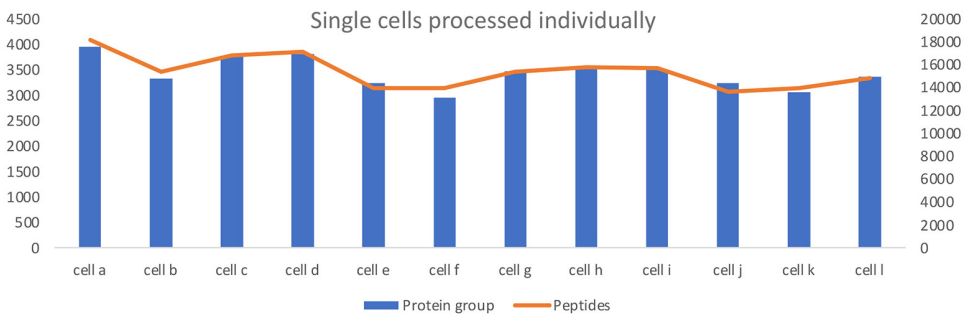


Figure 14. Number of quantified protein groups and peptides for individual single cells using directDIA (Spectronaut 18 software) for the 80 SPD method

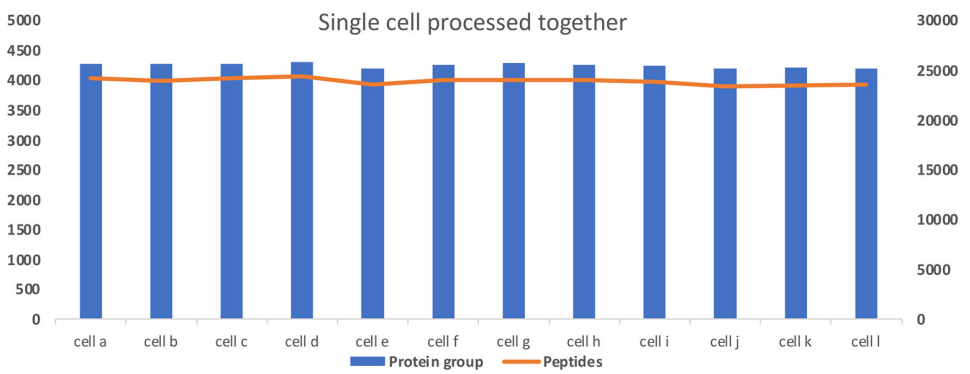


Figure 15. Number of quantified protein groups and peptides for individual single cells using directDIA (Spectronaut 18 software) for the 80 SPD method when all files are processed together

Figure 16 shows an overview of processing methods. In addition to the above-described processing methods, two library search-based methods were applied. The first has a library of 12 single cells, and the second has a library of three raw files of 20 single cells. There is almost no difference between processing all files together and using a library of 12 single cells. However, when using the library of 20 cells, the number of quantified protein groups and peptides increased to >5,370 and 30,770, respectively.

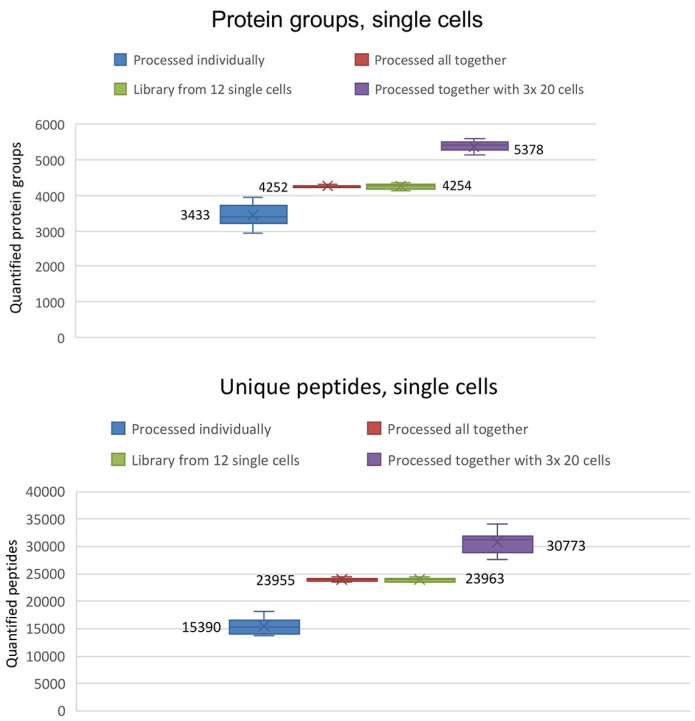


Figure 16. Overview of quantified protein groups and peptides with different processing methods using Spectronaut 18 software

To our knowledge, the identifications reported in this technical note are the highest ever reported on any commercial mass spectrometer for throughput of 50 SPD and 80 SPD using a library-free and library-based DIA approach. The quantitative precision and accuracy were impressive, with CVs <10% in the protein groups analyzed in a library-free search at each dilution point.

Conclusions

- The new Orbitrap Astral mass spectrometer combines an Orbitrap mass analyzer and a novel Astral mass analyzer to enable robust, reproducible, rapid, and sensitive deep proteome coverage from low sample amounts using DIA.
- High quality single-cell amount level data showed impressive quantitative accuracy and precision.
- The FAIMS Pro interface improves the signal-to-noise (S/N) ratio with decreased background ions, enhancing spectral quality for improved protein and peptide identifications for low sample amounts.
- Reproducible separations were achieved with a next generation 50 cm µPAC Neo Low Load column with consistent performance for 50 pg to 5 ng sample loads.
- The complete workflow is robust, showing an RSD of 2% for quantified protein groups and peptides between three different instruments, sites, and operators.

Learn more at **thermofisher.com/OrbitrapAstral**