Nanoscale Capillary LC/MS

LC/MS/MS

LC/LC/MS/MS
Liquid Chromatography and Mass Spectrometry

- Why bother with the added complexity and expense?
- What type of LC system?
- What type of stationary and mobile phases?
- How do I optimize my LC/MS/MS data acquisition?
- What type of results might be expected?
Liquid Chromatography and Mass Spectrometry
- why bother with the added complexity and expense?

- Goal - analysis of complex mixtures of peptides
  - single protein - 1 protein - 50 peptides
  - protein complex - 10 proteins - 500 peptides
  - cell fraction - 1,000 proteins - > 50,000 peptides
  - cell lysate - 10,000 proteins - > 500,000 peptides

- Problems
  - MS is a parallel process
    - all peptides in m/z range *may be* analyzed in a single MS scan
  - MS/MS is a serial process
    - one MS/MS spectrum of one peptide at a time
    - low duty cycle - waste of sample
  - ion suppression
    - ion current not evenly distributed across all peptides
    - discrimination between peptides
Liquid Chromatography and Mass Spectrometry  
- why bother with the added complexity and expense?

- LC enables the delivery of peptides to the mass spec with a reasonable degree of separation  
  - provides more time for MS/MS data acquisition  
    - better matches peptide delivery with data acquisition capabilities  
  - suppression effects minimized  
    - minimized coelution of high abundance and low abundance peptides  
  - peptide co-elution is to be expected  
    - data acquisition software critical (data dependent scanning)  
- LC delivers the peptides to the mass spec at high concentrations  
  - narrow, high concentration chromatographic peaks  
  - ESI is a concentration sensitive detector
Liquid Chromatography and Mass Spectrometry
- what type of LC system?

- ESI/MS is a concentration sensitive detector
  - best detection limits achieved using chromatographic systems yielding the highest peak concentrations (minimal dilution of analyte in mobile phase)
- Optimum column flow rate scales as the ratio of the square of the radius of the columns
  - going from a 4.6 mm ID column to a 320 um ID column
    - 1 mL/min. on 4.6 mm column equivalent to 4.8 uL/min. on 320 um column
      - \((1,000 \text{ uL/min})/(4.8 \text{ ul/min}) = 208\)
    - injecting the same amount of analyte on each column provides an increase in analyte peak concentration with the capillary column of 208 fold
      - an increase in MS signal of \(208\) fold
  - going from a 4.6 mm ID column to a 75 um ID column
    - an increase in MS signal of \(3,761\) fold
- These gains in sensitivity are why nanoscale capillary LC systems are used
Liquid Chromatography and Mass Spectrometry
- nanoscale capillary LC/MS considerations

- Advantages
  - significant improvement in ESI/MS detection limits
  - high chromatographic resolution can be obtained
  - low solvent consumption (acetonitrile shortage world wide)
  - low waste disposal cost
  - can be interfaced with both ESI (on-line) and MALDI (off-line)

- Disadvantages
  - great care must be taken to minimize dead volumes
    - greater attention must be paid in making system connections
    - specialized instrumentation required
      - “home-made” systems fairly easy to fabricate using flow splitter
        - cost vs reproducibility
      - fully automated commercial systems available
        - Split flow systems waste the majority of solvent
        - Direct flow systems and split flow systems MUST SHOULD have flow sensors to minimize changes in retention time
Liquid Chromatography and Mass Spectrometry
- what type of stationary phase and mobile phase?

• We get best peptide sensitivity with positive ion detection
  • need acidic mobile phases (pH<4) to produce positive peptide ions
    • protonation of C-terminal residue and Asp and Glu side chains
  • must use volatile buffer additives for MS work
  
  ◆ Positively charged peptides adsorb strongly to most stationary phases
    – trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA) additives often used to improve separation
      » strong ion pair formed between positively charged peptide and negatively charged ion pair reagent
  
  ◆ Positively charged peptide + strong ion pairing reagent yields zwitterions
    – such ions give poor mass spec detection limits
Liquid Chromatography and Mass Spectrometry
- what type of stationary phase and mobile phase?

