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QUALITATIVE AND QUANTITATIVE CHARACTERIZATION OF A NOVEL DIA METHOD FOR OMICS ANALYSIS AND ITS APPLICATION TO BIOMEDICAL ANALYSES

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OVERVIEW

- Bioanalytical characterization of a novel scanning quadrupole DIA method

- Application of SONAR™ DIA for discovery and targeted proteomics experiments

- Quantitative precision assessed through detailed study pool/cohort analyses

- The analysis of drug treated *Aspergillus fumigatus* shows applicability of the method by the qualitative and quantitative analysis of calcineurin proteins and protein complexes

INTRODUCTION

Quadrupole time of flight (Q-ToF) MS is a well-established tool for both discovery and quantitative applications. In discovery, Q-ToF instruments are generally operated in two distinct modes of operation, that is data-dependent acquisition (DDA) and data independent acquisition (DIA). In DIA methods, such as MS^E and ion mobility (IM) enabled MSE (HDMS^E), the quadrupole mass filter operates in wide pass mode. Other DIA methods use ion traps or stepped quadrupoles with resolving DC voltages having transmission windows typically in the range of 10-20 Da. Here we describe a mode of DIA operation whereby a resolving quadrupole is scanned repetitively over alternating low and elevated energy scans. This produces data in a similar format to IM enabled acquisitions. Evaluation of this methodology for the qualitative and quantitative proteomic analyses will be made via the differential analyses of protein/protein complexes of calcineurin (*Aspergillus fumigatus*) as a function of mechanism-of-action of several antifungal drugs[1,2].
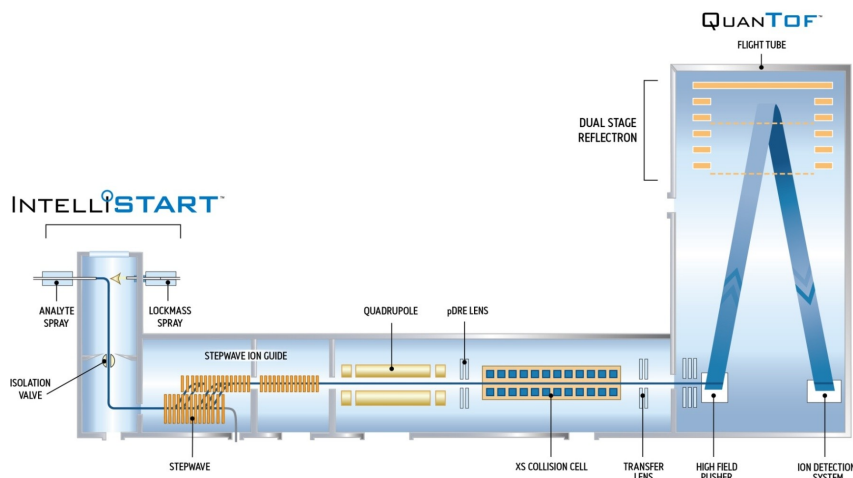

Figure 1. Xevo G2-XS QTof mass spectrometer instrument schematic.

METHODS

Sample preparation

Standards. A standard comprised a four protein digest mixture (Waters Corporation) spiked into 1 µg/µL E. Coli tryptic digest (Waters Corporation) matrix at a maximum concentration of 20 fmol/µL and then serially diluted to a lowest concentration of 100 amol/µL.

Biological samples. Proteins were extracted from immunoprecipitated *Aspergillus fumigates* samples and resuspended in 0.1% RapiGest SF (Waters Corporation) solution prior to reduction, alkylation and overnight digestion with trypsin.

LC-MS conditions

Label-free LC-MS was used for qualitative and quantitative peptide analyses. Experiments were conducted using a 90 min gradient from 5 to 40% acetonitrile (0.1% formic acid) at 300 nL/min using a M-Class system (Waters Corporation) and a HSS 1.8 µm C18 reversed phase 75 µm x 20 cm nanoscale LC column.
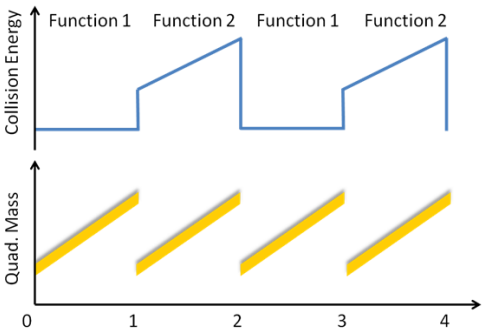
A Xevo G2-XS QToF (Waters Corporation), Figure 1, was operated in SONAR[TM] 2D MS/MS mode. The optimized quadrupole window and the other parameters employed for peptide analyses are described in Figure 2.

