01

Towards 1000 cells per day - scalable single cell analysis using the Evosep Whisper Zoom methods on the timsTOF Ultra 2

Lars Kristensen[1], Christoph Krisp[2], Dorte Bekker Jensen[3], Ole Bjeld Hørning[3], Nicolai Bache[3], Markus Lubeck[2]
[1] Bruker Nordic, [2] Bruker Daltonics GmbH, [3] Evosep Denmark

Scalability is one of the crucial factors for single cell proteomics to allow meaningful data collection with reliable and robust statistical power for near single cell tissue input applications. Multiplexing and fast chromatography with low overhead time aids in upscaling proteome analyses. Here, we demonstrate applicability of the new Whisper Zoom methods on the Evosep system for speeding up label free sample analysis up to 120 samples per day (SPD) and exceeding >1,000 samples per day in a multiplexing approach with data acquisition on the timsTOF Ultra 2.

Analyses of HeLa cell digest dilutions (Pierce) from 50 ng to 0.25 ng were performed to assess sensitivity of the different Whisper Zoom methods identifying more than 4,000 protein groups from 250 pg at 120SPD, 5,000 proteins at 80SPD and 5,500 protein groups at 40SPD speed and reaching 6,500 protein groups at 120SPD, 7000 protein groups at 80SPD and 7700 protein groups at 40SPD from the higher loads. From single Hela cells, protein identification rates correlated with gradient length, identifying on average 2,500 protein groups at 120SPD, 3,000 protein groups at 80SPD and 4,000 protein groups at 40SPD.

The 9-plex multiplexing approach run at Whisper Zoom 120SPD speed resulted across the 1,080 samples on average in the identification of 1,500 protein group per label/cell type. Excellent tip to tip chromatographic reproducibility as well as quantitative accuracy within and across TMT batches was observed, accurately separating the 360 HEK 293 samples from the 360 HeLa samples and the 360 K562 samples.

02

reagents with increased multiplexing capacity facilitate deep and high-throughput dia

Egor Vorontsov[1], Sasa Koncarevic[1], Gomathi Ayyasamy[1], Harald Legner[1], Nikolai Wöllmer[1], Karsten Kuhn[1], Ian Pike[2]
[1] Proteome Sciences R&D GmbH & Co. KG, [2] Proteome Sciences plc

Background: We demonstrate the performance of a six-plex subset of dedicated isotopologue amine-reactive reagents for DIA analysis with high multiplexing potential.

Materials and methods: Six amine-reactive isotopologue reagents for multiplexed DIA were synthesized in-house. Model systems consisting of mammalian and yeast tryptic digests were labelled and analysed using Thermo Orbitrap Tribrid and Exploris instruments. LC-MS data was processed using DIA-NN 1.9.

Results: Collision energy-dependent behaviour of the labelled peptides was studied in detail. Peptides labelled with the novel reagents demonstrate favourable chromatographic and fragmentation properties. Six-plex samples based on mixed proteomes with known spiking ratios demonstrate high specificity and accuracy of quantification coupled to acceptable proteome coverage at the throughput of up to 180 SPD. Low loading-amount samples with a carrier channel demonstrated deeper proteome coverage and higher throughput than comparable label-free samples at the single-cell loading level on the same Orbitrap instrument. Spectral libraries based on empirical data for labelled peptides improved performance and coverage of the highly resource-intensive computational analysis of the multiplexed DIA data.

Conclusion: We show favourable depth and quantitative performance of the six-plex subset of the new isotopologue reagents. We further discuss associated challenges and practical approaches for increasing the multiplexing rate and improving the performance and depth of the analysis.

03

Determining APOE ε4 carriership by NUcleic acid Linked Immuno-Sandwich Assay (NULISA)

Kübra Tan[1], Andrea Benedet[1], Bingqing Zang[2], Nesrine Rahmouni[3], Ilaria Pola[1], Guglielmo Di Molfetta[1], Kaj Blennow[1], Nicholas J. Ashton[1], Pedro Rosa-Neto[3], Henrik Zetterberg[1]

[1] University of Gothenburg, [2] Alamar Biosciences, [3] McGill University

The authors have chosen not to publish the abstract.

04

Discovery of diagnostic biomarkers for early detection of Non Small Cell Lung Cancer (NSCLC)

Irene Villanueva Sanz, Noora Sissala, Xiaofang Cao[1], Haris Babacic, Lars E. Eriksson, Janne Lehtiö, Maria Pernemalm, Lukas Orre[1,2]
[1] Scilifelab, [2] Karolinska Institute

Non-small cell lung cancer (NSCLC) represents the majority of lung cancer cases and remains the leading cause of cancer-related deaths worldwide. While surgical resection currently offers the main curative option, the unfortunate reality is that a significant number of patients are ineligible for surgery due to tumor spread resulting from late detection. Hence, the need for early detection methods in lung cancer is crucial.

In our previous research, we assembled an exploratory cohort of patients with suspected advanced-stage NSCLC (IIIA-IV) to detect plasma biomarkers that could assist in the early diagnosis of NSCLC. Utilizing in-depth proteomics through HiRIEF LC-MS/MS on plasma samples, we employed a machine learning pipeline to identify differentially expressed proteins that distinguish lung cancer patients' plasma from controls.

To broaden our findings in early-stage disease, an independent discovery cohort of 186 plasma samples representing controls and patients with early and late-stage lung adenocarcinoma (IA-IV) was included. Mass spectrometry analyses were conducted on these samples to explore the potential biomarkers identified in the initial study. Between 638-954 proteins were identified and quantified per sample across the cohorts. Ongoing data analysis will be presented at the conference.

This study focuses on exploring novel biomarkers for the early detection of NSCLC, emphasizing the potential of clinical proteomics. The integration of these techniques into clinical practice holds promise for enhancing the diagnosis and management of lung cancer patients.

05

Histone deacetylase inhibitor altering adipose derived stem cells fate transition toward epidermal lineage

Sallam Abdallah[1], Ana Maria Carrasco Del Amor[1], Ahmed Elserafy[1]
[1] Linköping University

The authors have chosen not to publish the abstract.

06

Primary human jejunal organoids for drug disposition studies

Evgeniya Mickols[1], Per Artursson[1], Mikael E. Sellin[2], Maria Karlgren[1], Volker Lauschke[3,4,5]

[1] Department of Pharmacy, Uppsala University, Sweden, [2] Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden, [3] Department of Physiology and Pharmacology, Karolinska Institutet, Sweden , [4] Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology (IKP), Stuttgart, Germany, [5] University of Tübingen, Tübingen, Germany

Background

Human intestinal organoids are gaining ground in studies of drug metabolism and disposition. 1 As of today, these in vitro cultures are maintained in the cell culture medium enriched with extensive bovine serum supplementation and high glucose and insulin content (17.49 mM and 10,000 ng/mL respectively). 2,3 Here we develop physiologically relevant cell culture medium and benchmark the performance of primary human jejunal organoids in this novel medium.

Materials and methods

Human Jejunal organoids have been cultured according to previously published protocols 3,4 and differentiated into basal- and apical-out jejunal organoids in suspension in either commercially available Organoid Differentiation Medium or using novel chemically defined normoglycemic differentiation medium. To accurately assess phenotypes, organoids were harvested and analyzed using label-free mass-spectrometry based proteomics on Orbitrap Q Exactive HF operating in data-dependent acquisition mode.

Results

In total, 6248 proteins were identified, of them 5040 proteins were quantified with at least two unique peptides. Differential analysis via DEqMS revealed that basal-out organoids cultured in the chemically defined medium exhibited significantly higher expression of clinically relevant drug metabolizing enzymes, including cytochrome P450s 3A4, 2C19 and 2C9. Conversely, proteomes of the basal-out organoids differentiated in commercial media exhibited enrichment in DNA replication and DNA repair pathways, typically associated with undifferentiated stem-cell state. Lastly, we observed that when cultured in an apical-out setting, organoids fully differentiate independently of the cell culture medium.

