Chimeric Spectra and Data-Driven DIA analysis

reSpect, DISCo, Quantic
reSpect

Finding chimeric peptides from high resolution MS/MS spectra
• **Standard Shotgun** (MS/MS) **Workflow**

- **Peptides**
- **Precursor Scan (MS)**
- **Fragmentation Scan (MS/MS)**

**Selection Window**
Chimeric MS/MS

- Chimeric spectra contain multiple precursor ions

Peptides

Precursor Scan (MS)

Fragments ions are seen for all precursor ions

Selection Window includes two peptides!
Factors Contributing To Chimeric Spectra

- Chimeric spectra are a common occurrence in LC-MS/MS
  - proportional to sample complexity
  - proportional to selection window size
  - influenced by chromatography conditions

Database search algorithms only report one peptide per spectrum!
Search Algorithms Do Not Find Chimeras

- **Selection window of the instrument is *much wider* than the peak resolution**
  - Search algorithms use only the measured precursor mass
    - For example: ±25 ppm

- **Selection window may include peptides outside the search mass**
  - Those peptides never considered during search
    - For example: different charge leads to mass outside ±25 ppm tolerance
Chimeric MS/MS

Selected Ion (A):
Mass = 615.74
Charge = 3+

Chimeric Ion (B):
Mass = 614.85
Charge = 2+

Database Search:
Peptides ~ 1844.22 Da
Peptide (A) Found!!

Not Searched:
Peptides ~ 1227.70 Da
Peptide (B) NOT Found!!
reSpect

- Allows identification of chimeric peptides from spectra.
- Works with existing search algorithms.
reSpect: The Approach

First Pass Search

PeptideProphet & iProphet

Search algorithms using standard, narrow mass tolerances and assumed charge states

TPP Statistical Tools are applied to identify the match probabilities

reSpect attenuates the matched peaks of PSMs. The resulting spectra are saved in a new mzML/mzXML

reSpect

Repeat Search

Searches repeated using wide mass tolerance and assuming all charges
reSpect: Attenuation Original $P = 0.999$
reSpect: Attenuation

\[ I_{rs} = (1-P)I_{\text{orig}} \]
reSpect: Second Match $P = 0.995$
reSpect: Mass Differences

First Pass Mass Differences

reSpect Mass Differences
Chimeric Depth

- Chimeric spectra may contain more than two precursors
- reSpect can be iteratively applied until novel PSMs are exhausted
reSpect: The Original
reSpect: The First Iteration
reSpect: The Second Iteration
reSpect: The Third Iteration
reSpect: No Fractionation

- 4 Replicate Yeast Q-Exactive Runs
- 347,000 spectra
- 30.3% Boost in Distinct Peptide Identification

![Venndiagram showing peptide identification numbers]
reSpect: Medium Fractionation

- iPRG2013 study
- 118,000 spectra
- Orbitrap Velos, 14 RP fractions of whole cell lysate of human peripheral blood mononuclear cells
- 8.1% Boost in Distinct Peptide identifications
reSpect: High Fractionation

- Human Cancer Cell Line
- Orbitrap Velos, 48 fractions
- 12.9% Boost in Distinct Peptide Identifications
Different Datasets, Different Chimeras

- Sample complexity and instrument settings have largest impact on chimeric spectra

Precursor analysis algorithms can estimate number of precursors to solve.

These numbers correlate with PSMs identified.
reSpect: Different Datasets

reSpect: Distinct Peptide Percentage Boost

\[ y = -17.18 \ln(x) - 0.1642 \]

\[ R^2 = 0.8371 \]
reSpect: Identifies More Proteins

Protein level performance on the Moritz Lab yeast dataset **BEFORE reSpect analysis.**

Protein level performance on the Moritz Lab yeast dataset **AFTER reSpect analysis.**
reSpect in the TPP
reSpect: Summary