• Since we do not want to form strong ion pairs, less chromatographically ideal modifiers are used
  • formic acid, acetic acid
  • give broader, tailing peaks with many stationary phases

• To improve chromatographic peak shape, special stationary phases have been developed for use with formic acid modifier
  • PepMap, BioBasic, Magic C18, Atlantis, Jupiter, BEH, etc.
  • highly deactivated columns to minimize adsorption of peptides in MS-friendly modifiers
    • Small amounts of HFBA may be added, but not required with most newer LC columns
    • Typically 0.1% to 0.2% FA used in both mobile phases
    • “Trapping-column LC system can use 0.1% TFA in injection buffer to improve peptide retention – TFA is removed via flushing the trap column
Liquid Chromatography and Mass Spectrometry
- what type of stationary phase and mobile phase?

- Typical conditions for simple protein mixtures (2D spots)
  - 300 um ID x 5 mm long trapping column
    - sample loading and desalting at 25 uL/minute
  - 75 um ID x 15 cm long analytical column
  - Gradient at 300 nL/min.
    - initial 5% MeCN in H2O (both w/0.1% Formic Acid)
    - 1 min 5% MeCN
    - 30 min 40% MeCN (30 minute analytical gradient)
    - 31 min 90% MeCN (column flushing)
    - 36 min 90% MeCN
    - 37 min 5% MeCN (column re-equilibration)
    - 50 minutes 5% MeCN

NOTE – proper timing of steps requires knowledge of system “dead time”
- time for gradient change at pump to reach LC column
- for more complex samples, extend the gradient time (60, 90, 120 minutes)
Nanoscale Capillary LC System

Autosampler with Chilled Sample Tray (sample in 5% MeCN, 0.1% TFA)

Sample Loading Pump 25 μL/min 0.1% formic acid

Micro-Precolumn 300 μm id x 5 mm length

Waste

Ultimate Gradient Pump 300 nL/min gradient flow MeCN/H₂O (0.1% formic acid)

Nanoscale Capillary Column 75 μm id x 15 cm length

To MS
Automation Aspects of Modern Nanoscale Capillary LC/MS/MS Systems

- Fully automated analysis - peptide separation and MS/MS sequencing
  - Automated sample introduction
  - Automated column switching
    - preconcentration and desalting using a 300 um ID, 5 mm long “trapping” column
      - allows for injection of large volumes onto a nanoscale system
  - gradient elution LC analysis
- Automated data acquisition
  - data dependent scanning (DDA, DIA, etc.)
    - DDA - MS to MS/MS switching based on peptide signal intensity
  - Complex samples can generate >> 1,000 MS/MS precursors per hour
  - Identification of multiple proteins/sample
    - typically 2 to 5 proteins identified per 2D gel spot
    - >100 proteins in a 1D gel band (our lab record)
    - 400 ++ (1,000+) proteins complex sample (cell lysate)
  - CAUTION – sophisticated data curation methods absolutely required
Nanoscale Capillary LC/MS
- Analysis of 125 fm BSA Digest

125 fmol BSA
qt04142 Sm (SG, 2x3)

Selected Ion Chromatogram of m/z 653.3

Base Peak Plot Chromatogram

Mass Spectrum of Chromatographic Peak

125 fmol BSA
qt04142 1438 (22.970) Cm (1434:1441-(1414:1422+1462:1463))

TOF MS ES+
653_654 233

TOF MS ES+
BPI 34.7
Automated Protein ID as a Function of Protein Amount
- BSA digest analyzed in triplicate by nanoLC/MS/MS with Mascot
Data Dependent Scanning (DDA) - automated MS to MS/MS switching

- The MS decides when to perform an MS/MS experiment based on a threshold ion intensity

- Because DDA is intensity driven, datasets tend to be biased towards detection of the highest abundance peptides (and therefore, proteins) in the sample

- Full automation allows for rapid switching during an LC gradient separation
  - >>1000 MS/MS spectra per hour

- SERENDIPITY PLAYS A MAJOR ROLE IN DDA
  - Subtle variations in ion intensity/retention time profiles will result in differences in datasets when the same sample is analyzed multiple times!
Nanoscale Capillary LC/MS/MS (DDA) Analysis of a Tryptic Digest of a 500 kD Protein from Cilia
Nanoscale Capillary LC/MS/MS (DDA) Analysis of a Tryptic Peptide from a 500 kD Cilia Protein

Sequence: YGYEYLGNSPR
Human Cytoplasmic Dynein
Typical User-Defined Parameters for a DDA Experiment: *Optimize with a **RELEVANT** Protein **Mixture**

- **MS->MS/MS switching**
  - What Threshold
  - Which Charge States (higher resolution analyzers)
  - How many precursors interrogated per switch

- **MS/MS->MS switching**
  - MS/MS TIC intensity (above or below threshold)
  - Time to stay in each MS/MS channel

- **Include Masses**
  - Targeted DDA (“MS Western”)

- **Exclude Masses**
  - Fixed Exclude
    - Known contaminants
  - Dynamic Exclude
    - Recently interrogated species
    - Must take into account average peak widths, don’t want to interrogate the same peptide multiple times!