Conclusions

Our results provide a novel protocol for the culture of physiologically relevant primary human jejunal organoids with maintained drug-metabolizing enzyme expression.

References

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2.Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 141, 1762–1772 (2011).

3.Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459, 262–265 (2009).

4.Co, J. Y., Margalef-Català, M., Monack, D. M. & Amieva, M. R. Controlling the polarity of human gastrointestinal organoids to investigate epithelial biology and infectious diseases. Nat Protoc 16, 5171–5192 (2021).

o8

Mass Spectrometry Histochemistry on FFPE samples with high-resolution instruments for top-down biomolecule analysis

Peter Verhaert[1,2], Gilles Frache[3], Dietmar Thal[1], Marthe Verhaert[2,4], Raf Sciot[1]

[1] Leuven University Hospital, [2] ProteoFormiX BV, [3] Luxembourg Institute for Science and technology, [4] Brussels University Hospital

Proteomics is developing continuously, with higher numbers of peptides being mapped to protein IDs in bottom-up analyses and more and more proteoforms being identifiable in top-down strategies. For these high performant approaches dedicated sample preparation procedures are worked-out resulting in stunning datasets. For clinical applications, however, not all sample preparation workflows are feasible, e.g., for ethical reasons.

We employ the increasing performance of modern analytical (MS) technologies to allow for deeper analysis of the available material archived in biobanks. We will show the latest data of our spatial mass spectrometry histochemistry (MSHC) approach which allows for top-down detection of endogenous peptides and small (bio)molecules in tissue sections of formaldehyde-fixed, paraffin-embedded (FFPE) tissues, and this with cellular lateral resolution.

Our results confirm that the power of modern high resolution MS analytics allows many more biomolecules to become accessible for direct localization and identification in FFPE tissues than could be imagined a few years ago using the conventional MALSI TOF based MSI technology.

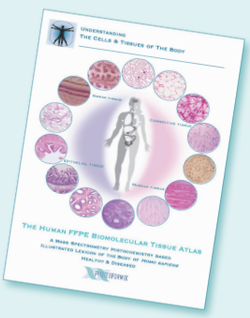
Mass Spectrometry Histochemistry on FFPE samples with high-resolution instruments for top-down biomolecule analysis

Peter Verhaert[1,2,3] Gilles Frache[4], Dietmar Thal[1], Marthe Verhaert[2,5], Raf Sciot[1]

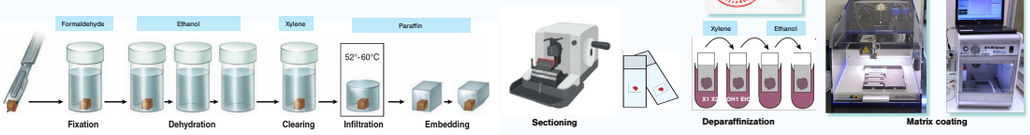
[1]ProteoFormiX, Vorselaar, Belgium; [2]University Hospital, Leuven, Belgium; [3]Integrated Molecular Physiology Research Initiative, University of the Witwatersrand, Johannesburg, South Africa; [4]Luxembourg Institute of Science and Technology, Belvaux, Luxembourg; [5]Free University of Brussels, Belgium

Goal

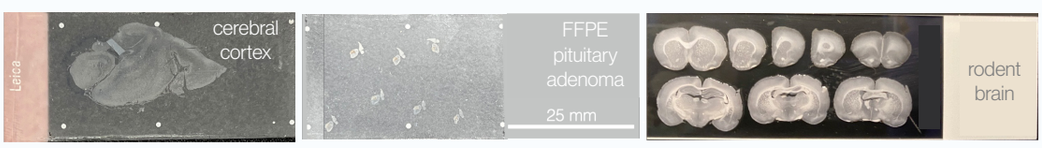
- To make formaldehyde-fixed, paraffin-embedded (FFPE) tissues available for spatial analysis of neuropeptides and other small molecules, we optimize mass spectrometry histochemistry (MSHC) protocols on brain and associated neuronal samples of Homo sapiens and other species[1].
- MSHC makes pathologically well-documented FFPE sample archives accessible for disease biomarker discovery research.
- Cross-platform comparative MSHC analyses on different generation Orbitrap instruments illustrate state-of-the-art.



Samples / Preparation for MSHC

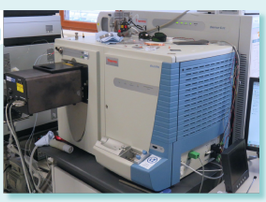


- Different types of biopsy / autopsy samples from Leuven University Hospital FFPE tissue bank

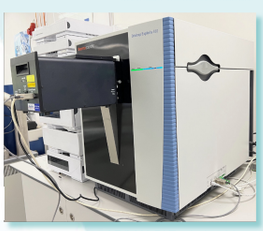


- Microtome sections (5 µm) mounted on regular microscope slides
- Short deparaffinization through xylene (X) and ethanol (EtOH) dips[1]
- Automated pneumatic MALDI matrix (DHB) application with Sunchrom™ or HTX M5™ Sprayer

UZ LEUVEN



AP/MALDI LTQ Orbitrap Elite Velos PRO™
MassTech / ThermoFisher Scientific

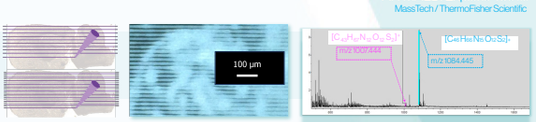


LIST

AP/MALDI Exploris 480™
MassTech / ThermoFisher Scientific

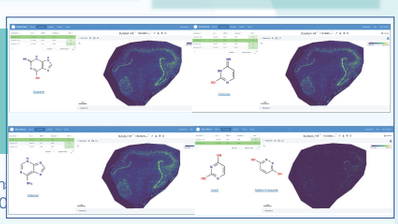
AP/MALDI HR MSHC imaging on different generations Orbitrap™

- Two-tier approach: 2 *m/z* analyses / sample: 1st pass: 'peptide' *m/z* range (150-2000); 2nd pass: 'metabolite' *m/z* range (50-200)



MSHC Data Processing

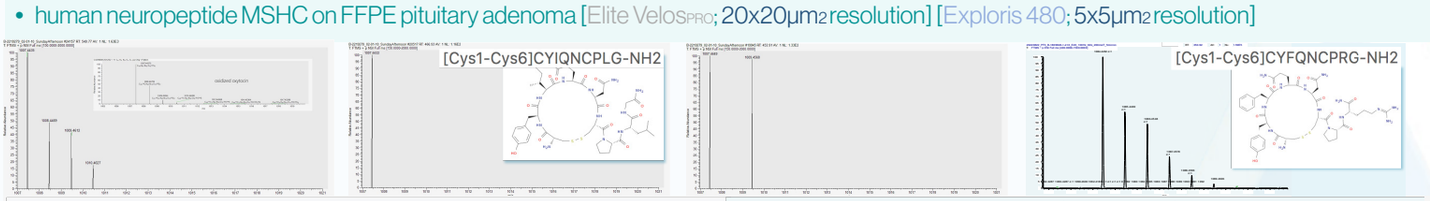
- HistoSnap™ [ProteoFormiX' imzML Heatmap Generator] for image based MSHC data browsing
- Mozaic™ MSI [Spectroswiss] & MSI Reader [MSI Software Solutions] for performant data processing
- Pyxis MSI™ [Mass Analytica] & METASPACE for metabolite annotation (HMDB, ChEBI, ...)
- NeXtProt [Swiss Institute for Bioinformatics] for annotation of known / novel human peptides



AP/MALD MSHC metabolite images in human hippocampus section annotated with METASPACE [Exploris 480]

