Metabolomica e Lipidomica Untarget,
Untargeted Metabolomics Takes the Lead

**Thermo Scientific™ MS instruments included:** Thermo Scientific™ Q Exactive™ MS, Thermo Scientific™ Q Exactive™ Plus MS, Thermo Scientific™ Q Exactive™ HF MS, Thermo Scientific™ Orbitrap™ Fusion Tribrid MS, Thermo Scientific™ Orbitrap™ Fusion Tribrid MS, Thermo Scientific™ TSQ Quantiva™ Quadrupole MS
Untargeted Metabolomics: Challenges

- Structural and physical diversity
- Isomeric / isobaric species
- Dynamic range
- Large sample sets

- Leucine
- Isoleucine
High Quality Data for High Quality Results

High Resolution
- Complex matrix
- Differentiate similar masses
- Fine isotopic pattern

Mass Accuracy
- Identification of unknowns
- Narrow mass tolerance
- Mass stability from peak to peak and run to run

Instrument Performance
- Scan-to-scan consistency
- Injection-to-injection reproducibility
- Robustness over extended time periods
Stable Mass Accuracy from Scan to Scan Across the Peak

Orbitrap MS provides confidence in peak detection

creatinetheoretical m/z 132.0768
Excellent Mass Accuracy Across the Molecular Weight Range

Metabolites identified from human plasma and verified against an authentic standard

Orbitrap MS delivers accurate mass measurements for all metabolites
Orbitrap MS: Unmatched Resolution

Superior resolution ideal for small molecule analysis

High Orbitrap MS resolving power for correct identification of isomeric and isobaric species
Mass Resolution and Scan Speed

Vanquish UHPLC and Q Exactive HF MS
D8-Phenylalanine spiked in human plasma

- **R = 30k**
  - Scans/peak = 67
  - Scan speed = 14 Hz
  - ~4.8 s

- **R = 60k**
  - Scans/peak = 36
  - Scan speed = 7.5 Hz
  - ~4.8 s

- **R = 120k**
  - Scans/peak = 19
  - Scan speed = 4 Hz
  - ~4.8 s
Higher Confidence For Unknown Extractable & Leachable Analysis

**Experiment vs. Theory for M+2**

- Fine isotope structure data was compared with simulated spectrum for the predicted elemental compositions.
- Only one out of the 6 predicted elemental compositions for M0 also matched M+2 fine isotope structure with the data.
- The selected composition along with MS/MS fragment analysis allowed structure elucidation for this unknown extractable.

**Proposed Structure**

ThP 362, Comstock et al.
Selective Component Identification In Complex Matrices

**LC-MS Analysis Of Flavonoid Conjugates In Orange Juice**

- The fine isotope structure obtained was utilized as a fingerprint for selecting compounds having C/O ratio between 1.5 and 3.

- The detected peak lists were exported as an inclusion list for subsequent MS^n characterization.

- 128 putative flavonoid conjugates were detected in orange juice matrix containing over 4,000 compounds.

Orange Juice Matrix
Base Peak Chromatogram

Extracted Ion Chromatogram for Putative Flavonoid Conjugates

WP 457, T. Stratton
Why Orbitrap Is Naturally A Better Tool

Higher resolution can view more metabolic peaks

Q TOF: 6294 features
QE Plus: 8960 features \( \rightarrow \) >42% more

Data courtesy Stanford University
High Resolving Power Increases Metabolome Coverage

Human plasma metabolites (positive mode)

<table>
<thead>
<tr>
<th>Resolution</th>
<th>Compounds Detected</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>30K</td>
<td>1106</td>
<td>43% increase</td>
</tr>
<tr>
<td>60K</td>
<td>1584</td>
<td>9% increase</td>
</tr>
<tr>
<td>120K</td>
<td>1719</td>
<td></td>
</tr>
</tbody>
</table>

Human plasma metabolites (negative mode)

<table>
<thead>
<tr>
<th>Resolution</th>
<th>Compounds Detected</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>30K</td>
<td>548</td>
<td>46% increase</td>
</tr>
<tr>
<td>60K</td>
<td>799</td>
<td>13% increase</td>
</tr>
<tr>
<td>120K</td>
<td>900</td>
<td></td>
</tr>
</tbody>
</table>
Great reliability and linearity is a must for quantitation

Data courtesy Stanford University
Lipid Nomenclature: Glycerophospholipids

Hydrogen (PA)
Choline (PC)
Ethanolamine (PE)
Serine (PS)
Glycerol (PG)
Inositol (PI)

Phosphate
Head group

Ester bond

Ether bond

Molecular species composition:
PC 18:0/18:3

Sum composition:
PC 36:3

Molecular species composition:
PC 18:1\(p/18:1\)\(e\)

Sum composition:
PC 36:2\(e\)
Resolving Isobaric Species Improves ID and Quan

Bovine Heart Extract
500ng/µL x 2µL injected
30min LC-MS run
Q Exactive HF

LPE 18:1
C_{23}H_{47}NO_{7}P
R = 83K

LPC 16:1e
C_{24}H_{51}NO_{8}P
R = 86K

Lyso PE 18:1

LPC 16:1e/16:0p
Confident Identification of Low Abundant PS Species

MS

PS (40:4)

M+H

MS-MS of m/z 840.5740

PS (18:0_22:4)
LipidSearch Batch Data Processing and Quantitation

**Processing Steps:**

1. Search each data file
2. Merge the search results
   - Pos. and Neg. ion
3. Report includes ID’s
   - a) Estimated Quan (IS) or
   - b) Rel. Amounts (no IS)
Triacylglycerol Lipids in Control Non-Diabetic Human Serum – Orbitrap Fusion MS

Data courtesy of Prof. Gavin Reid, Michigan State University
Resolving Isobaric TAGs with Ultra-high Resolution

Orbitrap Fusion MS

Data courtesy of Prof. Gavin Reid, Michigan State University
Accurate ID and Quan Made Possible by Ultra-high Resolution

Which Species—PC/PE (+1.8ppm) or PI (-4.6ppm)?