- Explained peaks are *attenuated* according to the quality of the match amongst medium and high scoring spectra
- Attenuated spectra are *researched* with wide mass window and assuming all charges
- Uses *same validation* with PeptideProphet and iProphet to estimate error rates
- *Automated* within the TPP framework
- Identifies new peptide sequences even in highly fractionated data
- Works best on *high mass accuracy MS*² data
  - Q-Exactive
  - Orbitrap Velos
  - QTOF
- Also works nicely on *standard mass accuracy MS*² data
  - LTQ
Principles of Data Independent Acquisition

Slides courtesy of Dr. Mukul Midha
Principle of sequentially windowed acquisition in DIA/SWATH-MS

Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial, Ludwig, C. et al Molecular Systems Biology 2018
SWATH-MS/DIA experiment setup.....So much to choose from!!

- Instruments......
- Nano flow or Micro flow?
- LC separation considerations?
- Sample datasets...
- Library free approach
- Spectral ion Libraries
- Fixed or Variable windows?
- Software, software, software.......
What are the critical acquisition attributes for DIA/SWATH-MS?

- High resolution MS/MS
- Cycle Time
- Q1 Isolation Windows
- Dynamic Range
DIA Acquisition – Quantitation

Data Points Per Peak (DPPP)

<7 DPPP = under sampling

7-10 DPPP = optimal sampling

>10 DPPP = over sampling

ex. 30 s peak width at base
3 s cycle will collect 10 DPPP
<3 s will over sample
>3 s will under sample
DIA/SWATH-MS precursor isolation windows

# windows x MS2 acquisition time = cycle time
42 (20 m/z width, 400-1200 m/z, 1 Da overlap) x 60 ms = 3.6 s

Choices are dependent on chromatography, application and platform.
DIA data analysis – retention time normalization

- **iRT spike**
  - set of non-endogenous peptides
  - Used to convert to iRT scale (linear)

- **Observed peptides/features**
  - High-precision iRT (non-linear)
  - Anchor points

![Graph showing Observed Retention Time vs Indexed Retention Time](image1)

\[ y = 0.8737x + 42.807 \]
\[ R^2 = 0.9977 \]
DIA/SWATH-MS: Library based and library free approach

Peptide-Centric Proteome Analysis: An Alternative Strategy for the Analysis of Tandem Mass Spectrometry Data, Ying S. Ting et al., MCP, 2015
TPP Software for Data-Driven DIA Analysis and Quantitation
Trans-Proteomic Pipeline a.k.a. TPP

RAW Data
- msConvert
- Vendor converter

Processed Spectra
- DISCO
- reSpect

PSM Identification
- SpectraST
- Comet
- X!Tandem
- KOJAK

PSM Validation
- PeptideProphe t
- PTMProphet

Peptide Validation
- iProphet

Quantitation
- ASAPRatio
- StPeter
- Libra
- Quantic

Protein Validation
- ProteinProphe t

Protein List
- SBEAMS
- Proteographer

Result Cataloguing
- SpectraST
- RTCatalog

RT Prediction
- SSRCalc
- RTCalc

Tool Specific
Highlights

- Works on tiMS-TOF PASEF DIA data
- DIA Windows is not required if isolation_window info available
- Multiple scan windows sizes for data point extraction
- Minimum amount of correlation required to select fragments depends on the scan window size (number of data points)
- Kernel Density smoothing of raw signals
- Better utilization of multi-threading resources
- Fail-safe mzML file reading
USAGE: DiscoFilter <OPTIONS> <mzML/mzXML_input_file>

OPTIONS:

TARGETS=<targets_file>  Use specified <target_file> to look for MS1 features  (default: use HardKlör internally)
UMPTARGETS=<targets_file> Use specified <target_file> to look for MS1 features  (default: use HardKlör internally)
HKCONFIG=<config_file>   Use specified <config_file> for HardKlör (default: use Hardklor.conf)
WINDOWS=<DIA_windows_file>
MAXSCANWINDOW=<number>: Use specified maximum number of scans over which to track (default=17). This option applies only when the UMPTARGETS are not specified, when UMPTARGETS are specified the SCANWINDOW is set to the maximum peak width defined in the UMPTARGETS file.
MINSCANWINDOW=<number>: Use specified minimum number of scans over which to track (default=7). This option applies only when the UMPTARGETS are not specified, when UMPTARGETS are specified the SCANWINDOW is set to the maximum peak width defined in the UMPTARGETS file.
AVGSCANHALF=<number>:   Use specified number of + - scans over which to average MS1 scans prior to feature detection, set to 0 for no averaging. (default=0)
MZPREC=<number>:        Set mz precision in 'points after the decimal', used for binning and averaging. Applies in combination with AVGSCANHALF= greater than 0. (default=2.1)
MININTENS=<number>:     Filter out peaks below minimum intensity factor in each spectrum, set as a non-negative number (default=off)
MAXPPM=<number>:        Maximum Allowed PPM signal offset best defined in powers of 2 (e.g. 2, 4, 8, 16, etc. default=32)
PPMFWHM=<number>:       Full Width at Half Maximum expected for Mass PPM profile of peaks (default=16)
IMFWHM=<number>:        Full Width at Half Maximum expected for Inverse Reduced Ion Mobility profile of peaks (default=0.05)
IONMOBBINS=<number>:    Set number of bins to partition Ion Mobility. (default=10)
SUFFIX=<string>:        Set suffix for output file (default='_ds')
THREADS=<number>:       Use specified number of threads (default=1).
STARTSCAN=<number>:     Starting scan to process (default=1).
ENDSCAN=<number>:       Ending scan to process (default: process until the end of the run).
DISCo matches identified **precursor** signals to fragment signals by looking for those that have a similar shape in Retention Time Space.

Fragment signals are sorted according to the following criteria, in order:  

*entropy* → *distance* → *correlation* → *intensity*

Type I Error Rate $\alpha$ and Type II Error Rate $\beta$, $N$ is sample size (points across peak).

For each MS$^1$ target the following MS$^2$ peaks are selected:

- Up to 1000 peaks with correlation $\geq r_{\text{Max}}$ ($\alpha = 0.01$, $\beta = 0.05$)
- Up to 500 peaks with correlation $\geq r_{\text{Mid1}}$ ($\alpha = 0.03$, $\beta = 0.1$)
- Up to 200 peaks with correlation $\geq r_{\text{Mid2}}$ ($\alpha = 0.05$, $\beta = 0.2$)
- Up to 100 peaks with correlation $\geq r_{\text{Min}}$ ($\alpha = 0.1$, $\beta = 0.333$)

Peak intensities are scaled by the correlation.
Data-Driven DIA Workflow

DIA.mzML Data

DISCO Results

DIA_ds.mzML

PeptideProphet

ProteomeProphet

Comet/X!Tandem

PepXMLViewer.cgi

comparePeptides

PepXMLViewer.cgi

Quantic

iProphet

ProteinProphet

compareProteins

Proteins

Peptides

RTCatalog

Spectral Library

RTCatalog Peptides

DIA_Q[123].mzML Data

Result Cataloguing

SpectraST

RTCatalog

Umpire Targets

HardKlör Targets

DISCO

PECAM \[123\]
For TTOF 6600 Converter Choices Matter!

ABSciex profile
32.7%

msconvert
8.3%

qtofpeakpicker
3.0%

msconvert centroid
11.9%

38.9%
## Benchmarking HeLa/Halo Dataset

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<tr>
<th>Sample</th>
<th>HeLa Background (ug)</th>
<th>Halo Variable (fmol)</th>
<th>Halo E/x Ratio</th>
<th>Halo log2 (E/x)</th>
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<td>A</td>
<td>3.52</td>
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<tr>
<td>B</td>
<td>3.52</td>
<td>6.25</td>
<td>64</td>
<td>E/B = 6</td>
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<tr>
<td>C</td>
<td>3.52</td>
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<td>16</td>
<td>E/C = 4</td>
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<tr>
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<td>100</td>
<td>4</td>
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<tr>
<td>E</td>
<td>3.52</td>
<td>400</td>
<td>1</td>
<td>E/E = 0</td>
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</table>
Hela/Halo TTOF6600:
DISCO - DIAUmpire - DISCO w/ Umpire Targets
DISCO - DIAUmpire - DISCO w/ DIAUmpire Targets

