BIOGNOSYS
NEXT GENERATION PROTEOMICS

APPLICATION NOTE

Proteomic analysis of acetaminophen toxicity in liver micro tissues using data independent acquisition on a quadrupole-Orbitrap™ mass spectrometer and Spectronaut™ 9 software

Abstract

Comprehensive data-independent acquisition (DIA) and targeted data analysis with retention-time-normalized spectral libraries have become the methods of choice for discovery and quantitative proteomics. We demonstrate here how thousands of proteins are reproducibly quantified from as little as 12'000 liver derived cells using Spectronaut™ 9.0. In this study, liver micro tissues were used to show that liver toxicity markers are up-regulated below the acetaminophen's therapeutic dose. In contrast, proteins expected to contribute to the therapeutic effect are only affected at concentrations higher than the therapeutic dose.

In case you have any questions about this application note or Spectronaut™ software please contact us at support@biognosys.ch.

Hyper Reaction Monitoring (HRM)
NEXT-GENERATION PROTEOMICS

Introduction

Mass spectrometry based discovery proteomic workflows have long relied on data dependent acquisition (DDA) to identify and quantify proteins of interest. In a classical DDA experiment, thousands of peptides and proteins can be identified but their quantitative analysis is usually limited by the low analytical reproducibility of the workflow. Due to the semi stochastic nature of DDA, only 30-60% of the peptides are reproducibly identified in technical replicates[1]. This is often compensated using the MS1 alignment of the different runs, however, currently available software solutions do not support FDR control on the MS1 alignment. This can lead to drastically inflated FDRs and consequently incorrect quantification resulting in accumulation of false positives in the candidate lists when mass and retention time tolerances are set sub optimally.
Recently, data-independent acquisition (DIA) has rapidly evolved as powerful mass spectrometric approach for comprehensive, reproducible and precise proteome quantification. In contrast to DDA where single precursor ions are isolated for further analysis, in DIA mode the mass spectrometer cycles through broad precursor windows fragmenting multiple peptide ions together. This results in a comprehensive digital map of all detectable peptides present in a sample and more importantly a highly reproducible data matrix. The DIA data generated is comprehensive but it is convoluted and the peptide signals can be extracted using retention-time-normalized spectral libraries. Hyper Reaction Monitoring (HRM-MS™)is a discovery proteomics workflow based on data independent acquisition and retention-time-normalized spectral libraries and allows for unmatched proteome coverage with reproducible and precise quantification of