Orbitrap Fusion MS

Data courtesy of Prof. Gavin Reid, Michigan State University
Unique Features of Orbitrap Fusion Lumos MS – A Tribrid Orbitrap Mass Spectrometer

- **HCD MS/MS CID MS^n**: LIPID IDENTIFICATION REQUIRES MORE THAN ONE TYPE OF DISSOCIATION TECHNIQUE
- **UVPD**: ION TRAP MS^n EXPERIMENTS PROVIDE MORE DEFINITIVE STRUCTURAL INFORMATION
- **1M**: ULTRA-HIGH RESOLUTION FOR FINE ISOTOPE STRUCTURE
- **Ultra-High Field Orbitrap Analyzer**: OPTIONAL ON NEW AND EXISTING SYSTEMS
- **Advanced Quadrupole Technology**: UNIQUENESS FOR LIPOIDOMICS CHARACTERIZATION
- **Dual-Pressure Linear Ion Trap**: OPTIONAL ON NEW AND EXISTING SYSTEMS

**New**
UVPD Implementation (Class 1 Laser System)

Compact Footprint

- UVPD source is embedded inside the instrument, directly connected to the dual-pressure linear ion trap
- UVPD source employs a 213 nm laser with 2.5 kHz repetition rate delivering >1.2 μJ/pulse
- UVPD is a field upgradable option

UVPD Source
The UVPD MS^n fragments are generated in the linear ion trap and can be detected by either the ion trap or Orbitrap
UVPD For Comprehensive Lipid Characterization

Locating Double Bonds

- HRAM UVPD MS$^2$ spectrum of [M+Li]$^+$ precursor ions of TG 16:0/16:0/18:1
- Fragments identify acyl chains
- UVPD unique fragments identify location of double bonds within the acyl chains
Questions?
Proteomics Analysis with Orbitrap

Leopoldo Dimiziani
Thermo Fisher Scientific
Verona 12/12/2017
Omics Studies – the Link between Genotype and Phenotype

environment

- genome
- transcriptome
- proteome
- metabolome

phenotype
Proteomics is Not Genomics

- Proteomics is not genomics where a single, whole genome sequencing experiment provides a relatively accurate picture of the genomic aspects of biology.
- Proteomics is (was?) complicated, expensive and time-consuming, we have been forced to limit the number of samples we process and restrict ourselves to a static view of biology.
- We have been looking at *snapshots*, when we really want to see *dynamics* as biology changes over time, across many samples.
- We want to see the important differences between closely related biological states, such as the stages of cellular development and differentiation or the cellular response to therapeutic intervention at a protein, PTM, or even proteoform level. And we want to quantify them.
What Are Biologists Researching?

- Molecular changes in the life cycle of plants and animals
- Comparison of normal and diseased tissues in animal and plants
- Study of molecular changes associated with particular genotypes
- Understanding the impact of the environment on species separated by, or located in, different geographies
- Understanding the evolution of species

All of these require the ability to measure multiple conditions or in other words measure over a time
What Are Biologists Researching?

Taking molecular snapshots does not provide the answers
What Are Biologists Researching?

Requires 100 quantitative protein comparisons (10 development stages x 2 mutants x 5 biological replicates/flies)

Observing 3500 proteins quantitatively using label-free technique requires: 100 injections with 180 min gradients = 13 days instrument time

Results: 350,000 protein detection events with 80% reproducibility between runs results in an overlap of <<2000 proteins that are reproducibly observed
High Quality Data for High Quality Results

**High Resolution**
- Complex matrix
- Differentiate similar masses
- Isobaric species
- Fine isotopic pattern

**Mass Accuracy**
- Identification of unknowns
- Narrow mass tolerance
- Mass stability from peak to peak and run to run

**Instrument Performance**
- Scan-to-scan consistency
- Injection-to-injection reproducibility
- Robustness over extended time periods
The Industry’s Leading Portfolio of MS Solutions

Transform Your Science

Quantitative
- Metabolomics
- Proteomics
- Bioanalysis

Applied Markets
- Food Safety
- Environmental
- Clinical Research/
  Forensic Toxicology

Non-targeted Analysis

HRAM

Q-Exactive MS

Orbitrap MS

Research Markets
- Biomarker Discovery
- Proteomics
- Metabolism

Targeted Analysis

MS, MSn

Triple Quads

Ion Traps

Qualitative
- Metabolomics
- PTM Analysis
- Lipidomics
Thermo Scientific™ Q Exactive™ Series Portfolio for Proteomics

**Thermo Scientific™ Q Exactive™ MS**
- Orbitrap analyzer
- Mass Range m/z 50 - 6000
- Mass Accuracy <1ppm
- Max. Mass Resolution >140,000
- Scan speed up to 12Hz
- Spectral Multiplexing
- Polarity switching <1 sec

**Thermo Scientific™ Q Exactive™ Plus MS**
- Orbitrap analyzer
- Mass Range m/z 50 - 6000
- Mass Accuracy <1ppm
- Max. Mass Resolution >140,000
- Scan speed up to 12Hz
- Spectral Multiplexing
- Polarity switching <1 sec
- Advanced Quadrupole Technology (AQT)
- Advanced Active Beam Guide (AABG)
- Opt. Enh Res.Mode (280k)

**Thermo Scientific™ Q Exactive™ HF MS**
- Ultra High Field Orbitrap analyzer
- Mass Range m/z 50 - 6000
- Mass Accuracy <1ppm
- Max. Mass Resolution >240,000
- Scan speed up to 18Hz
- Advanced Quadrupole Technology (AQT)
- Advanced Active Beam Guide (AABG)
- Spectral Multiplexing
- Polarity switching <1 sec

**Thermo Scientific™ Q Exactive™ HF-X MS**
- Ultra High Field Orbitrap analyzer
- Mass Range m/z 50 - 6000
- Mass Accuracy <1ppm
- Max. Mass Resolution >240,000
- Scan speed up to 40Hz
- High capacity transfer tube
- Electrodynamic ion funnel
- Advanced Quadrupole Technology (AQT)
- Advanced Active Beam Guide (AABG)
- Spectral Multiplexing
- Polarity switching <1 sec
- Advanced ddHCD algorithm
Q Exactive Plus/HF Mass Spectrometer

