High-resolution DIA proteomics workflow for single-cell samples on the Orbitrap Astral mass spectrometer

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Keywords

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

Goal

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 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 heterogeneous 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 deeper proteome coverage and faster throughput. It enables these capabilities 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 Duo 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 and 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 preparation platform were analyzed on the Orbitrap Astral mass spectrometer using DIA. The data was analyzed using Biognosys™ Spectronaut™ 18 software with library-free and library-based approaches and Thermo Scientific™ Proteome Discoverer™ 3.1 software.

Experimental

Recommended consumables

- Thermo Scientific™ Optima™ LC-MS Grade Water with 0.1% Formic Acid (FA) (Fisher Scientific Cat. No. LS118-500)
- Fisher Chemical™ Optima™ LC-MS Grade 80% Acetonitrile (ACN), 20% Water with 0.1% FA (Fisher Scientific Cat. No. LS122500)
- Fisher Chemical™ Optima™ LC-MS Grade Water (Fisher Scientific Cat. No. 10505904)
- Fisher Chemical™ Optima™ LC-MS ACN (Fisher Scientific Cat. No. A955-1)
- Fisher Chemical™ Optima™ LC-MS Isopropanol (Fisher Scientific Cat. No. A461-212)
- Thermo Scientific™ Pierce™ Sequencing Grade Trifluoroacetic Acid (TFA) (Cat. No. 28904)

Samples

- Pierce HeLa Protein Digest Standard (Cat. No. 88328)

HPLC system

- Vanquish Neo UHPLC System (Cat. No. VN-S10-A-01)
- Sonation Column Oven for µPAC Neo nanoLC Columns (Sonation Lab Solutions Cat. No. PRSO-V2-PF)

LC columns

- µPAC Neo HPLC Column, 50 cm (Cat. No. COL-NANO050NEOB)
- Evosep™ Biosystems Fused Silica Emitters, 10 µm I.D. (Evosep Cat. No. EV1111)

Mass spectrometer

- Orbitrap Astral Mass Spectrometer (Cat. No. BRE725600)
- Thermo Scientific™ FAIMS Pro Duo interface (Cat. No. OPTON-20068)
- Thermo Scientific™ EASY-Spray™ Ion Source (Cat. No. ES081)

Data analysis software

- Spectronaut 18 Software
- Proteome Discoverer 3.1 Software (Cat. No. B51001480)