- **Inclusion/Exclusion Windows**
  - Function of analyzer mass accuracy “on-the-fly”!
  - Include generally small enough to minimize spurious switching (say +/- 25ppm on Q-Tof)
  - Exclude generally large enough to prevent switching on isotopes of previously interrogated species (+/-2 Da on Q-Tof)

- **Collision Energy**
  - 1 fixed CE
  - CE “Profile” (step through 2 or more CE’s)
  - m/z and z dependent CE (Look-up Table – now the most common approach)

- **Scan times and interscan delays**
MS/MS Scans from Analysis of Mitochondrial Ribosomal Proteins

8 product ion spectra of precursors selected from a single MS scan

1\textsuperscript{st}, 4\textsuperscript{th} and 8\textsuperscript{th} product ion spectra of precursors from a single MS scan
Serendipity in DDA
- saturation of data acquisition due to sample complexity)
- triplicate runs of a mammalian mitochondrial ribosomal 39S subunit

- Three replicate runs with a three hour gradient
- Total number of MS/MS spectra
  - run 1 1013 average Mascot score = 46
  - run 2 1390 average Mascot score = 44
  - run 3 843 average Mascot score = 41
- Exact matches in database (nrdb and EST)
  - peptides observed in all three runs 53 (20%) avg. score = 50
  - peptides observed in two runs 66 (25%) avg. score = 43
  - peptides observed in only one run 141 (54%) avg. score = 37

- DDA ALONE IS NOT A GOOD TECHNIQUE FOR QUANTITATIVE COMPARISON OF SAMPLES UNLESS NUMBER OF MS/MS CYCLES AND CYCLE TIMES IS MINIMIZED
Advanced LC and MS Techniques

• Why Shotgun Proteomics?
• What are the limitations of LC/MS/MS?
  • Instrument Saturation – mixture too complex for data acquisition process being used
• What are the options for overcoming the limitations?
  • Instrument optimization
  • Targeted DDA (MS Western)
  • Fractionation
    • Protein Level Fractionation
      • GeLC/MS/MS
    • Peptide Level Fractionation
      • Multi-dimensional LC (LC/LC)
    • Gas Phase Fractionation
  • UPLC
  • Data Independent Acquisition (Multiplexed Data Acquisition)
Why Shotgun Proteomics?

- Ability of identify and quantify proteins at picomole to femtomole levels is a given
- The most biologically interesting proteins are often at very low copy numbers per cell
- How can one insure that sufficient coverage of all proteins will be obtained?
  - shotgun proteomics has proven improved proteome coverage over traditional techniques (i.e., 2D gels)
  - total proteolytic digestion yields tractable peptides from
    - very large and very small proteins
    - acidic and basic proteins (typical 2D gel pI 4-7)
    - hydrophobic proteins (e.g. membrane proteins)
  - no gel-based sample loss
    - improved digestion efficiency
    - minimized sample loss (no recovery losses from gel)
"Large-scale analysis of the yeast proteome by multidimensional protein identification technology (MuDPIT)"

LC/LC
SCX/RP (low pH)
Low Resolution/High resolution

Figure 2. Codon adaptation index (CAI) distribution of the identified *S. cerevisiae* proteome and the predicted *S. cerevisiae* genome. (A) CAI distribution of the proteins predicted in the *S. cerevisiae* genome. (B) Compare this to the distribution of the proteins identified in this study over CAI ranges. In both cases, the largest protein region is found between the CAI range of 0.11 and 0.2. (C) The average number of peptides identified for each protein in a particular CAI range was determined and plotted against CAI ranges.
Overcoming Saturation Effects when Only a Few Proteins in a Complex Mixture are of Interest
MS Western: Hypothesis Driven Protein Identification

- Method for selective detection of proteins of interest without an antibody
- Can be applied to any protein
- Multiple proteins detected in one analysis
- Detection of PTM’s possible
- Relative quantitation possible with use of isotope tagging
- Method of choice for detecting a single protein (or small number of proteins) in a complex background

- Detected ErbB2 @ 15K copies/cell
- from 5e7 cells (~1pmol)
How does the MS Western work?
How does the MS Western work?