- HCD Cell
- C-Trap
- HyperQuad Mass Filter with Advanced Quadrupole Technology (AQT)
- S-Lens
- Advanced Active Beam Guide (AABG)
- Ultra-High-Field Orbitrap Mass Analyzer
Key Technologies of Q Exactive HF MS

**Q Exactive HF MS**

**Key Technologies**

- **Ultra-High-Field Orbitrap**
  - Up to 18 Hz and standard 240k resolution
- **Smaller Size**
  - 1.8x frequency at the same voltage
  - 1.8x higher resolution over standard Orbitrap
  - New lenses for focusing ions in to the Orbitrap entrance
- **32 msec transient for fastest MS/MS data and max scan speed**
Standard Orbitrap Analyzer & Ultra-High-Field Orbitrap Analyzer- Real Size Cutaways

**STANDARD Orbitrap™ Analyzer**
- Thermo Scientific™ LTQ Orbitrap Classic/XL/ Discovery /Velos (Pro) MS
- Thermo Scientific™ (Q)Exactive Plus™ MS
- Thermo Scientific™ Exactive Plus EMR MS

**HIGH FIELD Orbitrap Analyzer**
- Thermo Scientific™ Orbitrap Elite™ MS

**ULTRA-HIGH-FIELD Orbitrap Analyzer**
- Thermo Scientific™ Orbitrap Fusion™/Lumos™ MS
- Thermo Scientific™ Q Exactive HF™/HF-X™ MS
## Q Exactive Plus MS: Specifications

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan rate</td>
<td>12 Hz</td>
</tr>
<tr>
<td>Max resolution</td>
<td>140K at m/z 200 (280k optional)</td>
</tr>
<tr>
<td>Quad isolation</td>
<td>Step-less from full mass range down to 0.4 amu</td>
</tr>
<tr>
<td>Mass Accuracy</td>
<td>3 ppm external, 1 ppm internal</td>
</tr>
<tr>
<td>Dissociation</td>
<td>Source CID, HCD</td>
</tr>
<tr>
<td>Multiplexing</td>
<td>Up to 10 precursor ions</td>
</tr>
<tr>
<td>Detectors</td>
<td>Orbitrap</td>
</tr>
<tr>
<td>Polarity Switching</td>
<td>1 sec cycle time (@ RES 35k)</td>
</tr>
<tr>
<td>Scan Functions</td>
<td>FS: Full Scan, AIF: All Ion Fragmentation, SIM: Selected Ion Monitoring, PRM: Parallel Reactin Monitoring, DIA: Data Independent Acquisition, ddHCD: data dependent HCD</td>
</tr>
<tr>
<td>Options</td>
<td>Intact Protein Mode</td>
</tr>
<tr>
<td></td>
<td>Enhanced resolution</td>
</tr>
</tbody>
</table>
Q Exative HF MS: Specification

### Unmatched Analytical Performance

- **240,000 Resolution** for best selectivity
- **18 Hz** for maximum MS/MS scan speed
- **Intact Protein Mode** for best S/N of intact proteins

<table>
<thead>
<tr>
<th>Mass Range</th>
<th>50 &lt; m/z &lt; 6,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution @ m/z 200</td>
<td>15,000 at 18Hz</td>
</tr>
<tr>
<td></td>
<td>30,000 at 12 Hz</td>
</tr>
<tr>
<td></td>
<td>60,000 at 7 Hz</td>
</tr>
<tr>
<td></td>
<td>120,000 at 3 Hz</td>
</tr>
<tr>
<td></td>
<td>240,000 at 1.5 Hz</td>
</tr>
<tr>
<td>Mass Accuracy</td>
<td>&lt; 1ppm RMS, Internal Calibration</td>
</tr>
<tr>
<td></td>
<td>&lt; 3ppm RMS, External Calibration</td>
</tr>
<tr>
<td>Polarity Switching</td>
<td>one full cycle in &lt;1 sec (one full positive mode scan and one full negative mode scan at a resolution setting of 60,000)</td>
</tr>
</tbody>
</table>
Comparison of Q Exactive Plus MS and Q Exactive HF MS

**HeLa – 1 µg sample load**

### Unique Peptides

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Q Exactive Plus MS</th>
<th>Q Exactive HF MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>8604</td>
<td>12872</td>
</tr>
<tr>
<td>60</td>
<td>16455</td>
<td>21103</td>
</tr>
<tr>
<td>120</td>
<td>22707</td>
<td>25054</td>
</tr>
</tbody>
</table>

- **Average Number of Unique Peptides**
  - Q Exactive Plus MS: 28.24%
  - Q Exactive HF MS: 10.33%

### Protein Groups

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Q Exactive Plus MS</th>
<th>Q Exactive HF MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1991</td>
<td>2827</td>
</tr>
<tr>
<td>60</td>
<td>3413</td>
<td>4105</td>
</tr>
<tr>
<td>120</td>
<td>4427</td>
<td>4621</td>
</tr>
</tbody>
</table>

- **Average Number of Protein Groups**
  - Q Exactive Plus MS: 41.96%
  - Q Exactive HF MS: 20.27%

**More IDs with shorter Gradients**

*ASMS 2014 Poster M186 Tabewang Arrey*
Same Identifications with **Half the Time**

![Graph showing protein groups and unique peptides for Q Exactive Plus and Q Exactive HF with 120 min and 60 min gradients.]

**Unique Peptides**
- Q Exactive Plus: 22707 (120 min gradient)
- Q Exactive HF: 21103 (60 min gradient)

**Protein Groups**
- Q Exactive Plus: 4427 (120 min gradient)
- Q Exactive HF: 4105 (60 min gradient)
More Identifications with Less Sample

What if I am sample limited?