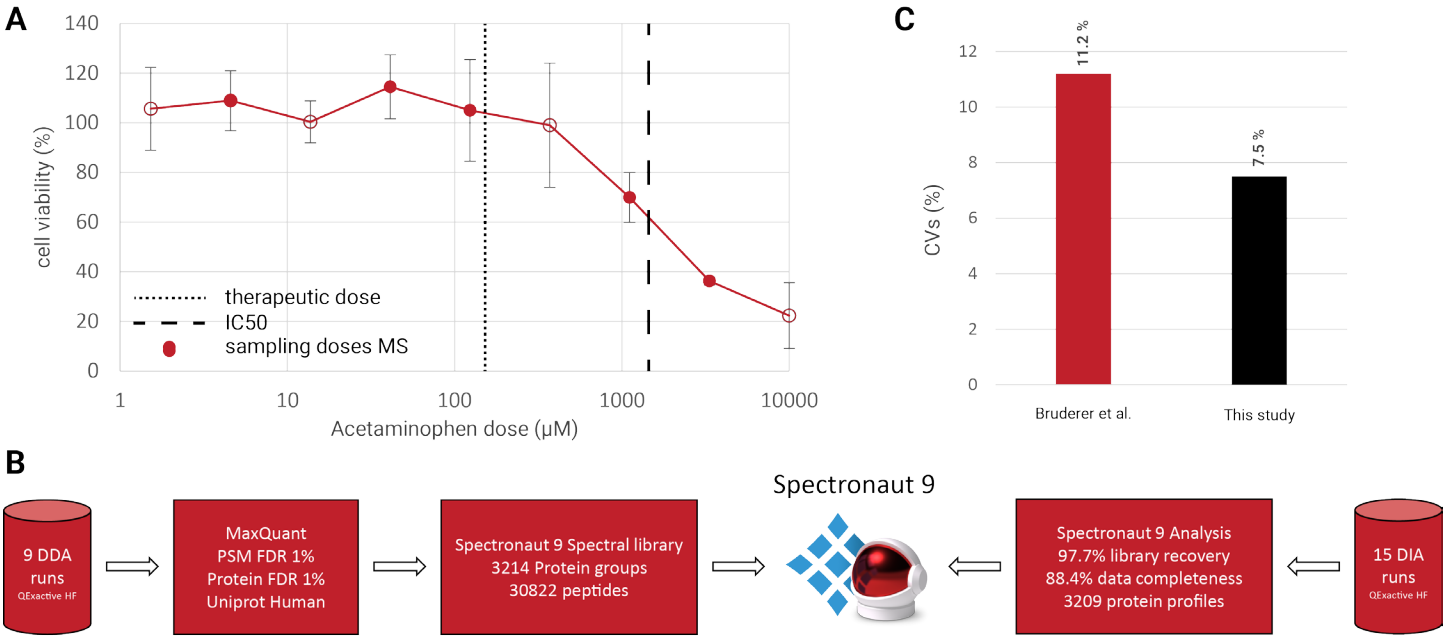

Figure 1: A) *Liver micro tissues were treated with different concentrations of acetaminophen for 72h and survival compared to the control (0 µM) was tested. At five critical concentrations (0 µM, 14 µM, 123 µM ~therapeutic, 1111 µM ~IC50 and 3333 µM) micro tissues were collected and triplicates of 12'000 cells were processed.* ***B)*** *For DDA spectral library generation the samples were pooled and measured in triplicates. This resulted in 9 DDA runs which were searched with MaxQuant and spectral library was generated with Spectronaut 9. All 15 DIA samples were processed with Spectronaut 9. Data completeness over 85% and spectral library recovery of almost 98% demonstrates the excellent data quality achievable with the HRM workflow using the newest quadrupole-Orbitrap mass spectrometer and Spectronaut 9 software.* ***C)*** *The results of this study was compared to the data set published by Bruderer et al[2]. Overall a major improvement, such as significantly lower median CVs (7.5% vs 11.25%) are observed.*

proteomes.

Acetaminophen (APAP), also known as paracetamol, is the most widely used pain killer in the western world. However, overdoses of APAP causes liver failure and is responsible for more than 26'000 hospitalizations per year in the USA alone[3]. However, the exact mechanism of toxicity is poorly understood. In this study, the HRM workflow was used for quantitative proteomic analysis of APAP induced changes using liver micro tissues treated with 5 different concentrations as a model system. To maximize the performance of the HRM we have acquired data using Thermo Scientific™ Q Exactive™ HF mass spectrometer. The resulting data was analyzed with the Biognosys' proprietary software Spectronaut™ 9. In single shot DIA measurements of 12'000 cells per sample we have reproducibly identified and quantified more than 3'000 proteins with median CVs as low as 7.5%.

Methods

Sample preparation: Detailed description of the sample preparation can be found in Bruderer et al[2]. Briefly, 3D InSight™ Human Liver Micro tissues (InSphero AG InSphero, Schlieren, Switzerland) were treated at day 5 with various concentrations (0, 13.7, 123, 1,111.1 and 3,333.3, µM) of acetaminophen for 3 days. This resulted in 12'000 cells per condition **(Figure 1A)**. Following cell lysis, tryptic digestion and desalting of the resulting peptides the Biognosys' HRM Calibration Kit was added to all of the samples according to manufacturer's instructions (required for the DIA analysis using Biognosys' Spectronaut software) Schlieren, Switzerland.

