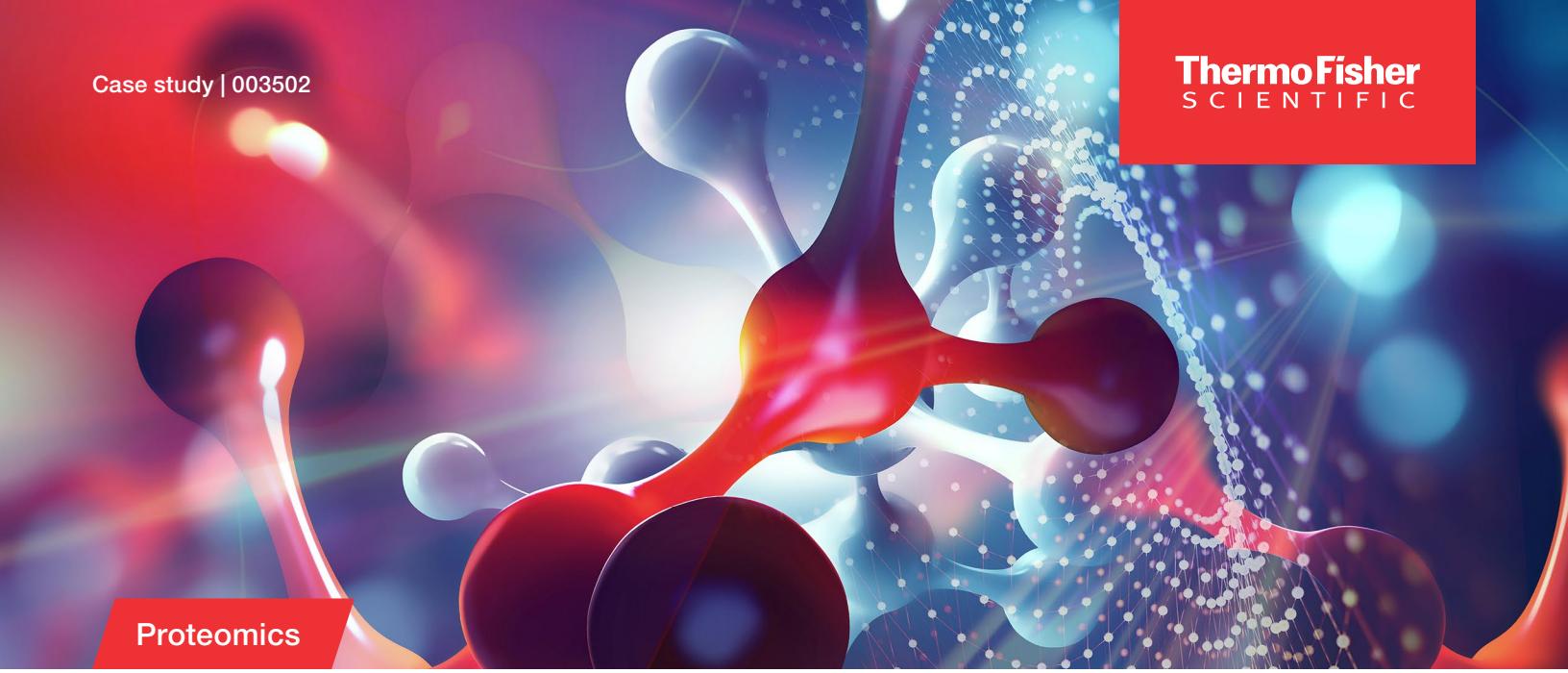

Case Western Reserve University case study:

Harnessing the power of quantitative precision and accuracy enabled by high performance Orbitrap technology to access proteome depth for discovery and research using data-independent acquisition (DIA)