HR MS Data

- human neuropeptide MSHC on FFPE pituitary adenoma [Elite Velos$_{PRO}$; 20x20µm^2 resolution] [Exploris 480; 5x5µm^2 resolution]



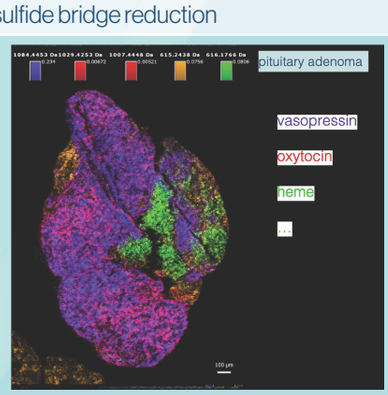
Single pixel mass spectra showing $[M_{oxytocin} + H]^+$ (*m/z* 1007.44373) at <1 ppm (on Elite Velos^{PRO}) and $[M_{vasopressin} + H]^+$ (*m/z* =1084.44513) at 55 ppb (on Exploris 480) mass measurement accuracy

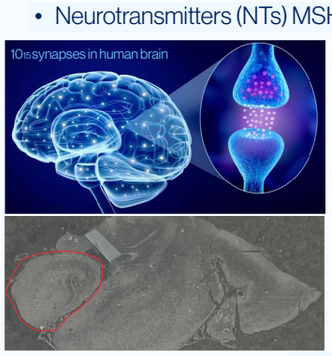
- on tissue isotopic envelopes vary with peptide abundance/pixel; some pixels show evidence of partial disulfide bridge reduction

Results

- Steadily expanding the Human FFPE Biomolecular MSHC Tissue Atlas (>400 Homo sapiens tissue datasets; among which various brain areas)
- Spatial multiomics data with cellular resolving power...
- Neurotransmitters (NTs) MSHC?

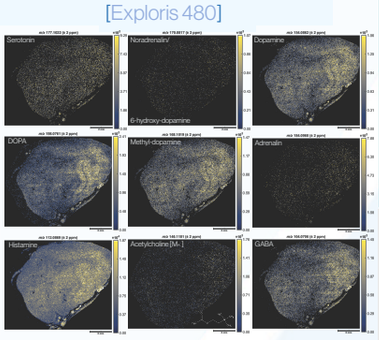
FFPE neuropeptide MSHC metabolite (human pituitary adenoma) [5x5µm^2 resolution] [Exploris 480]

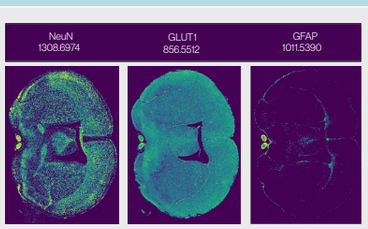




preliminary data based on (accurate mass of) 1st isotope [Exploris 480]

NT	formula	M	[M+H]+	detected	accuracy
serotonin	C10H12N2O	176.094963016	177.10223	✓	<1 ppm
noradrenalin	C8H11NO3	169.073893223	170.08171	✓	ppm <1
dopamine	C8H11NO2	153.078978601	154.08624	✓	ppm <1
(DOPA)	C9H11NO4	197.068807845	198.07607	✓	ppm <1
(6-hydroxy-dopamine)	C8H11NO3	169.073893223	170.08171	(✓)	ppm <1
(methyl-dopamine)	C9H13NO2	167.094628665	168.10189	✓	ppm <2
adrenalin	C9H13NO3	183.089543287	184.09681	?	ppm <1
histamine	C5H9N3	111.079647303	112.08691	✓	ppm <2
acetylcholine	C7H16NO2	146.118103761	147.12593	[M+] ✓	ppm 3
glutamate	C5H9NO4	147.053157781	148.06043	?	ppm <2
GABA	C4H9NO2	103.063328537	104.07060	✓	ppm

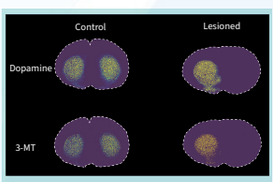




ongoing biological & analytical validation

AP/MALDI 6495D TQ™
MassTech / Agilent

- comparing different areas of the human brain rich in specific NTs
- checking MSHC correlations with catcholamine fluorescence HC and MSIHC "proteomic" images [www.ambergen.com], in particular using antibodies to NT receptors, transporters, synthesizing and/or metabolizing enzymes
- assessing AP/MALDI QQQ [www.agilent.com] for targeted high-sensitivity on-tissue MRM
- comparing known neurotransmitter images after on-tissue chemical derivatization (OTCD) with FMP-10 [www.tag-on.se] and imaging unilaterally lesioned mouse FFPE brain sections [collaboration with Prof. Per Andrén, Uppsala University]



AP/MALDI OTCD MSHC images of fresh/frozen rat brain sections [Exploris 480]

AP/MALDI MSIHC images of FFPE mouse brain sections [Exploris 480]

[1] Paine et al. (2018) Anal. Chem. 90: 9272

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09

Cerebrospinal fluid proteome profiling across the Alzheimer's disease continuum: A step towards solving the equation for 'X'

Sophia Weiner[1], Mathias Sauer[1], Laia Montoliu-Gaya[1], Andrea Benedet[1], Nicholas Ashton[2], Fernando Gonzalez-Ortiz[1], Joel Simrén[1], Ville Leinonen[3], Pedro Rosa-Neto[4], Kaj Blennow[1], Henrik Zetterberg[1], Johan Gobom[1]

[1] University of Gothenburg, [2] Banner Alzheimer's Institute and University of Arizona, [3] University of Eastern Finland, [4] McGill University Research Centre for Studies in Aging

Background

While the temporal profile of amyloid (Aβ) and tau biomarkers in cerebrospinal fluid (CSF) along the Alzheimer's disease (AD) continuum is well-studied, chronological changes of CSF proteins reflecting other disease-relevant processes, denoted 'X' in the ATX(N) framework, remain poorly understood.

Materials/Methods

Using tandem mass tag (TMT) mass spectrometry, we quantified over 1500 CSF proteins across the AD continuum in three independent cohorts, finely staged by Aβ/tau positron emission tomography (PET) (n=134), fluid biomarkers (n=467), or brain biopsy (n=73). Weighted protein co-expression network analysis identified protein clusters consistent across all cohorts that were further correlated with fluid biomarkers, PET imaging, and clinical parameters. Finally, cluster abundance profiles were modelled across the AD continuum.

Results

Protein cluster abundances changed sequentially with AD progression. Neurodegeneration-related proteins (e.g., SMOC1, PPIA, YWHAZ) showed strong correlations with fluid and imaging biomarkers ($P<0.01$), increasing early in the AD continuum. SMOC1 was associated with Aβ-pathology, while YWHAZ and PPIA were linked to both Aβ and tau pathology as indexed by PET ($P<0.01$). Endo-lysosomal proteins (e.g., HEXB) also increased early, followed by increases in metabolic proteins such as ALDOA at the mild cognitive impairment stage. Later stages of AD were marked by reductions in synaptic/membrane proteins (e.g., NPTX2).

Conclusions

Our study identifies candidate biomarkers for AD disease staging beyond Aβ/tau, highlighting the dynamic fluctuations of the CSF proteome with disease progression. The latter bears important implications for biomarker discovery design and biomarker interpretation in the context of the AD continuum.

10

Quantifying 500 proteomics samples per day with ZT Scan DIA

Tom Ruane, Patrick Pribil, Ihor Batruch, Yves Le Blanc

This poster describes improvements in the identification and quantitation of proteins and peptides with high throughput liquid chromatography-mass spectrometry (LC-MS) analyses using ZT Scan data-independent acquisition (DIA). ZT Scan DIA enhances proteomics performance compared to traditional discrete-window DIA (Zeno SWATH DIA), especially as sample loading and complexity increases. With these enhancements, up to 70% gains in the identification and quantitation of proteins and precursors was achieved.

