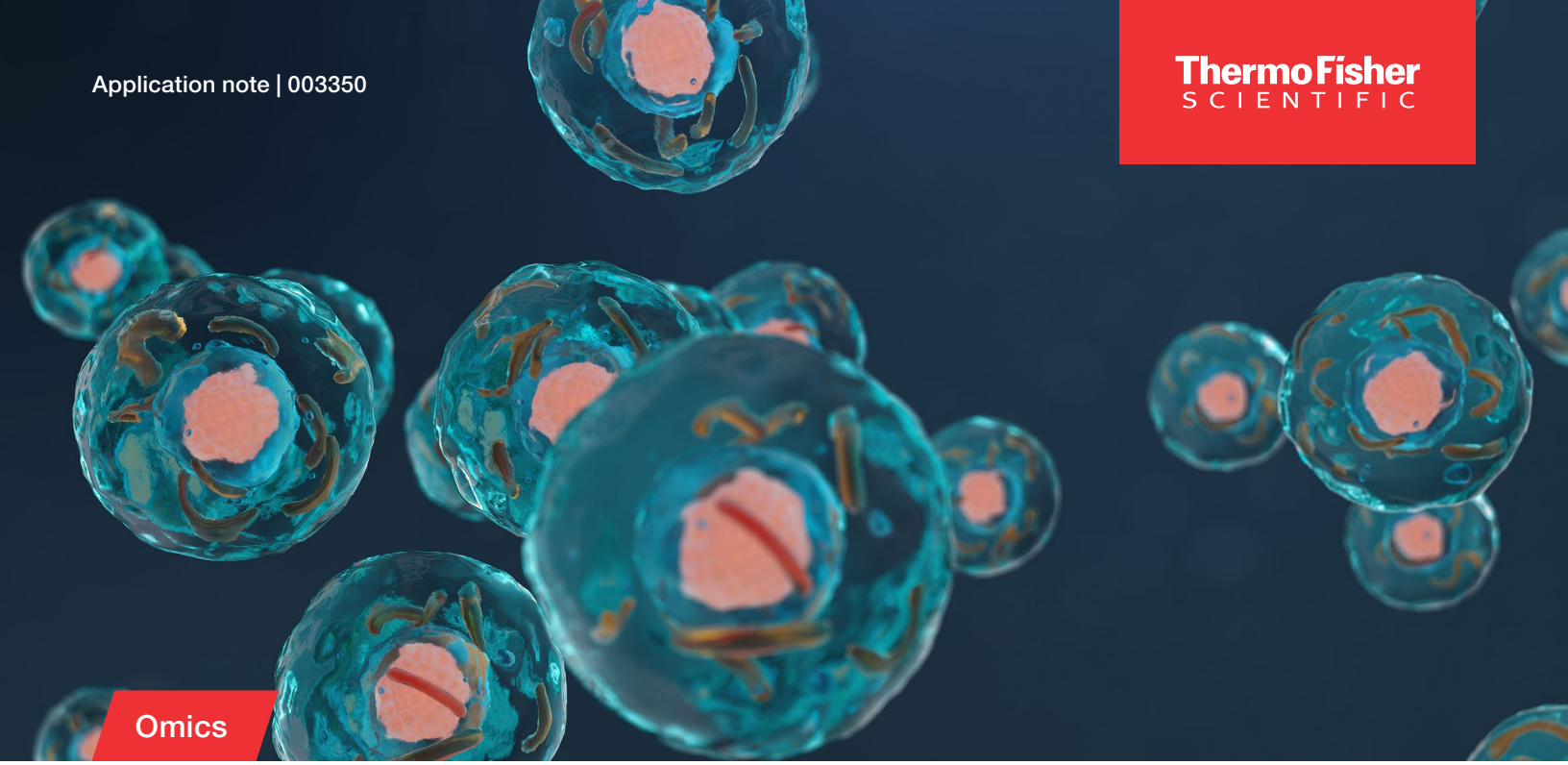
Application note | 003350



Omics

Orbitrap Astral mass spectrometer allows comprehensive proteome coverage at the single-cell level

Authors

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Keywords

Single-cell proteomics, SCP, Orbitrap Astral mass spectrometer, HeLa, Vanquish Neo UHPLC system, cellenONE, label-free, DIA, data-independent acquisition

Highlights

- The automatic single cell sorting pretreatment method is efficient, flexible, and stable.
- With a total gradient of 14 min, up to 80 single cell samples can be analyzed per day.
- Up to 7,200 protein groups can be identified from only 250 pg of HeLa digest using a library-based DIA approach.
- Up to 6,500 protein groups are identified from real single cells.
- The correlation between different single cells ranges from R=0.93–0.98, indicating good reproducibility.
- The dynamic range is more than five orders of magnitude, satisfying the need for protein quantitation.

Introduction

In contrast to traditional proteomics studies that analyze millions of cells simultaneously and present protein changes as a cumulative measurement, single-cell proteomics (SCP) analysis allows for a more detailed examination of protein composition and function at the single cell level. This advancement is highly significant in understanding cellular heterogeneity, functional diversity, cell development, tumor heterogeneity, disease progression and treatment, as well as the identification of potential drug targets.

However, the development of SCP technology faces significant technical challenges related to sample preparation, diverse protein types, low protein content, and the absence of known methods for artificial amplification of proteins to increase detection. Therefore, to fully explore this diversity, it is essential to develop robust, high-throughput, and user-friendly sample preparation techniques, as well as high-sensitivity data acquisition and data processing workflows.

To address the issue of extremely low input amounts in single cell analysis, various methods have been developed, including manual, semi-automated, and fully automated sample preparation techniques.[1-8] The cellenONE™ system (Cellenion) offers a solution by integrating single cell isolation and nanoliter dispensing into a single device, eliminating the need for additional transfer steps. This system allows for single cell isolation and picoliter dispensing, enabling sample volumes to be reduced to low nanoliters and protected from evaporation using an oil layer. Furthermore, the system is compatible with all labeling reagents, easily adaptable to customized workflows, and suitable for both multiplexed and label-free sample preparation. The specialized proteoCHIP design or standard 96- and 384-well plate enables direct injection of single cells through a standard autosampler.

Chromatography plays a crucial role in single cell analysis, and advancements in chromatography, such as ultra-high-performance liquid chromatography (UHPLC) and columns, have greatly improved the sensitivity and throughput of LC-MS-based single cell proteomics. An excellent example is the Thermo Scientific™ Vanquish™ Neo UHPLC System, which offers exceptional retention time precision spanning from 100 nL/min to 100 µL/min at pressures of up to 1,500 bar. When used in conjunction with narrow bore HPLC/UHPLC columns, the high-pressure capability of the system enables rapid loading, washing, and equilibration procedures. Moreover, the wide flow range of the system allows for lower flow rates, thereby enhancing sensitivity in single cell proteomics.