Introduction

Data-independent acquisition (DIA) in mass spectrometry (MS) is a technique in which all ions within a specified mass range (*m/z*) are sequentially analyzed through MS/MS fragmentation in a repeated cycle (Figure 1). The use of DIA for label-free quantitative (LFQ) proteome profiling leveraging high resolution accurate mass (HRAM) Thermo Scientific™ Orbitrap™ technology has been on the rise. The approach offers a more comprehensive and consistent capture of a broader range of information during sample analysis. Additionally, it provides enhanced sensitivity and reproducibility, leading to greater data completeness (or fewer missing values). Traditional data-dependent acquisition (DDA) approaches, commonly used for LFQ experiments, often suffer from run-to-run inconsistencies due to the stochastic triggering of precursors based on intensity. This can result in undersampling, especially of low-abundant proteins. As sample size increases, the likelihood of missing values also rises, making DIA a preferred technique for large-scale quantitative analyses. Ensuring quantitative precision and accuracy with DIA measurements is crucial to provide the fundamental reliability of data necessary for biological and statistical assessments, enabling the tracking of proteins over space, time, and across laboratories. The mere presence or absence of a protein is frequently insufficient to delineate relevant perturbations in a biological system. An accurate assessment of the relative level of the protein is necessary.

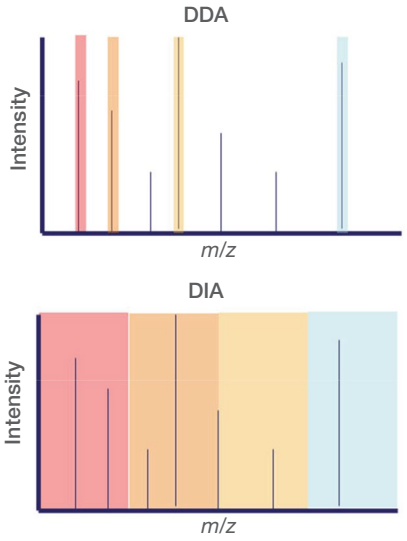

Figure 1. DDA vs DIA

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An interview with Dr. Daniela Schlatzer, Case Western Reserve University

Daniela Schlatzer is the Assistant Director of the Proteomics and Small Molecule Mass Spectrometry Core of the Case Center for Proteomics and Bioinformatics at Case Western Reserve University (CWRU).

Q. Who is using the Velocity DIA workflow?
A. CWRU Proteomics and Small Molecule Mass Spectrometry Core in collaboration with the Case Center for Proteomics and Bioinformatics (CPB) was established in 2006. The Core provides state-of-the-art proteomic instrumentation, computational resources, and software for systems biology, proteomic methods development and applications, small molecule mass spectrometry, consultation, and training. The Core utilizes the Velocity DIA workflow[1] to support label-free quantification for shotgun proteomics and phosphoproteomics.

Q. What project work are they doing?
A. Core staff actively collaborates in grant proposals and development of novel technologies that apply proteomics/mass spectrometry to biomedical research. Current proteomic applications include quantitative global (shotgun) proteomics and global phosphoproteomics for diabetes, cancer, immunology, and neurodegeneration research; targeted proteomics and chemoproteomics for drug discovery; and protein interaction mapping and technology development for structural biology research using hydroxy-radical based MS foot printing.

Q. What are the main challenges in this area of research?
A. Developing a robust, reliable, and sensitive data acquisition method that can provide deep proteome coverage is essential to all our applications. In addition, providing these in a high-throughput manner is critical in a core laboratory facility where demands for instrument time are high.

Q. What challenges or struggles were they having with their existing or previous workflows?
A. Our existing DDA workflows were time-consuming and didn't provide sufficient depth of coverage when performing large-scale global proteomic analyses.

Q. What positive results have they seen?
A. Velocity DIA workflows have made a significant impact in both quantitative protein and phosphoprotein analyses. The Velocity DIA workflow has improved our studies' accuracy, depth of coverage, and speed. On average, we observe a 2-fold improvement in accuracy (average coefficient of variation from 35% to 15%) and a 1.75-fold improvement in protein coverage (from 4,000 proteins to 7,000 proteins) in half the analysis time.