DIA has become the cornerstone of MS-based proteomics analysis, enabling the identification and quantification of large numbers of proteins and peptides to enhance the understanding of the complex biological mechanisms of various diseases. A critical aspect of disease research is analyzing large sample cohorts to increase the statistical significance of observed trends and paint a broader picture of the disease mechanisms involved. As such, the ability to analyze large numbers of samples faster, with easy-to-implement methods and improved data quality, is of great value to researchers. The power of

Zeno SWATH DIA for fast, high-quality quantitative proteomics has been repeatedly demonstrated. ZT Scan is presented as the next step in the evolution of DIA. Using a scanning quadrupole coupled tofast, sensitive, time-of-flight (TOF) analysis improves specificity over existing discrete-window DIA methods for accurately and precisely identifying and quantifying analytes across a given mass range.

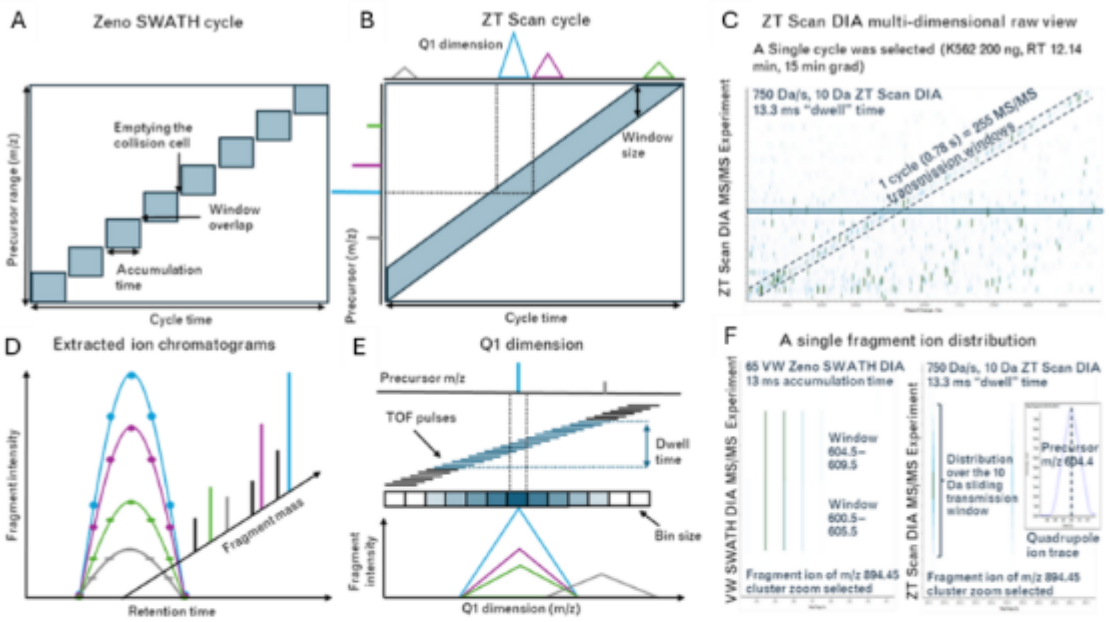

Figure 1. Overview of ZT Scan DIA and the added Q1 dimension. Visualization of (A) a conventional Zeno SWATH DIA cycle showing data is collected in a stepped manner, and (B) a ZT Scan DIA cycle where Q1 scans the mass range and adds this dimension to the data. (C) Raw data showing a single ZT Scan cycle selected (K562 200 ng, RT 12.14 min, 15 min gradient) (D) Extracted ion chromatogram generated for each fragment according to retention time. (E) MS/MS data collected from TOF pulses are binned according to the precursor m/z. Fragments can be distinguished from chimeric MS/MS spectra and aligned to their precursor ions using the Q1 dimension. (F) Fragment ion distribution from a Zeno SWATH DIA experiment and the ZT Scan DIA shown in (C) above.

11

Structural and functional characterization of streptococcal SpnA-host interactions

Isabella Andersson Bennig[1], Simon Ekström[1,2], Lotta Happonen[1,2,3]
[1] Lund University, [2] SciLifeLab IBS, [3] BioMS

Every major health organization has identified infectious diseases as serious global threats. We address these problems by studying the Streptococcus pyogenes bacterium; an important human pathogen, ranking globally among the top ten causes of mortality from infectious diseases with an estimated >150 000 annual deaths. During an infection, S. pyogenes encodes for a variety of virulence factors; many of these virulence factors have not been thoroughly studied. One of these virulence factors is the S. pyogenes nuclease A, SpnA, originally described as a cell- wall associated DNAse degrading host neutrophil extracellular traps (NETs) via its C-terminal endo/exonuclease domain.

We study the structure and function of SpnA by combining quantitative and structural proteomics mass spectrometry with integrative structural biology methods. Our results combining AlphaFold modeling, single-particle cryoEM and hydrogen-deuterium exchange mass spectrometry demonstrate that SpnA harbors disordered regions in its N-terminus. We have demonstrated by combining affinity-purification and bacterial surface adsorption mass spectrometry, that in human blood plasma SpnA binds the complement system membrane attack complex (MAC). By combining affinity-purification with crosslinking mass spectrometry we have been able to determine that SpnA specifically targets the MAC assembly intermediate C5b-C7. We hypothesize that SpnA acts like another streptococcal virulence factor SIC, streptococcal inhibitor of complement, which targets the C5b-C7 complex preventing its insertion into the streptococcal membranes, hence preventing bacterial lysis.

Our results indicate that in addition to degrading host DNA in NETs, SpnA mediates other central functions in host immune evasion in streptococcal pathogenesis.

12

N-linked glycopeptide identification using nanoflow LC and electron activated dissociation (EAD) MS/MS fragmentation
Mats Garmer

This technical note describes the identification of N-linked glycopeptides using nanoflow liquid chromatography (LC) separation with electron activated dissociation (EAD) fragmentation on the ZenoTOF 7600 system. >1,000 Nglycosylated peptides were identified in glycopeptide-enriched samples from human plasma digests with an EAD-based data-dependent acquisition (DDA) approach. EAD on the ZenoTOF 7600 system is a tunable electron capturebased fragmentation technique that produces unique peptide fragment ions, allowing for the unambiguous assignment of the types and sites of glycosylation post-translational modifications. Data processing using a combination of PEAKS GlycanFinder software and SCIEX OS software enables robust qualitative and quantitative analysis of the rich spectra generated by the ZenoTOF 7600 system.

N-linked glycopeptide identification using nanoflow LC and electron activated dissociation (EAD) MS/MS fragmentation

Remco van Soest[1], and **Patrick Pribil**[2]
[1]SCIEX, USA; [2]SCIEX, Canada

P-III-0908

ABSTRACT

This technical note describes the identification of N-linked glycopeptides using nanoflow liquid chromatography (LC) separation with electron activated dissociation (EAD) fragmentation on the ZenoTOF 7600 system. >1,000 N-glycosylated peptides were identified in glycopeptide-enriched samples from human plasma digests with an EAD-based data-dependent acquisition (DDA) approach. EAD on the ZenoTOF 7600 system is a tunable electron capture-based fragmentation technique that produces unique peptide fragment ions, allowing for the unambiguous assignment of the types and sites of glycosylation post-translational modifications. Data processing using a combination of PEAKS GlycanFinder software and SCIEX OS software enables robust qualitative and quantitative analysis of the rich spectra generated by the ZenoTOF 7600 system.

