Increasing the depth of single shot proteomics with enhanced data acquisition and processing strategies

David Bergen[1], JingJing Huang[1], David Horn[1], Daniel Hermanson[1], Romain Huguet[1], Bernard Delanghe[2], Daniel Zolg[3], and Martin Frejno[3]
[1]Thermo Fisher Scientific, San Jose, CA, USA; [2]Thermo Fisher Scientific GmbH, Bremen, Germany; [3]MSAID GmbH, Garching b Munchen, Germany

ABSTRACT

Purpose: Single-shot proteomics depth of coverage has been enhanced by multiple advancements. Here we focus on the use of the Thermo Scientific™ Proteome Discoverer™ software with the CHIMERYS™ intelligent search algorithm paring with wide window acquisition to optimize the depth of proteome coverage in single-shot proteomics data.

Methods: We performed comparative analyses of standard runs using various gradient lengths, sample loads, and data-dependent acquisition MS^2 isolation widths. Data was acquired using a Thermo Scientific™ Orbtrap Eclipse™ Tribrid™ mass spectrometer with or without a Thermo Scientific™ FAIMS Pro Duo™ Interface. Data was processed using Proteome Discoverer 3.0 software.

Results: Increasing the precursor isolation width for data-dependent acquisition in tandem with processing using the CHIMERYS intelligent search algorithm results in improved protein and unique peptide identifications for both Orbitrap and Ion Trap MS^2 acquisition. These results are unique to the CHIMERYS intelligent search algorithm, as processing with Sequest™ HT using the same data does not provide substantial improvements.

INTRODUCTION

Advances in the online separation of complex proteomics samples including ultra-high performance liquid chromatography (UHPLC), ultra-high resolution separation columns, and high-field asymmetric waveform ion mobility spectrometry (FAIMS) enable a deeper mining of the proteome with single-shot methods. In addition to the improved separation of complex proteomes with single-shot methods described above, Proteome Discoverer software with the CHIMERYS intelligent search algorithm unlocks the ability to deconvolute the chimeric spectra that still arise from the co-isolation and fragmentation of multiple peptides in tandem mass spectra from both Orbitrap and ion trap mass analyzers. Here we employ all of these strategies together in single shot proteomics workflows for improved speed, sensitivity, and depth of coverage compared to current acquisition methods and previous search strategies.

MATERIALS AND METHODS

Sample Preparation and Liquid Chromatography

Thermo Scientific™ Pierce™ HeLa Digest Standard (20 µg/vial) was reconstituted by adding 200 µL of 5% ACN in 0.1% formic acid (FA) in water. Sample was aspired and pipetted approximately 10 times and then transferred to an autosampler vial for injection onto a Thermo Scientific™ µPac™ Neo column. Samples were separated at 300 nL/min using a Thermo Scientific™ Vanquish™ Neo UHPLC system. Gradient lengths between 30 minutes and 3 hours were used to evaluate single shot proteomics performance.

Mass Spectrometry Methods

Data were collected using a FAIMS Pro Duo interface and Orbitrap Eclipse Tribrid mass spectrometer in data-dependent acquisition mode with full scan data collection using Orbitrap detection with and fragmentation data collection using either Orbitrap (OT) or Ion trap (IT) detection. For OT/IT data collection the full scan was collected with 240,000 resolution and Turbo IT scans. For OT/OT data collection the full scan was collected with 60,000 resolution and fragmentation data collected at 15,000 resolution. Quadrupole isolation widths for MS^2 acquisition were varied between 0.4 Th and 3 Th. Maximum injection time (MaxIT) was varied between 10 and 25 ms for OT/IT acquisition and 11 ms and 35 ms for OT/OT acquisition. The scan range used was 375-1500 *m/z*. Dynamic exclusion was set to 10 ppm with a 30 second duration. Fragmentation was performed using HCD with a fixed collision energy of 30. For two compensation voltage (CV) experiments the FAIMS Pro Duo interface CV was set to -50 and -70. For three CV experiments the FAIMS Pro Duo interface was set to -40, -60, and -80.

Data Analysis

The acquired raw data files were processed with Proteome Discoverer 3.0 software using the default Sequest HT_Percolator, INFERYS_Rescoring_SequestHT_Percolator, and CHIMERYS_Percolator workflows paired with a standard consensus workflow.

RESULTS

Optimization of isolation window for data-dependent acquisition

To optimize acquisition strategies for use with the CHIMERYS intelligent search algorithm we investigated the impact of isolation window on both OT/OT and OT/IT acquisition with an Orbitrap Eclipse Tribrid mass spectrometer with a FAIMS Pro Duo Interface using -50 and -70 CV. These results demonstrated a clear benefit to increasing isolation windows for both acquisition strategies with an isolation window of 1.5 Th providing the best results for OT/IT acquisition and between 2-4 Th providing the best results for OT/OT acquisition.

Figure 1. Comparison of the number of unique peptides and protein groups identified on average from OT/OT data-dependent acquisition with 3 replicates of HeLa cell lysate digest with a 1-hour gradient and variable isolation widths using different search strategies in the Proteome Discoverer software framework demonstrates an increase in unique peptides and protein groups for isolation widths between 2-4 Th when using the CHIMERYS intelligent search algorithm.