Bioinformatics

SONAR™ DIA data were processed using Progeneseis QI, Progenesis QI for proteomics (PQIp) (Nonlinear Dynamics) and ProteinLynx Global Server (Waters Corporation) using optimized threshold and search parameters. Quantitative analysis was performed with Scaffold (Proteome Software) or Skyline (University of Washington) using libraries derived from PQIp and PLGS protein database searches. Pathway analysis and GO annotation were conducted with Reactome[3] and Panther[4].

Experimental design

Two biological replicates of a control and three independent and different drug treated *Aspergillus fumigates* samples were analyzed each. A study pool, as shown in Figure 3, was created by combining all eight samples and run prior and during the experiment to condition the LC-MS system and act as a QC.

Figure 3. Experimental design calcineurin (Aspergillus fumigates) study.

Quad Scan (Da)	Quad Window (Da)	ToF Scan (Da)	Function Integration Time (s)	Fn 1 CE (V)	Fn 2 CE (V)
400 to 900	23	50 to 2000	0.5	6	14 to 40

Figure 2. SONAR[TM] DIA method and acquisition parameters.

Qualitative and Quantitative Characterization of a Novel DIA Method for Omics Analysis and its Application to Biomedical Analyses

2

RESULTS

Principle acquisition method

Next to the information shown in Figure 2 on the principle of the method, Figures 4 and 5 illustrate the type of information that is obtained with SONAR™ DIA. Figure 4 illustrates the multi-dimensional nature of the data by plotting product ion intensity as a function of both precursor (quadrupole) and ToF (fragment) *m/z*. The plot was produced by summing ion detections resulting from all peptides eluting during a 1 min time window. The detection of features using open source informatics is shown in Figure 5. All ToF detections are shown on the left-hand side, while the corresponding figures on the right-hand side show data in a selected precursor *m/z* window.
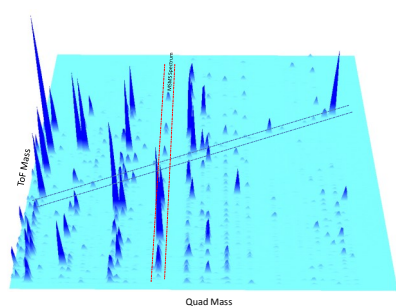

Figure 4. SONAR™ DIA ToF vs. quadrupole m/z data, showing product ions (vertical bands) from peptides eluting during a 1 min window and the quadrupole sweep (diagonal line).
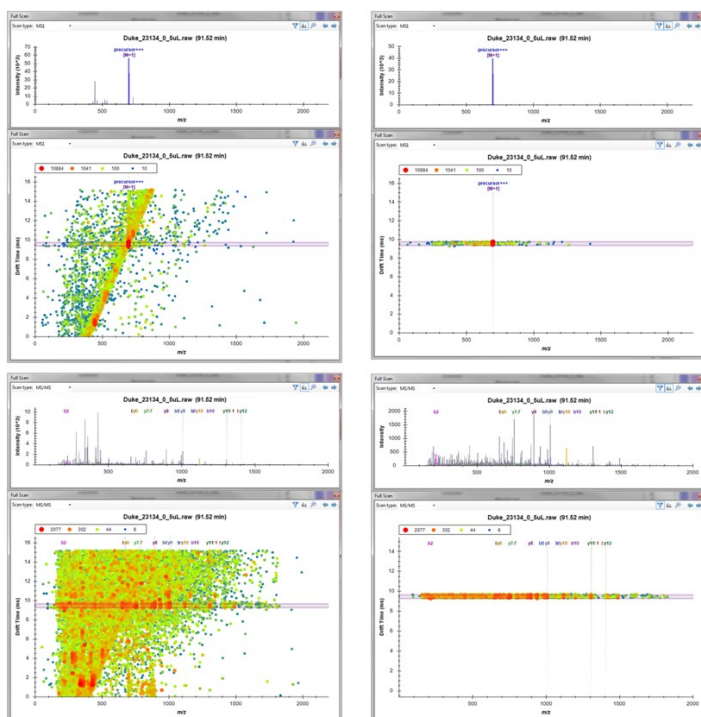

Figure 5. Single MS1 SONAR™ DIA scan (top left) and the peptides that reside within one quadrupole isolation window, represented by the grey bar (top right). The corresponding MS2 data are shown below.