Q Exactive HF™
60 min gradient

<table>
<thead>
<tr>
<th>Sample Size (ng)</th>
<th>Average Number of Unique Peptides</th>
<th>Average Number of Protein Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>464</td>
<td>172</td>
</tr>
<tr>
<td>1</td>
<td>1069</td>
<td>335</td>
</tr>
<tr>
<td>10</td>
<td>5322</td>
<td>1078</td>
</tr>
<tr>
<td>100</td>
<td>14343</td>
<td>2439</td>
</tr>
<tr>
<td>500</td>
<td>19702</td>
<td>2439</td>
</tr>
<tr>
<td>1000</td>
<td>22833</td>
<td>3438</td>
</tr>
<tr>
<td>2000</td>
<td>27705</td>
<td>4087</td>
</tr>
</tbody>
</table>

Peptides

Protein
Q Exactive HF-X – new architecture

- Optimized Scan Matrix with accelerated HCD 40 Hz MS/MS
- Advanced DDA for bottom-up and top-down
- HyperQuad Mass Filter with Advanced Quadrupole Technology (AQT)
- C-Trap
- HCD Cell
- Dedicated transient for TMT 10plex
- Improved S/N for Native mAB
- Ultra-High Field Orbitrap Mass Analyzer
- Advanced Active Beam Guide (AABG)
- Electrodynaic Ion Funnel
- High Capacity Transfer Tube (HCTT)
- Brighter ion beam
- More sensitive

ThermoFisher Scientific
Improvement of sensitivity – direct infusion

Electrometer current

Injection times

Ion intensities

ASMS’17: TP 389, T.N. Arrey et al. New innovations implemented on the Q Exactive HF mass spectrometer.
Sensitivity and Linear Dynamic Range in Quantitation

• tSIM, Resolution 60K, maxIT 119ms, Iso 8 amu
• 3 replicates
• Sample: Alprazolam spiked into crashed plasma
• LOQ = 10 fg/µL = 10 ppt
• Range = 10 - 200,000 pg/mL

>10^4 linear dynamic range - LOQ at low ppt level

ASMS’17: TP 389, T.N. Arrey et al. New innovations implemented on the Q Exactive HF mass spectrometer.
Analysis of Intact Trastuzumab under Native Conditions in HMR Mode

Improved S/N ratio on the Q Exactive HF-X by a factor of ~5-10.

SEC-LC/MS analysis of intact Trastuzumab monoclonal antibody using Acclaim SEC column, 4.6 x 300 mm, 300 µl/min flow rate, 50 mM ammonium acetate. Full MS, HMR mode, m/z 2500–8000, resolution setting 30k, 10 µscans. Spectra show an average of 3 scans (10 µscans each).
Brighter ion beam, reduced scan overhead, and accelerated HCD (aHCD) is boosting acquisition speed.

Advantage for both MS and MS/MS mode.

Fast and high quality MS/MS acquisition up to 40 Hz with new 16 msec transient (7,500 resolution setting).

Q Exactive HF

- 18-22 Hz
- 32 ms
- Longer fill time
- Reduced scan overhead

Q Exactive HF-X

- 28 Hz
- 32 ms
- Similar fill time
- Reduced scan overhead

MS/MS

- 32 ms
- MaxIT

Comparative fill time
Reduced scan overhead
aHDC

ThermoFisher Scientific
Ultra Fast MS/MS Scan Speed > 40 Hz

Duty cycle: 1.05 s
Scan rate (1 FS + 40 MS2): 38.1 Hz
40 MS2: 45.1 Hz

Duty cycle: 1.03 s
Scan rate (1 FS + 40 MS2): 38.8 Hz
40 MS2: 45.8 Hz

Duty cycle: 1.02 s
Scan rate (1 FS + 40 MS2): 39.2 Hz
40 MS2: 46.1 Hz

Duty cycle: 1.09 s
Scan rate (1 FS + 40 MS2): 36.6 Hz
40 MS2: 42.8 Hz

1 full scan (60,000 @ m/z 200) and 40 MS² scans 7,500@ m/z 200) at LC time scale in 1 second. 30 min gradient, MS2 max IT: 11 ms

ASMS’17: TP 389, T.N. Arrey et al. New innovations implemented on the Q Exactive HF mass spectrometer.
Protein identification faster than ever

- Maximizing protein identifications
- Same protein identifications in half the analysis time
- Faster, with same high quality results

Sample: 1 ug Pierce HeLa digest

ASMS’17: TP 389, T.N. Arrey et al. New innovations implemented on the Q Exactive HF mass spectrometer.
From Discovery to Quantification - do it all with a Q Exactive

**Qual-Quan**

- **Discovery**
  - Precursor accurate mass
  - MS/MS spectrum

- **HR/AM Targeted Quan**
  - Accurate Mass (AM)
  - Retention Time (RT)
  - Isotope Pattern
  - MS/MS spectrum
  - MS/MS Transitions

- **Target Confirmation**
  - Precursor accurate mass
  - MS/MS spectrum

- **Target Quantification**
  - LC Peak Area
  - Precursor accurate mass
  - MS/MS spectrum

- **Discovery Quan**
  - Precursor accurate mass
  - MS/MS spectrum

- **All Targeted Quan**
  - TMT
  - Label free
  - SILAC

- **Protein / peptide ID**

**ThermoFisher Scientific**
The Power of Q Exactive to Access the Low-abundant Proteins

*Yeast cellular protein copy numbers are from Weissman and co-workers, Nature, 2003, 16, 737-41.

*Yeast cellular protein copy numbers are from Weissman and co-workers, Nature, 2003, 16, 737-41.
Low-abundant Protein Identified from Low Sample Load

High confidence identification from 10 ng of Yeast Digest

Peptide of YOR020C, \textbf{149} copy number, identified from \textbf{10 ng} yeast digest
Larger dynamic range leads to deeper sequencing

Q Exactive raises the challenge in discovery proteomics to the next level – identifying the proteins that matter.