DISCO

19.9%

DIAUmpire

5.6%

DISCO w/ DIAUmpire Targets

9.2%

5.8%

2.9%

44.3%
# Mass-Spectrometry Setups

<table>
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<th>Q Exactive HF</th>
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<td>DIA</td>
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<td># Windows</td>
<td>100 variable</td>
<td>100 fixed (6m/z)</td>
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<td>MS1 m/z range</td>
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<td>400-1000</td>
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<td>MS1 scans- Accumulation time/Max fill time</td>
<td>250ms</td>
<td>200ms</td>
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<td>MS2 scans- Accumulation time/Max fill time</td>
<td>25ms</td>
<td>10ms</td>
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<tr>
<td>Cycle Time (sec)</td>
<td>2.92</td>
<td>4.45</td>
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</table>
DISCO Peptide Identification: TTOF 6600 vs QE HF

*modified peptides, no charge;
combining 2 search engines (Comet and X!Tandem)
DISCO Quantitation Options

- **MS1**
  - For each spectrum coming from an MS1 feature, DISCO stores the integrated precursor peak intensity stored as `precursor_intensity` in `mzML`.

- **MS2 using QuanticParser**
  - For each PSM, Quantic computes the **Annotated Ion Current** (antic_score) by summing the intensities of annotated fragments in the spectrum.

- **Hybrid – Combined MS1 and MS2 Quantitation**
  - Average of MS1 and MS2 quantitation results
DISCO Peptide Quantitation from PSMs

**MS1**

- For each run $r$, for each peptide ion $s$ compute
  - **maximum MS1 precursor intensity** over all PSMs in run $r$ matching peptide ion $s$
    
    $$I_s^r = \max(I_x: \forall \text{PSMs } x \text{ in } r \text{ where } x \text{ matches } s)$$

  - **probability weighted MS1 intensity** over all PSMs in $r$ matching $s$,
    
    $$W_s^r = \frac{\sum_{\forall \text{PSMs } x \text{ in } r \text{ matching } s} p_p(x)p_i(x)I_x}{\sum_{\forall \text{PSMs } x \text{ in } r \text{ matching } s} p_p(x)p_i(x)}$$

  where:

  - $I_x$ is the precursor intensity of PSM $x$
  - $p_p(x)$ is PeptideProphet probability and $p_i(x)$ is iProphet probability, of PSM $x$
MS2
- For each run $r$, for each peptide ion $s$ compute
  - $\max MS1 \text{antic\_score}$ over all PSMs in run $r$ matching peptide ion $s$
    \[
    A_s^r = \max(A_x : \forall (\text{PSMs } x \text{ in } r \text{ where } x \text{ matches } s))
    \]
  - probability weighted $\text{antic\_score}$ over all PSMs in $r$ matching $s$,
    \[
    a_s^r = \frac{\sum \forall (\text{PSMs } x \text{ in } r \text{ matching } s) p_p(x)p_i(x)A_x}{\sum \forall (\text{PSMs } x \text{ in } r \text{ matching } s) p_p(x)p_i(x)}
    \]
where:
- $A_x$ is the $\text{antic\_score}$ of PSM $x$
- $p_p(x)$ is PeptideProphet probability and $p_i(x)$ is iProphet probability, of PSM $x$
DISCO Peptide Quantitation from PSMs

Hybrid

- For each run $r$, for each peptide ion $s$ compute
  - Compute Hybrid intensity

\[ H^r_s = \frac{1}{2}(I^r_s + a^r_s) \]

where:

- $I^r_s$ is the maximum precursor_intensity of peptide ion $s$ in run $r$
- $a^r_s$ is the weighted antic_score of peptide ion $s$ in run $r$
DISCO Peptide Quantitation Sample Means

- For each peptide ion $s$ over $n$ replicates $r_1, \ldots, r_n$ of classification $c$, compute sample means:

- **MS1**

$$\overline{W}_s^c = \frac{1}{n} \sum_{i=1}^{n} W_{s}^{r_i}$$

- **MS2**

$$\overline{a}_s^c = \frac{1}{n} \sum_{i=1}^{n} a_{s}^{r_i}$$

- **Hybrid**

$$\overline{H}_s^c = \frac{1}{n} \sum_{i=1}^{n} H_{s}^{r_i}$$
DISCO Halo Quantitation (not corrected for background)

QExactive HF

TTOF 6600
DISCO Halo Quantitation (corrected for background)

QExactive HF

TTOF 6600
Spectronaut Halo Quantitation (corrected for background)

QExactive HF

TTOF 6600
Conclusions

• Choice of data converter and centroiding options are important to ensure identifications are not lost due to the file converter

• DISCO identification rate improves upon DIAUmpire
  - Combining approaches maximizes data-driven identifications

• DISCO quantitation computes accurate ratios, reflecting the ground truth of the dataset
  - Hybrid quantitation reduces variance in ratios
  - Correcting ratios using the median background ratio is important for accurate ratio estimation
QuanTic

Applied to CID Cleavable Isobaric Tag Quantitation
A Collision-Induced Dissociation Cleavable Isobaric Tag for Peptide Fragment Ion-Based Quantification in Proteomics

Xiaobo Tian, Marcel P. de Vries, Hjalmar P. Permentier, and Rainer Bischoff

ABSTRACT: Quantifying peptides based on unique peptide fragment ions avoids the issue of ratio distortion that is commonly observed for reporter ion-based quantification approaches. Herein, we present a collision-induced dissociation-cleavable, isobaric acetyl-isoleucine-proline-glycine (Ac-IPG) tag, which conserves the merits of quantifying peptides based on unique fragments while reducing the complexity of the b-ion series compared to conventional fragment ion-based quantification methods thus facilitating data processing. Multiplex labeling is based on selective N-terminal dimethylation followed by derivatization of the ε-amino group of the C-terminal Lys residue of LysC peptides with isobaric Ac-IPG tags having complementary isotope distributions on Pro-Gly and Ac-Ile. Upon fragmentation between Ile and Pro, the resulting y ions, with the neutral loss of Ac-Ile, can be distinguished between the different labeling channels based on different numbers of isotope labels on the Pro-Gly part and thus contain the information for relative quantification, while b ions of different labeling channels have the same m/z values. The proteome quantification capability of this method was demonstrated by triplex labeling of a yeast proteome spiked with bovine serum albumin (BSA) over a 10-fold dynamic range. With the yeast proteins as the background, BSA was detected at ratios of 1.14:5.06:9.78 when spiked at 1:5:10 ratios. The raw mass data is available on the ProteomeXchange with the identifier PXD 018790.

KEYWORDS: isobaric labeling, tandem mass spectrometry, fragment ion, quantitative proteomics, stable isotope
Concept of the Ac-AG-tag

Fig. 1. Schematic view of the Ac-AG approach.

A) Isobaric labeling steps;

B) LC-MS/MS concept map for a mixture of triplex labeled samples;

C) Functional design of the Ac-AG-PNP tag (\(^{13}\)C isotope locations are marked with“**”).
Studying peptide fragmentation with various NCEs

NCE 18 for producing peptide-coupled reporter-ion
NCE 28 for formation of fragments of the peptide backbone
Calculating peptide ratios based on top 3 most intense PSMs.
Calculating protein ratios based on top 3 most intense peptides.

