Single-cell proteomics

Advancing low flow LC/MS for single cell proteomics with variable flow and 50 cm microfabricated pillar array columns

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Abstract

Purpose: Demonstrate the impact of optimized chromatographic separation for low-input sample and single cell analysis workflows.

Methods: Single HeLa cells, HeLa cell titrations, and dilution series of Thermo Scientific™ Pierce™ HeLa Protein Digest Standard were analyzed using a Thermo Scientific™ Vanquish™ Neo UHPLC System connected to a Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer. Variable flow rate methods for 50 cm Thermo Scientific™ µPAC™ Neo Plus Columns were optimized towards maximum sensitivity and minimal impact on instrument productivity.

Results: By optimizing the connectivity of 50 cm µPAC Neo columns, improved chromatography and enhanced proteome coverage were achieved. The most significant impact was observed at the lowest flow rate of 100 nL/min. With an optimized gradient and MS acquisition method, over 2000 protein groups were successfully identified from single HeLa cells.

Introduction

Recent LC-MS advancements have greatly enhanced sensitivity and enabled nearly lossless automated sample handling, crucial for standardized workflows and large-scale single-cell MS studies. Capillary bore LC columns at ultra-low flow rates achieve high sensitivity but are limited in throughput and robustness. We describe an innovative pillar array column-based workflow compatible with both direct injection and trap-and-elute modes, allowing deep coverage and high throughput analysis of low input samples and true single cells.

Materials and methods

Sample preparation

HeLa cell digests were resuspended in 0.1% TFA, 10% DMSO to obtain 100 ng/µL stock solutions. The solution was sonicated, diluted in 0.1% TFA to 5 ng/µL, sonicated again, and vortexed. HeLa single cells were prepared from pre-frozen aliquots of 500,000 cells per mL. Thawed aliquots were washed with DPBS, resuspended in 2 mL degassed DPBS, and processed using the cellenONE™ platform (Cellenion SASU). Cells were stained with 1 µg/mL DAPI, incubated for 2 minutes, and loaded into the cellenONE. Cells sized were isolated at 10 ºC and 50% relative humidity. Isolated cells were placed into wells of an Eppendorf 384-well LoBind® plate with 1 µL of lysis and digestion mix[1]. The plate was centrifuged, re-inserted into the cellenONE, and incubated at 50 ºC and 85% RH for 30 minutes with continuous dispensing of LC-MS grade water. After 30 minutes, 0.5 µL of trypsin/Lys-C solution was added, followed by another 1.5-hour incubation. Samples were acidified with 3.5 µL of 0.5% TFA to a final volume of ~5 µL containing 0.35% TFA. Plates were sealed with Axygen® AXYMAT™ silicone sealing mats (Corning, Inc.) before insertion into the LC sampler.

LC-MS configuration

Samples were analyzed using a Vanquish Neo UHPLC system with a 50 cm µPAC Neo Plus column positioned in an external heating device (Sonation, PRSO-V2_PF) and directly mounted onto a Thermo Scientific™ EASY-Spray™ Source that was coupled to an Orbitrap Exploris 240 mass spectrometer. Variable flow rate LC methods with initial flow at 600 nL/min and eluting flow rates of 100 and 200 nL/min were used in a back flush trap and elute workflow (µPAC C18 trap column). Data independent acquisition (DIA) parameters used are listed below.

Full Scan		MS² scan			
Resolution	Max IT	**Resolution**	**Precursor mass range**	Isolation window	Max IT
120k	Auto	60k	375-675 m/z	30 Th	118 ms
120k	Auto	120k	375-675 m/z	60 Th	246 ms

Data analysis

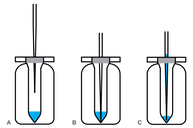
LC-MS data were analyzed either using Thermo Scientific™ Proteome Discoverer™ 3.1 Software with CHIMERYS™ (MSAID GmbH) or with Spectronaut® 19 (Biognosys AG). Results shown have been filtered to a 1% FDR.

Single cell sample preparation, transfer and separation

- Cell isolation with cellenion cellenOne.
- One-pot sample preparation protocol into Eppendorf LoBind 384 well plates.
- Sample aspiration directly from 384 well plate with Vanquish Neo UHPLC system.

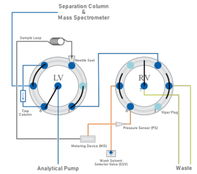
Vial bottom detection

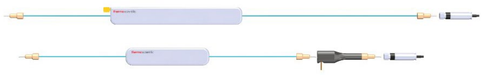
- Samples can be fully aspirated by setting injection volume with some margin (1-2µL added).
- Compatible with conventional sample vials, 96 and 384 well plates.



Back flush trap and elute workflow

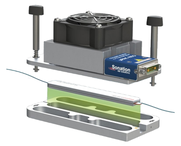
- Single cell samples vary in volume (between and within experiments) – sample volume range 1-10 µL
- Sample uptake + loading can be reduced to 2.4 min.
- Additional air drawn into sample loop will not affect ionization / analytical column performance.





Optimized chromatography

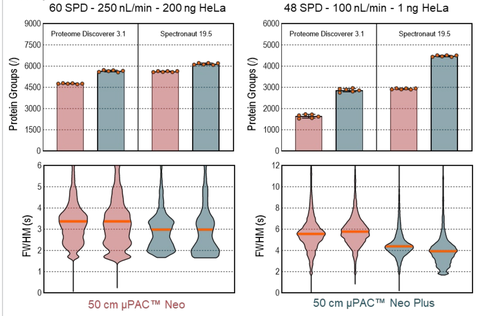
- µPAC Neo Plus configuration minimizes pre and post column dispersion.
- A 10 µm ID voltage spacer is used to connect the column outlet to the emitter.
- The column is placed directly in front of the mass spectrometer with the aid of an external column heater (Sonation, PRSO-V2_PF).