Mass spectrometric analysis: For the DDA analysis the samples were randomly pooled and measured in triplicates with a modified protocol of the "fast" method from Kelstrup[4]. For DIA all samples were measured in technical triplicates resulting in 15 DIA runs. To avoid cross-contamination between the runs from different dosing points, one blank wash was acquired in between the runs. The samples were analyzed on an EASY-Spray™ column (25 cm x 75 µm ID, PepMap C18 2 µm, Thermo Scientific™) at 40 °C and connected to a Q Exactive™ HF mass spectrometer (Thermo Scientific™) by an Easy-nLC™ 1000 system. The peptides were separated by a non-linear 75min gradient (2% to 28% ACN with 0.1% formic acid in 65mins, 28% ACN to 35% in 10mins, 35% ACN to 80% ACN, hold for 5min and back to 2% within 5mins). For DDA acquisition, a modified protocol of the "fast" method from Kelstrup was used[4].

For DIA one MS1 full scan (400-1200m/z, 30'000 resolution, target value 3e6) was followed by DIA MS2 scans (15'000 resolution, target value 1e6, isolation width 20Da, normalized collision energy of 30%) spanning the entire MS1 mass range.

Mass spectrometric data analysis: Spectral library generation: The nine individual DDA runs were searched against the human proteome (uniprot_sprot_2015-08-28_HUMAN) using MaxQuant 1.5.3.30 with a 1% FDR cut off at the protein level. Spectral libraries were generated in Spectronaut™ 9 using the default factory settings. The resulting spectral library consisted of 30'822 precursors and 3'214 protein groups **(Figure 1B)**.

DIA data analysis: DIA data analysis was performed using Spectronaut™ 9, a mass spectrometer vendor independent software platform from Biognosys. Proteins with a fold-change >50% and a Q-value of <0.05 in a pairwise-t-test among two conditions were considered statistically significant. For further downstream analysis the candidates were exported and analyzed using fuzzy c-means clustering followed by pathway enrichment analysis using DAVID Bioinformatics Resources 6.7. Further, pathways were visualized using the STRING interaction network resource.

Results and discussion

In data independent acquisition (DIA), using targeted data analysis with spectral libraries generated from shotgun proteomics measurements, a comprehensive digital map of the sample is created. Due to its high proteome coverage DIA is well suited to quantify 100-1000s of proteins in many samples across varying biological conditions with minimal sample amount needed. We show here how the HRM workflow is used to quantify proteomic changes in liver micro tissues at five different concentrations of acetaminophen (APAP). Acetaminophen is the most commonly used medication for the treatment of pain and fever. It is known that very high doses of APAP can lead to liver failure and even death. In a recent publication several mitochondrial proteins being modified with the reactive APAP phase I metabolite NAPQI and thereby adding substantial knowledge to elucidate the toxicity pathways of APAP[2].

This study shows global liver proteome changes at various APAP concentrations. Overall we quantified 37'141 sparse profiles that corresponds to a spectral library recovery of 98% at the precursor level. Data completeness of 88% with <10% CVs for the technical replicates demonstrates the excellent suitability of HRM-MS workflow for quantitative proteomics with minimal sample amount needed **(Figure 1C)**. In total 1'069 proteins were quantified statistically significant within at least two conditions. Significant up or down regulation of 57 proteins was already observed in the pairwise comparison of 0 µM APAP compared to 14 µM APAP and increased with increasing dose of acetaminophen.

