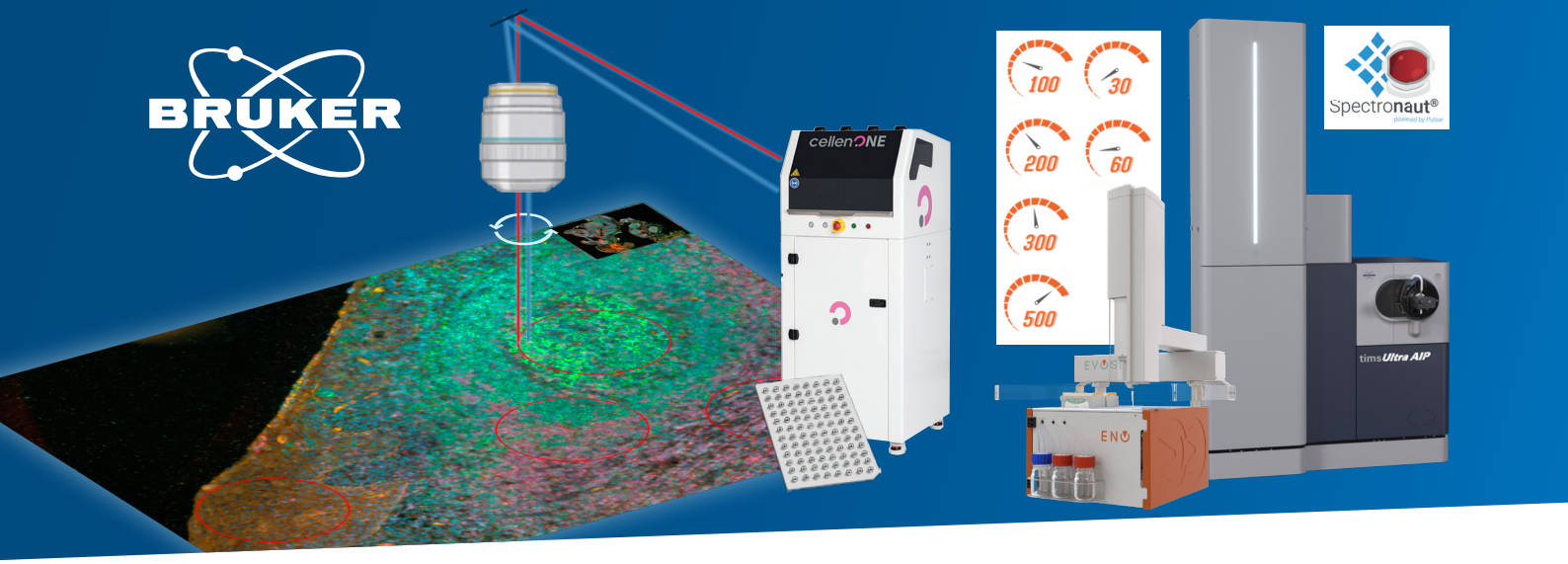


Scalable spatial proteomics with preserved bio insights using the Evosep Eno standard gradients on the timsUltra AIP

Benchmarking of laser microdissected (LMD) human FFPE tonsil tissue using miniaturized sample preparation (cellenONE), with the standardized gradients on the Evosep Eno, and the high sensitivity and robustness of the timsUltra AIP® demonstrates excellent scalability, consistency, and reproducibility, enabling comprehensive large cohort investigations.

Abstract

Characterizing heterogeneous tissues in a spatially preserved manner requires analyzing a large number of samples to understand biological processes in phenotypically diverse locations across the tissue specimen. Advances in ultra-high sensitivity MS combined with miniaturized sample preparation and fast chromatography enables proteome depth and sample throughput, crucial factors for large clinical cohort studies. Here, we assess performance, scalability, and reproducibility of cellenONE-based sample preparation, fast standardized peptide separation with the Evosep Eno system coupled to ultra-high sensitivity mass spectrometry on the timsUltra AIP.

Contours from human FFPE tonsil tissue of different niches of interest (T-cell zone, B-cell zone, mixed zone of B-cells and T-cells, and epithelial zone) demonstrated distinct biological differences between cell types at all gradient lengths with good quantitative accuracies. Proteome depths increased from about 2000 protein groups across the different cell-type niches at 500 samples per day (SPD) by a factor of three to 6000 protein groups at 30 SPD. Among differentiating proteins, well-known marker proteins were identified (e.g. epithelium: Cadherin1, T-cells: CD3: B-cells: CD19)- These results demonstrate excellent scalability and reproducibility of the cellenONE, Evosep Eno, and timsUltra AIP workflow, enabling comprehensive large cohort investigations.