Q. What specific instrument features have been particularly beneficial in achieving these workflow improvements?
A. Orbitrap mass analyzers provide high mass resolution and accurate mass with a high stability of precision in mass analysis. Due to the increased complexity of DIA spectra, high resolution is critical to discern closely related overlapping ions. Stable and reliable mass accuracy reduces the likelihood of mass assignment errors with interpretation of more complex spectra during data analysis. The stability of performance of the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer ensures that quantitative measurements remain consistent and reliable across multiple runs and different experimental conditions. In most biological systems, a wide dynamic range of proteins is present and intensity of ions in DIA can vary extensively. Orbitrap analyzers offer the wide dynamic range required to detect and analyze ions across a broad range of intensity without loss of data quality (including accurate mass measurement). Further, the fundamental characteristics of the Orbitrap MS enable high quantitative precision and accuracy crucial for DIA analysis.

Q. How have these improvements helped the customer to accelerate their work/research? Why are these improvements critical to advancing their work?
A. The increased proteome coverage observed in the Velocity DIA workflow provides deeper proteome characterization of complex samples in multiple disease models, capturing the much greater breadth of pathway dysregulation. Data collected using this workflow provides important knowledge of driving bioinformatics data integration and identifying novel pathways, speeding biological insights that will accelerate the development of new interventions.

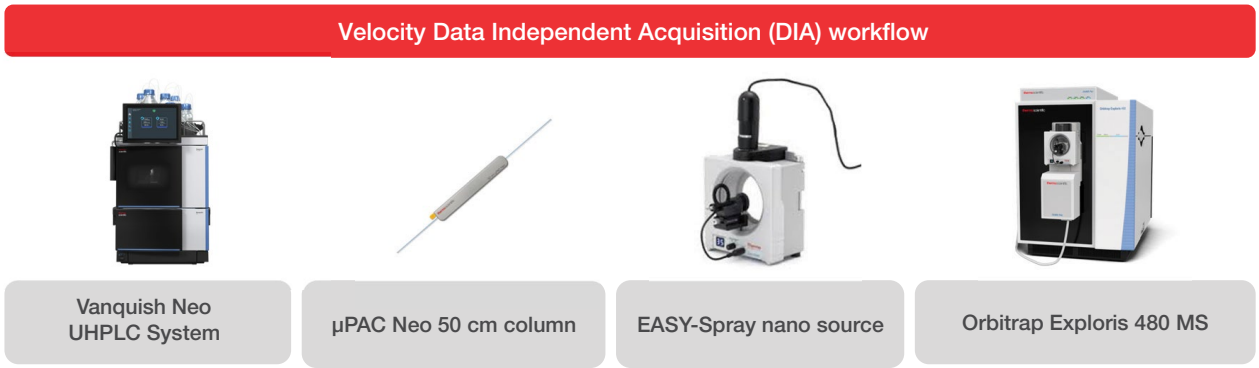

Figure 2. Velocity DIA workflow

Exemplary results

Sample preparation

Lung tissue was dissected from gender-matched mouse treated with either hyperoxia or room air. Proteins were then extracted and digested into tryptic peptides using the Thermo Scientific™ EasyPep™ MS sample prep kit following the manufacturer's instructions. The resulting peptides were reconstituted in a solution of 0.1% formic acid (FA).

Test methods

Mouse tissue digests were loaded onto a 50 cm Thermo Scientific™ µPAC™ Neo HPLC column and separated at a 350 nL/min flow rate in direct injection mode using a Thermo Scientific™ Vanquish™ Neo UHPLC system with an active LC gradient of 60 minutes before being transferred into the Orbitrap Exploris 480 mass spectrometer. Source parameters, including spray voltage and ion transfer tube temperature, are tunable parameters and must be optimized for the individual setup. The details of the LC gradient, LC parameters, and mass spectrometric method are reported in our technical note.[1]

Data analysis

Acquired data has been processed by Spectronaut™ software (Biognosys, v18) using the directDIA approach with default settings. Peptide-spectrum match (PSM) and protein identifications were filtered for 1% FDR, and a Q-value cutoff of 1% was used for the DIA analysis. FASTA files for human proteome were downloaded from Uniprot™. The resulting candidate tables were exported to tabular data formats, which were then processed with Python™ for downstream data analysis and visualization.