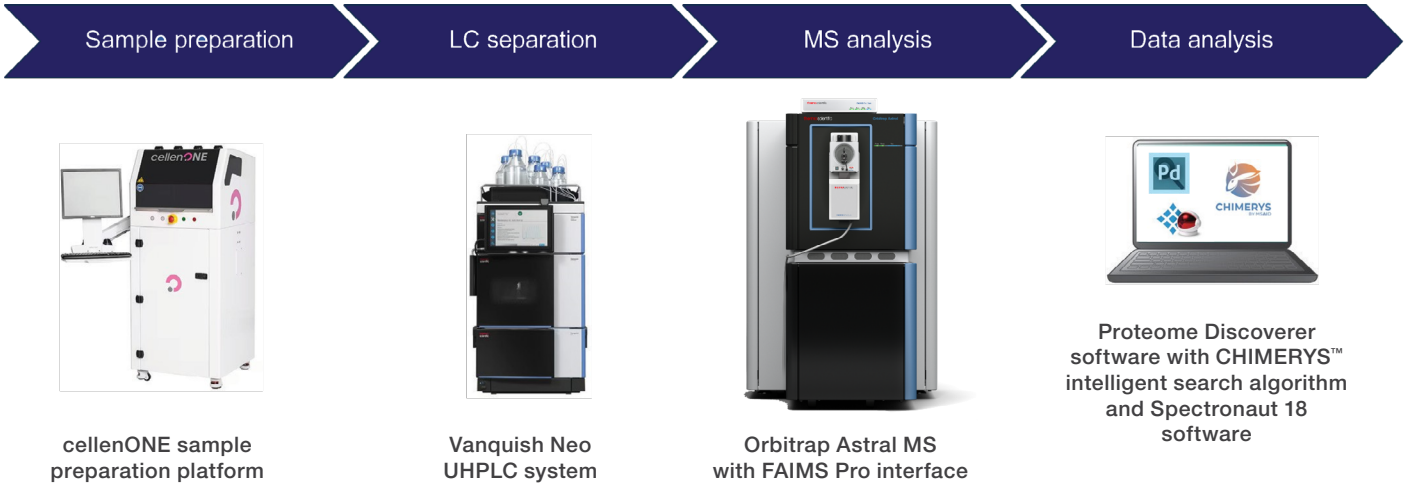

Figure 1. Single-cell proteomics workflow with the Orbitrap Astral mass spectrometer for a new standard in sensitivity, depth of coverage and quantitative precision and accuracy. Library-based searches were processed with Spectronaut 18 software. Spectral libraries were generated using DIA data with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

HeLa digest standard

The Pierce HeLa protein digest standard was resuspended as follows: 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 make a final concentration of 5 ng/µL, 95 µL of 0.1% TFA were transferred to a low bind 96-well plate, followed by the addition of 5 µL of the peptide resuspension at 100 ng/µL. The plate was placed on the autosampler for consecutive injections.

Table 2. 80/72 SPD method (µPAC Neo HPLC 50 cm column)

	Time (min.)	Duration (min.)	%B	Flow rate (uL/min.)
	Run			
Active gradient	0.0	0.0	4.0	0.75
	0.2	0.2	8.0	0.75
	2.0	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	0.4	99.0	0.18
	16.0	8.0	99.0	0.18
	Stop run			

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

LC conditions

To evaluate the performance of our workflow, an 80 samples-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. For single-cell samples, the injection-to-injection cycle time was 20 minutes, as the sample injection volume was 3.5 uL, providing a throughput of 72 SPD.

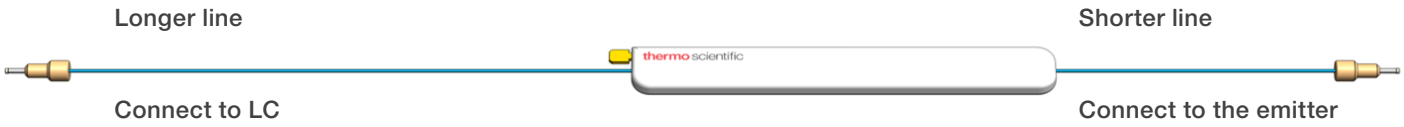

Figure 2. µPAC 50 cm column set up direction. Shorter outlet line should be connected to the emitter to reduce signal dispersion of low input samples. This column can be used with the Sonation oven or any other type of external column oven.

MS parameters

The Orbitrap Astral mass spectrometer was operated with the parameters shown in Table 3.

The DIA isolation window and maximum injection time used in these experiments were varied based on the sample load as shown in Table 4.

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 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 3. MS parameters

Source parameters	
Spray voltage	1.9 kV
Capillary temperature	275°C
FAIMS CV	–48 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%

Table 4. DIA isolation windows and maximum injection times for the dilution series

Sample amount	DIA isolation window (*m/z*)	Maximum injection time (ms)
<250 pg	20	60
≤500 pg	20	40
≤1 ng	10	20
≤2 ng	8	14
≥5 ng	5	10

Table 5. DIA isolation windows and maximum injection times for the single cells

Number of cells	DIA isolation window (*m/z*)	Maximum injection time (ms)
1 cell	20	40
20 cells	5	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 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 bulk HeLa digest dilution series consisting of 50 pg, 100 pg, 150 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 3) 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.

