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Pushing the Limits of Sensitivity: Micropillar Array-Based Chromatography Coupled to a Quadrupole Orbitrap Mass Spectrometer and FAIMS for Low-Input Proteomics

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ABSTRACT

Purpose: Enhancing sensitivity of a state-of-the-art mass spectrometer by high-field asymmetric waveform ion mobility spectrometry (FAIMS) Pro™ with a pillar array-based µPAC™ column prototype optimized for low sample loads.

Methods: Highly diluted HeLa and K562 cell lysate digests were separated under conventional nano-flow conditions on a non-porous column followed by FAIMS-assisted ion sampling and mass spectrometric analysis on a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer.

Results: The superior chromatographic performance of the non-porous µPAC™ column paired with improved sampling of analyte ions by FAIMS in the gas phase in front of the ion inlet significantly enhances sensitivity and leads to reliable and deep proteome profiling of very low sample amounts, paving the way for single cell applications.

INTRODUCTION

Mass spectrometry (MS)-based proteomics aims at the characterization of complex biological samples such as cell and tissue lysates. Although deep proteome profiling has advanced considerably over the last decade, typically such analyses require high numbers of cells. However, important biological information on cellular heterogeneity or rare cell populations cannot be fully elucidated[1]. Major problems with "low-input" proteomics include insufficient chromatographic sensitivity, and signal suppression of analyte ions by background noise. Here, we demonstrate that outstanding chromatographic performance of a novel column prototype designed for single cell proteomics combined with FAIMS technology can be applied to tackle these challenges.

MATERIALS AND METHODS

Sample Preparation

Thermo Scientific™ Pierce™ HeLa and K562 cell lysate digests (Promega) were serially diluted from 10 ng down to 500 pg mimicking the protein amount equivalent to 2-4 HeLa cells[2].