Results

The Velocity DIA workflow (Figure 2) on the Orbitrap Exploris 480 MS allows for the identification of close to 7,000 protein groups and over 70,000 peptide groups from mouse tissue digests in a 60-min active gradient method (Figures 3 and 4), highlighting the deep proteome coverage.

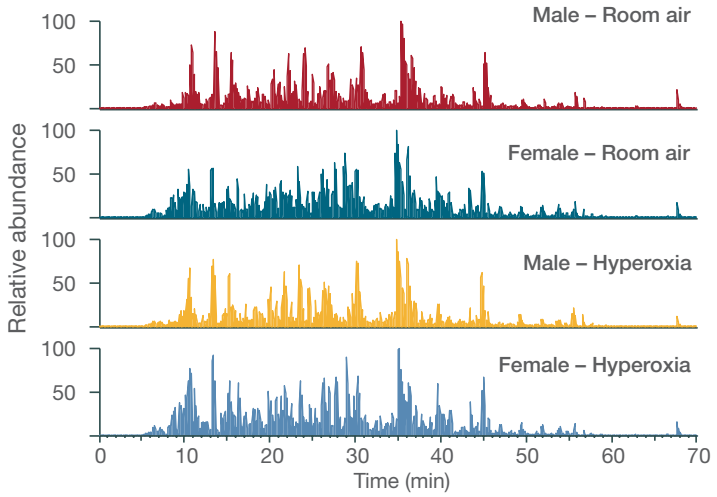

Figure 3. Base peak of example runs for each of the testing conditions

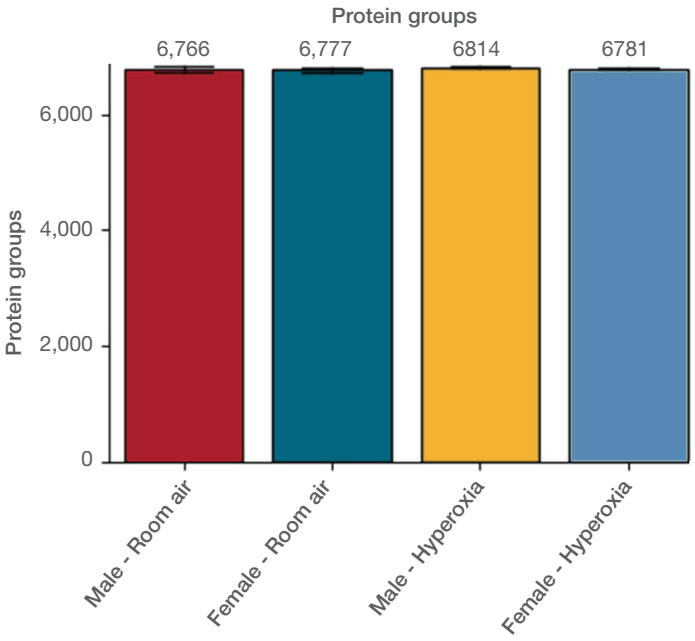

Figure 4. Bar chart showing the number of protein groups

Moreover, 97.7% of protein groups were systematically identified from all samples, indicating the sensitivity and reproducibility of the method (Figure 5). In addition to identification, quantitative data is necessary to accurately discover biology insights. We observed an average coefficient of variation (CV) of 10% from biological replicates, suggesting high quantitation precision (Figure 6).