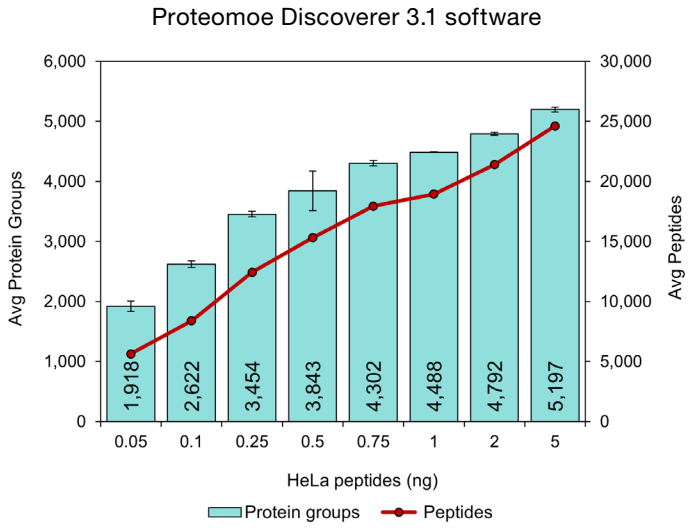

Figure 3. Average protein groups and peptides (n=3) identified and quantified when processing all files together using Proteome Discoverer 3.1 software