Keywords:
DVP, LMD, Evosep Eno, timsUltra AIP, Tonsil tissue

Christoph Krisp[1,2], Melissa Klingeberg[2,3,4], Fabian Coscia[2,3], Dorte Bekker-Jensen[5], Ole Bjeld Hørning[5], Nicolai Bache[5], Daniel Hornburg[6]; [1]Bruker Daltonics GmbH & Co. KG, Bremen, Germany; [2]MDC-Bruker Center of Excellence for Single Cell Omics, Max-Delbrück Center – Berlin Institute of Systems Biology (BIMSB), Berlin, Germany; [3]Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), Spatial Proteomics Group, Berlin, Germany; [4]Humboldt University Berlin, Berlin, Germany; [5]Evosep, Odense, Denmark; [6]Bruker Scientific LLC, Billerica, MA, USA.

Introduction

In the last decade, analyzing formalin fixed paraffin embedded (FFPE) tissues have become feasible to be investigated with LC-MS/MS techniques with proteome coverage comparable to fresh frozen studies (1, 2). This gives access to half a century worth of archived human samples in healthy and diseased settings contexts. Several large-scale proteomics studies have been performed to date. However, bulk proteomics often capture only parts of the biological processes in these tissues. Recent advances in tissue-based imaging, laser microdissection, miniaturized sample preparation, fast and standardized chromatography, and ultra-high sensitivity mass spectrometry have paved the way for highly resolved, deep proteome profiling in a spatial manner. The Deep Visual Proteomics (DVP) approach (3), which was rapidly recognized by Nature Methods as the "Method of the Year 2024" (4), allows the dissection of single-cell shapes or few-cell shapes belonging to specific niches of interest (e.g., tumor invading front, immune cell rich areas) enabling deep biological insights into the tissue's biology.

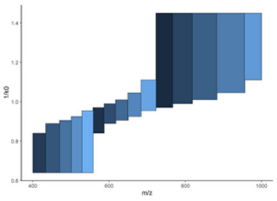
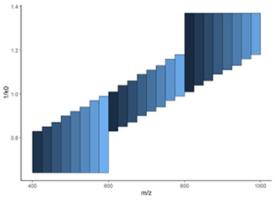
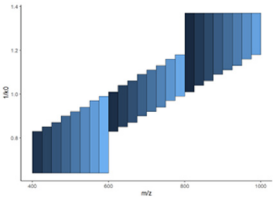
A challenge of this methodology is the large number of regions that need to be analyzed, often reaching into the hundreds. Therefore, large cohort style studies require sample turnarounds in realistic time frames. Klingeberg *et al.* (5) recently demonstrated a spatial proteomics workflow where they achieved deep characterization of a head and neck squamous cell carcinoma tissue at a speed of 100 spatial proteomes per day including sample preparation and LC-MS/MS based acquisition. They utilized the proteoCHIP EVO96 for gravity-based sample collection after laser microdissection and reported a capturing success rate of >95%. The semi-automated sample preparation workflow inside the cellenONE robot allowed for a fast 2.5 h process from tissue lysis to ready-to-inject peptide samples. Analysis at Evosep's Whisper Zoom 120 SPD allowed for the profiling of 100 spatial proteomes within a day and revealed deep insights into the cancer's biology.

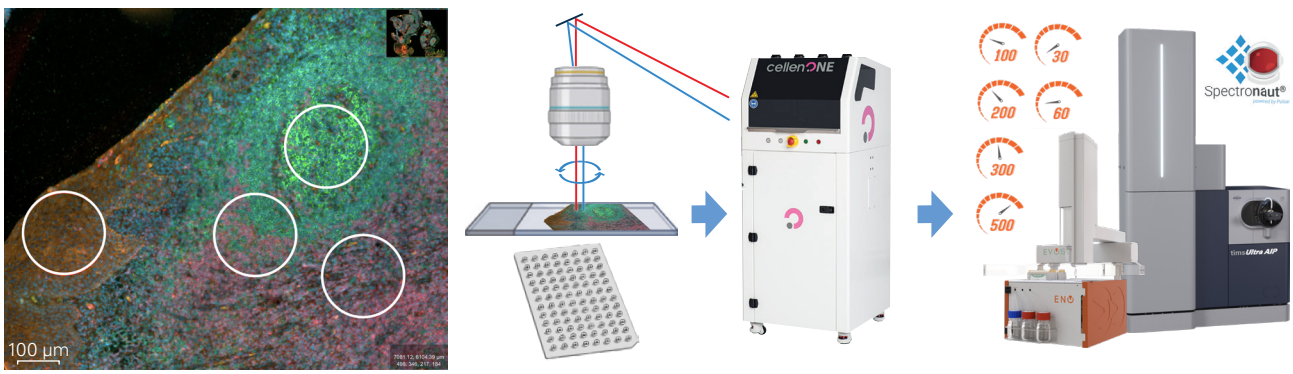
Motivated by these findings we set out to perform a performance benchmarking of the Evosep ENO standard gradients at 30, 60, 100, 200, 300, and 500 SPD on human FFPE tonsil tissue dissected by niches of T-cells, B-Cells and epithelial zones and detection in dia-PASEF mode on a timsUltra AIP.