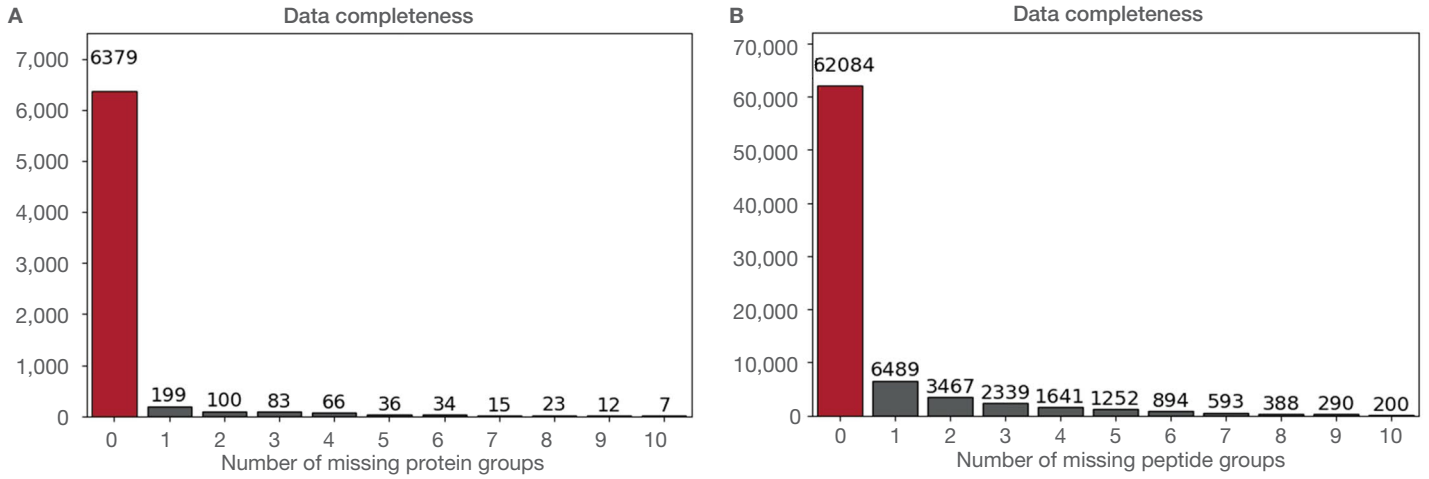

Figure 5. Bar charts showing the reproducible identification

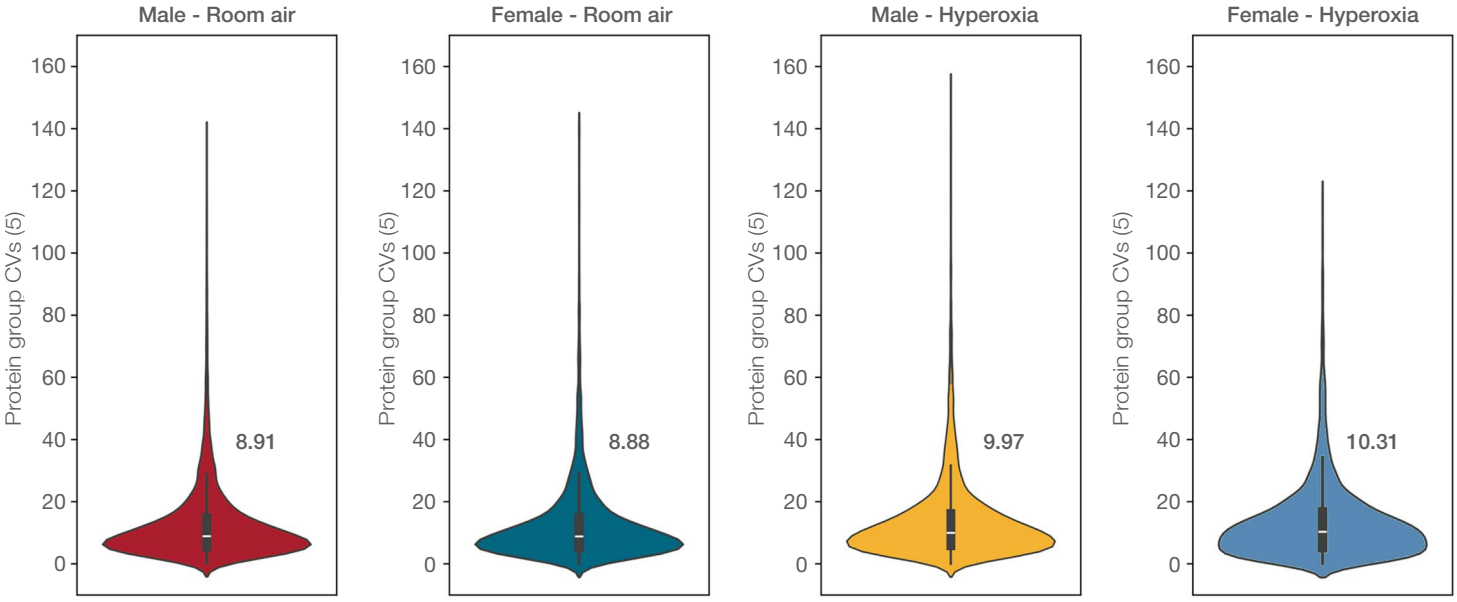

Figure 6. Violin plots showing the CV of LFQ-DIA

The comprehensive proteome coverage and precise quantitation provided by the Orbitrap Exploris 480 MS facilitates in-depth and reliable biological discovery. Principal component analysis (PCA) revealed that 80% of the data variance is explained by the first three components (Figure 7). Additionally, the samples predominantly clustered according to hyperoxia treatment, highlighting the effectiveness of the Velocity LFQ-DIA workflow in biological studies. The volcano plot shows