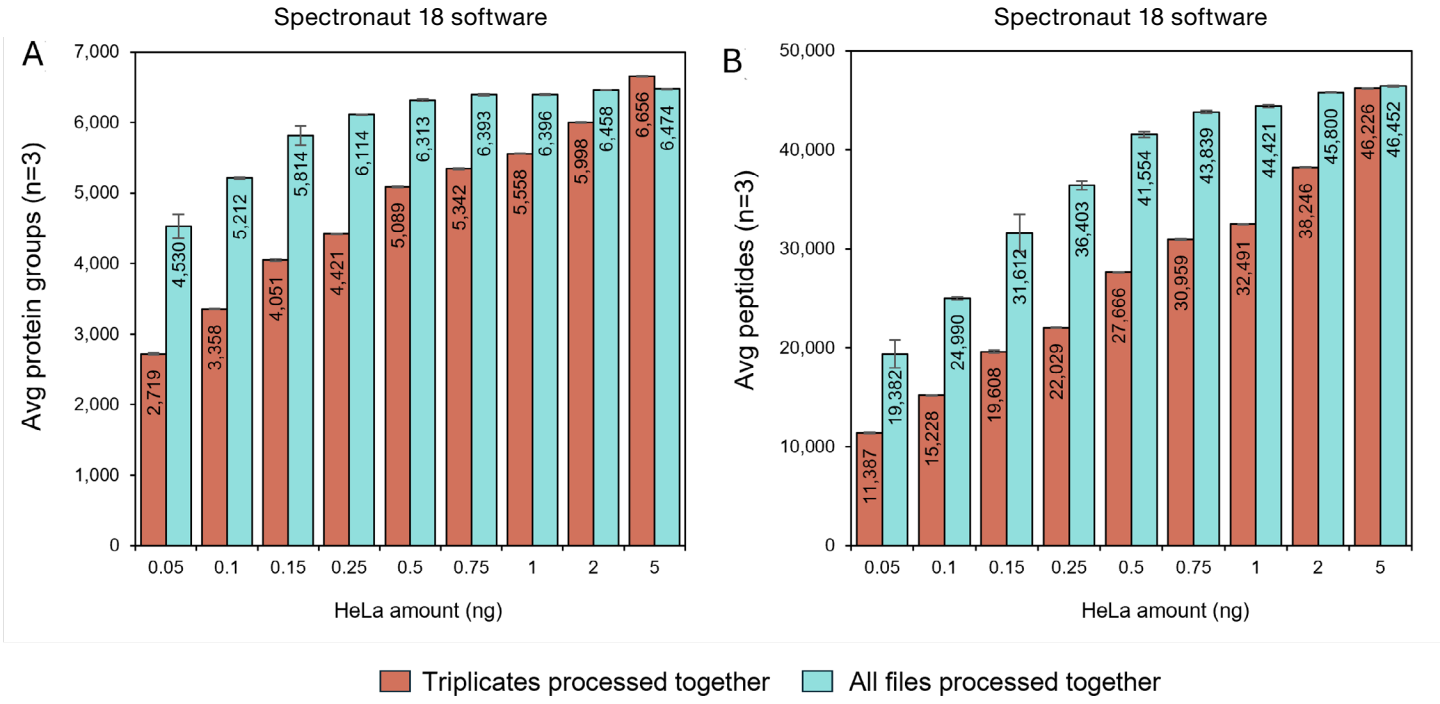

Figure 4. Average protein groups (A) and peptides (B), n=3, identified using two processing strategies on Spectronaut 18 software. Red bars represent search results when files from triplicates were searched together and blue bars represent search results when all files from the dilution series were processed together.

Precision, accuracy and linearity of the workflow

The median protein group compensation voltages (CVs) of all the dilution points are below 10% and 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 it 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 (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, e.g., 1:10 to 1:100.