Application results

The objectives of calcineurin study involved the qualitative and quantitative characterization of changes in calcineurin complexes as function of drug treatments. In particular, to examine the differential interactors with calcineurin in the presence of anti-fungal drugs, reveal the mechanism of action of calcineurin control over cell wall stress, and identify potential new targets for drug treatment. Calcineurin controls hyphal invasion into host tissue and commonly used anti-fungal therapeutic drugs include azoles and echinocandins.
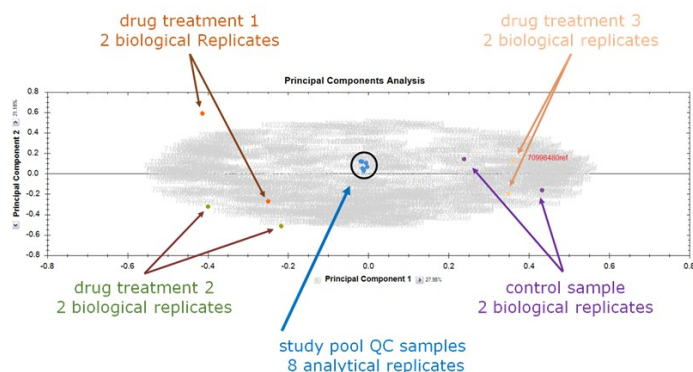

Figure 6. Quantitative analysis PCA SONAR™ DIA LC-MS data of drug treated Aspergillus fumigates.

- total proteins   538   10.7% CV in study pool QC (8 analyses)
- proteins >1 peptide   430   7.5% CV in study pool QC (8 analyses)

	control *vs.* DT1	control *vs.* DT2	control *vs.* DT3
fold change > 2	145	120	49
p < 0.05	84	137	48
both	59	65	14

(peptide score > 5, mass accuracy better than 10 ppm)

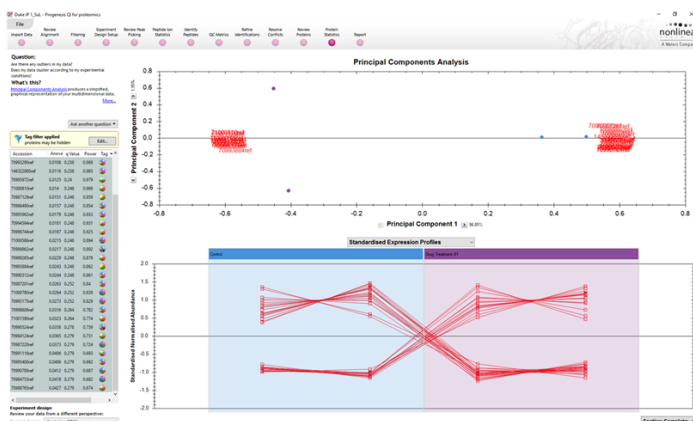Figure 7. Qualitative and quantitative label-free experiment wide results overview drug treated Aspergillus fumigates.

Figure 8. Quantitative analysis SONAR™ DIA LC-MS results of drug treated (#1) vs. control Aspergillus fumigates filtered by ANOVA p (<0.05) and fold change (≥2).

Figures 6 and 7 summarize the qualitative results and the quantitative precision of the label-free DIA calcineurin (Aspergillus fumigates) experiment. As can be observed from the PCA results, the drug treatment samples are significantly different in terms of their quantitative abundances *vs.* the study pool, control, and each other. Moreover, the precision was found to be better than 10% when a least two peptides were available for quantitation.
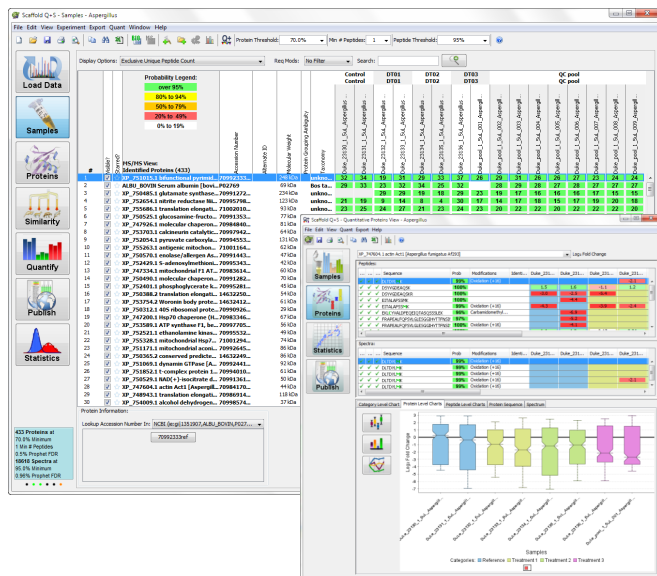