Methods

A human FFPE tonsil tissue section on a PPS frame slide (Leica) at 5 µm thickness was characterized by immunofluorescence, staining with human anti-CD3 (T-cell marker), anti-CD19 (B-cell marker) and anti-cadherin 1 antibodies. Niches of T-cell, B-cell, mixed T-cell and B-cell, and epithelial zones were selected and regions of 30,000 μm^2 (150,000 μm^3 = 150 pL of tissue or ~60 HeLa cells) were excised using laser microdissection (LMD; Leica LMD7) with sample collection by gravity into proteoCHIPs EVO96. Plate was placed into the cellenONE, lysis buffer consisting of 20% ACN, 0.1% n-dodecyl β-D-maltoside (DDM), 0.1 M triethylammonium bicarbonate (TEAB), 5 mM tris(2-carboxyethyl)phosphine (TCEP), and 20 mM chloroacetamide (CAA) was dispensed onto the tissue pieces, plate was heated to 65°C at 85% humidity and rehydration enabled for 1 h. After cooldown, 1 µL of 10 ng Trypsin/LysC (Promega) was added and proteins digested for 1 h at 37°C at 85% humidity and rehydration enabled. Digested samples were transferred onto Evotips by direct transfer by centrifugation from the proteoCHIP EVO96. Peptide separation was performed on an Evosep Eno using the standard gradients at 500, 300, 200, 100, 60, and 30 SPD speed with column temperature set to 40°C and eluted into a timsUltra AIP. Peptide analysis was performed in dia-PASEF mode using gradient length specific methods to match cycle times to peak widths (table 1). Acquired data were processed in Spectronaut® 21 using directDIA™. Samples from the same gradient type were processed together without method evaluation (Figure 1).

Table 1. dia-PASEF acquisition method overview.
dia-PASEF acquisition methods tailored to gradient length and column-dependent peak properties. For 500 – 200 SPD (4 cm PepSep column) a pyDiAID (6) method with 5 MS/MS PASEF cycles of 3 variable mass ranges each. For 100 – 60 SPD (8 cm PepSep column) and 30 SPD (15 cm PepSep column) a method with 8 MS/MS PASEF cycles of 3 fixed 25 Da mass ranges each.

	500-200 SPD	100-60 SPD	30 SPD
dia-PASEF window scheme			
Mass range MS1 (*m/z*) Mass range MS2 (*m/z*)	100-1700 400-1000	100-1700 400-1000	100-1700 400-1000
Ion mobility range (1/K_0)	0.7-1.3	0.7-1.3	0.7-1.3
Accumulation and ramp time (ms)	70	70	100
Cycle time (s)	0.46	0.64	0.96
AIP profile	800-0 Vpp slope 25 Vpp/µs	800-0 Vpp slope 25 Vpp/µs	800-0 Vpp slope 25 Vpp/µs
Storage base time MS1 (µs)	15	15	15
Storage base time MS2 (µs) ramped from 0.8-1.4 1/K_0	14-16	14-16	14-16
Spray voltage (V)	1400	1400	1400
Source temperature (°C)	200	200	200



30,000 µm² regions
Epithelial (CDH1), B-cell (CD19), T-cell (CD3), mixed (B / T-cell) niches

Laser microdissection
Gravity-based collection into proteoCHIP EVO96

2.5 h sample preparation
Lysis & denaturing at 65°C
Reduction & alkylation
digestion

LC-MS/MS
Separation with Evosep Eno standard gradients
Data acquisition in diaPASEF
Data processing in directDIA

Figure 1. Sample preparation workflow.
Schematic workflow representation from region of interest selection of immunofluorescence characterized human FFPE tonsil tissue, laser microdissection (LMD) and gravity-based sample capture into proteoCHIPs EVO96, tissue lysis and digestion inside the cellenONE dispensing robot, sample cleanup and transfer onto Evotips Pure, LC-MS/MS based acquisition with peptide separation on Evosep Eno 500, 300, 200, 100, 60, 30 SPD standard gradients and peptide detection in dia-PASEF mode on a timsUltra AIP, and data processing in directDIA using Spectronaut 21.