A) Log2-normalized ratio distribution at the PSM level at a mixing ratio of 1:1:1; B) Log2-normalized ratio distribution at the peptide level at a mixing ratio of 1:1:1; C) Log2-normalized ratio at the peptide level at a mixing ratio of 10:5:1; D) Log2-normalized ratio at the peptide level at a mixing ratio of 1:5:10. Expected values for log2-normalized mixing ratios are shown as dotted lines.
QuanTic: New Option for Quantitation

K:311.17:-155.1:-156.1:-157.1

<amino acids, n, or c>:<mass_shift>:<neut_loss1>:...:<neut_lossN> Specify modifications with neutral losses for quantitation (default: off)

*Quantify the intensity of neutral loss peaks associated with a modification for all fragments retaining the modification, when all are available for a given fragment ion, skip y1
QuanTic  Normalized Results 1:1:1
A Versatile Isobaric Tag Enables Proteome Quantification in Data-Dependent and Data-Independent Acquisition Modes

Xiaobo Tian, Marcel P. de Vries, Hjalmar P. Permentier, and Rainer Bischof

ABSTRACT: Quantifying proteins based on peptide-coupled reporter ions is a multiplexed quantitative strategy in proteomics that alleviates the problem of ratio distortion caused by peptide cofragmentation, as commonly observed in other reporter-ion-based approaches, such as TMT and iTRAQ. Data-independent acquisition (DIA) is an attractive alternative to data-dependent acquisition (DDA) due to its better reproducibility. While multiplexed labeling is widely used in DDA, it is rarely used in DIA, presumably because current approaches lead to more complex MS2 spectra, severe ratio distortion, or to a reduction in quantification accuracy and precision. Herein, we present a versatile acetyl-alanine-glycine (Ac-AG) tag that conceals quantitative information in isobarically labeled peptides and reveals it upon tandem MS in the form of peptide-coupled reporter ions. Since the peptide-coupled reporter ion is precursor-specific while fragment ions of the peptide backbone originating from different labeling channels are identical, the Ac-AG tag is compatible with both DDA and DIA. By isolating the monoisotopic peak of the precursor ion in DDA, intensities of the peptide-coupled reporter ions represent the relative ratios between constituent samples, whereas in DIA, the ratio can be inferred after deconvoluting the peptide-coupled reporter ion isotopes. The proteome quantification capability of the Ac-AG tag was demonstrated by triplex labeling of a yeast proteome spiked with bovine serum albumin (BSA) over a 10-fold dynamic range. Within this complex proteomics background, BSA spiked at 1:5:10 ratios was detected at ratios of 1.00:4.87:10.13 in DDA and 1.16:5.20:9.64 in DIA.
**COUPLED**

- Option that restricts the quantitation to using only the unfragmented precursor-coupled neutral losses for Quantic

**DIAMODE**

- Option that allows deconvolves quantitative values for multiple isotope, problem common to DIA data
• Combine Quantic PSM quantitation to compute protein level quantities using PSM stats weighted by intensity

• Fully integrated with ProteinProphet
  - Input and output are the same protXML file

• Requires no user input other than the input file

• Visualized by ProtXMLViewer.cgi
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<th>Main Enr Accession</th>
<th>Probability</th>
<th>NMT</th>
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Deconvolves overlapping Isotopes of DIA data to extract quantitative signals, uses Mike’s Mercury8 code to predict theoretical isotopic distributions of identified peptides.
Quantic allows the user to specify PTM molecular formulas for better isotopic distribution estimation with Mercury8.

"K[300]C7H12N2O3,M[147]C5H9N02S,n[29]N2H" vs No PTM Molecular Formula Adjustments
QuanTic: Protein Level DIAMODE
Acknowledgements

- Mukul Midha
- David Campbell
- Zhi Sun
- Mike Hoopmann
- Kristian Swearingen
- Luis Mendoza
- Eric Deutsch
- Rob Moritz

- Thank you for your attention!