Downstream analysis of the proteomic changes

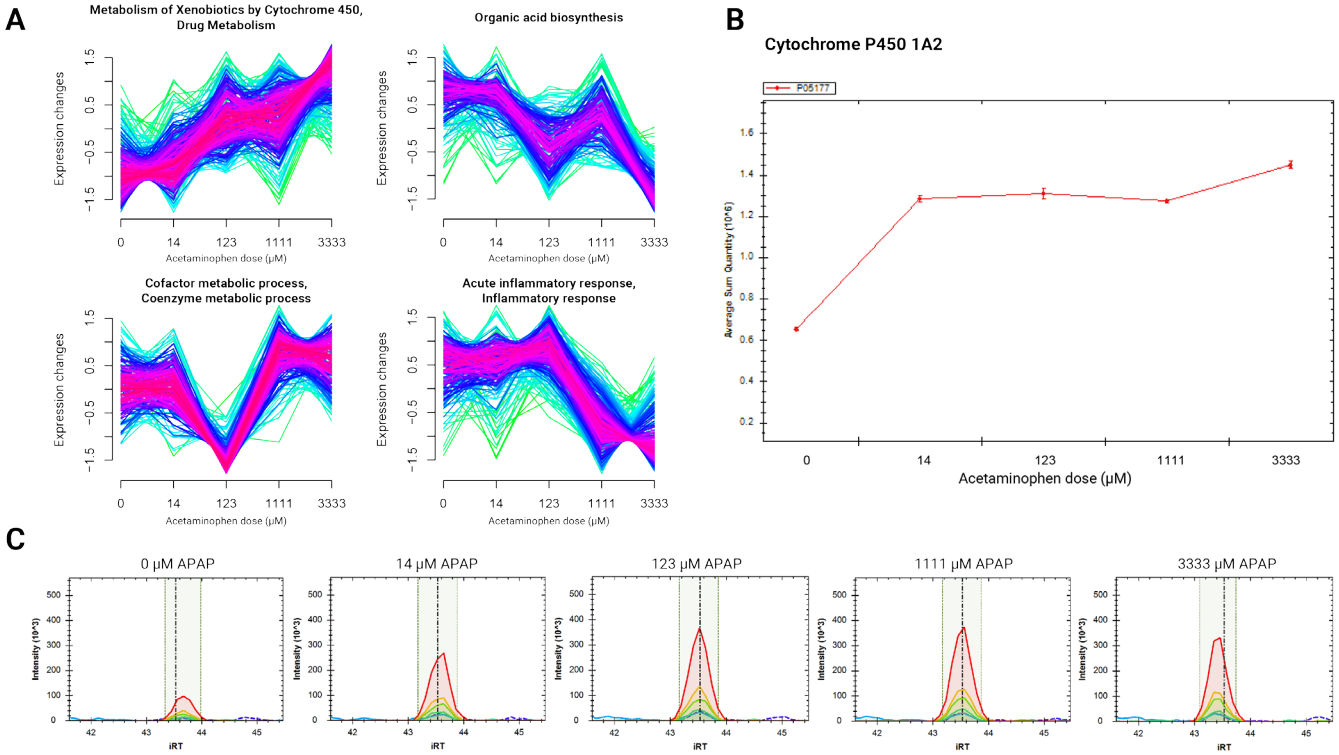

Figure 2: A) *Proteins significantly enriched (Q-value<0.05, avg-log2-FC>0.58) were subjected to fuzzy c-means clustering and pathway enrichment analysis. Proteins belonging to drug metabolism and metabolism of Xenobiotics by Cytochrome P450 built one prominent cluster* ***(top left)****. The second cluster involved proteins responsible for organic acid biosynthesis* ***(top right)****. Proteins related to cofactor metabolic process and coenzyme metabolic process made up the third cluster* ***(bottom left)****. The last cluster represents the therapeutic cluster containing proteins of the acute inflammatory response* ***(bottom right)****.* ***B)*** *For every cluster representative proteins can be extracted from Spectronaut to investigate the expression changes over the different concentrations. In this case it can be seen that Cytochrome P450 1A2 is already significantly upregulated at 14 µM acetaminophen.* ***C)*** *This can also be observed by looking at individual peptides of a certain protein.*

using fuzzy c-means clustering and pathway enrichment analysis shows that the metabolism of APAP is already induced at concentrations as low as 14 µM by the upregulation of proteins involved in the metabolism of xenobiotics via Cytochrome P450 pathway (e.g. Cytochrome P4501A2) **(Figure 2A top left, B and C)**. At 123 µM APAP, which mimics the therapeutic dose, toxicity markers are already significantly up regulated. In contrast, therapeutic markers like acute inflammatory response proteins are downregulated at concentrations higher than 123 µM APAP **(Figure 2A bottom right)**. Interestingly right at the therapeutic dose a cluster of proteins involved in cofactor metabolomics processes is being downregulated **(Figure 2A bottom left, Figure 3)**. The mechanism of this down regulation at only this dose needs to be further investigated. A similar but less drastic behavior is observed for proteins involved in organic acid biosynthesis **(Figure 2A top right)**. In summary, the data provided in here elegantly demonstrates the power of DIA based HRM-MS workflow for investigation of proteomic changes after external stimuli (e.g. drug administration), enabling the identification of individual proteins as biomarkers for pathways involved in drug toxicity.