that hyperoxia induces differential expression of a substantial number of proteins (Figure 8). Notably, many of these proteins are associated with mitochondria, consistent with current scientific paradigms. Furthermore, only a minor difference in lung proteome composition between genders was observed, further validating the robustness and confidence of the workflow in protein identification and quantitation.

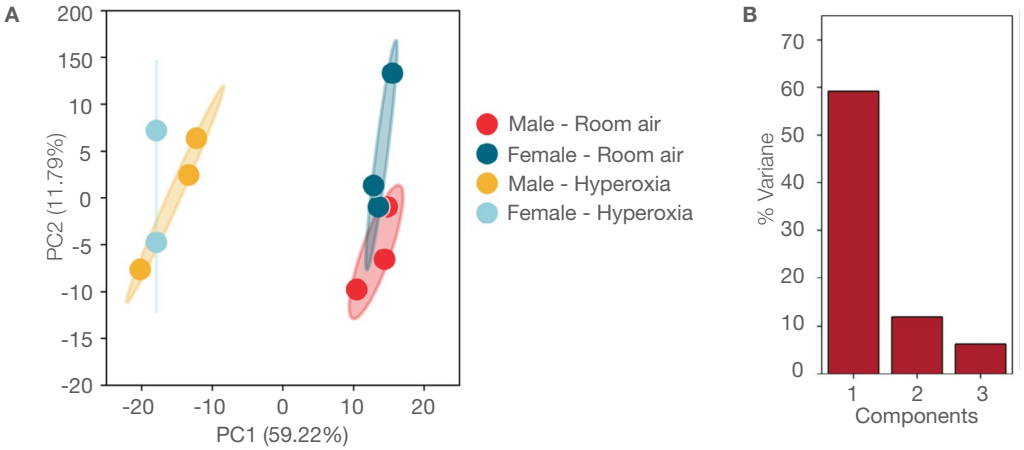

Figure 7. PCA demonstrates that samples are clustered based on hyperoxia treatment. (A) Scatter plot showing the PCA results. (B) Bar chart illustrating the percentage of variance explained by each of the PCA components.