Precursor intensity of identified peptides (normalized to the most intense peak)
From Discovery to Quantification - do it all with a Q Exactive

**Qual-Quan**

**Discovery**
- Precursor accurate mass
- MS/MS spectrum
- Protein / peptide ID

**Target Confirmation**
- Accurate Mass (AM)
- Retention Time (RT)
- Isotope Pattern
- MS/MS spectrum
- MS/MS Transitions

**Target Quantification**
- LC Peak Area
  - Precursor
  - MS/MS Transitions

**Discovery Quan**
- Precursor accurate mass
- MS/MS spectrum
- TMT
- Label free
- SILAC

**HR/AM Targeted Quan**

**All Targeted Quan**
Problem: Quantitative information about expression level of a protein is essential to understanding its biological role in response to change or disease.

Alterations in expression can reveal a meaningful biological pattern not apparent in a pure identification experiment, which provides only a list of detected proteins.

Add another dimension to any experiment by determining the relative abundance of each identified protein.
Several well established pipelines for the quantitation of label-free data from a data dependent (or DDA informed DIA experiment) exist. Among these:

**SIEVE 2.2**

- Multiple LC/MS Runs
- Compare a few conditions
- Requires replicate sample material
**Problem:** Requires multiple LC/MS analyses and is thus sample intensive

A differential analysis of 2 biological conditions with 3 technical replicates each would require **six** LC/MS injections and analyses:

**Problem:** Substantial instrument time to compare only a few conditions simultaneously

Comparing just two conditions with a two hour gradient would take more than 14 hours of instrument time

**Problem:** Irreproducibility due to less than 100% sample overlap

Even with 85% overlap run to run **AND** 4000 proteins identified in each run

...**less than 2500 common proteins**
Improving Quantitation Throughput: SILAC

SILAC Workflow

- Multiple LC/MS Runs
- Compare several conditions
- Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)
- Low variation between samples
- Requires Hi-Res Mass Spectrometry
- Compare up to 3 conditions
- Applicable to cell culture
- Peptide ID not required

SILAC MS1 Quantitation

Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

- Low variation between samples
- Requires Hi-Res Mass Spectrometry
- Compare up to 3 conditions
- Applicable to cell culture
- Peptide ID not required

Problem: Increases MS1 Spectral Complexity

High resolution and intelligent precursor selection (i.e. selection of only one SILAC labeled peptide per pair or triad) is required for best quantitative results.

Problem: Requires cell labeling in culture

Proteins must be able to be metabolically labelled and thus is not suitable for all organisms/conditions.

With SILAC began a trend towards increased multiplexing…
A Better Multiplexing Method—Isobaric Mass Tagging

- Less MS1 Complexity
- Increased Throughput
  - Concurrent MS analysis of multiple samples
  - Less consumed samples and less instrument time
- Fewer Missing Values
  - Identification and quantification achieved in a single run
  - No worries about irreproducibility
- Sample Origin Flexibility
  - Samples can be derived from cells, tissues or biological fluids
- Increased Multiplexing
  - Compare more than 3 conditions
- Multiple Comparisons and Improved Statistics
  - Incorporate replicates with multiple conditions: dose-response, time-course, multiple tissues, subcellular fractions, etc
Thermo Scientific Tandem Mass Tag (TMT) Isobaric Tag Family

TMT<sup>0</sup>
Method Development & SRM

- 13C and 15N labeled reporter
- Isotopes balanced between linker region and reporter region keeping all tags exactly isobaric
- Fragments by ETD or HCD
- Up to 10 different tags
- Other reactive tags: Iodo TMT and Aminoxy TMT
The Multiplexing Revolution – Not Only Consumables

- **SILAC**
  Compare 3 Conditions

- **TMT6plex**
  Compare 6 Conditions in MS² with amine reactive tags

- **iTRAQ8plex**
  Label and compare 8 Conditions

- **TMT8 and TMT10**
  Concurrently quantify up to 10 sample conditions

- **Orbitrap Classic**
  High Resolution Orbitrap Mass Analyzer

- **Orbitrap Velos**
  New Axial Field HCD Cell for Improved MS²

- **Orbitrap Elite**
  Hybrid; Single Notch MS²; P1

- **Orbitrap Fusion**
  Tribrid, Parallelized Analysis, Multinotch

- **Orbitrap Fusion Lumos**
  Newest Tribrid, highest sensitivity and selectivity

**ThermoFisher Scientific**
Result: Get accurate quantitation using the high resolution of Orbitrap Mass Analyzer

High Resolving Power is Essential for Accurate Quantification of the TMT10PLEX Reagents
A Real Example

**Sample:** Mouse mitochondrial extract untreated or treated with phosphatase inhibitor

**Orbitrap Elite**
- 75 um x 50 cm PepMap C18
- 210 min gradient: 250 min run
- 1 ug of sample on column

**Quantified**
- 1423 protein groups
  - in 1.04 days
  - using 6 ug material

**Quantified**
- 1310 protein groups
  - in 4.16 hours
  - using 1 ug material

Thermo Poster Note: Liver Mitochondria Proteomics Employing High-Resolution MS Technology; J.Ho. et al
**Problem:** Quantitation of low-abundance proteins in a complex background is distorted by co-isolated interfering precursor ions

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**Figure:**

- **Graph 1:**
  - Title: Isolation window
  - X-axis: m/z
  - Y-axis: Relative intensity
  - Data points:
    - 710, 710.5, 711, 711.5, 712, 712.5
  - Two ions: Contaminating ion (red) and Target ion (blue)

- **Graph 2:**
  - Title: TMT reporter ions
  - X-axis: reporter ion number
  - Y-axis: Intensity
  - Data points with interference:
    - 126, 127, 128, 129, 130, 131
    - Interference indicated

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**References:**

- Ow, S.Y. et al. 2009. JPR 5347-5355
TMT10plex and SPS MS³ for Quantitative Proteomics

Achieving accurate and precise quantitation using SPS MS³
Co-isolation of Interfering Ions Affects Accuracy

Results: Best possible accuracy and precision by reducing co-isolated interfering ions.