Results and discussion

Processing of human FFPE tonsil tissue across four niche types (T-cells, B-cells, mixed B-cell/T-cell regions, and epithelium) demonstrated robust proteome coverage that is primarily governed by gradient length, while preserving strong biological differentiation across all conditions (Figure 2A). The highest throughput setting (500 SPD), approximately 2000 protein groups per niche and ~2800 protein groups in total were consistently identified, already enabling clear discrimination of tissue-specific proteomic signatures. Increasing gradient length led to a systematic and predictable increase in proteome depth across all niche types. Relative to 500 SPD, protein identifications increased by ~25% at 300 SPD and ~50% at 200 SPD at the individual niche level, corresponding to gains of ~500 and ~1000 protein groups, respectively, in total identifications.

Further extensions of the gradient continued to improve proteome coverage, with approximately two-fold higher protein group identifications observed at 100 SPD compared to 500 SPD. At the lowest throughput settings (60 and 30 SPD), proteome depth reached up to ~5500–6000 protein groups per niche and ~6500–7000 protein groups in total.

Across all gradient conditions, the epithelial niche consistently yielded slightly lower protein group identifications compared to immune cell–rich regions. Importantly, despite differences in proteome depth, quantitative consistency remained high. Protein abundance profiles showed excellent Pearson correlations across all niches and gradient lengths (Figure 2C), with highest concordance observed within identical methods and only gradual deviations across the full throughput range.

Principal component analyses (PCA) for dimension reduction were performed (Figure 3A) at each standard gradient individual filtered for proteins identified across all samples. In all