Mass spectrometry (MS)-based SCP employs labeled (multiplexed, e.g., TMT)[16-17] or label-free methods[9-13], using either data-dependent acquisition (DDA) or data-independent acquisition (DIA) approaches. These methods face challenges due to the complexity of the proteome and low protein content within individual cells.[6] Hence, high-sensitivity and high-resolution mass spectrometers are crucial for SCP to achieve comprehensive coverage and enable the extraction of quantitative information from cells.

Thermo Fisher Scientific has recently introduced the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer, an advanced instrument that combines Thermo Scientific™ Orbitrap mass analyzer with an asymmetric track lossless (Astral) analyzer. This state-of-the-art device offers features such as >200 Hz MS/MS scanning speed, high resolving power, sensitivity, and low-ppm mass accuracy. It ensures nearly lossless ion transmission efficiency and a wide dynamic range. Leveraging this innovative mass spectrometer, we have developed a complete workflow solution for SCP that delivers exceptional sensitivity and throughput. Our automated workflow involves utilizing a proteoCHIP EVO 96 chip from Cellenion and standard 96- and 384-well plates for single cell sorting and sample pretreatment. Separation is carried out using an Aurora™ TS column from IonOpticks, in conjunction with the Vanquish Neo UHPLC system. The column is coupled with an Orbitrap Astral MS equipped with a Thermo Scientific™ FAIMS Pro Duo interface. The Orbitrap Astral MS operates in a data-independent acquisition (DIA) mode, and the resulting raw files are analyzed using Thermo Scientific™ Proteome Discoverer™ software with CHIMERYS™ intelligent search algorithm or Spectronaut™ 18 software for protein identification and quantitation.

Experimental

Recommended reagents and consumables

- Fisher Scientific™ LC-MS grade formic acid (FA) (P/N A117-50)
- Fisher Scientific™ Optima™ LC-MS grade water (P/N 10505904), degassed
- Fisher Scientific™ Optima™ LC-MS acetonitrile (ACN) (P/N A955-1)
- Fisher Scientific™ Optima™ LC-MS isopropyl alcohol (P/N A461-212)
- Fisher Scientific™ LC-MS grade water with 0.1% FA (P/N LS118-500)
- Fisher Scientific™ LC-MS grade 80% ACN/0.1% FA (P/N LS122500)
- Thermo Scientific™ Pierce™ TFA, sequencing grade (P/N 28904)
- Thermo Scientific™ n-dodecyl-β-D-maltoside DDM (P/N 89902)
- Thermo Scientific™ triethylammonium bicarbonate (TEAB), 1M (P/N 90114)

- Thermo Scientific™ trypsin, MS grade (P/N 90057)
- Thermo Scientific™ PBS (P/N 10010023, 500 mL), degassed
- Fisher Scientific™ dimethyl sulfoxide cell culture grade (DMSO, P/N 3672-0050, 50 mL)
- Cellenion™ proteoCHIP EVO 96 Set (PP) (P/N C-PEVO-96-10)
- Cellenion™ proteoCHIP EVO 96 cellenONE™ Holder Base (P/N C-PEVO-96-CHB)
- Cellenion™ proteoCHIP EVO 96 Centrifugation Dummy (P/N C-PEVO-96-CD)
- Eppendorf™ twin.tec™ 96-Well LoBind™ PCR Plates, Skirted (P/N E0030129512)
- Axygen™ AxyMats™ 96 Round Well Sealing Mat for PCR Microplates (P/N AM-96-PCR-RD)
- Thermo Scientific™ SureSTART™ WebSeal™ 384-well plate (P/N 60180-P340)
- Thermo Scientific™ SureSTART™ WebSeal™ 384-well plate silicone sealing mat (P/N 60180-M150)

Sample

- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (P/N 88328)

LC columns

- IonOpticks Aurora Elite 15 cm × 75 µm ID, 1.7 µm C18 (P/N AUR3-15075C18-TS)
- Column heater (P/N COLHTR01)

HPLC system

Thermo Scientific™ Vanquish™ Neo UHPLC system consisting of:

- Vanquish Neo Binary Pump N and Split Sampler NT (P/N VN-S10-A-01)
- Vanquish User Interface (P/N 6036.1180)
- Vanquish Column Compartment (P/N VN-C10-A-01, optional)

Mass spectrometer

- Orbitrap Astral mass spectrometer (P/N BRE725600)
- Thermo Scientific™ EASY-Spray™ ion source (P/N ES081)
- FAIMS Pro Duo interface (P/N FMS03-10001)

Data analysis software

- Spectronaut 18 software (Biognosys AG)
- Thermo Scientific™ Proteome Discoverer™ software with CHIMERYS™ intelligent search algorithm by MSAID

HeLa digest standard preparation

200 µL of 0.1% TFA/0.015% DDM was added to a vial containing 20 µg of lyophilized HeLa digest. The resulting mixture was then sonicated at room temperature for 5 minutes. Following this, 5 µL of a 100 ng/µL standard solution was added to a 96-well plate containing 95 µL of a 0.1% TFA/0.015% DDM solution. This addition was made to achieve a final concentration of 5 ng/µL in the plate.

Cell collection

HeLa cells were cultured in DMEM (Gibco, Invitrogen), supplemented with 10% fetal bovine serum, 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), at 37 °C, in a humidified incubator with 5% CO_2. Cells were harvested at approximately 50–60% confluence by washing three times with PBS (Gibco, Life technologies). Cells were then resuspended in degassed PBS at 200 cells/µL for isolation within the cellenONE system.

Buffer preparation

Master mix buffer (0.2% DDM, 100 mM TEAB, 20 ng/µL Trypsin) was used for cell lysis and digestion.

- **1% DDM:** The 10 mg DDM was dissolved in 990 µL degassed double-distilled water (ddH_2O) and stored at -20 °C for further use.
- **100 ng/µL Trypsin:** 20 µg Trypsin was dissolved in 200 µL resuspension buffer and stored at -20 °C for further use.
- **500 µL master mix:** The 250 µL degassed ddH_2O was added to the mixture of 100 µL of 1% DDM, 50 µL of 1 M TEAB, and 100 µL of 100 ng/µL Trypsin and stored at -20 °C for further use.
- **0.1% TFA/1% DMSO:** 100 µL of DMSO was added to 10 mL of 0.1% TFA and stored at 4 °C for further use. 0.1%TFA/ 1% DMSO was used to resuspend reaction systems, prepared for samples loading.