Include List
696.32
559.82
638.83
452.24
669.83
825.72
cetc.
(250 total)

P004762 - Rat Catalase

10 Counts

QT2U00156 2072 (43.538)
Methods for improving proteome coverage
- improved fractionation at the protein level

- Solution-based methods
  - Chromatographic methods
    - Reversed-phase, ion exchange, size exclusion,
  - Electrophoretic methods
    - Isoelectric focusing
    - “Rotofor”

- Issues
  - Adsorptive losses
  - “Smearing” across fractions
  - Difficult because of the wide range of physicochemical differences between proteins
    - Hydrophobicity, size, pI, etc.

- Advantage
  - Peptides from high abundance proteins constrained to only a few fractions, rather than being distributed across all fractions as with peptide level fractionation
Methods for improving proteome coverage - improved fractionation at the protein level

- Gel-based methods
  - 1-D gels
    - “Lane walking”, “GeLC/MS/MS”
  - Immobilized pH gradient (IPG) strips
    - Gel-based separation based on pI

- Comments
  - Methods generally work well
  - Proteins stable in gel matrix
  - High resolution protein separation
  - Standard in-gel digestion methods apply
Methods for improving proteome coverage - improved fractionation at the peptide level

• “Gas-Phase” separations
  • MS level
    • easily accomplished without any instrument modifications
  • Ion mobility coupled with LC/MS/MS

• Improved peptide fractionation
  • LC/LC - multidimensional chromatography

• Affinity labeling/affinity fractionation

• Data Independent Analysis

• Why work at the peptide level?
  • Peptides as a whole are generally more chemically homogeneous than their parent proteins – easier to manipulate than proteins
Gas Phase Fractionation for Improved Proteome Coverage
-multiple LC/MS/MS runs with narrow MS survey ranges
Spahr et al. (2001), Electrophoresis, 21, p1635.

Fig. (2). Increasing the numbers of ions selected for MS/MS. If sufficient sample is available, multiple LC-MS/MS runs can be conducted in series with ion selection from narrow overlapping m/z ranges to increase the number of ions selected. Example mass ranges are shown, but the number can be decreased or increased depending upon the amount of sample available. This is also referred to as gas phase fractionation (Spahr et al., 2000). In the LC chromatogram, TIC denotes total ion current while using a mass spectrometer and UV denotes absorbance while using an UV detector to follow the elution of peptides.
What is LC/LC?

- Initial fractionation using one mode of chromatography
  - typically ion exchange is used for first dimension

- Additional fractionation of fractions from first dimension separation
  - typically reversed phase is used for second dimension
Peak Capacity of Comprehensive Multidimensional Separations

- Peak capacity of a multidimensional separation:

\[ n_{c2d} = n_{c1dx} \otimes n_{c1dy} \]

- Two 1D separation systems combined into a 2D system
  - if each system has a peak capacity of 100
    - “only” 40,000 plates each system
  - yields a 2D system with a peak capacity of 10,000
    - requires a separation efficiency of 400,000,000 plates
    - currently unachievable by any 1D method

- Such an LC/LC system capable of separating 1,800 “ideal” peptides
Why Improve Peptide Fractionation? 
- to maximize information content from analysis

• Two figures of merit
  • sample throughput on mass spectrometers
    • peptides/hour to mass spectrometer
  • proteome coverage within each sample
    • (peptides analyzed/peptides present)

• There is a trade-off between these figures of merit
  • balance throughput with information content
Difference in Fundamental Analysis Process of Different Sample Types

- Rate of delivery of peptides to mass spectrometer should be identical for all sample types (peptides/minute)
  - want to supply peptides to MS at a rate matched with data acquisition rate
  - maximizes efficiency of mass spectrometer
  - maximizes information obtained from the sample
- Total analysis time differs based on sample complexity
  - more complex sample
    - longer total analysis time needed
- LC/LC effectively increases total analysis time
  - number of peptides presented to MS per unit time decreases
Different Approaches to LC/LC