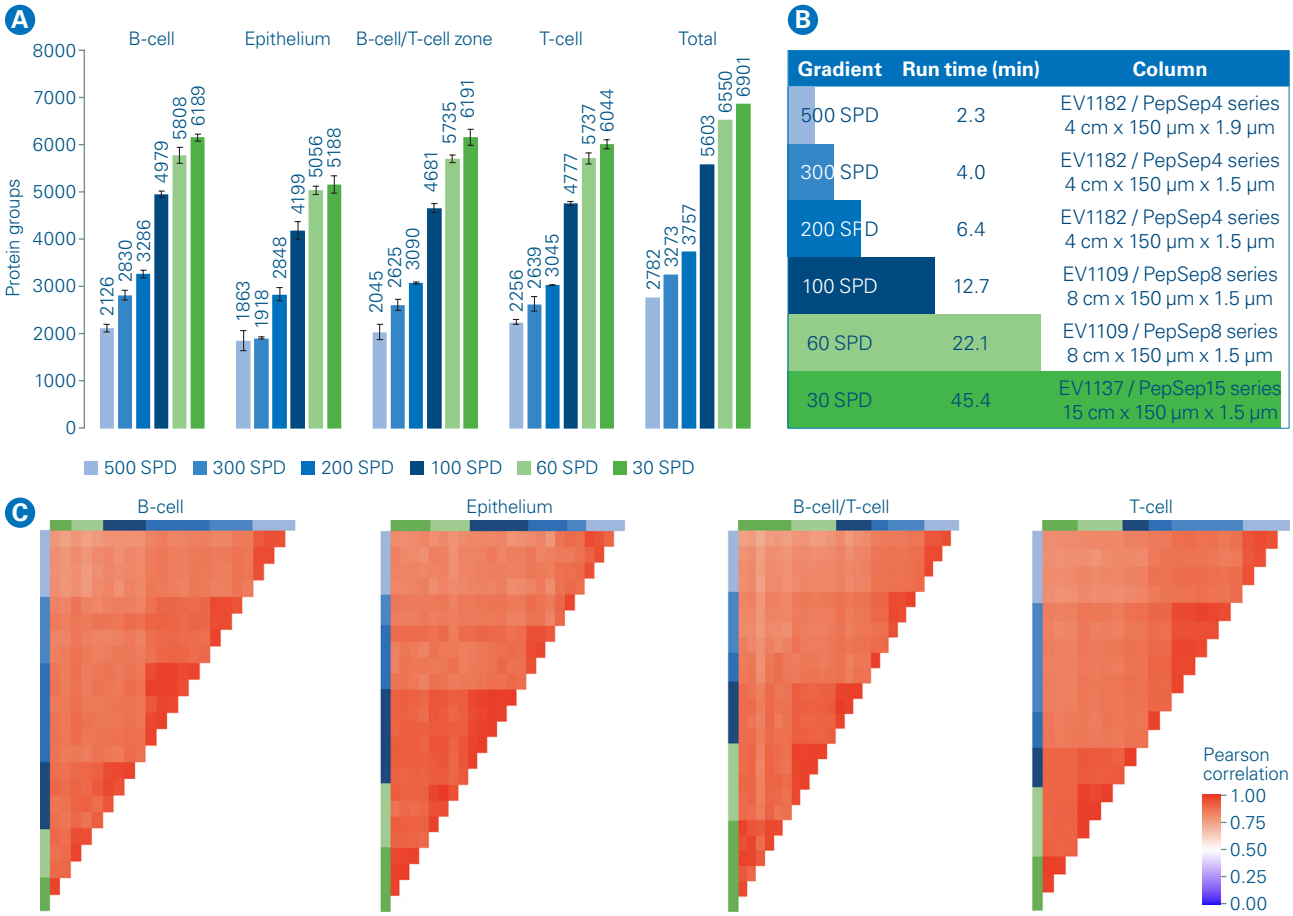


Figure 2. Protein identification metric, Gradient information and protein abundance correlation.
Ⓐ Protein group identification rates obtained from 150 k µm³ human FFPE tonsil tissue contours using Spectronaut 21 in directDIA mode with processing of all samples run with same standard gradients of the Evosep Eno together without method evaluation; Ⓑ Evosep Eno standard gradient conditions; Ⓒ Pearson correlation matrix of protein abundance profiles of the same cell type niche samples across all 6 Evosep Eno standard gradients.

conditions, principal component 1 distinctly differentiated the immune cell niches from the epithelial niche, component 2 differentiated in most gradient conditions the T-cells from the B- cells however, the mixed niche frequently clustered with the T-cell niche, assuming higher content of T-cells in these niches. Reducing the protein matrix to all proteins identified in all samples across all gradient lengths (914 protein groups) component 2 became the differentiating factor between the immune cell niches and the epithelial niche whereas component 1 differentiated based on gradient lengths (Figure 3B). As the different niches selected in this study comprises different cell types, filtering for proteins commonly identified across all samples will omit cell type specific proteins. Hence, data imputation based on a downshifted normal distribution was performed on the dataset comprising all samples across all gradient lengths. Like the PCA in Figure 3B, PCA of the dataset with data imputation preserved the distinct differentiation of the epithelial niche from the immune cell niches in component 2, though at different extend based on the gradient length. Component 1 still differentiates based on gradient length (Figure 3C).