- 500 fmol of BSA in 500 ng HeLa digest
- Narrow precursor isolation with 4 hour gradient

Expected vs. Observed

- Constant background
- Less precision
- Less accuracy (ratio distortion)

Co-isolation of Interfering Ions Affects Accuracy

<table>
<thead>
<tr>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMT8 HeLa, 500 ng</td>
<td></td>
</tr>
<tr>
<td>BSA alone</td>
<td></td>
</tr>
<tr>
<td>BSA+ HeLa SPS MS3</td>
<td></td>
</tr>
<tr>
<td>BSA+ HeLa MS2 (1.2 amu isolation)</td>
<td></td>
</tr>
</tbody>
</table>

126 127N 127C 128N 128C 129N 129C 130N 130C 131

BSA alone

BSA+ HeLa SPS MS3

BSA+ HeLa MS2 (1.2 amu isolation)
Orbitrap Fusion Tribrid Mass Spectrometer

Unmatched Analytical Performance

Revolutionary Performance

Exceptional Versatility

Unprecedented Usability
Orbitrap Fusion Tribrid Mass Spectrometer

- Reduced noise and increased robustness
  - Active beam guide prevents neutrals from entering the quadrupole and improves robustness.

- Unsurpassed resolution and speed
  - Ultra-high-field Orbitrap mass analyzer offers resolution exceeding 500,000 and scan speeds up to 18 Hz.

- Maximum throughput by massive parallelization
  - Ion-routing multipole facilitates parallel analysis and performs HCD and EThcD at any fragmentation stage.

- Excellent sensitivity and selectivity
  - Quadrupole precursor selection at isolation widths down to 0.4 amu improves sensitivity and selectivity.

- MSn and sensitive base analysis
  - Dual-pressure linear ion trap provides MSn CID and ETD fragmentation and fast, sensitive mass analysis.

- Compact ETD source
  - Uses Townsend discharge, making it more reliable and easier to use.
Orbitrap Fusion Tribrid Mass Spectrometer

Unmatched Analytical Performance

500,000 Resolution to remove spectral interferences

CID/HCD/ETD/EThcD detected by the Ion Trap or Orbitrap analyzer at any level of MS^n for maximum experimental flexibility

Powered by the new Dynamic Scan Management architecture that ensures efficient operation of the mass spectrometer

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan rate OTMS^2</td>
<td>18 Hz</td>
</tr>
<tr>
<td>Scan rate ITMS^2</td>
<td>20 Hz</td>
</tr>
<tr>
<td>Max resolution</td>
<td>500,000 at m/z 195</td>
</tr>
<tr>
<td>Quad isolation</td>
<td>down to 0.4 amu</td>
</tr>
<tr>
<td>Ion trap isolation</td>
<td>down to 0.2 amu</td>
</tr>
<tr>
<td>Mass Accuracy</td>
<td>3 ppm ext, 1 ppm int</td>
</tr>
<tr>
<td>Dissociation</td>
<td>CID, HCD, ETD, EThcD</td>
</tr>
<tr>
<td>MS^n</td>
<td>Up to MS^{10} in ion trap or Orbitrap analyzer</td>
</tr>
<tr>
<td>Analyzers</td>
<td>Q, OTMS, ITMS</td>
</tr>
<tr>
<td>Detectors</td>
<td>Ion Trap, Orbitrap</td>
</tr>
<tr>
<td>Compact</td>
<td>1186 x 674 x 650 mm (w, d, h)</td>
</tr>
</tbody>
</table>
Orbitrap Fusion Lumos Tribrid Mass Spectrometer

Unmatched Analytical Performance

- Revolutionary performance
- Exceptional versatility
- Unprecedented usability
- Highest sensitivity

Tribrid: three Mass Analyzers working together to produce unmatched analytical results

2015
Orbitrap Fusion Lumos Tribrid Mass Spectrometer

- **Advanced Quadrupole Technology**: Segmented design improves transmission at higher resolution; symmetric transmission across the window.
- **Electrodynamic Ion Funnel**: Focuses ions after FT; broad tuning curves.
- **Advanced Active Ion Beam Guide**: Prevents neutrals and high velocity clusters from entering mass resolving quadrupole.
- **High Capacity Transfer Tube**: Increases ion flux into the mass spectrometer.
- **Ultra-High Field Orbitrap Analyzer**: Offers resolution >500K FWH and scan rates up to 20Hz at 15K FWHM.
- **ETD HD**: Improved dynamic range and detection limit of ETD event.
- **Dual-Pressure Linear Ion Trap**: MS^n and sensitive mass analysis of four fragmentation types: CID, HCD, ETD and EThcD.
- **Ion Routing Multipole**: Enables parallel analysis; performs HCD at any MS^n stage.
- **Advanced Vacuum Technology**: Reduces pressure in UHV region, improving transmission to the Orbitrap analyzer.
Human HTC116 cells were treated with a proteasome inhibitor (Bortezomib) for 16 h and analyzed with TMT 10-plex (5 treated vs. 5 untreated).

Two fractions were prepared:
- With higher amount
- With lower amount

25-73% more quantifiable peptides

ASMS Lecture: Rose et al. Isobaric labeling enables 10-Plex quantitative analysis of ubiquitylated peptides: A diagnostic ion to improve identification and quantification.
Results: Best possible accuracy by reducing co-isolated interferences.

1μg mixture, 4 hr gradient, median ratios
TMT SPS MS³ Publications Have Very High Impact

Impact Factor

Cancer Discovery 19.463
Cerebral Cortex 8.668
Analytical Chemistry 5.636
Plos One 3.234
Cell 5.011
Molecular & Cellular Proteomics 6.564
Journal of Proteome Research 3.780
Am. J. Physiol. - Cell Physiology 6.564
Molecular & Cellular Proteomics 10.402
Leukemia 10.402
Science 33.611
Blood 33.611
Nature 41.459
Nature 41.459
Cell Host & Microbe 12.609
Molecular Cell 14.013
Plos One 3.234
Science Signaling 6.279
Current Biology 9.571
Nature Communications 11.470
Nature Communications 11.470
Nature Communications 11.470
Nature Communications 11.470
Molecular & Cellular Proteomics 6.564
Molecular Cancer Therapeutics 5.693
Genes & Development 12.640
Nature Communications 11.470
Nature Communications 11.470
Nature Communications 11.470
Nature Communications 11.470
Molecular Cell 14.013