INTRODUCTION

Protein glycosylation is a critical post-translational modification (PTM) that affects protein folding and stability. It is also essential for cell-cell adhesion and, as such, plays a role in immune response, cancer, and numerous other diseases. Mass spectrometry (MS) instrument sensitivity is one of the significant limitations when analyzing glycopeptides due to the heterogeneity of glycan structures, resulting in multiple peptide isoforms with much lower abundances than their non-glycosylated forms. The challenges associated with low glycopeptide abundances can be overcome with strategies like enrichment of glycopeptides, a lower flow rate LC separation regime (i.e., nanoflow LC separation), and high-performance MS systems for glycopeptide detection and characterization.

Another challenge for glycopeptide characterization comes from the labile nature of the glycosylation PTM. CID fragmentation of glycosylated peptides often provides more limited peptide backbone information and typically results in fragments lacking the labile side chain modifications. Alternative fragmentation methods, such as EAD-based MS/MS, have been shown to yield more complete peptide backbone information. Additionally, EAD fragmentation provides site-specific PTM localization due to the retention of these modifications on the resulting fragment ions[1].

MATERIALS AND METHODS

Sample preparation:
Human pooled plasma K_2EDTA was acquired from BioIVT. Top 14 Abundant Protein Depletion Midi spin columns from Thermo Fisher were used for plasma depletion. Trypsin/Lys-C protease mix was purchased from Promega. PolyHYDROXYETHYL A 12 µm 300 Å HILIC resin from PolyLC was used for glycopeptide enrichment. After depletion of the top 14 most abundant proteins using the depletion spin columns (using the manufacturer's protocol), human plasma was digested following a filter-aided sample preparation (FASP) protocol described in the literature[2]. After digestion and solid phase extraction clean-up, the sample was enriched for glycopeptides using a PolyHYDROXYETHYL A Hydrophilic Interaction Chromatography (HILIC)-cotton column[3]. The resulting extract was evaporated to dryness and redissolved in water with 0.1% formic acid for analysis by LC-MS. Based on an estimated human plasma protein concentration of 80 mg/mL, and the manufacturer's estimate of removal of 95% of the top 14 abundant proteins, the depleted extract had an assumed peptide concentration equivalent to 7.5 µg protein/µL before digestion and enrichment.

Chromatography:
The samples were analyzed using a Waters ACQUITY M-Class system in trap and elute nanoflow LC mode. A Waters nanoEase M/Z Symmetry C18 100 Å, 5 µm, 180 µm x 20 mm trap column was used in combination with a Phenomenex Biozen Peptide XB-C18 100 Å, 2.6 µm, 75 µm x 25 cm nanoLC column. Injection volumes of 1-10 µL sample were loaded on the trap from a 20 µL loop using 4 minutes of loading at 10 µL/min of 0.1% formic acid in water. A 60-minute gradient at 300 nL/min from 1-26% mobile phase B was run for the separation, using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The column and trap were washed at 80% mobile phase B for 5 minutes and re-equilibrated at 1% mobile phase B for 25 minutes. The column temperature was maintained at 50ºC.

Mass spectrometry:
The ZenoTOF 7600 system was used with an OptiFlow Turbo V ion source in nanoflow mode, using the OptiFlow nanoflow interface. Ion source and DDA method parameters were as previously described[4]. Replicate injections were performed for each sample as indicated.

Data processing:
Glycopeptide identification was done using PEAKS GlycanFinder 2.0 software (Bioinformatics Solutions Inc)[5]. This software identifies peptides and glycopeptides as described in Figure 2. A UniProt reviewed human plasma protein database downloaded on April 2, 2024 was used, in combination with the structural glycan database included in PEAKS GlycanFinder software consisting of 1,867 N-linked glycan structures. Search parameters used, were as previously described[4]. Additional quantitative data processing was done using SCIEX OS Analytics software.

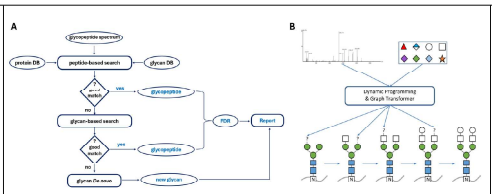

Figure 1. Workflow for glycopeptide database search and de novo sequencing with PEAKS GlycanFinder software. (A) Glycopeptide spectra can be processed in three stages: peptide-based search, glycan-based search, and de novo sequencing. If spectra cannot be identified in one stage, it proceeds to the next stage. Once a candidate glycopeptide is identified, the false discovery rates (FDRs) at both peptide and glycan levels are calculated; (B) An N-linked glycan tree is constructed from the N-linked core by iteratively adding monosaccharides to the tree. At each iteration, a dynamic programming algorithm coupled with a Graph Transformer neural network is used to predict the next monosaccharides based on the input spectrum and the partial tree obtained from the previous iteration.

RESULTS

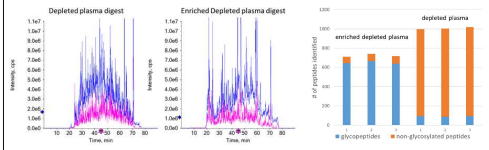

Figure 2. Results of depletion and enrichment of glycopeptides. (LEFT) Total ion chromatograms (TICs) of TOF MS (blue) and MS/MS (pink). The panel on the left shows the depleted sample (the equivalent of 0.5 µg protein on-column), while the panel on the right shows the enriched depleted sample (the equivalent of 37.5 µg protein on-column). (RIGHT) Fraction of identified glycopeptides versus non-glycosylated peptides in the enriched (left) and non-enriched depleted plasma samples

Table 1: Optimization of maximum number of candidates for the EAD-DDA method. The total scan time was kept constant at 2 seconds. The amount injected was the equivalent of 37.5 µg depleted plasma.

Method	# MS/MS spectra	# GlycoPSMs *	# Glycopeptides
50 ms - 36 candidates			
Replicate injection 1	45,760	1,048	491
Replicate injection 2	46,385	970	449
100 ms - 18 candidates			
Replicate injection 1	26,625	1,400	569
Replicate injection 2	26,811	1,457	574
200 ms - 9 candidates			
Replicate injection 1	14,589	1,949	710
Replicate injection 2	14,563	1,816	661

* Glycopeptide spectral match.

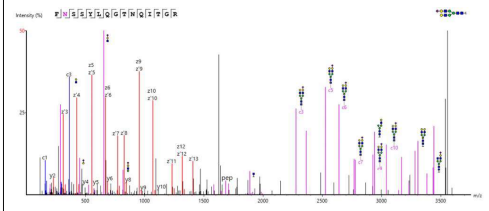

Figure 3. EAD MS/MS spectrum of glycopeptide FN[(HexNAc)$_4$(Hex)$_5$(NeuAc)$_1$]SSYLQGTNQITGR from Apolipoprotein B-100, processed and annotated using PEAKS GlycanFinder software. The peptide backbone is fully sequenced, while several peptide-glycan fragments are evidence for the proposed glycan structure.

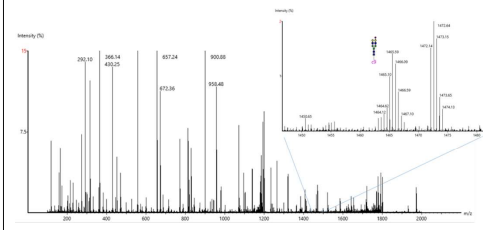

Figure 4. Raw MS/MS spectrum from Figure 3. EAD fragmentation provides information-rich spectra for glycopeptides, with fragments of both the peptide backbone and glycan. As an example, the inset shows the isotope cluster for the +2 charge state of the c9-glycan fragment.