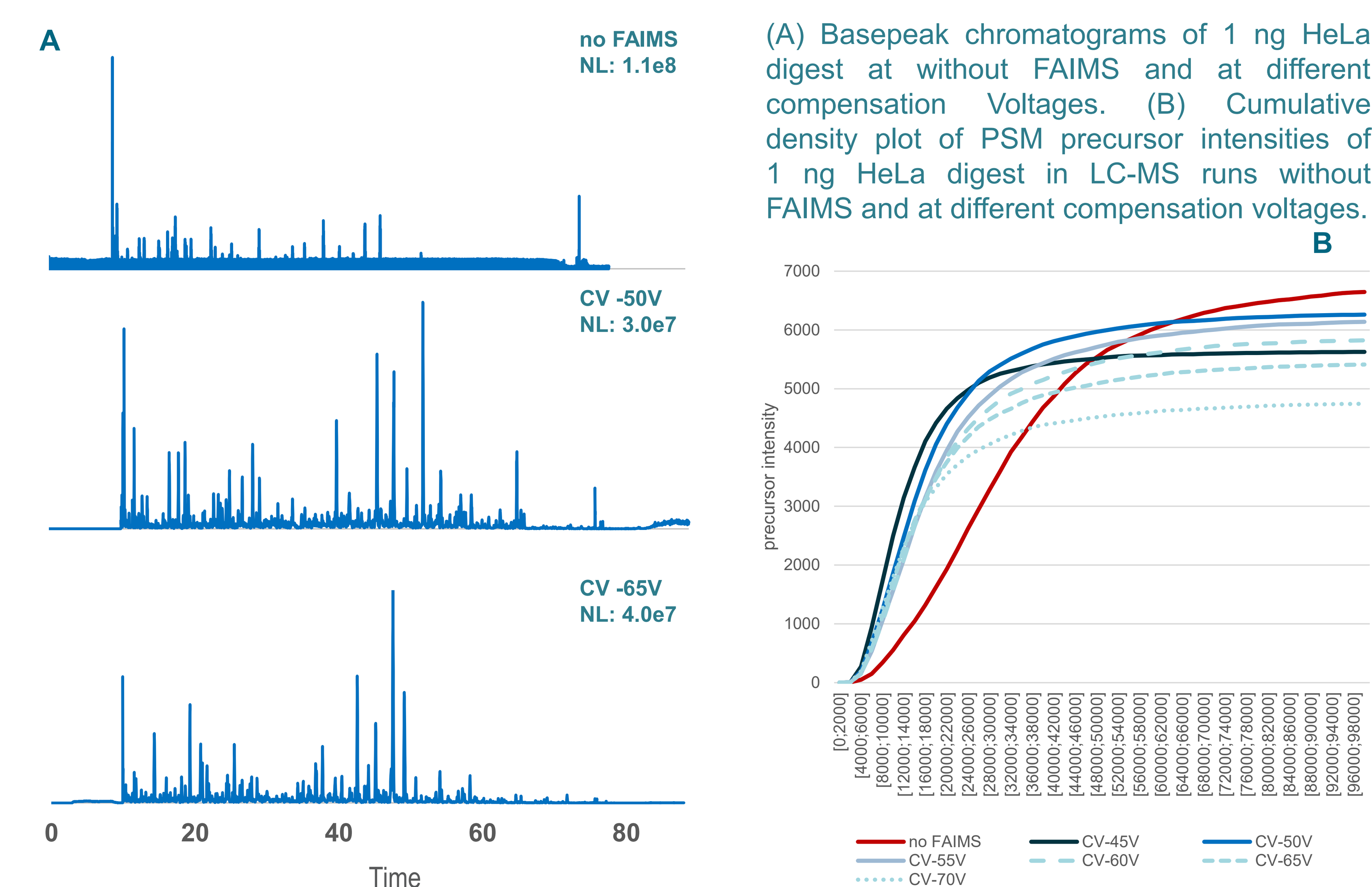
Instrumentation

The instrumental setup comprised an Orbitrap Exploris 480 equipped with a FAIMS Pro interface. Standard cell lysate digests were separated on a non-porous µPAC™ column prototype (PharmaFluidics). The column was operated at 50° C at a flow rate of 250 nL/min, using a direct full 1 µL sample loop injection setup. For pre-experimental optimization of the FAIMS method and setup, a 25 cm IonOptics column was used with the same LC-setup.

Data Analysis

Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 2.5 using both Sequest conservative database-search with and without multiple peptide search (MPS) and spectral library search with a predicted spectral library by the Prosit prediction tool[4].

Figure 1. FAIMS Pro Effectively Filters Background Ions and Significantly Improves Sensitivity.



(A) Basepeak chromatograms of 1 ng HeLa digest at without FAIMS and at different compensation Voltages. (B) Cumulative density plot of PSM precursor intensities of 1 ng HeLa digest in LC-MS runs without FAIMS and at different compensation voltages.

RESULTS

Gas Phase Fractionation Facilitated by FAIMS Pro Improves Sensitivity for Low Sample Loads