Average impact factor
>500,000 Resolution on Orbitrap Fusion MS

**Timolol**: glaucoma drug, beta blocker 0.3 ppm

**Cocaethyline**: Ethanol + Cocaine 1.6 ppm

This resolution is an order of magnitude better than any competitive instrument.
Dynamic Scan Management Ensures Efficiency

Data Dependent Experiment: OTMS > CID ITMS$^2$

- Full Orbitrap MS scan
- 1$^{st}$ parent ion isolation in Q1
- 1$^{st}$ MS$^2$ scan: CID in the Ion Trap
- 2$^{nd}$ parent ion isolation in Q1
- 2$^{nd}$ MS$^2$ scan: CID in the Ion Trap
- Full Orbitrap MS scan
Dynamic Scan Management Ensures Efficiency

Data Dependent Experiment: OTMS > HCD ITMS²

- Full Orbitrap MS scan
- 1st parent ion isolation in Q1 and HCD
- 1st MS² scan: Ion Trap Detection
- 2nd parent ion isolation in Q1 and HCD
- 2nd MS² scan: Ion Trap Detection
- Full Orbitrap MS scan
Ion Trafficking and Dynamic Scan Management

Full Scan

Parent Ion Q1 isolation

Ion Injection

Full OTMS Scan

Ion Trap Detection of the Fragments
Speed = Many More Points Across LC Peak

1 ug HeLa, 140 min run

Thermo Scientific™ Orbitrap Elite™ MS

Orbitrap Fusion MS

Top Speed

Scans in 40s

MS

MS/MS

16

245

76
Protein Groups

1 ug HeLa

Orbitrap Fusion MS
Outperforms Orbitrap Elite MS in Half the Time!

- Orbitalp Elite MS
- Orbitalp Fusion MS

<table>
<thead>
<tr>
<th>Time</th>
<th>Orbitrap Elite MS</th>
<th>Orbitrap Fusion MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>140 Min</td>
<td>3604</td>
<td>4996</td>
</tr>
<tr>
<td>70 Min</td>
<td>2861</td>
<td>3939</td>
</tr>
</tbody>
</table>
Principle of ETD

Multiply charged analyte \((n \geq 2)\)

Fluoranthene radical anion

\(+ n+ \quad + \quad - \quad \xrightarrow{\text{Electron-transfer}} \quad (n-1)+\)

Odd-electron protonated peptide

Cleavage of N-C\(\alpha\) bond
ETD versus CID

**ETD**
- electron transfer surpasses internal heating
- rapid bond cleavage (no energy dissipation)
- random fragmentation of peptide backbone
- leaves labile bonds like from PTMs intact
- N-Cα bond cleavage yields c- and z-ion
- preferable charge state $z > 2$

**Conventional (resonant) CID**
- via several collisions with Helium precursor ion is internally heated
- preferences for weak bond cleavages
- nearby selected amino acids (E, D, P) backbone cleavage is preferred
- b- and y-ions (and internal fragments)
- best fragment spectra from 2+ ions
Tube lens is replaced with discharge ion source
Discharge Ion Source Detail

- Reagent Entry Aperture
- Anion Exit Aperture
- Electron Entry Aperture
- Ion Axis
New Front Reagent Source: ETD and Internal Calibration

- Compact
- Townsend Discharge Ionization, No Filament
- Stable Reagent Ion Flux
**Data Dependent Experiment: OTMS > ETD ITMS**

- Full Orbitrap MS scan
- 1st parent ion isolation in Q1
- 1st parent trapped in the ion trap
- Injection of ETD reagent
- 1st parent: ETD dissociation
- Full Orbitrap MS scan
Advanced PTM Analysis

Challenges Associated with Glycopeptide Analysis

- More than one glycan attached at a single site. Can be up to 100 glycans.
- Difficult to detect by MS in the presence of non-glycopeptides

Huge glycopeptide impact:
Four publications, including PNAS, JBC, and Anal. Chem.

Glycosylation profile and site occupancy cannot be predicted!
Modes of MS Operation for Glycoproteomics

**Unmatched Analytical Performance**

- **Glycan/Glycopeptide Sequencing**
  - FT ITMS\(^n\) (HCD)

- **Glycopeptide Detection/Sequencing**
  - FT FTMS\(^2\) (HCD) pd-CID/ETD/HCD

- **Isobaric Glycopeptide Quantification**
  - FT ITMS\(^2\) (ETD) SPS MS\(^3\) HCD

- **Glycopeptide Sequencing Using Y1 Ion**
  - FT FTMS\(^2\) (HCD) ITMS\(^3\) CID
Ultimate in Flexibility: HCDpd “Any MS2”

HCD-pd-(CID+ETD)

HCD for selective trigger
ETD for peptide sequencing
CID for glycan sequencing

Wu et al., Anal. Chem., Just Accepted
A novel LC-MS product dependent parallel data acquisition function and data analysis workflow for sequencing and identification of intact glycopeptides

Sz-Wei Wu, Tsung-Hsien Pu, Rosa Viner, and Kay-Hoi Khoo

Anal. Chem., Just Accepted Manuscript • Publication Date (Web): 05 May 2014
Improving ETD-SPS Quantification of Glycopeptides

Precursor Ion

Synchronous Precursor Selection

HCD MS\(^3\), Orbitrap Analyzer

ThermoFisher Scientific
Synchronous Precursor Selection

**Precursor Ion**

**Synchronous Precursor Selection**

**HCD MS³, Orbitrap Analyzer**

**ITMS² ETD SPS FTMS³ Quantified TMT6 Serum Glycopeptides**

- Total
- Glycopeptides
- Quantified Glycopeptides

Unique to Orbitrap Fusion MS
Internal Calibration: LC/MS of Omeprazole Metabolites
Internal Calibration of MS and MS\(^2\) scan