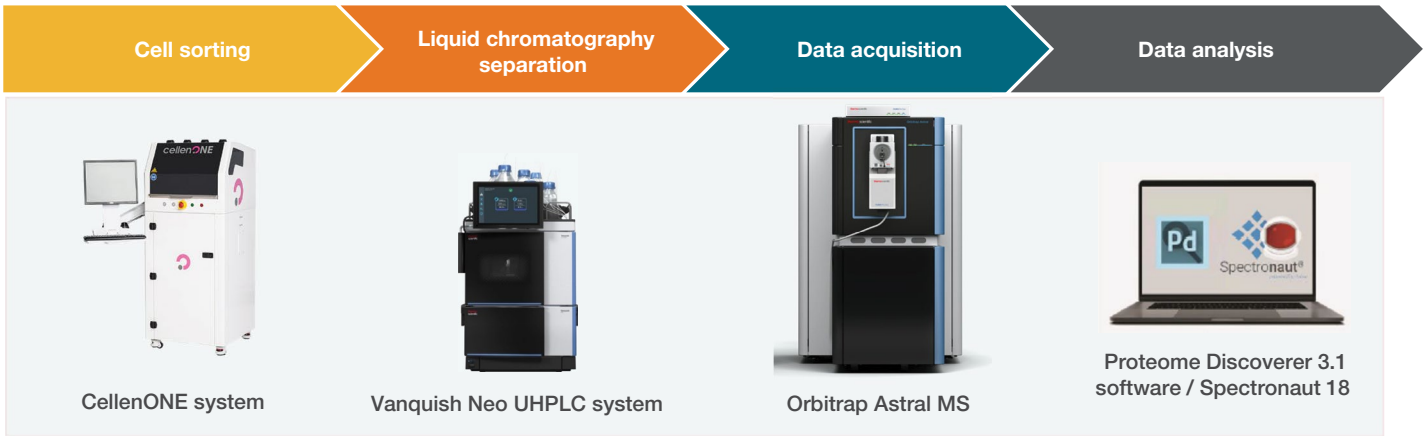

Figure 1. Workflow of SCP

Single cell sample preparation

Reaction condition 1: Using the Cellenion proteoCHIP EVO 96 chip, as shown in Figure 2

- Cell collection: HeLa cells were diluted with degassed PBS to approximately 200 cells/µL.
- Cell seeding: Using the proteoCHIP EVO 96 inside the CellenONE system, 300 nL of master mix buffer was automatically injected into each well of the chip, which was then positioned on the target plate.
- Mapping: 5–10 µL of cells were picked up by the CellenONE system via a standard autosampler to determine the morphology, density, and diameter of cells suitable for SCP analysis, such as 15–35 µm diameter, as shown in Figure 3A.
- Sorting: If cells met the criteria, the CellenONE system was used to sort individual cells based on a diameter range and elongation factor of 1.5–1.8 into the wells, and the green spots indicated target cells (Figure 3B–C).
- Digestion: The proteoCHIP was then subjected to a controlled incubation phase at 50 °C with 85% relative humidity for 1 h within the environment of the instrument. The automatic cycle system involved the addition of 192 nL of water to each well until the process was complete.
- Cooling: After incubation, the temperature of the system was reduced to 20 °C to stabilize the conditions post-reaction.
- Quenching: Upon completion of the incubation, the proteoCHIP EVO 96 was taken out of the CellenONE system and processed further. The 3.5 µL of 0.1%TFA/1% DMSO was manually added to the wells.
- Transferring: The chip was transferred to a new 96-well plate, and then centrifuged at 800 g for 5 min at 4 °C.
- Sealing: The plates were sealed with matching 96-well plate covers and placed in the Vanquish Neo UHPLC system, with a final volume of 4 µL in each well.

Reaction condition 2: Using the standard 96/384-well plate, as shown in Figure 4

- Cell collection: HeLa cells were diluted with degassed PBS to 100–200 cells/µL.
- Cell seeding: Using the 96/384-well plate inside the CellenONE system, 1 µL of master mix buffer was automatically injected into each well of the chip, which was then positioned on the target plate.
- Mapping: 5–10 µL of cells were picked up by the CellenONE system via a standard autosampler to determine the morphology, density, and diameter of cells suitable for SCP analysis, such as 15–35 µm diameter, as shown in Figure 3A.
- Sorting: If cells met the criteria, the CellenONE system was used to sort individual cells based on a diameter range and elongation factor of 1.5–1.8 into the wells, and the green spots indicated target cells (Figure 3B–C).
- Adding liquid: After sorting, the 500 nl of master mix buffer was injected into each well of the plate by the CellenONE system via a standard autosampler.
- Digestion: The 96/384-well plate was then subjected to a controlled incubation phase at 50 °C with 85% relative humidity for 1 h within the environment of the instrument. The automatic cycle system involved the addition of 192 nL of water (500 nL for a whole 384-well plate) to each well until the process was complete.
- Cooling: After incubation, the temperature of the system was reduced to 20 °C to stabilize the conditions post-reaction.
- Quenching: Upon completion of the incubation, the 96/384-well plate was taken out of the CellenONE system and processed further. The 3.5 µL of 0.1%TFA/1% DMSO was manually added to the wells.
- Sealing: The plates were sealed with matching 96/384-well plate covers and placed in the autosampler of the Vanquish Neo UHPLC system, with a final volume of 4 µL in each well.

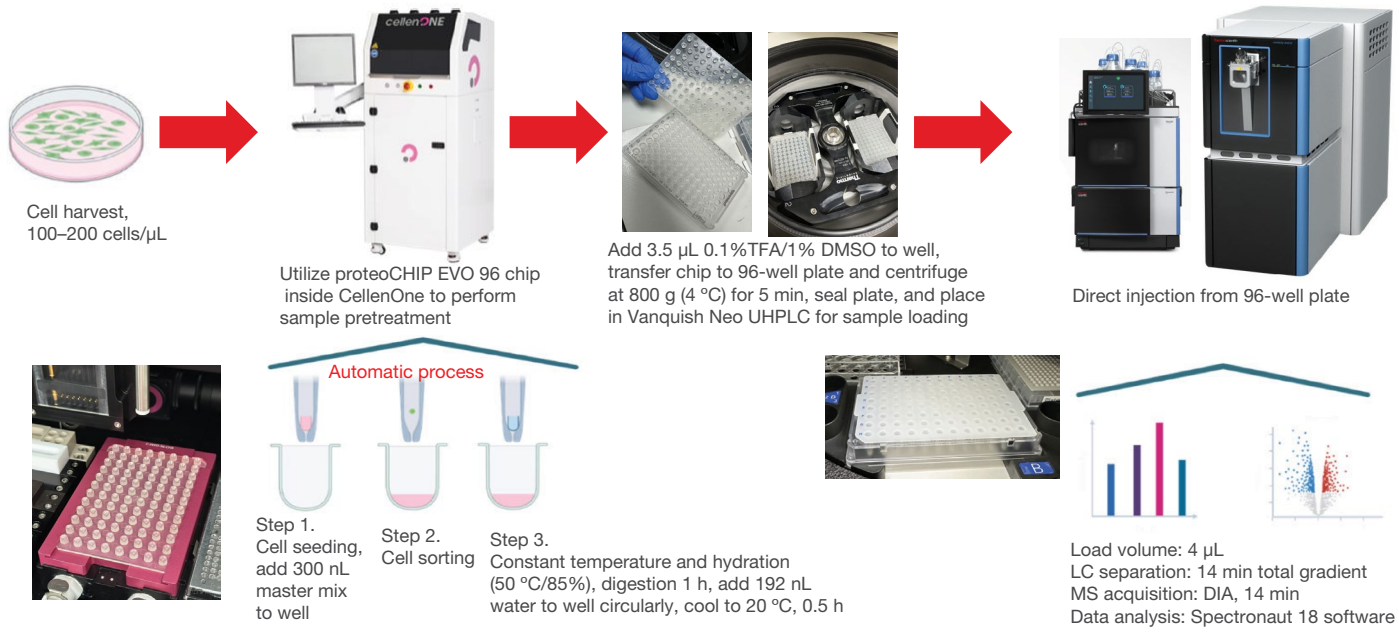

Figure 2. The workflow of SCP processing and analysis using the Cellenion proteoCHIP EVO 96 chip