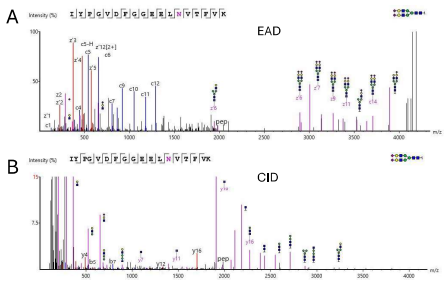

Figure 5. Comparison of EAD versus CID MS/MS spectra of a glycopeptide from Kallikrein B. (A) EAD spectrum, with the peptide backbone fully sequenced with c- and z-ion fragments, while several peptide-glycan fragments are evidence for the proposed glycan structure. (B) CID spectrum of the same glycopeptide, which, while showing some evidence of the glycan, lacks many peptide backbone fragments. Spectra were processed and annotated using PEAKS GlycanFinder software. Glycosylation identified on this protein was as previously described in the literature[6].

Table 2: Result statistics of data as presented by PEAKS GlycanFinder software. Results are from three replicate injections of the equivalent of 37.5 µg of plasma protein before enrichment.

	#Scans				#Features	Identified				#Glycopeptides	#Peptides	#Sequences	#Proteins		
	MS1	MS/MS	#Refined	#Glycan	#Chimera		#GlycoPSMs	#PSMs	#Scans	#Features				Groups	All	Top
Total	47481	42895	108407	101196	39075	517249	5160	353	5453	2363	1002	95	797	98	104	101
Sample 1	14322	14229	35775	33541	12931	171209	1678	105	1752	779	642	69	251	95	100	98
Sample 2	14081	14173	36711	33681	13033	174701	1803	173	1900	815	666	74	254	93	99	96
Sample 3	14078	14345	36419	33974	13061	172339	1679	125	1781	769	639	79	249	91	96	94

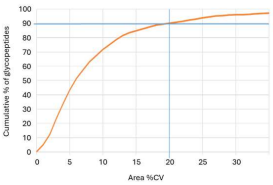

Figure 6. Cumulative % of glycopeptides quantified by Analytics based on the TOF MS data. Data was analyzed using SCIEX OS Analytics software with the Autopeak integration method. The monoisotopic m/z was used, with a precursor ion XIC width of 0.02 m/z. The graph indicates that >90% of the quantified precursor ions had CVs of <20%.

CONCLUSIONS

- EAD allows for the comprehensive structural characterization and localization of the glycan PTM for glycopeptides
- PEAKS GlycanFinder software uses information from EAD spectra for the identification of the glycopeptide, including confirmation and localization of the glycan structure
- >1,000 glycopeptides were identified in a depleted, digested human plasma sample that was enriched for glycosylated peptides
- SCIEX OS software allows for easy setup of data-dependent acquisition (DDA) EAD MS/MS methods, and the Analytics module can be used for highly accurate and precise analyte quantitation

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TRADEMARKS/LICENSING

13

Overnight enzymatic digestion? Try it at room temperature!

Lazaro Hiram Betancourt Nunez[1], Anahita Bakochi[1], Aniel Sanchez[1], Sven Kjellström[1]
[1] Lund University

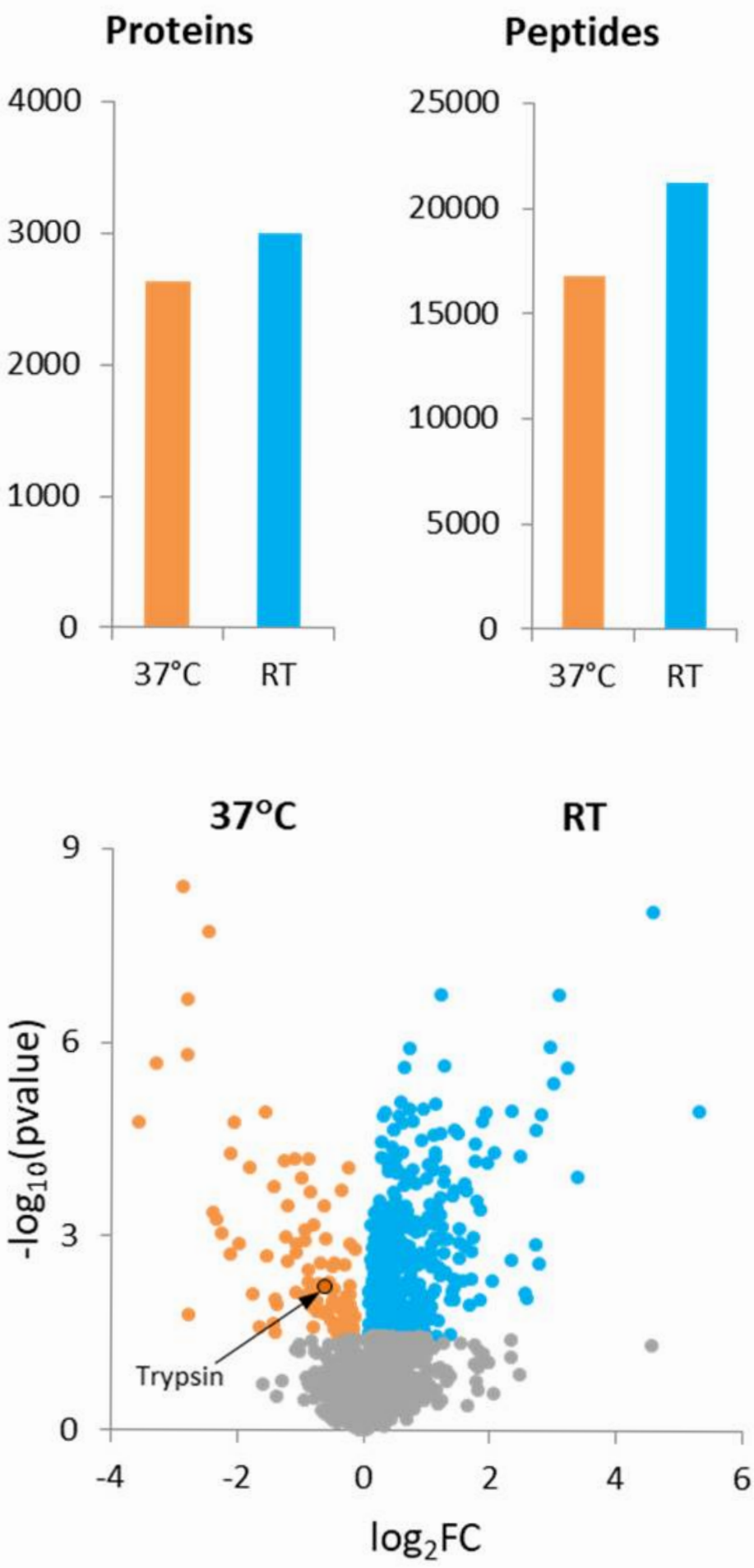
Background: Bottom-up proteomics primarily relies on trypsin or Lys-C/trypsin digestion to generate peptides, typically performed overnight at 37°C, the optimal temperature for trypsin activity. We previously found that overnight urea in-solution Lys-C/trypsin digestion at room temperature (RT) outperforms digestion at 37°C (1). Building on these results, we compared various sample preparation methods using both temperatures.

Materials and Methods: Cell lysates and plasma samples were processed using Urea, GuCl, and SDC in-solution digestion, FASP, MED-FASP, and S-trap methods. Samples were digested overnight at either 37°C or RT and analyzed using an Evosep One LC system coupled to a timsTOF Pro mass spectrometer. Data were acquired using DDA-PASEF and DIA-PASEF methods and searched with FragPipe (v20.0), applying a 1% FDR cutoff at the precursor and protein levels.

Results: RT digestion consistently outperformed 37°C, with more peptides and proteins identifications, higher peptide intensities, and a greater number of proteins exhibiting increased relative abundance. RT digestions also resulted in improved reproducibility, evidenced by comparable or lower protein coefficients of variation (CVs), particularly in the low-abundance range. These advantages were accompanied by fewer or minimal increases in the number of missed cleaved peptides. Reduced autolysis of trypsin and Lys-C most likely contributed to the superior performance of RT digestions.