- Favored chromatographic combinations
  - ion exchange LC with reverse phase LC
    - Low resolution LC with high resolution LC
  - reverse phase LC with reverse phase LC
    - High resolution LC with high resolution LC
- On-line coupling
  - coupled columns with step gradient salt elution of SCX column
    - two columns
    - one column with two packing beds (Yates’ MuDPIT)
      - lower total sample capacity (amount of digest loaded)
  - minimal sample handling; minimal losses
- Off-line coupling
  - perform 1st dimension separation, collect fractions, reanalyze fractions without rerunning entire sample
  - more sample handling but higher total sample capacity
    - larger 1st dimension column
Proteome Analysis of Low-Abundance Proteins Using Multidimensional Chromatography and Isotope-Coded Affinity Tags
S. P. Gygi, B. Rist, T. J. Griffin, J. Eng, and R. Aebersold
J. Proteome Research, 1, 47, 2002

- 1D = RP only
- 2D = SCX + RP
- 3D = SCX + avidin + RP
- MS1
  - (1 MS + 1 MSMS)/cycle
  - 1 hr gradient
- MS2
  - (1 MS + 5 MSMS)/cycle
  - 2 hr gradient

- Use of additional dimension of LC leads to much improved sample coverage
  - most improvement with low abundance proteins
Sequential Comprehensive Multidimensional Nanoscale Capillary LC/LC/MS/MS - SCX/RP

Reversed Phase Micro-Precolumn
300 um id x 5 mm length

Famos Autosampler
with Chilled Sample Tray
“Samples” are Step Salt Gradients

Sample Loading Pump
25 uL/min
0.1% formic acid

Ultimate Gradient Pump
200 nL/min
gradient flow
MeCN/H₂O
(0.1% formic acid)

Waste

Ion Exchange Micro-Precolumn
300 um id x 5 mm length
SCX

Reversed Phase Nanoscale Capillary Column
75 um id x 15 cm length

To MS
LC/LC with RP/RP (High pH/Low pH)

High Resolution Separation

Neutral
Acidic
Basic
Acidic
Basic

20mM ammonium formate, pH 10
0-42% acetonitrile in 50 min

0.2% Formic acid, pH 2.6
0-42% acetonitrile in 50 min

nanoACQUITY UPLC
2D w/Dilution
Step 5 - Step gradient to elute fraction 1 onto trap column
LC/LC with RP/RP (High pH/Low pH)
High Resolution Separation/High Resolution Separation

2.4 ug E. coli, 72 fmol ADH, 2D run, 1/5, 11.1%, 90 min

Time
20.00 30.00 40.00 50.00 60.00 70.00 80.00 90.00
%
0
100

695 Proteins
(90% increase)
7,961 Peptides
(177% increase)

4.8 ug E. coli, 48 fmol ADH, 2D run, 1/11, 7.6%, 90 min

Time
20.00 30.00 40.00 50.00 60.00 70.00 80.00 90.00
%
0
100

778 Proteins
(113% increase)
9,415 Peptides
(228% increase)

Note – one obtains diminishing returns as the number of fractions increase
- True for any combination of LC/LC
- Note that the increase in peptides > increase in proteins
- Should always be true – one will identify more peptides to proteins already identified rather than identifying more new proteins
LC/LC with RP/RP (High pH/Low pH)
High Resolution Separation/High Resolution Separation

Protein

Concentration (ng/µg)

1D
2D (5 fraction)
2D (10 fraction)

E.coli standard
Increasing Separation Capabilities for Increased Proteome Coverage - RP/RP Two-Dimensional LC

“Standard” Conditions:

5-fraction or 10-fraction RP/RPLC – HDMSE
3 ug (5 fraction) or 5 ug (10-fraction) loading
Total Cycle time 6 hr (5 fxn) or 11.5 hr (10 fxn)

First Dimension:
5 Fractions at pH 10 (300 um x 5 mm XBridge BEH130)
(10.8%, 14.0%, 16.7%, 20.4%, and 50.0%)

Second Dimension:
1.7 mm Acquity BEH130 C18 75 um x 150 mm column
37 minute gradient 7-35% MeCN,
0.1% Formic Acid, 0.5uL/min, 35C

HDMS<sup>E</sup> acquisition:
0.6 sec HDMS (6V), 0.6 sec HDMS<sup>E</sup> in Transfer (27-50V)

Data analysis:
PLGS 2.5 RC7
Rosetta Elucidator v3.3
RP/RP 2DLC as a Robust Alternative to SCX/RPLC