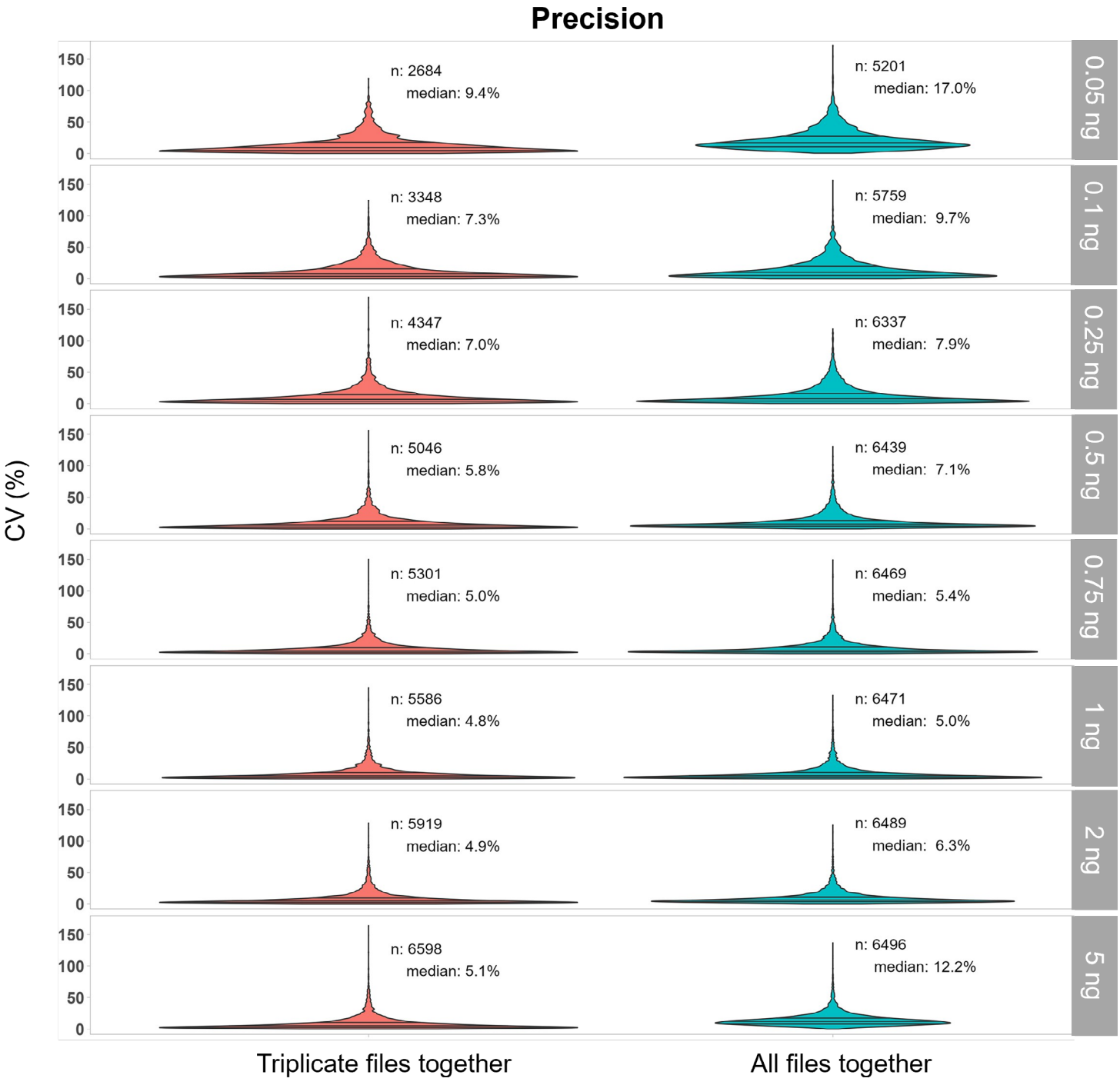

Figure 5. Precision of the two processing strategies using Spectronaut 18 software. The coefficient of variation (CV%) for triplicates is represented on the y-axis for different HeLa peptide loading amounts ranging from 0.05 to 5ng.