Conclusion: Room temperature digestion yields superior results compared to 37°C when analyzing several metrics across multiple preparation methods and samples, supporting its broader adoption for overnight enzymatic digestions with trypsin or Lys-C.

(1) Betancourt et al. J. Proteome Res. 2018, 17, 2556–2561.


Proteins
4000
3000
2000
1000
0
37°C
RT
Peptides
25000
20000
15000
10000
5000
0
37°C
RT
37°C
RT
9
6
3
0
-log10(pvalue)
Trypsin
-4
-2
0
2
4
6
log2FC

14

Exploring AI-based Digital Pathology and Proteomics to Decode Melanoma Progression

Jessica Guedes[1], Nicole Woldmar[1], Leticia Szadai[2], András Kriston[3], Ede Migh[3], Ferenc Kovacs[3], Henriett Oskolás[1], Roger Appelqvist[1], Kun Hsing Yu[4], Eugene R. Semenov[4], Johan Malm[1], Peter Horvath[3], István Balázs Németh[2], Jeovanis Gil[1], György Marko-Varga[1]

[1] Lund University, Lund, Sweden , [2] University of Szeged, Szeged, Hungary , [3] Biological Research Centre, Szeged, Hungary. , [4] Harvard Medical School, Boston, USA

Background: Melanoma, the deadliest form of skin cancer and the fifth most common cancer in Sweden exhibits significant heterogeneity that drives its aggressive progression. This study integrates AI-based digital pathology with proteomics to investigate the mechanisms behind melanoma progression.

Methods: We analyzed high-resolution HE images with AI to identify tumors, stroma areas, and melanoma subclones. These regions were isolated using laser microdissection and subjected to proteomic analysis alongside whole tissue analysis. Over 100 samples from 55 patients were examined and categorized into three groups: (i) patients with disease recurrence within 5 years, (ii) those with metastases at diagnosis, and (iii) patients without progression after 5 years.

Results: The AI analysis revealed distinct histopathological signatures in tumor cells and their surroundings, including melanoma subclones. The integration of AI tools and proteomics approaches identified mitochondrial dysfunction and immune pathway dysregulation as key drivers of melanoma recurrence. Additionally, we observed molecular heterogeneity in melanoma subclones, and upregulated proliferative pathways, and downregulated histidine metabolism as the disease advanced. These findings point to potential new drug targets for metastatic melanoma treatment. Moreover, key morphological markers such as microerosion, budding formations, melanocytic atypia, and the absence of regression areas were identified as significant risk factors for melanoma recurrence and progression.

Conclusion: Our findings show that integrating AI-based digital pathology with proteome profiling is a powerful approach to mapping melanoma progression, identifying new therapeutic targets, and discovering markers of recurrence. This strategy could reveal early prognostic factors and pave the way for personalized melanoma therapy.

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PRM method development for Non-Small Cell Lung Cancer patient classification.

Georgios Mermelekas, Olena Berkovska, Lukas Orre, Janne Lehtiö

Background: We have identified six distinct proteome subtypes with expected clinical utility, showed differences in immune microenvironments, cancer driver,s and related protein expression (Lehtiö et al. 2021).

Materials and methods:We are developing a targeted proteomics method (PRM) of 200 peptides corresponding to 100 proteins. Data-driven bioinformatics methods identified the protein biomarkers distinguishing the subtypes, complemented with literature research and clinical insights. The LC-MS method was developed using a one-hour gradient (5-40% ACN) in Ultimate 3000 RSLCnano System coupled to Orbitrap Exploris 480.

Results: i) Method development and evaluation

Reproducibility and repeatability of the SureQuant PRM were assessed by evaluating retention time variation, dot product, and light-to-heavy peptide ratio precision. A set of NSCLC subtype-representative tumor samples was analyzed with technical replicates, after repeated sample preparations on distinct days or using different SIL peptide stocks. More than 90% of peptides fulfilled the high-confidence identification and quantification criteria corresponding to all proteins in the panel. A set of housekeeping and contaminant proteins was also included in the list, for further QC.

ii) Analysis of an early-stage NSCLC cohort

Tumour samples (n = 141) previously by various omics methods, (Lehtiö et al. 2021) were re-analyzed using our targeted PRM method. The existing subtype annotations for the samples allowed the panel to refine and include the most informative peptides that enable accurate classification of the samples.

Conclusions: The developed PRM assay shows stability and accuracy of measurements over time in addition to separating the NSCLC subtypes like the previous analyses.

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Clinical Proteomics Mass Spectrometry and Global Proteomics and Proteogenomics Facility.

Georgios Mermelekas[1], Xiaofang Cao[1], Eduardo Araujo[1], Mahshid Zarrineh[1], Henrik Johansson[1], Lukas Orre[1], Janne Lehtiö[1]
[1] Karolinska Institutet

Clinical Proteomics Mass Spectrometry (MS) and Global Proteomics and Proteogenomics at SciLifeLab, is a core facility (CF) that provides expertise and services in a broad range of MS-based proteomics applications to encompass the needs of the Swedish life science community, including quantitative proteomics and proteome profiling, in-depth proteomics, interaction studies by immunoprecipitation MS (IP-MS) and post-translational modification (PTM) analysis. The facility also offers expert support in experimental design as well as downstream analysis to ensure a high-quality service.

Proteogenomics is another service that the facility offers, this rapidly developing field in biological mass spectrometry combines proteomics information with sample-specific genomic and transcriptomics information. Applications in proteogenomics include the discovery of novel protein-coding regions to improve genome annotation; detection of variant and mutated proteins based on DNA and/or RNA sequence data; discovery of cancer neoantigens and evaluation of the impact of genomic changes (e.g. copy number alterations, SNPs, mutations, hypermethylation) on the proteome.

In addition, we continuously strive to develop and implement cutting-edge research methods in our services. This is exemplified by the level of data produced by HiRIEF methods used for in-depth proteomics, and by our continuous development of bioinformatics methods for optimal use of generated data.

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A comprehensive human pan-disease blood atlas based on next generation protein profiling

María Bueno Álvez[1], Fredrik Edfors[1], Mathias Uhlén[1], Human Disease Blood Atlas Consortium
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Background

This study aims to create an open-access resource for exploring protein profiles in blood across a wide range of diseases.