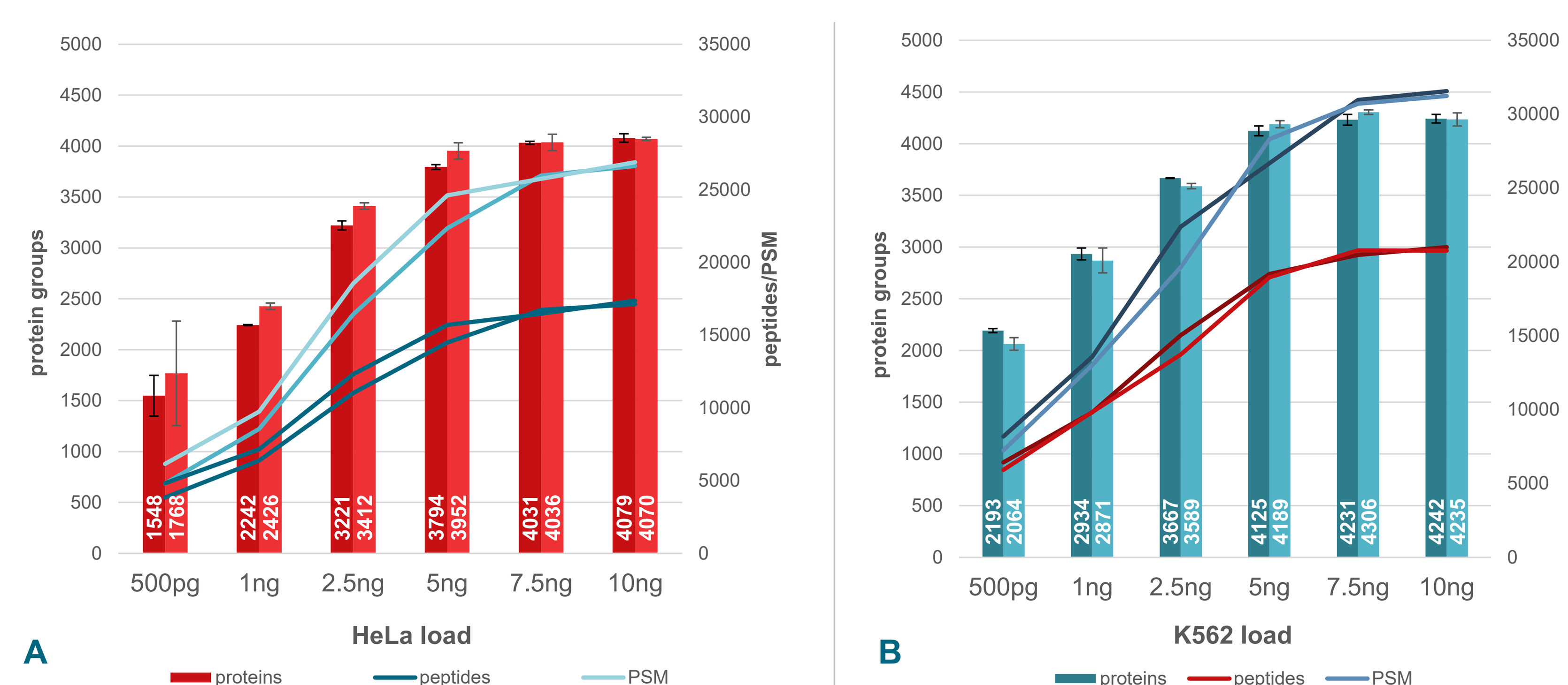
FAIMS favors the transmission of multiply charged ions at certain compensation voltages (CV), i.e. singly-charged solvent ions can be effectively prevented from entering the MS. At low sample loads, predominantlybackground ions are the detected, resulting in basepeak chromatograms with a high noise level (Fig. 1A). Applying one CV over a 60 min gradient gives rise to a multitude of peptide ion basepeaks emerging from the reduced noise band, though slightly reducing overall sensitivity. This does not affect the number of peptide-spectrum-matches (PSM): in the FAIMS runs, PSM numbers for lower precursor abundances are higher than without FAIMS (Fig. 1B). Nevertheless, the total PSM number with CV -50 V matches almost the baseline without FAIMS, i.e. higher intensities do not translate into more meaningful PSM when it comes to protein identifications.

Deep and Robust Proteome Profiling of Low Sample Amounts – Highest Performance While Maintaining Reproducibility

The LC-MS setup shown here lead to the reliable identification of more than 1500 protein groups from only 500 pg of HeLa digest (Fig. 2A). Three technical replicates of each sample load generally have a low standard deviation, and even in non-consecutively acquired data sets protein and peptide IDs are very similar. Even more proteins are identified in the more complex human cell line K562 (Fig. 2B).

Figure 2. High and Reproducible Proteome Coverage at Low Sample Amounts from Different Cell Lysate Digests.

Protein and peptide identifications, and PSM in low amounts of HeLa (A) and K562 (B) from two non-consecutive LC-MS analysis with technical replicates n = 3.