- Injection for full Orbitrap MS scan
- Internal calibrant injection
- Transfer of ions to Orbitrap analyzer
- Parent ion isolation in Q1 and HCD
- Internal calibrant injection
- Full Orbitrap MS scan with internal calibrant
- Transfer of ions to Orbitrap analyzer
- MS\(^2\) scan: Orbitrap detection with internal calibrant
**Data Dependent Experiment: OTMS > ETHcD OTMS²**

- Full Orbitrap MS scan
- 1st parent ion isolation in Q1
- 1st parent trapped in the ion trap
- Injection of ETD reagent
- 1st parent: ETD dissociation
- ETHcD dissociation
Glycopeptide Sequencing Using EThcD
Intact Protein Mode Principles of Operation

- Reduced pressure in the IRM>CTRAP>Orbitrap region
- Calibration of ion transfer at reduced pressure
- Push-button operation once calibrated
- Can be used via Tune or in the Method (Global Parameter)
- Good for signal conservation with longer transients necessary to obtain isotopic resolution of large intact proteins 25-50kDa
- Unnecessary for Intact IgGs analyzed at low resolution

Intact Protein Mode Enolase, 47+

46,641.39 Da

Standard Pressure Mode
Intact IgG: Seven Major Glycosylated Forms

<table>
<thead>
<tr>
<th>Deconvoluted Mass, Da</th>
<th>Reported Mass, Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>148018.5</td>
<td></td>
</tr>
<tr>
<td>148220.6</td>
<td>148220.4</td>
</tr>
<tr>
<td>148383.0</td>
<td>148382.5</td>
</tr>
<tr>
<td>148546.0</td>
<td>148544.6</td>
</tr>
<tr>
<td>148709.6</td>
<td>148706.7</td>
</tr>
<tr>
<td>148871.2</td>
<td>148868.8</td>
</tr>
<tr>
<td>149035.7</td>
<td></td>
</tr>
</tbody>
</table>

Why mass shifts?
41+: Higher Resolution Reveals Multiple Isoforms

High Resolution is Essential
Top Down of IgG: ETD, EThcD, CID, HCD
Top Down of IgG: ETD, EThcD, CID, HCD

46% bond coverage

Glycosylated Heavy Chain, 42+
Top Down of IgG: ETD, EThcD, CID, HCD

CID, HCD, ETD
77% bond coverage

24182.62 Da

Light Chain, 20+
Top Down of IgG: ETD, EThcD, CID, HCD

High Bond Coverage with Orbitrap Fusion MS

CID, HCD, ETD

77% bond coverage

CID, HCD, ETD

77% bond coverage

Glycosylated Heavy Chain, 42+

Light Chain, 20+
Top Down MS\textsuperscript{n} of Carbonic Anhydrase

HCD MS\textsuperscript{2}

36+
Combined Sequence Coverage

ETD

HCD

ETD MS\(^3\)

(only unique z fragments are shown)
Alla ricerca della Massa esatta
Definita come la capacità di riuscire a distinguere due ioni aventi rapporti m/z diversi.

All’aumentare della risoluzione aumenta la capacità di distinguere e misurare ioni con segnali m/z anche molto vicini.
Per una miglior comprensione sarebbe utile riferire il valore di risoluzione ad una massa di riferimento.

**Max Resolving Power**
140,000 @ $m/z$ 200

$R = \frac{M}{\Delta M}$

**Full Width Half Maximum**

$\Delta M = \text{ampiezza a metà altezza del picco} \approx 0,003$

Definita come la capacità di riuscire a distinguere due ioni aventi rapporti $m/z$ diversi. All’aumentare della risoluzione diminuisce la differenza che deve esistere tra due ioni affinché diano due segnali $m/z$ distinti.

$\text{FWHM} = 292,04 / 0,003 = 97.3460$
Resolution and Mass Accuracy

- Massa accurata

\[
(\text{ppm}) = \frac{m_{\text{true}} - m_{\text{measured}}}{m_{\text{true}}} \cdot 10^6
\]

L’ACCURATEZZA
nella misura di massa, ovvero la differenza tra la massa ottenuta sperimentalmente e quella teorica
Esempio: Molecole Isobariche

Thiamethoxam

\[ [\text{M+H}]^+ = 292.02656 \]

Parathion

\[ [\text{M+H}]^+ = 292.04031 \]

\( \Delta m \)

0.0138 Da
Resolution 15,000

m/z

292.04031 C_{10}H_{15}O_5 N P S

292.02656 C_8 H_{11} O_3 N_5 Cl S

Relative Abundance

100
90
80
70
60
50
40
30
20
10
0

0 10 20 30 40 50 60 70 80 90 100

291.98 292.00 292.02 292.04 292.06 292.08

m/z

15,000 (Mix 1:3)
20,000 (Mix 1:3)

Resolution 20,000
Resolution 25,000
35,000 (Mix 1:3)

Resolution 35,000
Resolution 50,000

292.04031
C_{10} \text{H}_{15} \text{O}_5 \text{N}_5 \text{P}_5 \text{S}

292.02656
C_8 \text{H}_{11} \text{O}_3 \text{N}_5 \text{Cl}_S

50,000 \text{ (Mix 1:3)}
100,000 (Mix 1:3)

Resolution 100,000

m/z

Relative Abundance

292.04031
C_{10}H_{15}O_{5}N_{P}S

292.02656
C_{8}H_{11}O_{3}N_{5}ClS

Resolution 100,000
Risoluzione e accuratezza

Thiamethoxam

\([M+H]^+ = 292.02656\]

\(\Delta m = 0.0138 \text{ Da}\)

Parathion

\([M+H]^+ = 292.04031\]
La tecnologia Orbitrap™ applicata alla LCMS
nello sito produttivo di Brema venne quindi prodotto l’ Orbitrap
Dove gli ioni ruotano in un campo elettrico…

"Fast" Injection

Elettrodo esterno
2 coni isolati

Elettrodo interno

...ed il segnale è elaborato dalla Fourier-transform
In breve Orbitrap significa:

- Altissima Risoluzione
- Accuratezza di massa e sua stabilità
- Sensibilità
- Range dinamico
- Acquisizioni Full Scan
- Analisi retrospettiva