Materials and Methods

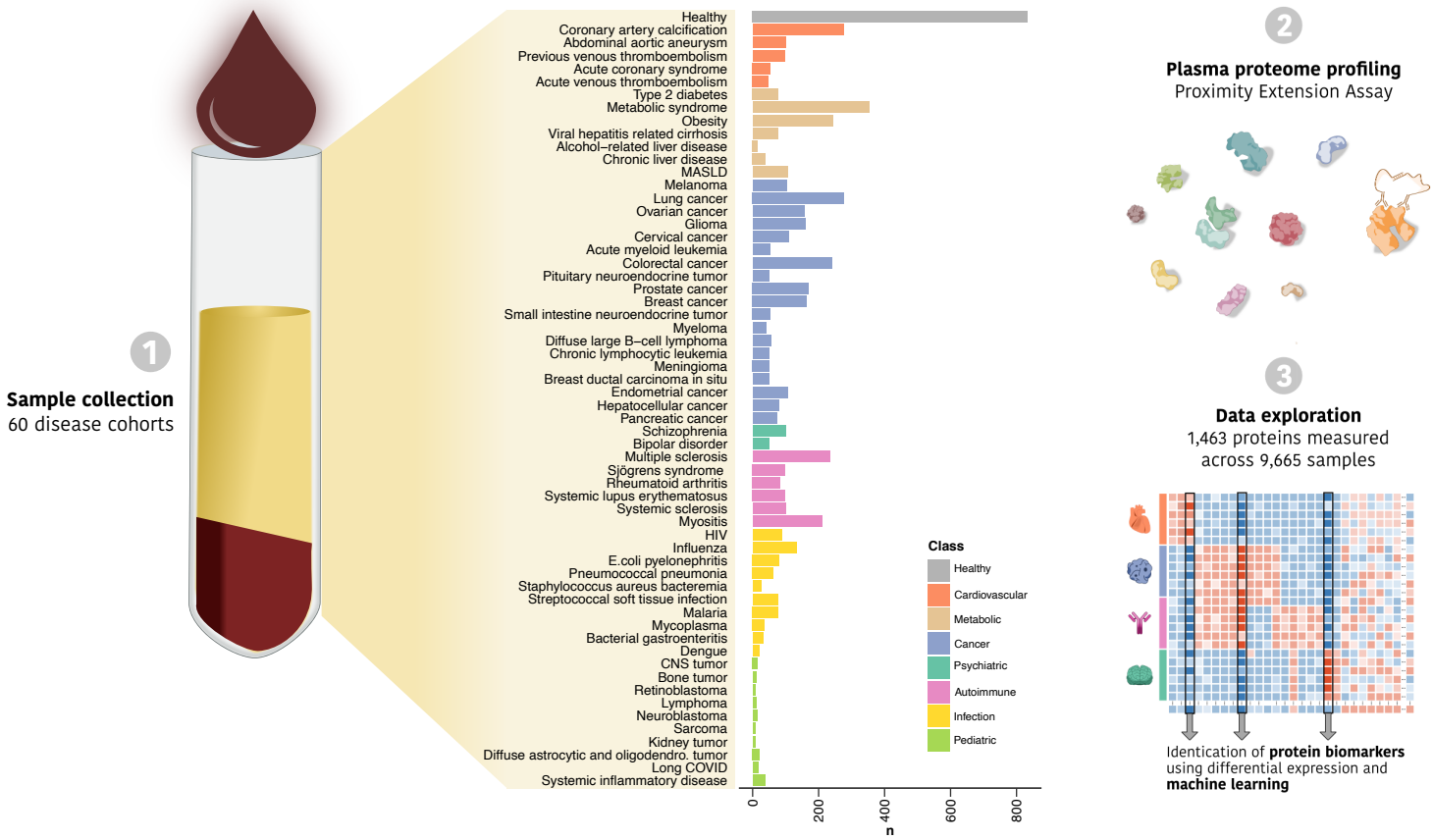
Using a highly sensitive multiplex assay, 1,463 proteins were analyzed in blood from patients with 59 diseases and healthy controls. Protein levels for each disease were compared against three control groups: healthy individuals, all other diseases, and diseases within the same disease class, providing comprehensive differential expression results.

Results

The study identified proteins correlating with age, BMI, and sex differences. Molecular signatures were discovered across a wide range of diseases, including cardiovascular, metabolic, cancer, autoimmune, infectious, and pediatric disorders. Additionally, cross-disease markers, like those linked to inflammation, were identified across cancer, autoimmune, and infectious diseases. The robust differential expression analysis enabled a deeper understanding of overlapping protein patterns across diseases, particularly inflammation-related proteins.

Conclusion

This resource provides valuable insights into biomarker discovery and improves the understanding of protein behavior across various diseases, underscoring the power of a pan-disease approach in identifying both disease-specific and cross-disease protein patterns.


1
Sample collection
60 disease cohorts
Healthy
Coronary artery calcification
Abdominal aortic aneurysm
Previous venous thromboembolism
Acute coronary syndrome
Acute venous thromboembolism
Type 2 diabetes
Metabolic syndrome
Obesity
Viral hepatitis related cirrhosis
Alcohol–related liver disease
Chronic liver disease
MASLD
Melanoma
Lung cancer
Ovarian cancer
Glioma
Cervical cancer
Acute myeloid leukemia
Colorectal cancer
Pituitary neuroendocrine tumor
Prostate cancer
Breast cancer
Small intestine neuroendocrine tumor
Myeloma
Diffuse large B–cell lymphoma
Chronic lymphocytic leukemia
Meningioma
Breast ductal carcinoma in situ
Endometrial cancer
Hepatocellular cancer
Pancreatic cancer
Schizophrenia
Bipolar disorder
Multiple sclerosis
Sjögrens syndrome
Rheumatoid arthritis
Systemic lupus erythematosus
Systemic sclerosis
Myositis
HIV
Influenza
E.coli pyelonephritis
Pneumococcal pneumonia
Staphylococcus aureus bacteremia
Streptococcal soft tissue infection
Malaria
Mycoplasma
Bacterial gastroenteritis
Dengue
CNS tumor
Bone tumor
Retinoblastoma
Lymphoma
Neuroblastoma
Sarcoma
Kidney tumor
Diffuse astrocytic and oligodendro. tumor
Long COVID
Systemic inflammatory disease
0
200
400
600
800
n
Class
Healthy
Cardiovascular
Metabolic
Cancer
Psychiatric
Autoimmune
Infection
Pediatric
2
Plasma proteome profiling
Proximity Extension Assay
3
Data exploration
1,463 proteins measured
across 9,665 samples
Identication of protein biomarkers
using differential expression and
machine learning

18

Comparing two workflows for DIA-based MS global plasma proteomics on timsTOF Pro2 and Orbitrap Astra
Xiaofang Cao, Haris Babačić, Rui Branca, Maria Pernemalm, Janne Lehtiö

The authors have chosen not to publish the abstract.

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A multiplex mass spectrometry method to quantify microglia-associated proteins

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Background

This study aimed to develop a method for reliably quantifying microglia-associated proteins across various biological matrices.

Materials and methods

We developed a multiplex mass spectrometry (MS) method targeting proteins associated with microglial activation. Two systems were employed: (1)Nano-liquid chromatography (LC) with electrospray ionization (ESI) high-resolution hybrid quadrupole-orbitrap MS, utilizing a Dionex 3000 system and a Q-Exactive (Thermo Fisher Scientific); (2)Micro-high-performance LC-MS/MS system (6495 Triple Quadrupole LC/MS, Agilent Technologies). Detection performance and variance were assessed using replicates of pooled CSF. Protein levels were further evaluated in CSF from AD patients (n=21) and non-AD controls (n=18). Aditionally, the Q-Exactive system was used to quantify panel proteins in cell material from human induced pluripotent stem cell (hiPSC)-derived microglia treated with various stimuli to mimic disease conditions.

Results

28 peptides (representing 12 proteins) were detected in CSF. The Agilent 6495 Triple Quadrupole LC/MS system demonstrated superior detection and accuracy (27 peptides, average CV=8.86%), compared to the Q-Exactive system (14 peptides, average CV=22.43%). In AD CSF, the levels of most panel proteins were increased, some with statistical significance, despite the limited sample size. Additionally, material from hiPSC-derived microglia was analyzed, quantifying 15 peptides (representing 9 proteins) with clear treatment-specific effects.

Conclusion

In CSF, the established method successfully quantifies 12 proteins associated with microglial activation. The observed differences in AD versus non-AD CSF, and the treatment-specific effects in hiPSC-derived microglia, further validates the relevance of our selected panel proteins in the context of microglial activation and neurodegenerative disease.

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Blood proteome profiling using proximity extension assay in patients with acute myeloid leukemia

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Acute Myeloid Leukemia (AML) is the most common form of acute leukemia in adults. Plasma proteomic profiling represents an attractive way to assess biomarkers and screen for early diagnosis in malignant diseases, but studies remain scarce in AML. This study was conducted by analyzing 1 463 plasma proteins in 52 AML patients at diagnosis using the Olink Explore 1536 platform. Both differential expression analysis and feature selection by machine learning were applied to find the most significant proteins to distinguish AML from 867 healthy individuals and 1 734 patients of varying cancer types, including different hematological malignancies.