8-fraction Analysis of NIST Yeast Lysate

Even peptide distribution across fractions and high fraction uniqueness yields a high rate of return on time investment for 2DLC
Introduction to Ultra-high Pressure LC (UPLC)

• Ultra-high pressure liquid chromatography
  • either
    • faster analysis
      • Up to 10x improvement over “traditional” HPLC
    • high resolution
      • Up to 10x improvement over “traditional” HPLC
      • equivalent to CE and CEC
  • high sensitivity analysis
  • “logical” control of separation process via gradient elution
Ultra-High Pressure Liquid Chromatography - Theoretical Predictions

- Required pressure

\[ \Delta P \propto \frac{1}{d_p^3} \]

- the pressure required to achieve the same linear velocity with a 1.5 u particle is 37x that required for a 5 u particle
- 400 psi required for a 5 u column
- 14,800 psi required for a 1.5 u column
UPLC Nanoscale Capillary LC/MS -depleted human plasma digest

3,726 peptides detected
3 of 3 injections

4,706 peptides detected
3 of 3 injections

80% of peptides detected in ½ analysis time.
Peptides not detected with shorter gradient had highest variation in long gradient.
Sample Complexity Leads to Multiple Peptides Being Sampled by DDA in a Single “Bin”
- only one peptide will be identified; multiple product ions may preclude obtaining a significant MS/MS quality score

+/- 2 Daltons
Data Independent Acquisition (MS$^E$)
-An approach for multiplexed data acquisition

• Parallel LC/MS analysis – accurate mass measurement of all detectable precursor and product ions. Alternating MS and elevated collision energy MS (MS$^E$) acquisition.
• Chromatographic alignment of precursor and product ion data reduces miss assignment of product ions to similar mass/ret. time precursor ions.
• All Charge-States and All Isotopes All of the Time
• Identifications are confirmed by parent and product ion accurate mass.
Data Independent Acquisition - DIA or MS$^E$

1. Parallel LC/MS analysis – accurate mass measurement of all detectable precursor and product.

MS$^E$ is a PARALLEL process.
Data Independent Acquisition
- alignment of low energy (LE) and high energy (HE) ions by selected ion chromatogram of all precursors and product ions

- Gas cell collision energy alternates between low (5 eV) and elevated energy (linear ramp from 15 eV - 42 eV)
- LC-MS$^E$ alternate scanning provides quantitative accurate mass measurement for all detectable peptide precursor and product ions throughout UPLC separation
DDA vs MS\(^E\): Improved Coverage for a Simple Protein Mixture (15 min gradient, 28 min LC cycle time)

<table>
<thead>
<tr>
<th>Mode</th>
<th>Injection</th>
<th>Enolase (200fmol)</th>
<th>Phosphorylase B (100fmol)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td># Peptides</td>
<td>Average Mascot Score</td>
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<tr>
<td>DDA</td>
<td>1</td>
<td>8</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td>MS(^E)</td>
<td>1</td>
<td>14</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12</td>
<td>56</td>
</tr>
</tbody>
</table>

2X more peptides with comparable data quality
Quadrupole – IMS – Tof Instrumentation for HDMS
Instrument Resolution as Function of m/z

- Singly Charged Ions
- Multiply Charged Ions
- Synapt G2 High Resolution Mode
- Orbitrap Velos 1Hz
- Orbitrap Velos 3 - 4 Hz
Ion Mobility Adds Unique Dimension of Peptide Separation

Precursor ions separated by IMS

Precursor and products share same drift time
Improved Qualitative Coverage using Ion Mobility

Improved Database Search results due to increased specificity in High Energy Spectra
Alignment of DDA, MSE, and HDMSE by AMRT Gives Maximum Information Content

DDA, 3515 peptides (175 unique)
MSE, 6371 peptides (1813 unique)
HDMSE, 9942 peptides (4872 unique)

NIST Yeast Lysate, Analyzed by 8-fraction LC/LC
Alignment of DDA, MSE (15-40V), and HDMSE (27-50V) data collections in Rosetta Elucidator
All spectra processed through PeptideTeller and ProteinTeller Algorithms
Annotation at ~0.5% Peptide FDR
LC/LC-HDMSE Impact on Biomedical Applications
(5-hour single-sample pilot studies)

Platelet Lysate, Healthy Volunteer Proteins (0.5% FDR)