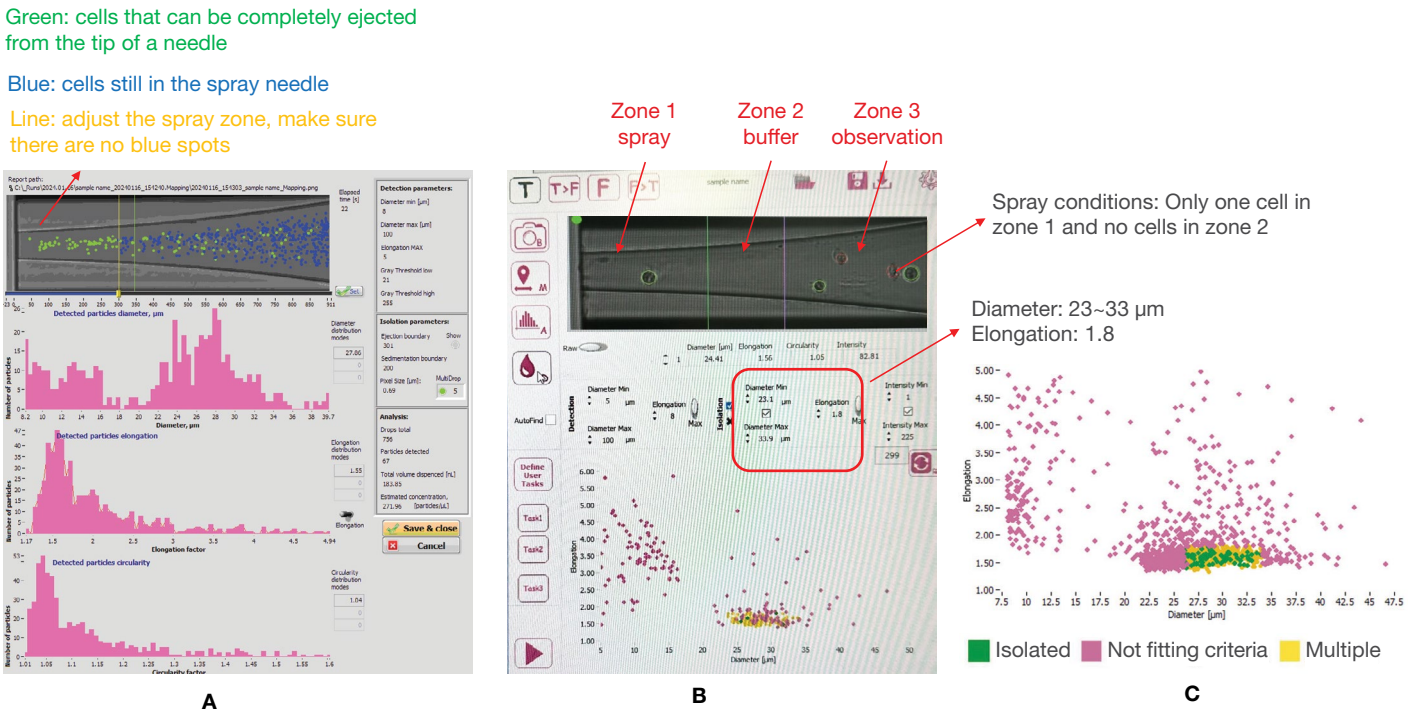

Figure 3. The cell sorting using the CellenONE system. (A) Cell Mapping reports, (B) parameter setting and process of cell sorting, (C) cell sorting results

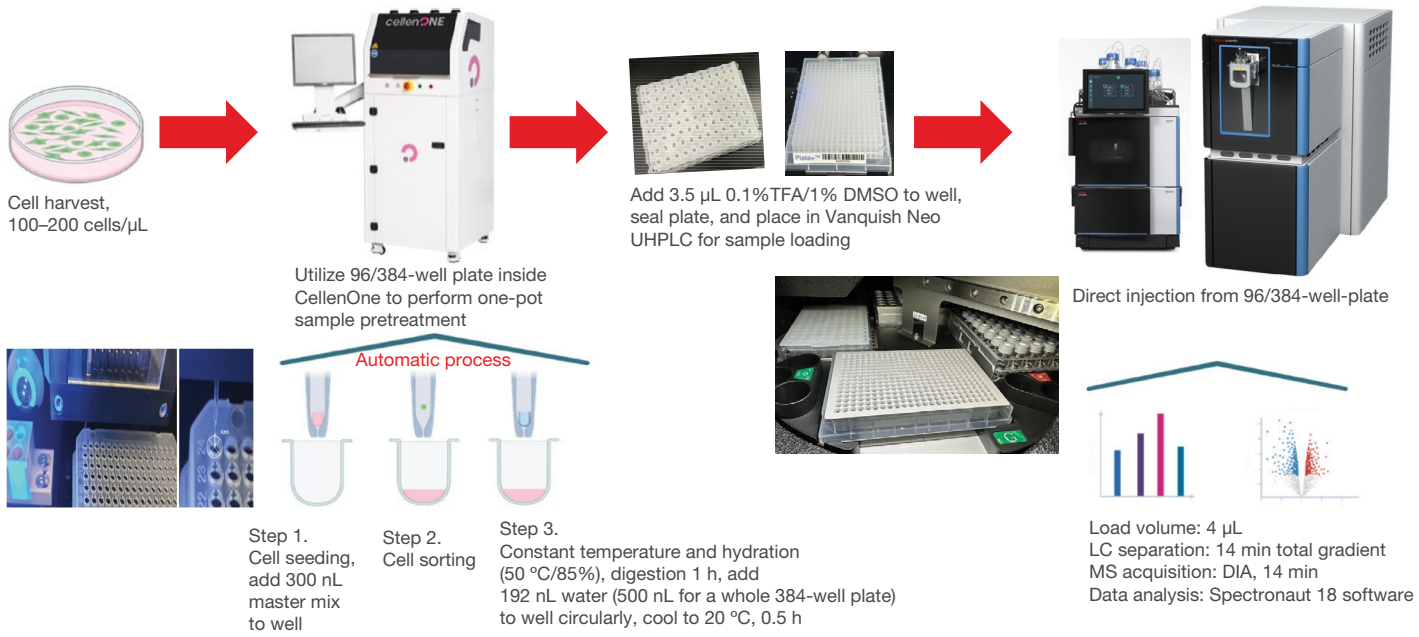

Figure 4. The workflow of SCP processing and analysis using the standard 96/384-well plate

LC conditions

The LC separation was carried out using a Vanquish Neo UHPLC system configured in a direct injection mode and an Aurora Elite TS analytical 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 80 samples per day (SPD) was employed, with a total runtime of 18 minutes from injection to injection. The gradient method used for the separation is detailed in Table 1.

