Microflow 4D-Proteomics™ for robust, high-throughput sample analysis

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Introduction

A high level of reliability in combination with adequate depths of analysis are essential for high-throughput proteomics. For this application, we therefore evaluated a combination of:

(1) Robust microflow-based chromatography

(2) Efficient ionization at microflow rates

(3) Ion mobility separation and time-of-flight detection

Methods

(1) **Chromatography:** LC-40B X3 and Nexera Mikros (Shimadzu) **Column:** Luna Omega, 1.6 µm Polar C18, 100 Å, 150x1mm (Phenomenex) unless stated otherwise. **Solvents**: water with 0.1% formic acid and 3% DMSO (A) and acetonitrile with 0.1% formic acid and 3% DMSO (B)

(2) **Ionization:** VIP-HESI source with 50 µm inner diameter emitter (Bruker)

(3) **Mass spectrometer:** timsTOF HT (Bruker)

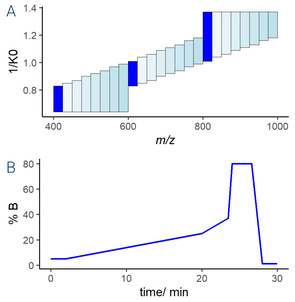

Figure 1. (A) 25 *m/z* DIA windows; 8x3 schema acquisition. Non-overlapping windows were used to efficiently target a peptide-specific area in 1/K0 – *m/z* dimension for fragmentation. (B) LC-gradient for peptide separation. The flowrate was set to 50 µl/min. The total gradient length was adjusted proportionally to the total runtime (17 min, 30 min, and 60 min). Peptide separation was performed at 50 °C.

Results

1 Consistency across iterative sample injections

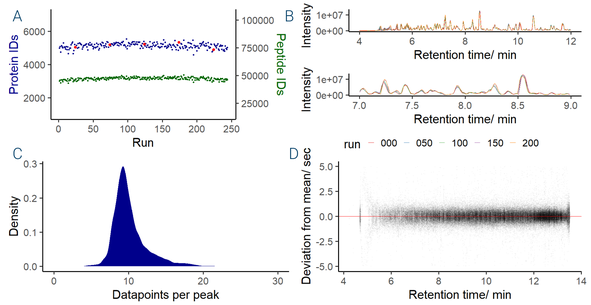

Figure 2. 1200 ng HeLa tryptic digest were separated on a ReproSil Saphir C18, 100 Å, 150x1mm (Dr. Maisch) column (17 min runtime) and analyzed by dia-PASEF (50 *m/z* DIA windows; 6x2 schema). (A) Consistent identification of ~5000 proteins and 45000 peptides. Data were analyzed with PaSER 2023 (Bruker). (B) Overlay of 5 chromatograms corresponding to runs 0, 50, 100, 150, 200 (highlighted in red in A) indicate little change in chromatographic performance over time. (C) Distribution of the number of datapoints per peak. An average of ~10 datapoints are acquired per eluting peptide ion, which is generally sufficient for quantitation. (D) Small deviation from mean (average <<1 s) across gradient for runs 0, 50, 100, 150, 200 confirm consistent and reliable long-term chromatographic performance.

2 Influence of gradient length on identifications

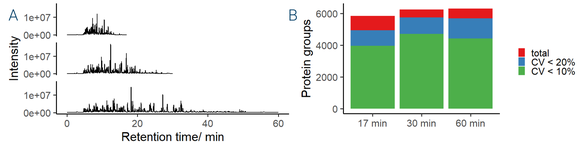

Figure 3. 1200 ng HeLa tryptic digest were separated at different gradient lengths and analyzed by dia-PASEF (25 *m/z* DIA windows; 8x3 schema). (A). Base peak chromatograms corresponding to a total runtime of 17 min, 30 min, and 60 min. (B) Quantification of ~5000 proteins groups with CV < 20% for a total runtime of 17 min. Using longer gradients provides little benefits regarding the overall number of identified protein groups, albeit quantification accuracy is slightly increased. Data were analyzed with Spectronaut 17 (Biognosys).