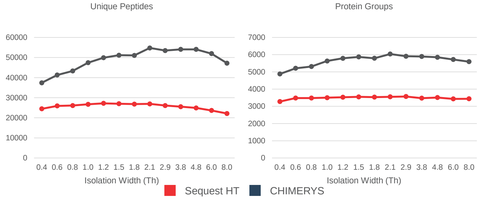


Figure 2. Comparison of the number of unique peptides and protein groups identified on average from OT/IT data-dependent acquisition with 3 replicates of HeLa cell lysate digest with a 1-hour gradient and variable isolation widths using different search strategies in the Proteome Discoverer software framework demonstrates an increase in unique peptides and protein groups for isolation widths between 1.2-1.5 Th when using the CHIMERYS intelligent search algorithm.

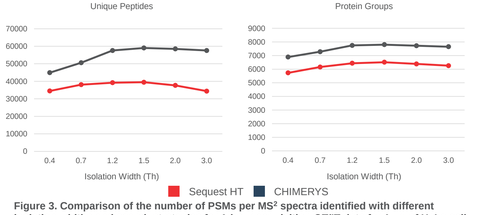


Figure 3. Comparison of the number of PSMs per MS^2 spectra identified with different isolation widths and search strategies for 1-hour acquisition OT/IT data for 1 ug of HeLa cell lysate digest. Wider isolation widths increase instrument utilization in combination with CHIMERYS processing by identifying more PSMs per spectrum.

Performance improvements for longer gradients

To determine if improvements for 1-hour gradients also applied to longer gradients we also compared the impact of isolation window and search strategy for 2- and 3- hour gradients using variable isolation windows for OT/IT acquisition with an Orbitrap Eclipse Tribrid mass spectrometer with a FAIMS Pro Duo Interface using -50 and -70 CV. These results demonstrated a clear benefit to increasing isolation windows for both acquisition strategies with an isolation window of 1.5 Th providing the best results for OT/IT acquisition and between 2-4 Th providing the best results for OT/OT acquisition.

Figure 4. Comparison of the number of unique peptides and protein groups identified on average from OT/IT data-dependent acquisition with 3 replicates of HeLa cell lysate digest with a 2-hour gradient and 3 FAIMS CVs and variable isolation widths and a 3-hour gradient and 3 FAIMS CVs using different search strategies in the Proteome Discoverer software framework demonstrates an increase in unique peptides and protein groups even for longer gradients when using the CHIMERYS intelligent search algorithm compared to Sequest HT.

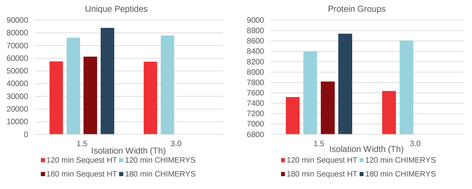
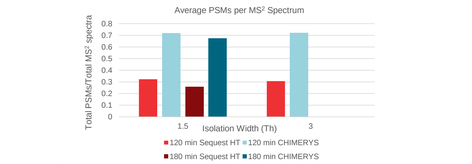


Figure 5. Even with 2-hour and 3-hour gradients using 3 FAIMS CVs, CHIMERYS dramatically increases the average number of PSMs identified per MS^2 spectrum for OT/IT data-dependent acquisition for 3 replicates of 1 ug HeLa cell lysate digest when compared to Sequest HT.



Optimization of maximum injection time

To further determine the impact of other acquisition parameters that impact MS^2 precursor selection and acquisition we also tested modifications of the MS^2 scan maximum injection time (MaxIT) for both OT/OT and OT/IT acquisition. This determined that MaxIT values of 15 ms for OT/IT and 22 ms for OT/OT provided incremental improvements versus 10 ms and 11 ms, respectively. Longer MaxIT values of 20 or 25 ms for OT/IT and 35 ms for OT/OT showed decreased identification due to fewer MS^2 spectra being collected with these longer MaxIT settings.

Figure 6. Comparison of the number of unique peptides and protein groups identified by OT/IT data-dependent acquisition with 1 ug HeLa cell lysate digest with a 1-hour gradient using variable isolation widths and different MaxIT values processed with Proteome Discoverer software and the CHIMERYS intelligent search algorithm. These results suggest using a slightly higher MaxIT value of 15 ms for OT/IT along with a 1.5 Th isolation window provides slight performance improvements.