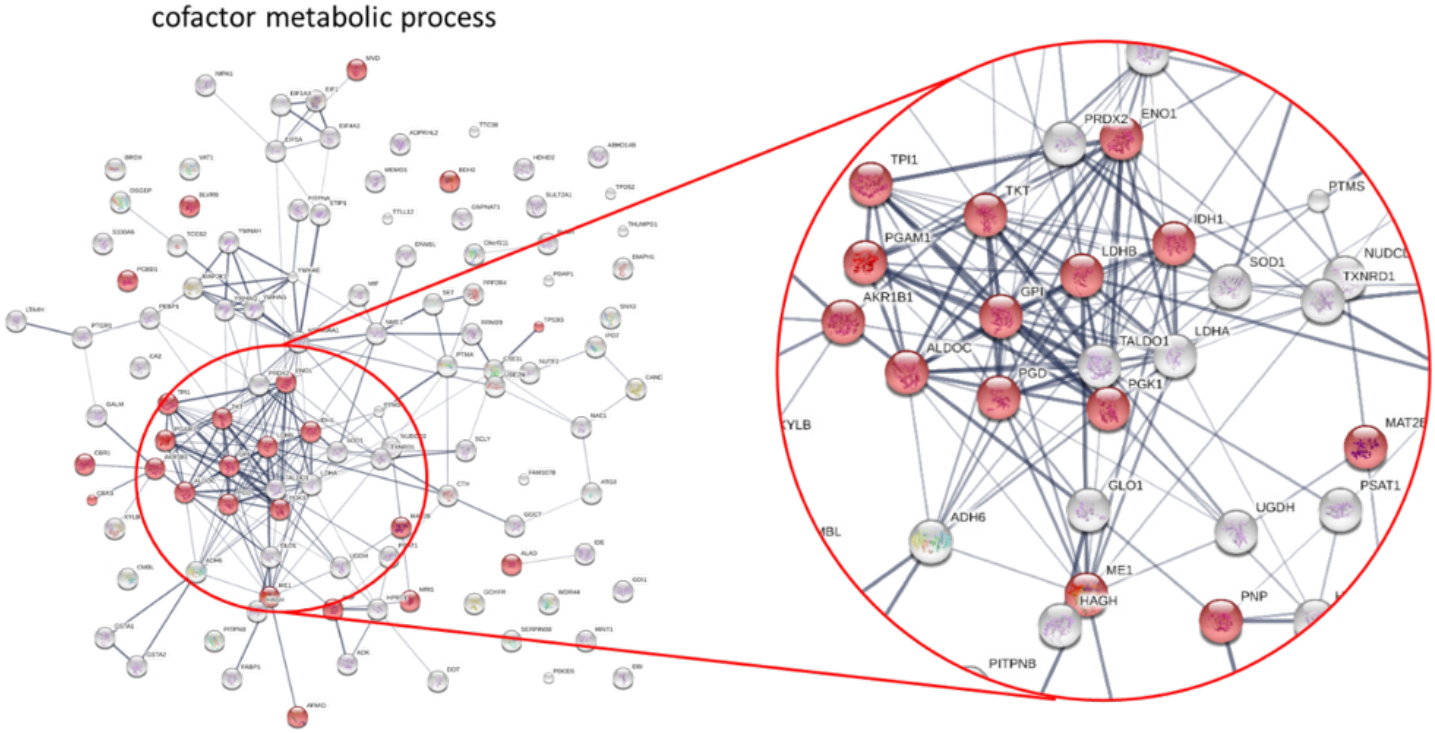

Figure 3: *Candidates of the cluster with proteins involved in metabolic process were further visualized in String-DB using their implemented KEGG pathway analysis. A clear cluster of proteins is identified and shows a high enrichment of members of cofactor metabolic process.*

References

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