MS acquisition

MS data acquisition was conducted using the Orbitrap Astral MS. To enhance sensitivity for identifying low-abundance peptides, the FAIMS Pro Duo interface was utilized to reduce background interference. The specific MS parameters employed for the analysis are provided in Table 2. The MS acquisition parameters for the DIA isolation window and maximum injection time were adjusted accordingly to accommodate different loading amounts or numbers of cells on column. The corresponding details can be found in Table 3.

Table 1. Chromatography conditions

Parameter	Value			
Mobile phase A	0.1% FA			
Mobile phase B	80% ACN/0.1% FA			
Flow rate (active gradient)	0.2 µL/min			
Chromatographic column	IonOpticks Aurora Elite TS 15 cm × 75 µm ID, 1.7 µm C18			
Column temperature	50 °C			
AS sample tray temperature	7 °C			
Needle wash	Strong wash: 80% ACN/0.1% FA Weak wash: 0.1% FA			
Needle wash mode	After draw			
Gradient (80 SPD)	Time	Duration] [min]	Flow rate [uL/min]	% B
	0.0	0.0	0.450	4
	0.1	0.1	0.450	4
	1.6	1.5	0.200	12
	1.7	0.1	0.200	12
	9.7	8.0	0.200	28.5
	11.2	1.5	0.200	40
			Column wash	
	12.0	0.8	0.300	99
	14.0	2.0	0.300	99
			Stop run Column equilibration	

Table 2. MS parameters

Part A

Parameter	Value
Source properties	
Spray voltage	1.9 kV
Capillary tube temperature	275
*FAIMS CV	-48
*FAIMS carrier gas	3.8
Orbitrap full scan properties	
Orbitrap resolution	240,000
Scan range	400–800
FAIMS CV	-48
Normalized AGC target (%)	500
Max injection time (ms)	100
FAIMS carries gas (L/min)	3.8
Data-independent acquisition properties	
Precursor mass range (*m/z*)	400–800
Isolation width	See Part B below
Window placement	On
NCE (%)	25
Scan range (*m/z*)	150–2,000
Injection time	See Part B below
AGC target (%)	800
Loop control	Time
Time (s)	0.6

Part B

Isolation width [Th]	Injection time [ms]	Sample amount
5	10	5–10 ng
8	14	2–5 ng
10	20	1–2 ng
20	40	250–500 pg
20	60	<250 pg
20	60+	True single cell

Results and discussion

System evaluation using HeLa digest standard

To assess the performance of the LC-MS setup before analyzing actual mammalian single cells, we conducted an evaluation using a commercially available HeLa digest standard. A dilution series ranging from 50 pg to 10 ng of HeLa digest was created and analyzed. The dilutions were prepared by injecting the appropriate volume from a 5 ng/µL stock solution. For example, injecting 50 nL from the 5 ng/µL stock solution allowed us to obtain a 250 pg sample. The dilution series was then subjected to separation and analysis using the Orbitrap Astral MS in DIA mode. The DIA isolation width and maximum injection time were adjusted based on the sample amount.

The raw data obtained were analyzed using Spectronaut 18 software, utilizing both library-free and library-based approaches. In the library-free approach, triplicate raw files of each sample amount were processed together against a human protein database containing 20,563 sequences. Additionally, the potential of utilizing a spectral library was explored. For the library-based approach, triplicate runs of a 10 ng HeLa digest were used to generate libraries in Spectronaut 18 software, with slight adjustments to the DIA *m/z* range (150–2,000), while keeping all other default parameters constant.

Both the library-free and library-based approaches employed MS1 for quantitation, with oxidation and carbamidomethylating of cysteine set as variable and fixed modifications, respectively. The results obtained from the analysis are as follows: In the library-free DIA approach, more than 4,200 protein groups were identified from a mere 50 pg sample, and over 5,500 protein groups from a 250 pg sample (Figure 5). By employing the library-based approach, up to 6,100 protein groups were identified from a 50 pg HeLa digest, and more than 7,200 protein groups were identified from a 250 pg HeLa digest (Figure 6). The median coefficient of variation (CV) for protein groups in technical replicates was below 10% (Figure 7). The results obtained from the HeLa dilution not only demonstrate the impressive depth of coverage achieved but also highlight the quantitative capabilities of the workflow.

Subsequently, each raw file obtained from the different sample amounts was processed using Proteome Discoverer software with CHIMERYS intelligent search algorithm. The data was searched against a human protein database containing 20,563 sequences. Oxidation of methionine was selected as a variable modification and carbamidomethylating of cysteine as a static modification. To ensure reliability, all protein and peptide groups were filtered for a 1% false discovery rate (FDR). The average number of identified protein and peptide groups for each sample amount (n=5) is presented in Figure 8.

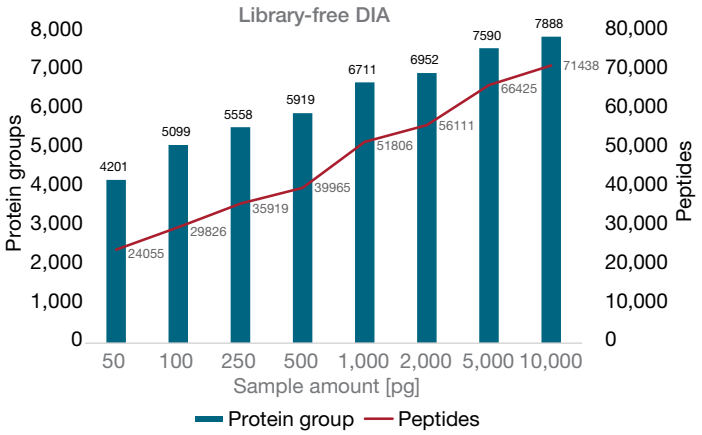

Figure 5. The identification of protein groups and peptides in the 80 SPD method 14 min gradient under library-free search mode

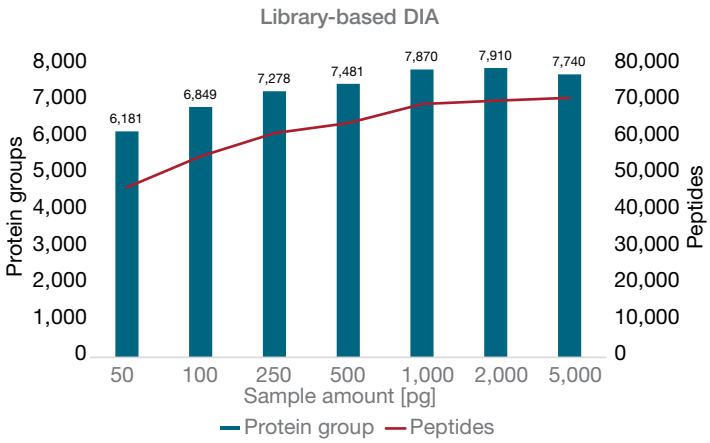

Figure 6. The identification of protein groups and peptides in the 80 SPD method 14 min gradient under library-based search mode

