

Introduction to LC-MS/MS Analysis

Bottom Up Proteomics

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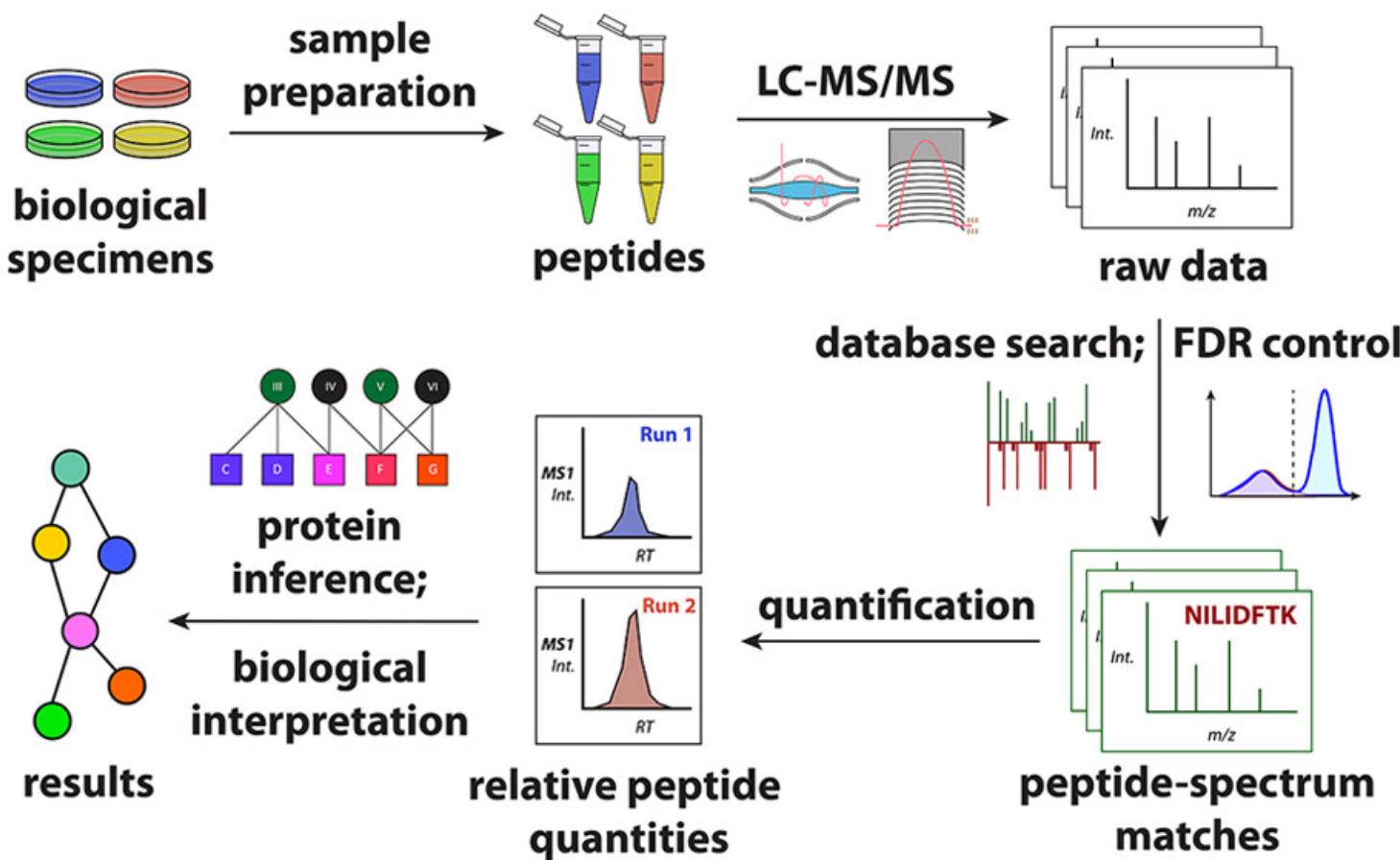
BYU

Chemistry &
Biochemistry

Learning Objectives

- Understand LC Principles and C18 Columns
- Understand MS vs MS/MS
 - Peptide information
 - Fragmentation
 - DDA vs DIA analysis
- Understand MS instrument Fundamentals
 - Astral
 - Bruker TIMS TOF