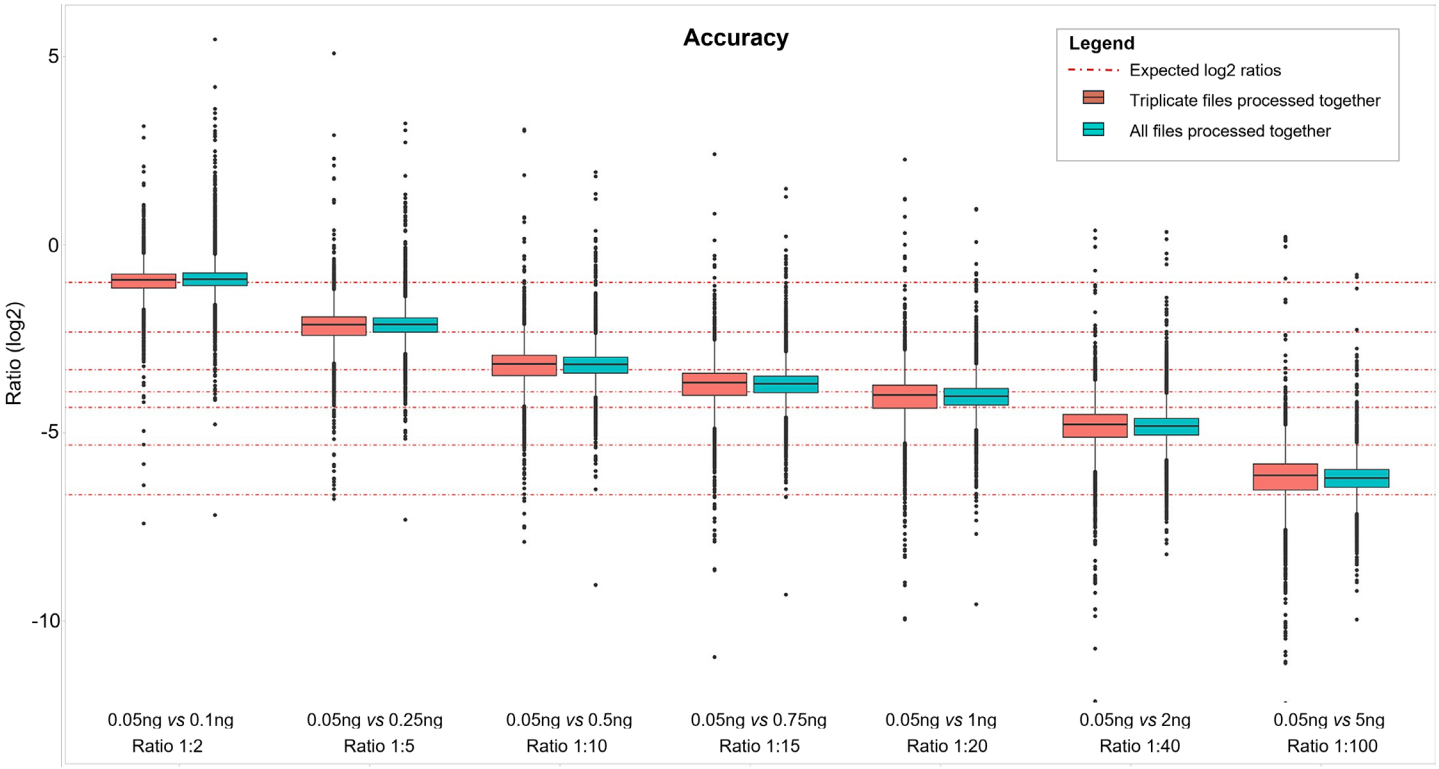

Figure 6. Accuracy of the protein ratios using both processing strategies on Spectronaut 18 software. The y-axis represents the log2 ratio values for each dilution comparison. Dashed lines represent the expected log2 ratio values.

Single-cell data

Using the 72 SPD method, 12 HeLa single cells were analyzed. The result of the individually processed raw files can be found in Figures 7 and 8. On average, >3,400 protein groups and 15,300 peptides were quantified using directDIA from each cell (Figure 8). Processing the 12 single cells together on average resulted in the quantification of >4,250 protein groups and >23,550 peptides (Figure 8).

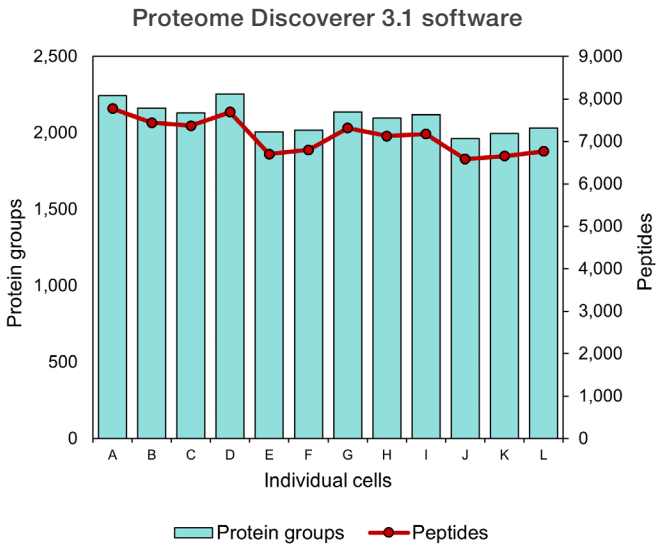

Figure 7. Number of quantified protein groups and peptides for HeLa single cells using Proteome Discoverer 3.1 software. All files were processed together.