Figure 9. Quantitative analysis SONAR™ DIA LC-MS results of drug treated (#1) vs. control Aspergillus fumigates assessed through conventional statistics.

Example results of one of the quantitative comparisons (DT#1 *vs.* control) is shown in Figures 8 and 9, illustrating the normalized abundance profiles for significantly regulated proteins and . The biological interpretation, i.e. effect of drug treatment, of these results is summarized in Figures 10, highlighting DNA replication and cell cycle as the most enriched pathways, and 11, overviewing the key calcineurin binding interactors.
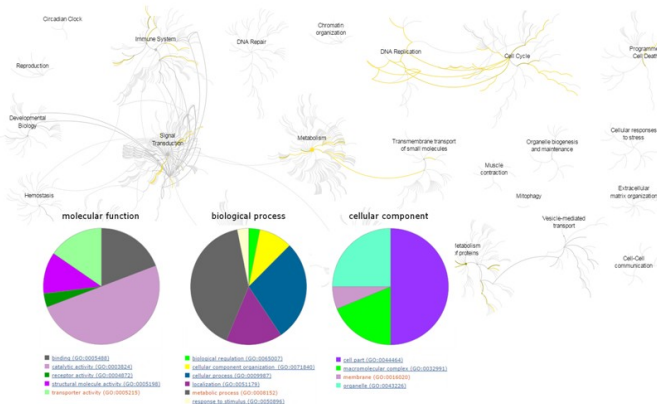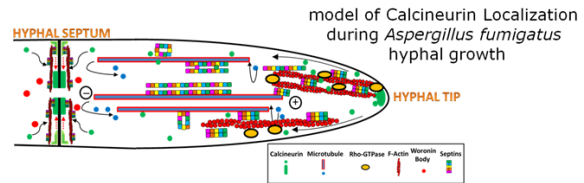

Figure 10. Overrepresentation pathway analysis results of drug treated (#1) vs. control Aspergillus fumigates with GO annotation shown inset.

- Control *vs.* DT1 or DT2
  - DT1 or DT2 *reduces* the binding of calcineurin to α- and β-Tubulins
    - α- and β-Tubulins & actins polymerize into microtubules which enable Calcineurin localization
  - DT1 or DT2 *reduces* the binding of calcineurin to Rho GTPase Rac
    - Rho proteins act as intermediaries between external stimuli and Actin organization
- Control *vs.* DT2
  - DT2 *reduces* the binding of calcineurin to Woronin body protein
    - Woronin body protein is a stress protein that protects the hyphae during damage/stress
- Control *vs.* DT3
  - DT3 *increases* the binding of calcineurin to Cyp51A
    - Cyp51A is involved in the biosynthesis of the fungal plasma membrane

Figure 11. Effect of anti-fungal therapeutic drug treatment on protein abundance expression in Aspergillus fumigates.

CONCLUSION

- **Quadrupole scanning DIA was applied for the analysis of quantitative differences of drug treated Aspergillus fumigates, identifying significant abundance changes in approximately 10% of the detected protein complement**
- **Calcineurin biding to various protein and protein complex interactors were identified as a function of drug treatment type, including tubulins, GTPases, and several stress-related proteins, providng mechanistic insights**
- **The obtained SONAR™ DIA data were used for both discovery and targeted identification informatics strategies, revealing treatment type associated proteins and pathways**
- **Precision of the novel quadrupole scanning DIA LC-MS technique was to be found better than 10%, affording the detection of mild protein abundance changes**

Qualitative and Quantitative Characterization of a Novel DIA Method for Omics Analysis and its Application to Biomedical Analyses

4

References

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4. PANTHER: a library of protein families and subfamilies indexed by function., Thomas PD et al. Genome Res. 2003 Sep;13(9):2129-41.