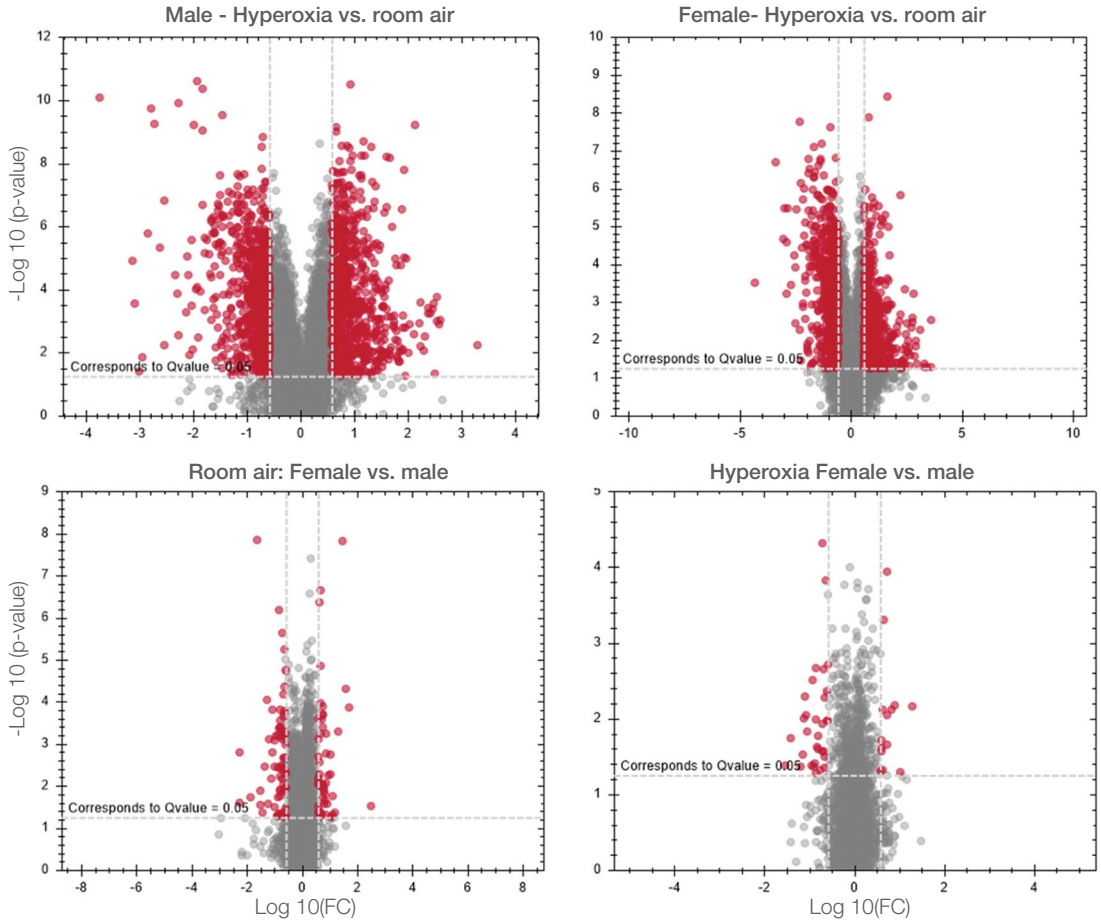

Figure 8. Volcano plots demonstrate that hyperoxia exposure but not gender leads to significantly altered protein expression

Summary

- The label-free Velocity DIA workflow allows for deeper proteome coverage with higher reproducibility of analysis for quantitative measurements on the Orbitrap Exploris 480 MS coupled with the Vanquish Neo UHPLC system equipped with µPac Neo micropillar nanopore columns.
- The HRAM measurement, stability, sensitivity, and dynamic range of the Orbitrap analyzer enables quantitative precision and accuracy for DIA mass spectrometry proteome profiling.

Acknowledgements

Daniela Schlatzer[1], Kevin Yang[2], Mark R. Chance[1], Anantha Krishnan Harijith[1], Filipa Blasco[1], Tonya Pekar Hart[2], Andrew Clark[2], Amirmansoor Hakimi[2]

[1]Case Western Reserve University, Cleveland, OH, U.S.A.
[2]Thermo Fisher Scientific, San Jose, CA, U.S.A.

Reference

1. High-throughput high-resolution data-independent acquisition workflow on an Orbitrap Exploris 480 Hybrid mass spectrometer for accurate label-free quantitation, Thermo Fisher Scientific, Technical Note 002684.

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