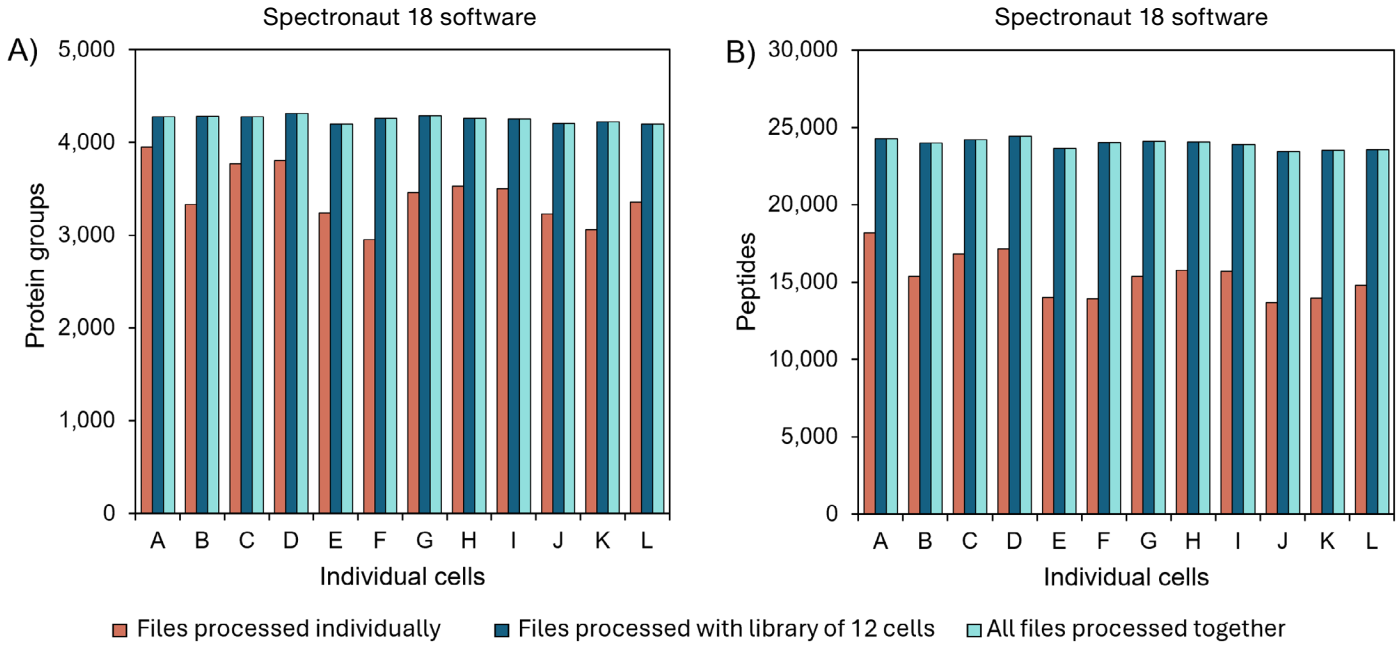

Figure 8. Number of protein groups (A) and peptides (B) quantified in HeLa single cells using library-free and library-based searches on Spectronaut 18 software. Red bars represent results when each file was processed individually (library-free); dark blue bars represent files processed with a library of 12 cells; light blue bars represent all files processed together.

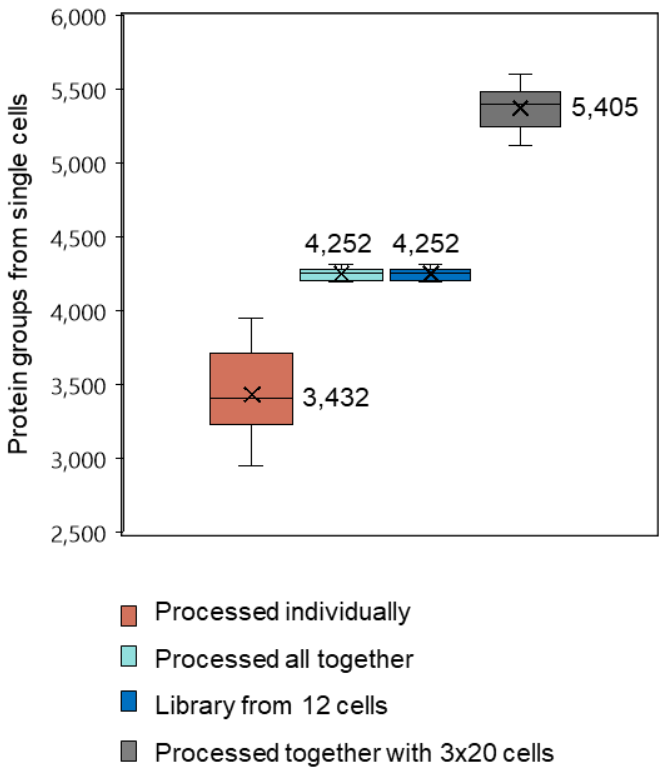

Figure 9. Quantified protein groups from HeLa single-cell injections processed using different searching strategies on Spectronaut 18 software

Figure 9 shows an overview of processing methods. In addition to the processing methods previously described, two library search-based strategies 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 increased to 5,405.

Conclusions

Recommended consumables

- 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 column with consistent performance for 50 pg to 5 ng sample loads

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