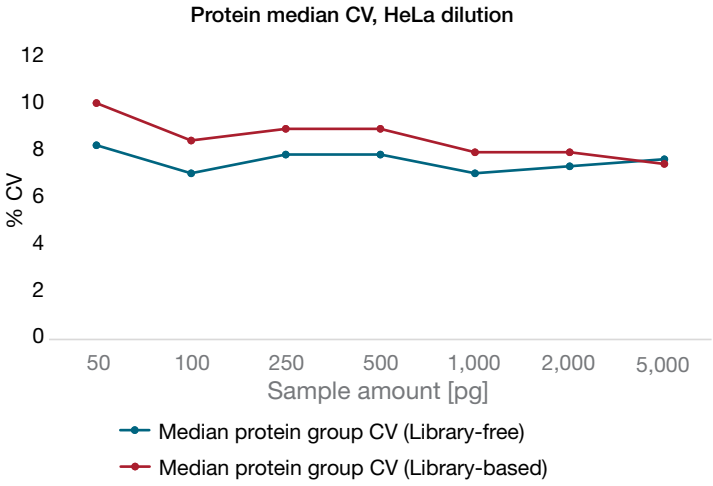

Figure 7. Precision of the library-free and library-based DIA experiment for the HeLa dilution series using Spectronaut 18 software

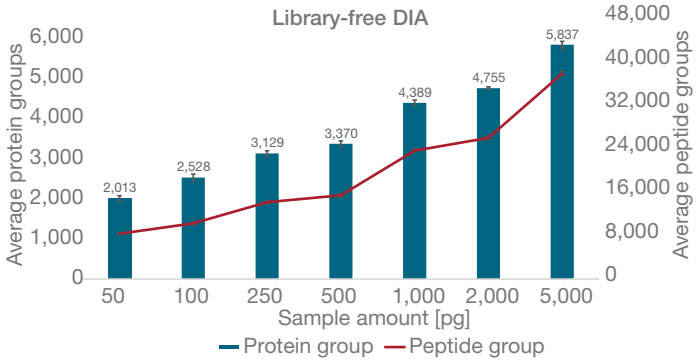

Figure 8. The average number of protein and peptide groups identified from a HeLa digest dilution series with library-free search on Proteome Discoverer software with CHIMERYS intelligent search algorithm

Single cell sample

HeLa cells were chosen as a representative model for validating this workflow due to their widespread use in cell cultures, tumor studies, and other biological experiments. To ensure diversity, HeLa cells with diameters ranging from 15 to 22 µm and 23 to 32 µm were isolated using the CellenONE system. The cells were sorted into standard 96- or 384-well plates or a proteoCHIP Evo 96 and prepared for further analysis. The separation was carried out using an Aurora 15 cm × 75 µm column, utilizing the 80 SPD method. Subsequent MS analysis was performed using the Orbitrap Astral MS operating in DIA mode.

384-well plate

Using the CellenONE system, single cells were isolated and distributed into various positions of a 384-well plate. The cells were then lysed, digested, and subjected to LC-MS analysis. A total of 77 raw files were processed together in Spectronaut 18 software, using the same parameters as in the HeLa dilution series experiments mentioned earlier. No library or booster raw files from concentrated HeLa digest or bulk number of cells were used for this data processing. The results revealed that, on average, 34,894 peptides were assigned to approximately 5,977 protein groups (Figure 9).

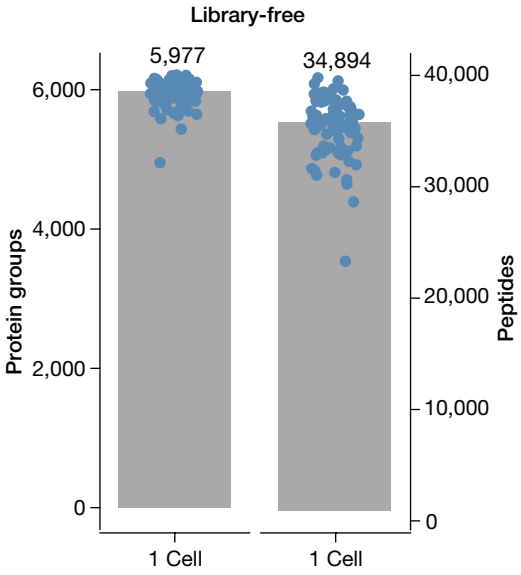

Figure 9. The identification of protein groups and peptides by using the standard 384-well plate

To assess the variability in the measurements, we constructed a correlation matrix. This involved consistently processing the identification results of 20 individual single cell samples. The Pearson correlation coefficients were calculated, revealing a high level of reproducibility, with R values ranging from 0.93 to 0.98 (Figure 10A). Furthermore, the protein abundance demonstrated a wide dynamic range, spanning more than five orders of magnitude (Figure 10B).