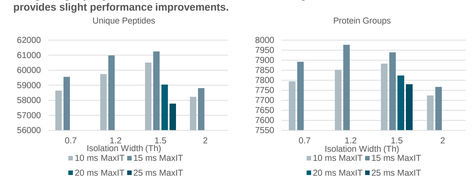
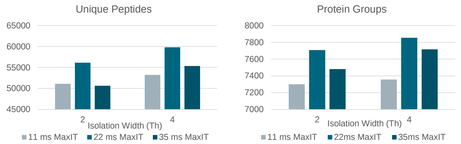


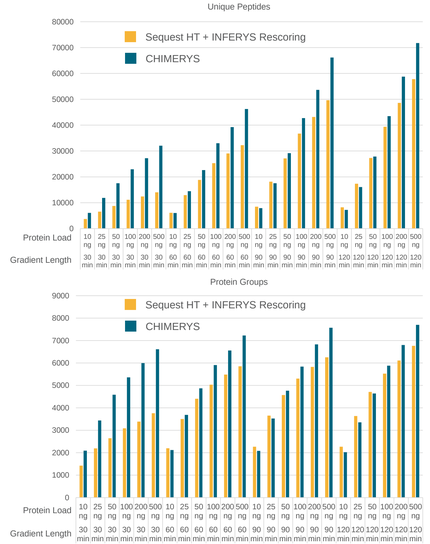
Figure 7. Comparison of the number of unique peptides and protein groups identified by OT/OT data-dependent acquisition with 1 ug HeLa cell lysate digest with a 1-hour gradient using variable isolation widths and different MaxIT values processed with Proteome Discoverer software and the CHIMERYS intelligent search algorithm. These results suggest using a slightly higher MaxIT value of 22 ms for OT/OT along with isolation windows between 2-4 Th provides slight performance improvements.



Generalization of CHIMERYS improvements

To determine if wide window acquisition with Proteome Discoverer software and CHIMERYS intelligent search algorithm processing applies broadly, we also compared multiple protein loads and gradient lengths between Sequest HT with INFERYS Rescoring processing to CHIMERYS processing within the Proteome Discoverer software framework. These results show striking improvements across protein loads and run times, with similar results being produced by CHIMERYS processing with a quarter of the run time for comparable protein loads.

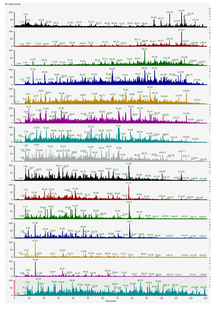
Figure 8. Comparison of Sequest HT with INFERYS Rescoring versus CHIMERYS intelligent search algorithm processing for various HeLa digest protein loads and gradient lengths with OT/OT data acquisition using a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer.



Deep proteome coverage through differential ion mobility with the FAIMS Pro Duo Interface

Typical proteomics runs using the FAIMS Pro Duo Interface use one, two, or three CV values in alternation throughout the run. To determine if CHIMERYS provides incremental improvements with extensive gas phase fractionation through single CV value runs we also compared the number of peptide and protein identifications between a single shot no FAIMS Pro Duo Interface run and multiple runs using single CV values with a FAIMS Pro Duo Interface. These results demonstrate that using the FAIMS Pro Duo Interface with individual CV values allows access to more peptides than without, and CHIMERYS provides further improvements even with extensive front end gas phase fractionation.

Figure 6. Impact of individual FAIMS CV value acquisition visualized by total ion current (TIC) chromatograms on unique peptides identified using an Orbitrap Eclipse Tribrid mass spectrometer. Processing with CHIMERYS in Proteome Discoverer 3.0 software (PD 3.0) increases the number of unique peptides identified in most individual CV runs as well as the no FAIMS run compared to processing with Sequest HT and INFERYS Rescoring (PD 2.5). Using no FAIMS Pro Duo interface identified 83,929 unique peptides while combined individual FAIMS CV values identified 110,078 peptides, an increase of 31%.



CV	PD 2.5	PD 3.0
-25	4652	3318
-30	14809	14644
-35	23939	27509 (+14.9%)
-40	30819	39201 (+27.2%)
-45	34108	45187 (+32.5%)
-50	33562	45673 (+36.0%)
-55	32089	43182 (+34.6%)
-60	28951	38352 (+32.5%)
-65	24533	30466 (+24.2%)
-70	19681	23813 (+21.0%)
-75	14919	16582 (+11.1%)
-80	9982	10401 (+4.2%)
-85	5999	5543
-90	2542	2053
No FAIMS	56766	83929 **(+47.9%)**

Total unique peptides from individual FAIMS CV runs: 110078 **(+31%)**

CONCLUSIONS

These results demonstrate that pairing optimized instrument acquisition strategies for both OT/OT and OT/IT leveraging UHPLC, ultra-high resolution µPac Neo columns, FAIMS Pro Duo interface gas phase fractionation, and advanced processing strategies with Proteome Discoverer software and the CHIMERYS intelligent search algorithm can synergistically improve the depth of proteome coverage and increase throughput.

- Increasing the MS^2 isolation width for data-dependent acquisition to between 2-4 Th for OT/OT and 1.2-1.5 Th for OT/IT acquisition along with processing using Proteome Discoverer software with the CHIMERYS intelligent search algorithm improves performance
- Wide window acquisition and data processing using Proteome Discoverer software with the CHIMERYS intelligent search algorithm improves performance across a wide range of protein loads and gradient lengths
- FAIMS Pro Duo Interface gas phase fractionation can access unique peptides in tandem with data processing using Proteome Discoverer software with the CHIMERYS intelligent search algorithm

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