Results

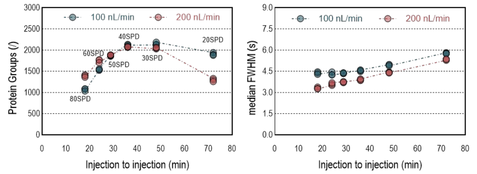
µPAC Neo Plus performance

Figure 5. Performance comparison between the 50 cm µPAC Neo and the 50 cm µPAC Neo Plus columns, utilizing a 10 µm ID voltage spacer. Left: 60 SPD, 250 nL/min flow rate, 200 ng sample load. Right: 48 SPD, 100 nL/min flow rate, 1 ng sample load.



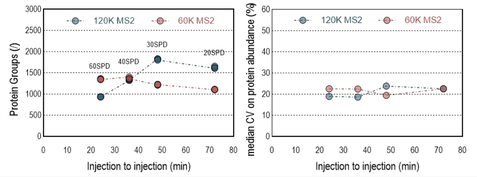
LC method optimization with single HeLa cells

Figure 6. Impact of elution flow rate and throughput on proteome coverage for single HeLa cell protein digest samples. n=3 per condition. Analyzed using the Orbitrap Exploris 240 MS.



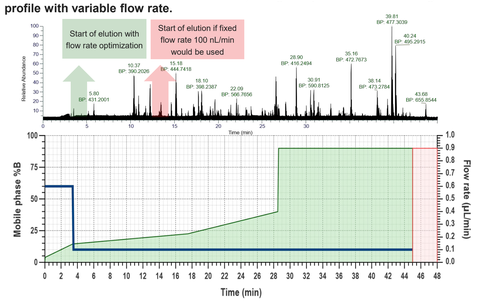
MS method optimization with single HeLa cells

Impact of throughput and MS2 acquisition resolution on proteome coverage for single HeLa cell protein digest samples. n=3 per condition. Analyzed using the Orbitrap Exploris 240 MS.



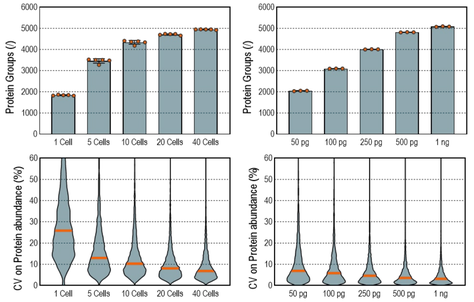
Bulk HeLa dilution series and single HeLa Cell titration at 30 SPD

Figure 8. Basepeak chromatogram for the separation of 250 pg HeLa digest standard at 30 SPD in trap-and-elute mode, using an optimized 30 SPD LC gradient profile with variable flow rate.



- The method yielding the deepest coverage, achieved at 30 SPD with an eluting flow rate of 100 nL/min, was employed to compare proteome coverage and quantitation between HeLa cell titration and bulk HeLa dilution.
- In the current evaluation, the protein content of a single HeLa cell most closely corresponds to 50 pg of bulk HeLa material.
- Coefficients of variation in protein abundance are significantly higher for single HeLa cells compared to pooled HeLa cells or diluted bulk HeLa, highlighting the inherent biological variability.

Figure 9. Proteome coverage and coefficients of variation for protein abundance obtained from a bulk HeLa dilution series compared to a HeLa cell titration ranging from 40 down to 1 cell. n=5 per condition. Analyzed using the Orbitrap Exploris 240 MS.



Effect of HeLa cell size on proteome depth

Figure 11. Microscopic images of HeLa cells captured by the cellenONE instrument, demonstrating precise isolation and selection of cells within predefined size bins.

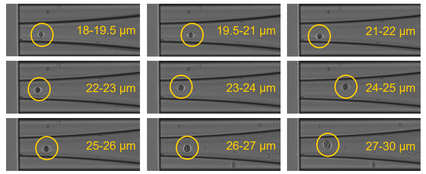
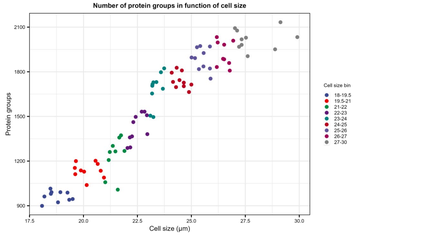


Figure 12. Proteome coverage for single HeLa cells plotted as a function of cell size. Nine different cell size bins were defined, with 10 cells isolated and processed within each bin. Analyzed using the Orbitrap Exploris 240.



Conclusions

- Improved connectivity of the µPAC Neo Plus configuration decreases peak width for peptides by approximately 10% at 250 nL/min and 30% at 100 nL/min. This results in a 25-40% increase in proteome coverage for low input samples.
- The deepest coverage for single HeLa cells was achieved using a 30 SPD LC method with elution at 100 nL/min. High-resolution MS2 isolation and increased fill times enhance sensitivity.
- Utilizing variable analytical flow and loading samples onto a trap column significantly boosts instrument productivity.
- More than 2000 protein groups were successfully identified from single HeLa cells, showing a strong correlation between protein coverage and cell size.

References

1. Matzinger, M.; Müller, E.; Dürnberger, G.; Pichler, P.; Mechtler, K. Robust and Easy-to-Use One-Pot Workflow for Label-Free Single-Cell Proteomics. Anal. Chem. 2023, 95 (9), 4435–4445.

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