- DDA/Mascot (370)
  - Peptides (0.1% FDR)
    - 1
    - 343
    - 262

- HDMSE/IdentityE (605)

Lung Epithelium, Cystic Fibrosis patient Proteins (0.7% FDR)

- DDA/Mascot (371)
  - Peptides (0.3% FDR)
    - 2
    - 369
    - 303

- HDMSE/IdentityE (672)

Samples courtesy of Deepak Voora, MD
Duke Institute for Genome Sciences & Policy

Samples courtesy of Bernie Fisher, DVM, PhD
Pediatric Pulmonary Medicine, Duke Univ Med Ctr.
Identification of Proteins in HepG2 Cell Lysate

NQLTSNPENTVFDAK from 78 kD Glucose Regulated Protein

PLGS 2.5 software – GPU enhanced

With Ion Mobility Separation  Without Ion Mobility Separation
Selectivity Gained from Orthogonal Ion-Mobility Separation can Produce Spectra Similar to Serial Precursor Isolation

MS/MS Fragmentation of \textbf{SGTDVDAANLR}
Found in \textbf{CASP3\_HUMAN}, Caspase-3 OS=I

MS/MS Fragmentation of \textbf{YDPTIEDSYR}
Found in \textbf{RAP1A\_HUMAN}, Ras-related protein Rap-1A

Examples from 5-fraction RP/RPLC Analysis of 3 ug Platelet Lysate (5 hours total)
Selectivity Gained from Orthogonal Ion-Mobility Separation Sometimes Resolves Chimeric DDA Spectra

MS/MS Fragmentation of **VELEDWNGR**
Found in **FIBG_HUMAN**, Fibrinogen gamma chain OS=Homo sapiens

**DDA Spectrum, Mascot Ion Score 5**
(clearly two peptides in MS/MS isolation window)

**DIA Spectrum acquired by HDMSE, Mascot Ion Score 62**

Examples from 5-fraction RP/RPLC Analysis of 3 ug Platelet Lysate (5 hours total)
High Spectral Quality: Searching Mobility-Assisted DIA Spectra Against Alternative Search Engines (MASCOT)

**ACQUIRE**
- Acquisition “Rules”: +2 to +4 CS, Top 3, >400 m/z, dynamic exclusion, etc
- No Acquisition “Rules”; Alternate 0.6 s MS with MS<sup>E</sup> and Real-Time IMS

**PROCESS**
- Mascot Distiller / PLGS Processing, Combine MS/MS spectra (.mgf / .pkl)
- Apex4D Processing, RT/IMS alignment, .pkl FILTERING (>800 M+H, Perl)

**SEARCH**

DDA/Mascot (1821)

HDMSE/Mascot (1982)

574 1247 735

Peptides from 5-fraction RP/RPLC Analysis of 3 ug Platelet Lysate (5 hours total)
DDA vs HDMS$^E$: Improved Coverage for a Complex Mixture (human platelets; Mascot DB search each; 2+ peps)

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet DDA</td>
<td>Platelet DDA</td>
</tr>
<tr>
<td>11</td>
<td>487</td>
</tr>
<tr>
<td>223</td>
<td>1158</td>
</tr>
<tr>
<td>22</td>
<td>586</td>
</tr>
</tbody>
</table>

HDMS$^E$ vs HDMS$^E$: Improved Coverage for a Complex Mixture (human platelets; Mascot DB vs IdentityE DB; 2+ peps)

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet HDMSE (Mascot)</td>
<td>Platelet HDMSE (Mascot)</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>357</td>
<td>1751</td>
</tr>
<tr>
<td>262</td>
<td>4579</td>
</tr>
</tbody>
</table>
High Definition MS\textsuperscript{e} Requires Significant Computational Power

- Home-Brew Supercomputer
  - 64-bit OS
  - Six Core 3 GHz Xeon Processor
  - 24 GB DDR3 1600 MHz RAM
  - C2070 Fermi Tesla GPU Card
    - 448 core 1.15 GHz processor
    - 6 GB GDDR5 RAM
  - Three TB Disk Space
  - USB 3.0 board
  - Nine Fans (keeps the lab warm)

‘Home-Brew’ Supercomputer capable of 515,000 MFLOPS
Original Cray-1 Supercomputer capable of 250 MFLOPS

‘Home-Brew’ Costs $8,000
Cray-1 Costs $8,860,000