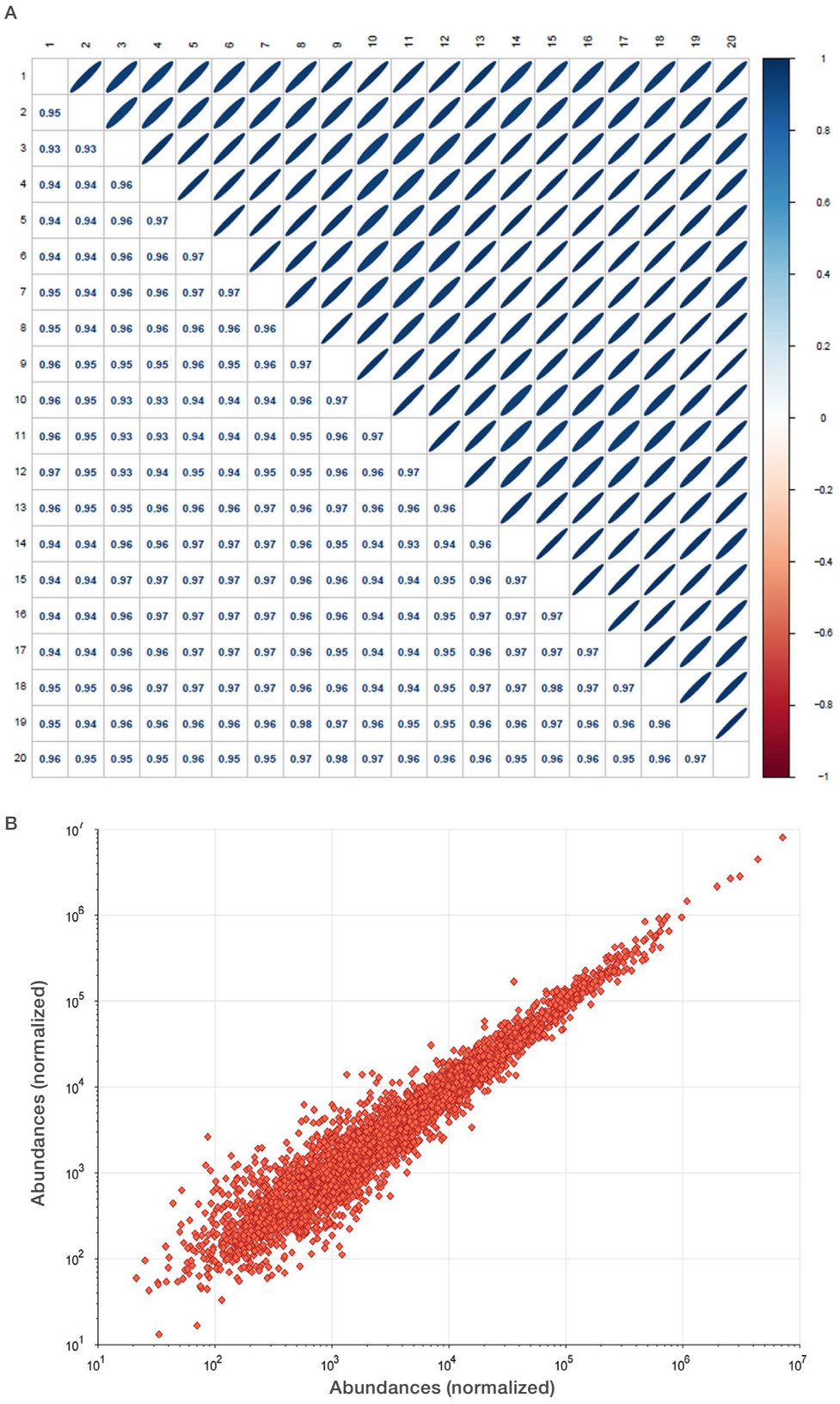

Figure 10. The evaluation of Pearson correlation coefficients and dynamic range

ProteoChIP EVO 96

In the proteoCHIP Evo 96, cells were sorted into different positions using the CellenONE system, with one cell, ten cells, and twenty cells per well. The sorted cells (ϕ15~22 µm) were then digested, transferred to a standard 96-well plate, separated, and subjected to analysis. The raw data files for each cell number were processed using a library-free DIA approach in Spectronaut 18 software. Specifically, 65 single cells were processed together without any booster raw files. On average, the analysis revealed that 24,877 peptides were matched to an average of 4,589 protein groups for the single cell per well, using the library-free DIA approach. For the wells with 10 and 20 cells, on average, 48,114 and 54,782 peptides were assigned to an average of 6,154 and 6,642 protein groups, respectively (Figure 11).

96-well plate

In the 96-well plate, different cell numbers (0, 1, 12, and 20 cells) were sorted into various positions, followed by digestion, separation, and analysis. Under library-free DIA processing conditions, an average of 42,821 peptides (n=80) were matched to an average of 6,556 protein groups (Figure 12). For the wells with 10 and 20 cells, an average of 56,552 and 60,804 peptides from 7,069 and 7,160 protein groups were identified, respectively. To assess the potential carryover and cross-contamination in this workflow during large single cell experiments, randomized blanks in the 96-well plate were also analyzed. The results showed that, on average, less than 180 protein groups were matched to less than 500 peptides. These findings indicate that there was no significant cross-contamination and minimal carryover observed in this workflow.

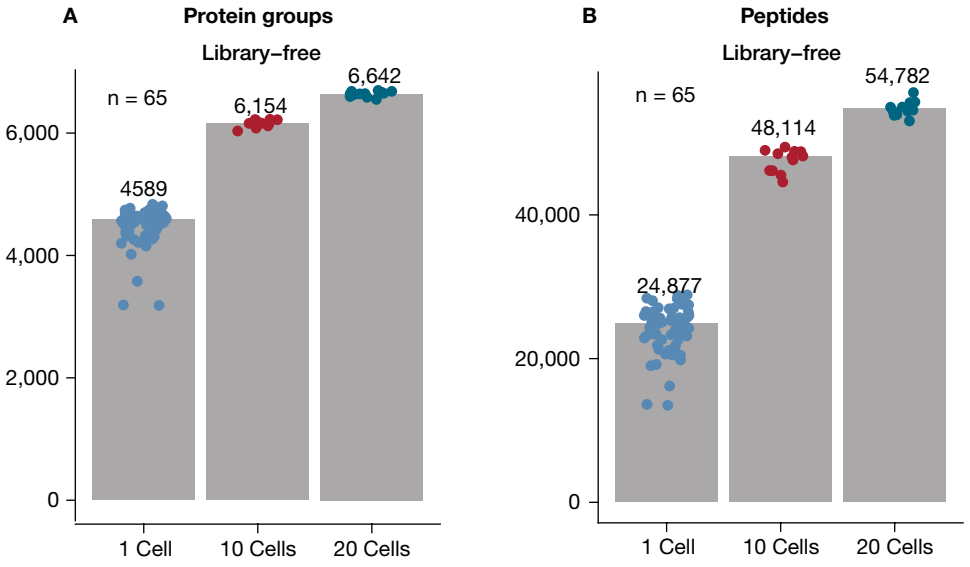

Figure 11. (A) The identification of protein groups and (B) peptides by using the Cellenion proteoCHIP EVO 96 chip

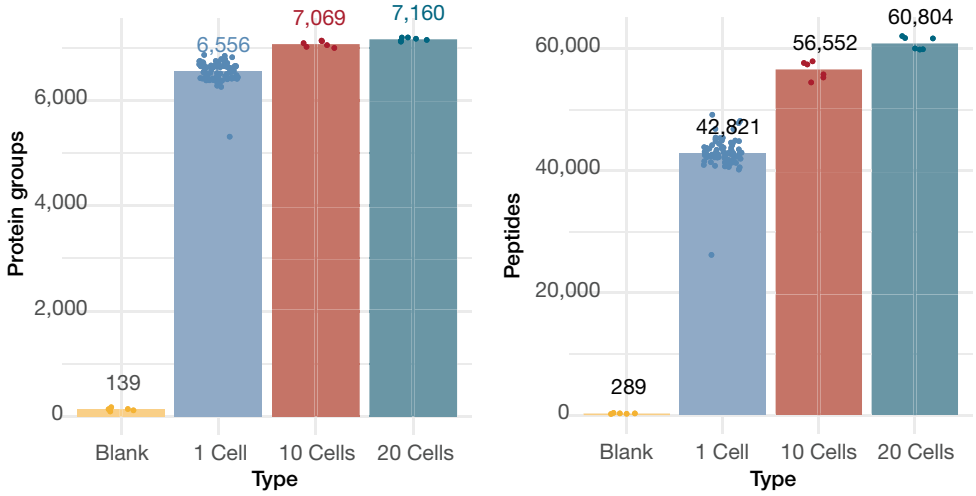

Figure 12. The identification of protein groups and peptides by using the standard 96 well plate

Membrane proteins play crucial roles in cellular communication, shape maintenance, triggered responses, and material transport. Their significance extends to drug development, as they serve as drug targets, influence drug permeability, and participate in drug transport mechanisms. To investigate the identification of membrane proteins, we processed three files with Proteome Discoverer software. The analysis revealed that over 22% of the average number (5,616) of proteins identified were membrane proteins (Figure 13).