Optimized Search Strategies Enhance Proteome Coverage

Operating at the low end of what can be detected emphasizes the importance of getting the most out of the data with dedicated processing workflows. In Fig. 3, the different outcomes of several search strategies are shown. Generally, the strict Sequest database-search gives lowest protein and peptide, but can be slightly boosted by multiple precursor search (MPS). The highest gain can be achieved by spectral library search with a high-quality predicted library (Prosit)[4].

Outstanding Chromatographic Performance

The µPAC™ non-porous column prototype is designed for highest performance at very low sample amounts. This is impressively demonstrated in the basepeak chromatograms shown in Fig. 4A. The precursor abundances increase significantly from 500 pg to 5 ng, and less so at higher sample amounts (Fig. 4B), underlining the column's suitability for single cell proteomics. Looking at average variations of peak area and shape, and retention times in Fig. 4D illustrates once more the excellent chromatographic performance.

Figure 3. Different Search Strategies Impact the Depth of Proteome Profiling

Data from one LC-MS run with 1 ng HeLa on separated in a 60 min gradient have been submitted to different search strategies. 1% FDR on both peptide and protein level.

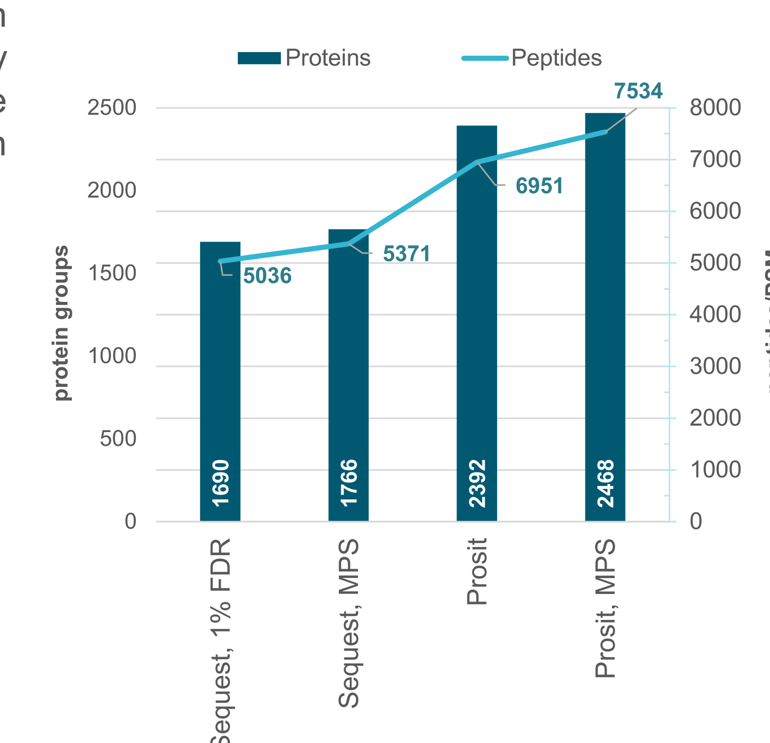
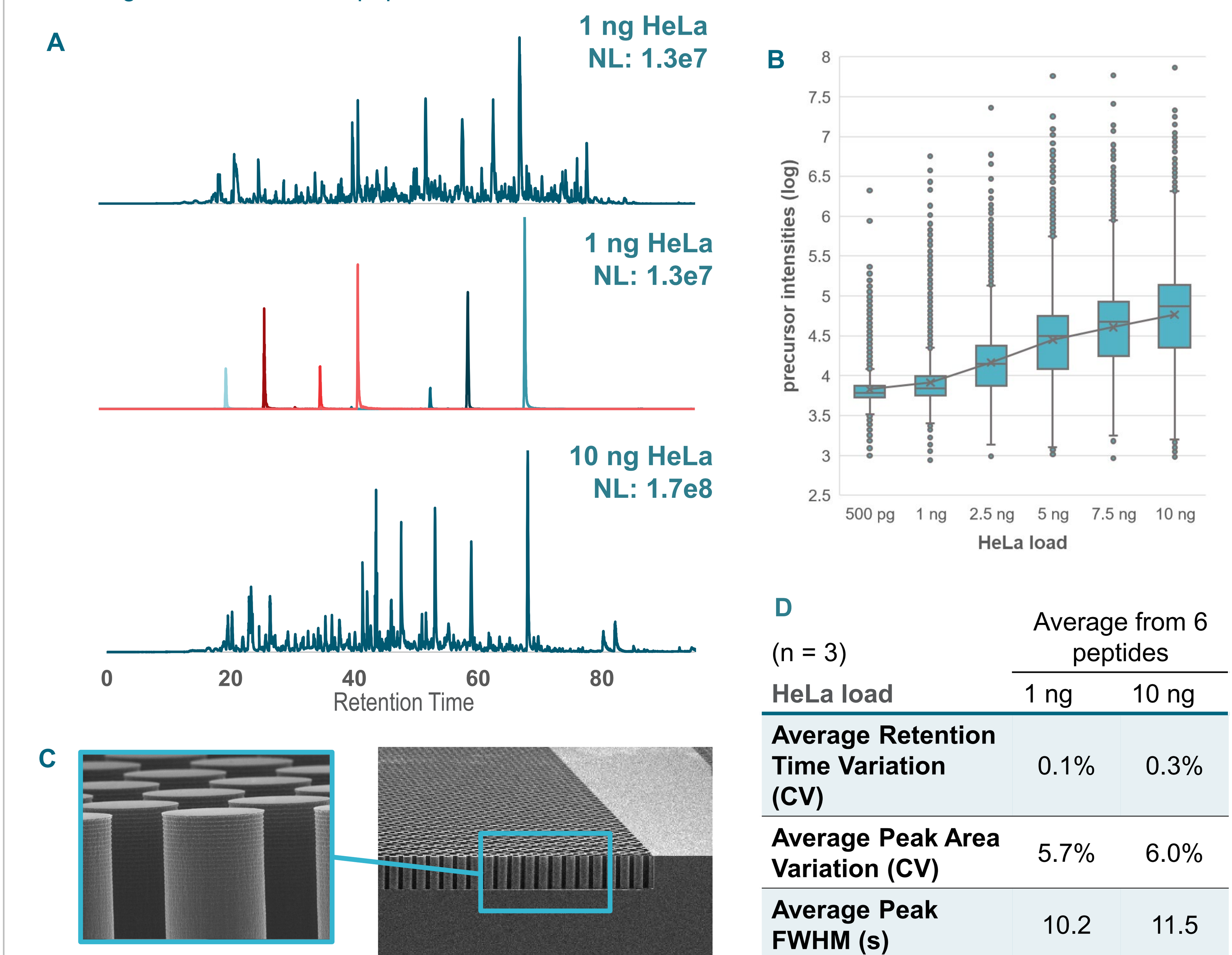