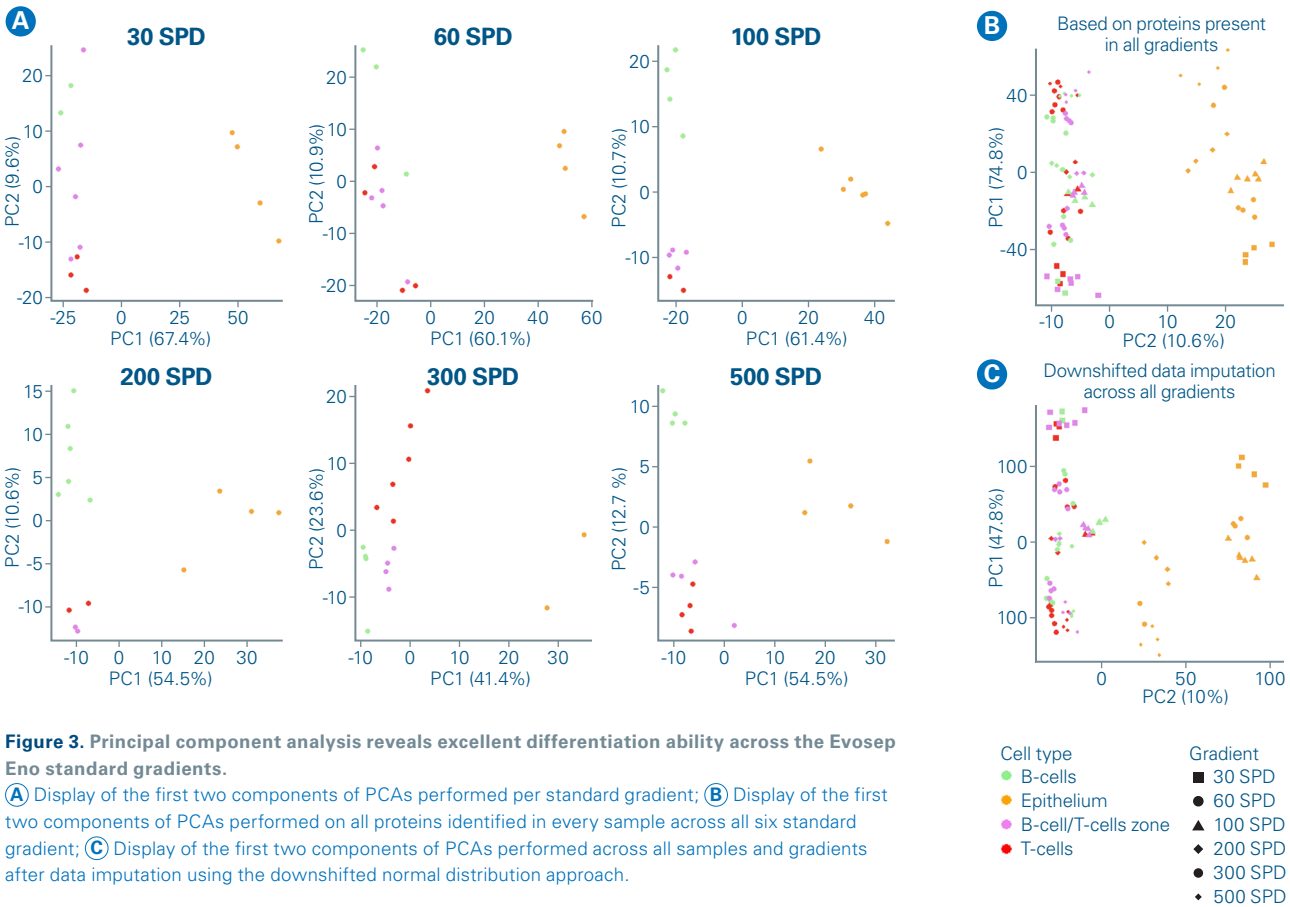


Figure 3. Principal component analysis reveals excellent differentiation ability across the Evosep Eno standard gradients.
Ⓐ Display of the first two components of PCAs performed per standard gradient; Ⓑ Display of the first two components of PCAs performed on all proteins identified in every sample across all six standard gradient; Ⓒ Display of the first two components of PCAs performed across all samples and gradients after data imputation using the downshifted normal distribution approach.

To evaluate biological insights at the various standard gradient lengths, protein groups identified at the specific gradient length were used, followed by downshifted data imputation to include cell type specific proteins in statistical analyses. Based on student's t-test p-value and log2 fold changes, volcano plots for epithelial niche vs. T-cell niche (Figure 4A), B-cell niche vs. T-cell niche (Figure 4B) and epithelial niche vs. B-cell niche (Figure 4C) were generated. Boundaries for significance selection was -log10(p-value > 1.3 and log2(fold change) < -1 or > 1. Markers used for T-cells were CD3D, CD3E, CD3G, CD4, CD5, CD8A and CD99, for B-cells were CD19, CD72 and CD79B, and for the epithelium were CDH1, Keratin 4 and 13, and MUC21. Most of these markers, when identified, were present in the clouds of proteins associated with the cell type niche. Across the gradient lengths, significance values varied, reflecting the differences in sample numbers per conditions, whereas fold changes remained comparable. As expected, decreasing gradient length resulted in the loss of some markers, however, canonical markers such as CD3, CD19 and CDH1 remained detectable and significantly differential among the proteins associated with the cell type niche.