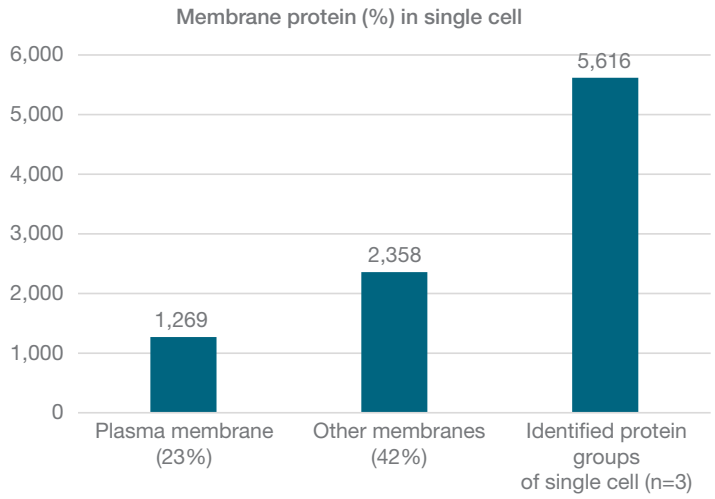

Figure 13. Membrane proteins identified in a single cell

Conclusion

In this study, a comprehensive workflow was developed for SCP analysis. The workflow incorporates the use of the CellenONE system, which enables automatic cell sorting, lysis, and digestion, as well as the Vanquish Neo UHPLC system, which provides precise flow rates and high-pressure capabilities for efficient sample loading and increased method development flexibility. The Orbitrap Astral MS, coupled with the FAIMS Pro Duo interface, was employed for SCP analysis, offering high resolution, sensitivity, and dynamic range.

The workflow was evaluated in two steps. First, a HeLa dilution series ranging from 50 to 10,000 pg was analyzed using DIA mode. With a library-free analysis, more than 4,200 protein groups were identified from a 50 pg HeLa digest. Increasing the sample amount to 250 pg resulted in the identification of over 5,500 protein groups. Processing the same raw files with a library-based method further increased the number of identified protein groups to over 6,180 and 7,270 for the 50 pg and 250 pg samples, respectively. The median coefficient of variation (CV) for protein groups in both DIA approaches was less than 10%.

In the second step, a total of 222 single cells, along with a few wells containing 10 or 20 cells, were analyzed. These cells were prepared on three different plates using two sample pretreatment conditions. Based on the plate type and number of single cells analyzed, an average of 5,977, 4,589, and 6,556 protein groups were identified from the 384-well plate, proteoCHIP Evo 96, and 96-well plate, respectively. Furthermore, the protein abundance exhibited a wide dynamic range spanning more than five orders of magnitude. These results highlight the impressive sensitivity and depth of coverage achievable with the Orbitrap Astral MS in both HeLa dilution series and single-cell analyses.

References

1. Budnik, B., Levy, E., Harmange, G. & Slavov, N. SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. *Genome Biol. 19,* 161 **(2018)**.
2. Leduc, A., Huffman, R.G., Cantlon, J., Khan, S. & Slavov, N. Exploring functional protein covariation across single cells using nPOP. *Genome Biol. 23,* 261 **(2022).**
3. Ctortecka, C. et al. An automated nanowell-array workflow for quantitative multiplexed single-cell proteomics sample preparation at high sensitivity. *Mol. Cell. Proteomics MCP* 100665 **(2023)** doi:10.1016/j.mcpro.2023.100665.
4. Zhu, Y. et al. Proteomic Analysis of Single Mammalian Cells Enabled by Microfluidic Nanodroplet Sample Preparation and Ultrasensitive NanoLC-MS. *Angew. Chem. Int. Ed. 57,* 12370–12374 **(2018).**
5. Liang, Y. et al. Fully Automated Sample Processing and Analysis Workflow for Low-Input Proteome Profiling. *Anal. Chem. 93,* 1658–1666 **(2021).**
6. Johnson, K.R., Gao, Y., Greguš, M. & Ivanov, A. R. On-capillary Cell Lysis Enables Top-down Proteomic Analysis of Single Mammalian Cells by CE-MS/MS. **Anal. Chem. 94,** 14358–14367 **(2022).**
7. Matzinger, M., Müller, E., Dürnberger, G., Pichler, P. & Mechtler, K. Robust and Easy-to-Use One-Pot Work-flow for Label-Free Single-Cell Proteomics. *Anal. Chem. 95,* 4435–4445 **(2023).**
8. Ye, Z. et al. One-Tip enables comprehensive proteome coverage in minimal cells and single zygotes. 2023.08.10.552756 Preprint at doi:10.1101/2023.08.10.552756 **(2023).**
9. Matzinger, M., Mayer, R.L. & Mechtler, K. Label-free single cell proteomics utilizing ultrafast LC and MS instrumentation: A valuable complementary technique to multiplexing. *PROTEOMICS 23,* 2200162 **(2023).**
10. Guzman, U.H. et al. Narrow-window DIA: Ultra-fast quantitative analysis of comprehensive proteomes with high sequencing depth. 2023.06.02.543374 Preprint at doi:10.1101/2023.06.02.543374 **(2023).**
11. Ye, Z. et al. High-throughput and scalable single cell proteomics identifies over 5000 proteins per cell. 2023.11.27.568953 Preprint at doi:10.1101/2023.11.27.568953 **(2023).**
12. Zheng, R. et al. A High-Sensitivity Low-Nanoflow LC-MS Configuration for High-Throughput Sample-Limited Proteomics. *Anal. Chem. 95,* 18673–18678 **(2023).**
13. Stewart, H.I. et al. Parallelized Acquisition of Orbitrap and Astral Analyzers Enables High-Throughput Quantitative Analysis. *Anal. Chem. 95,* 15656–15664 **(2023).**
14. Arrey, T. et al. Deeper proteome coverage and faster throughput for low input samples on the Thermo Scientific Orbitrap Astral mass spectrometer. Thermo Fisher Scientific Technical Note 002255 **(2023).**
15. Bubis, J.A. et al. Challenging the Astral mass analyzer — going beyond 5200 proteins per single-cell at unseen quantitative accuracy to study cellular heterogeneity. bioRxiv preprint doi: https://doi.org/10.1101/2024.02.01.578358 (2024).
16. Cheung. T.K. et al. Defining the carrier proteome limit for single-cell proteomics. *Nature Methods. 18,* 76–83 **(2021).**
17. Ye, Z. et al. A deeper look at carrier proteome effects for single-cell proteomics. *Communications Biology volume 5,* Article number: 150 **(2022).**

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