Figure 4. Outstanding Chromatographic Performance and Signal Intensities of µPAC C18 SCP Column Prototype for Highly Diluted Samples

(A) Basepeak chromatograms and extracted ion chromatogram (XIC) of seven peptides from 1 ng and 10 ng HeLa. (B) Box and whisker charts of precursor intensities from different amounts of HeLa. (C) Electron microscopic image of µPAC™ pillar structure. (D)Average chromatographic parameters for 60 min gradient of six HeLa peptides.



(n = 3)	Average from 6 peptides	
HeLa load	1 ng	10 ng
Average Retention Time Variation (CV)	0.1%	0.3%
Average Peak Area Variation (CV)	5.7%	6.0%
Average Peak FWHM (s)	10.2	11.5

CONCLUSIONS

- Non-porous µPAC™ columns deliver high intensities of extremely low sample loads while showing sharp peaks and high reproducibility of retention times and peak areas. These properties make them ideal for future real-life single cell applications.
- FAIMS effectively filters out unwanted background ions that mask low abundant analyte ions in highly diluted samples, thereby greatly enhancing the sensitivity of an Orbitrap Exploris 480™.
- Tailoring the method to most exhaustive search strategies as offered by Proteome Discoverer™ 2.5 and implemented third-party nodes can significantly increase the proteome coverage of very low sample amounts.

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