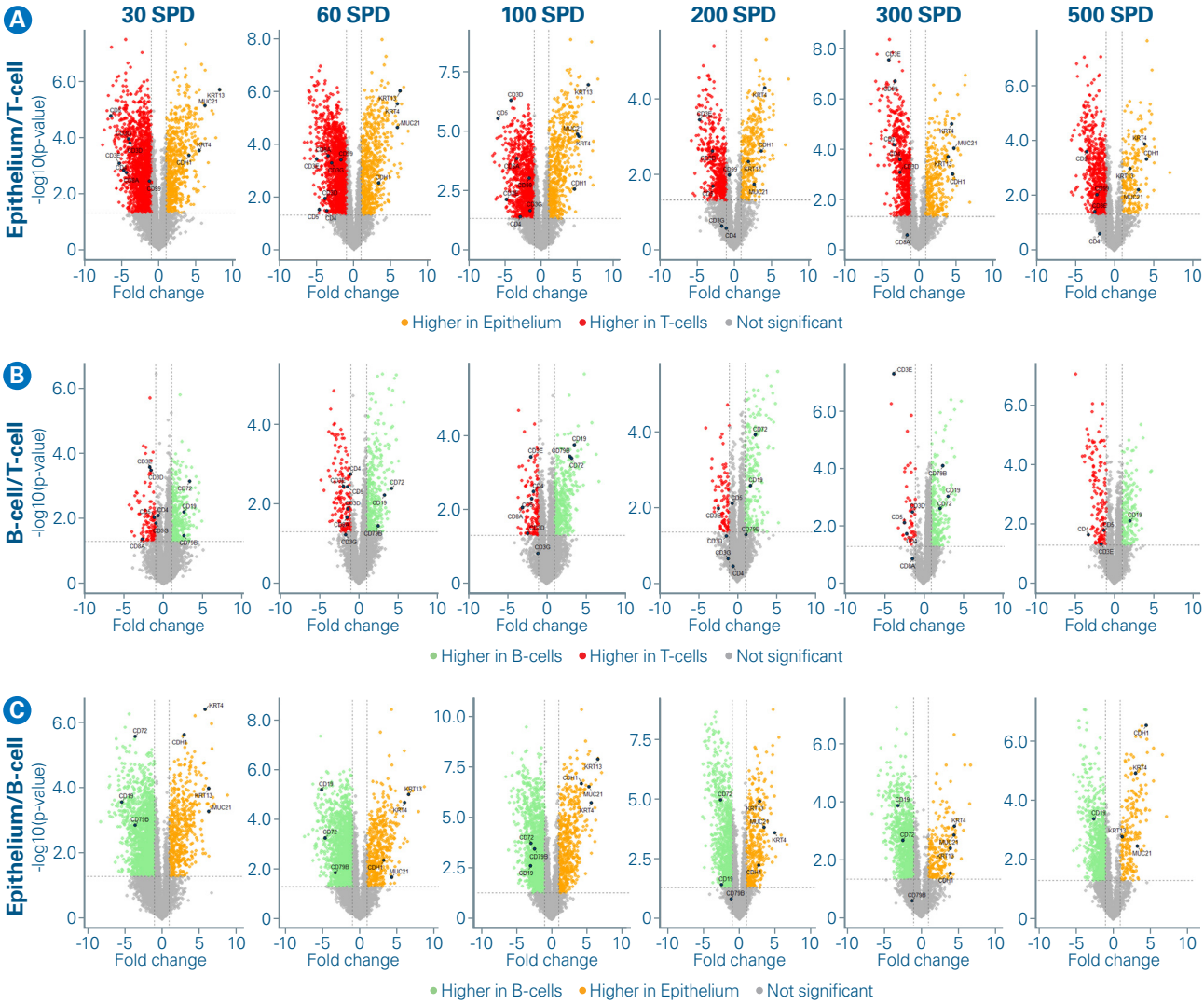


Figure 4. **Consistent identification of cell type specific profiles across gradients.**
Volcano plot representation of statistical analysis performed after data imputation by downshifted normal distribution per standard gradient comparing Ⓐ epithelial niche (orange) against T-cell niche (red) Ⓑ B-cell niche (light green) against T-cell niche (red) and Ⓒ epithelial (orange) niche against B-cell niche (light green). Cell type specific marker proteins highlighted; CD3D, CD3E, CD3G, CD4, CD5, CD8A, and CD99 used for T-cells, CD19, CD72, and CD79B used for B-cells and CDH1, Keratin 4, Keratin 13, and MUC21 used for epithelium.

Depicted in the principal component analyses in Figure 3A, most mixed B-cell/T-cell niches group close to T-cell niches. Nonetheless, statistical analyses confirm the presence of both B-cell and T-cell marker proteins in the mixed niches. Protein abundance of the surface markers CD19 (B-cell), and CD3E and CD5 (T-cell) plotted for the different gradient lengths and cell type niches (Figure 5) demonstrate that protein abundances of these markers in the mixed B-cell/T-cell zone are quantitatively intermediate between the B-cell niche and the T-cell niche.

In summary, this benchmarking study demonstrates excellent scalability, consistency, and reproducibility when combining miniaturized sample preparation in the cellenONE, with the standardized gradients on the Evosep Eno, and the high sensitivity and robustness of the timsUltra AIP. This workflow is enabling comprehensive large cohort investigations.