3 Influence of peptide amount on peak widths and number of identifications

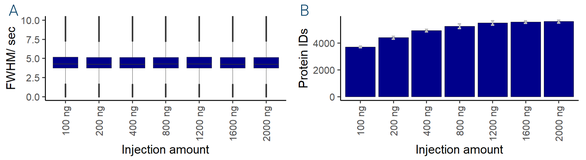

Figure 4. 100 ng to 2000 ng HeLa tryptic digest were separated (17 min runtime) and analyzed by dia-PASEF (25 *m/z* DIA windows; 8x3 schema). (A) The median full width half maximum (FWHM) of eluting peptides is below 5 sec. Increasing the injection amount up to 2000 ng doesn't negatively affect the FWHM of eluting peptides, indicating a sufficiently high column capacity. (B) 100 ng are sufficient to identify more than 3500 proteins, and ~ 5000 proteins can be identified from 1200 ng injected peptide. Data were analyzed with PaSER 2023 (Bruker).

4 Quantitation accuracy based on mixed proteome samples

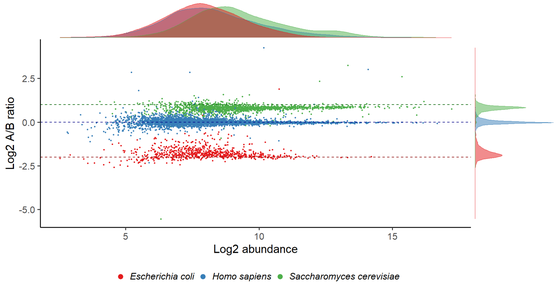

Figure 5. 4 µg tryptic digest mixtures HYE124 were separated on a ReproSil Saphir C18, 100 Å, 150x1mm (Dr. Maisch) column (30 min runtime) and analyzed by dia-PASEF (25 *m/z* DIA windows; 16x2 schema). Determined protein quantities were used to calculate log2 A/B ratios for mixtures corresponding to 1:1 for *H. sapiens*, 2:1 for *S. cerevisiae* and 1:4 for *E. coli* (indicated by dashed lines). Data are based on triplicate measurements. Data were analyzed with Spectronaut 17 (Biognosys)

5 Application of microflow 4D-Proteomics for plasma

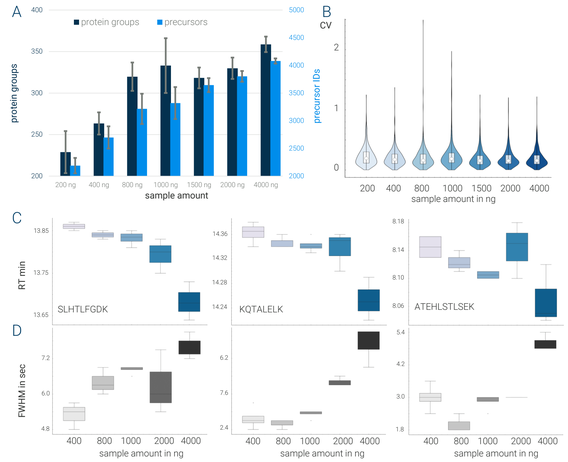

Figure 6. (A) Protein groups and precursor identifications from different loading amounts of tryptic digest of human plasma (from Biomex and Sigma-Aldrich) onto the microflow HPLC-MS system. All samples were analyzed with a 30 min gradient. A steady increase in identifications is observed with increased sample loading. For plasma proteomics, it is important to acknowledge that the total number of identifications significantly depends on additional factors like source and quality of plasma, sample preparation, and further data processing. Therefore, when solely comparing technical aspects of LC-MS, it is more appropriate to focus on relative changes and quantitative accuracy. (B) Reproducibility of quantitation is demonstrated between 200 and 4000ng plasma loading; average CV values for all samples are ~15%. (C) Retention times of 3 selected high abundant plasma peptides. (D) Peak width of the previously shown plasma peptides.

Summary

A total of 17 min run-to-run is sufficient to identify ~5000 proteins from HeLa. Furthermore, more than 300 proteins can be detected from challenging human plasma samples with a 30 min gradient using a combination of Mikros-LC (Shimadzu) and timsTOF HT equipped with a VIP-HESI source and 50 µm emitter (Bruker).

A combination of microflow chromatography, electrospray ionization with low inner diameter emitter and ion mobility mass spectrometry is suitable for routine analysis of proteomics samples, even for sub-microgram sample quantities

Technology

Innovation with Integrity