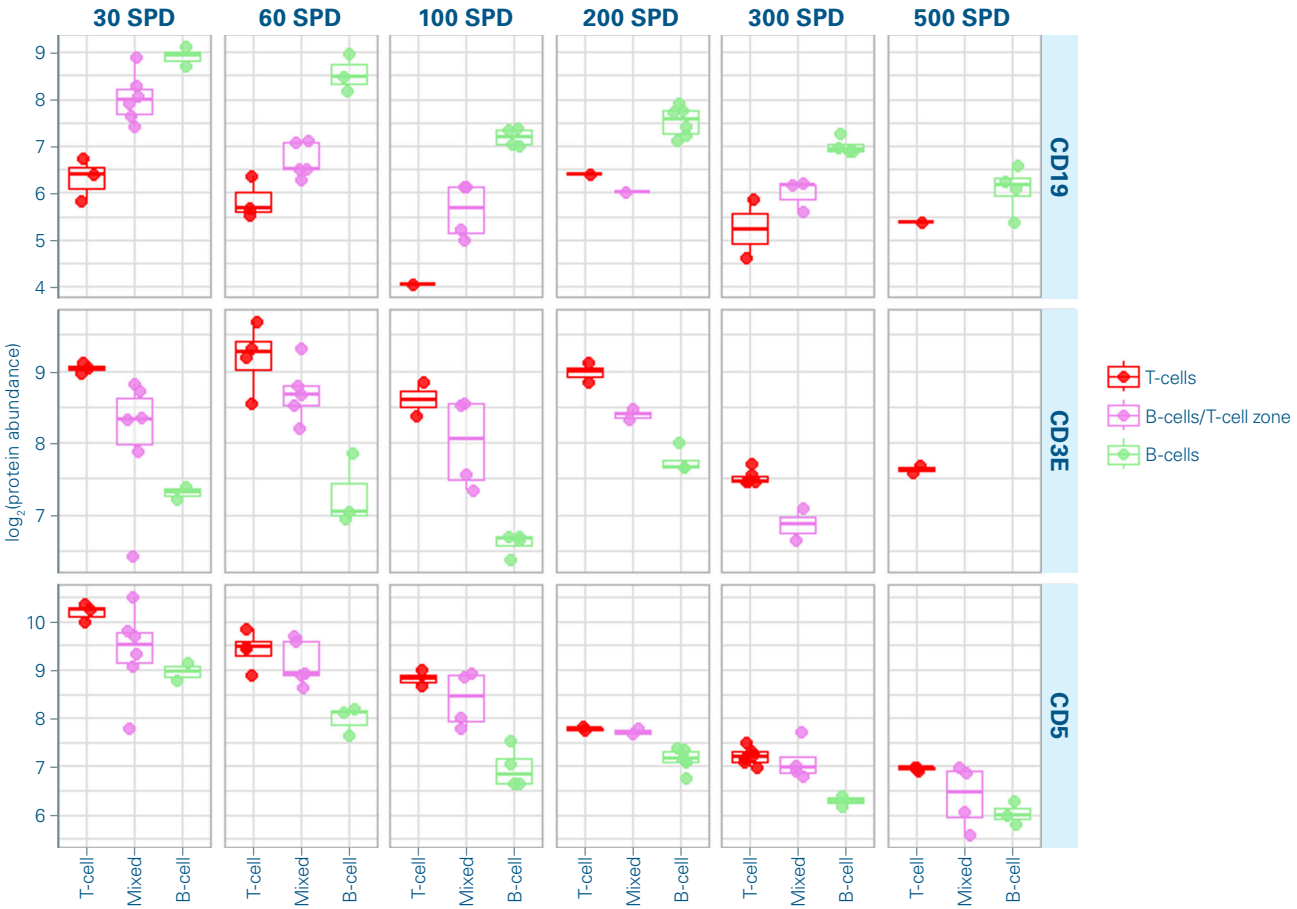


Figure 5. **B-cell/T-cell mixed niches represent markers from both, T-cell and B-cell niches.**
Box plot representation of log_2(protein abundance) of CD3D, CD5 and CD19 across the 6 standard gradients for T-cell niche (red), B-cell/T-cell mixed niche (violet) and B-cell niche (light green); no data imputation.

Conclusion

- Spatial proteomics at scale going from 30 SPD throughputs to 500 SPD and maintain distinct tissue region information
- timsUltra AIP enables deep proteome profiling from limited FFPE tissue material and preserving spatially distinct features even at 2.5 min run times
- Cell type specific marker proteins detected at all gradient speeds

References

[1] Piehowski et al. (2018). Clin Proteom, **15**, 26

[2] Marchione et al. (2020). J Proteome Res, **19**(2), 973-983

[3] Mund et al. (2022). Nat Biotechnol, **40**, 1231–1240

[4] Nat Methods, 2024. **21**, 2195–2196

[5] Klingeberg et al. (2026). Mol Cell Proteom, (1):101489

[6] Skowronek et al. (2022). Mol Cell Proteom, (9):100279

Bruker Daltonics is continually improving its products and reserves the right to change specifications without notice. © Bruker Daltonics 05-2026, LCMS-262, 1929349

For Research Use Only. Not for use in clinical diagnostic procedures.

Bruker Switzerland AG

Fällanden · Switzerland
Phone +41 44 825 91 11

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com

Learn more at **www.bruker.com/timsUltra-AIP**