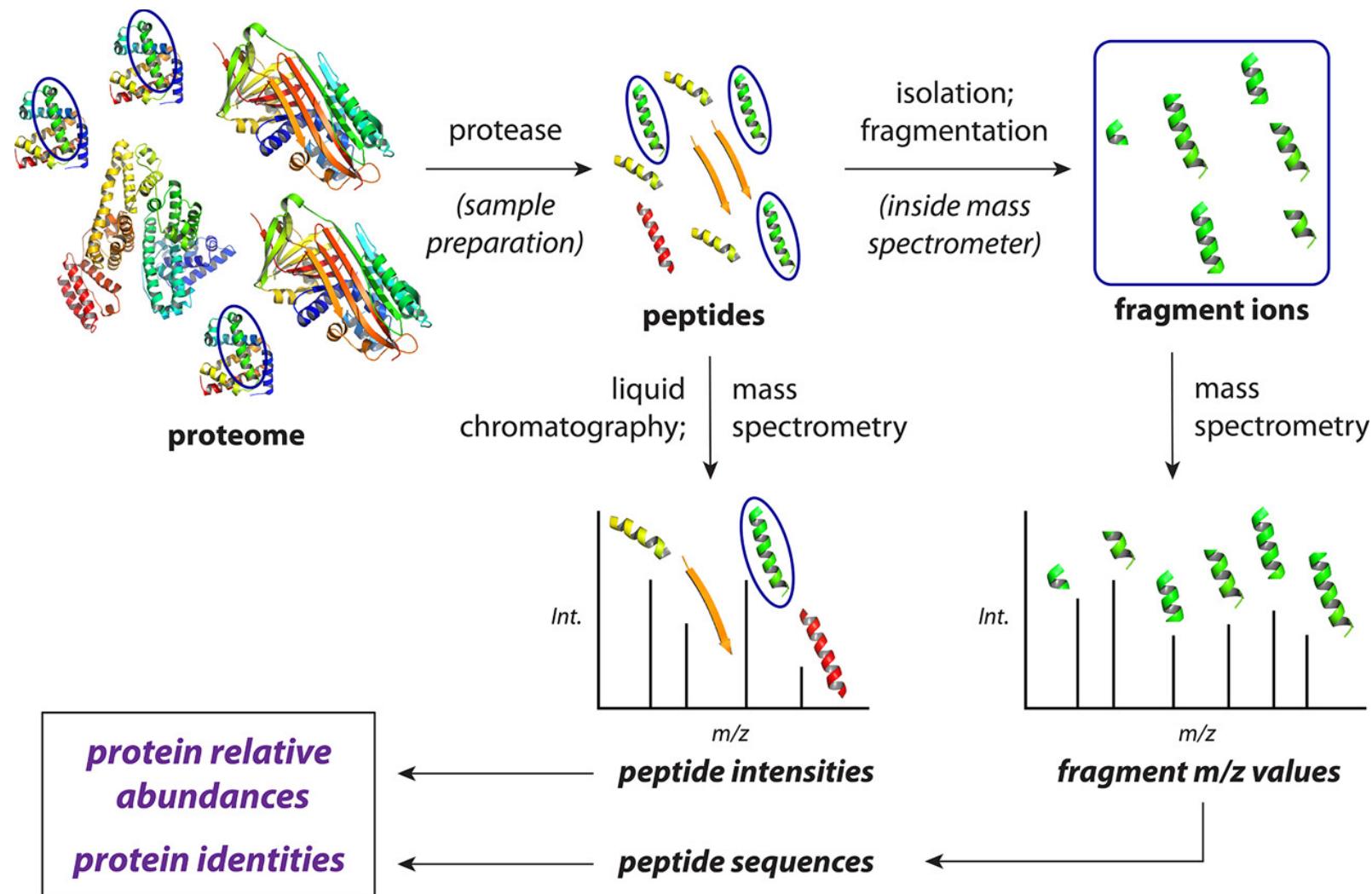
Bottom-up Proteomic Workflow



- Why Peptides?

- Better chromatographic behavior
- Easier Fragmentation
- More interpretable spectra

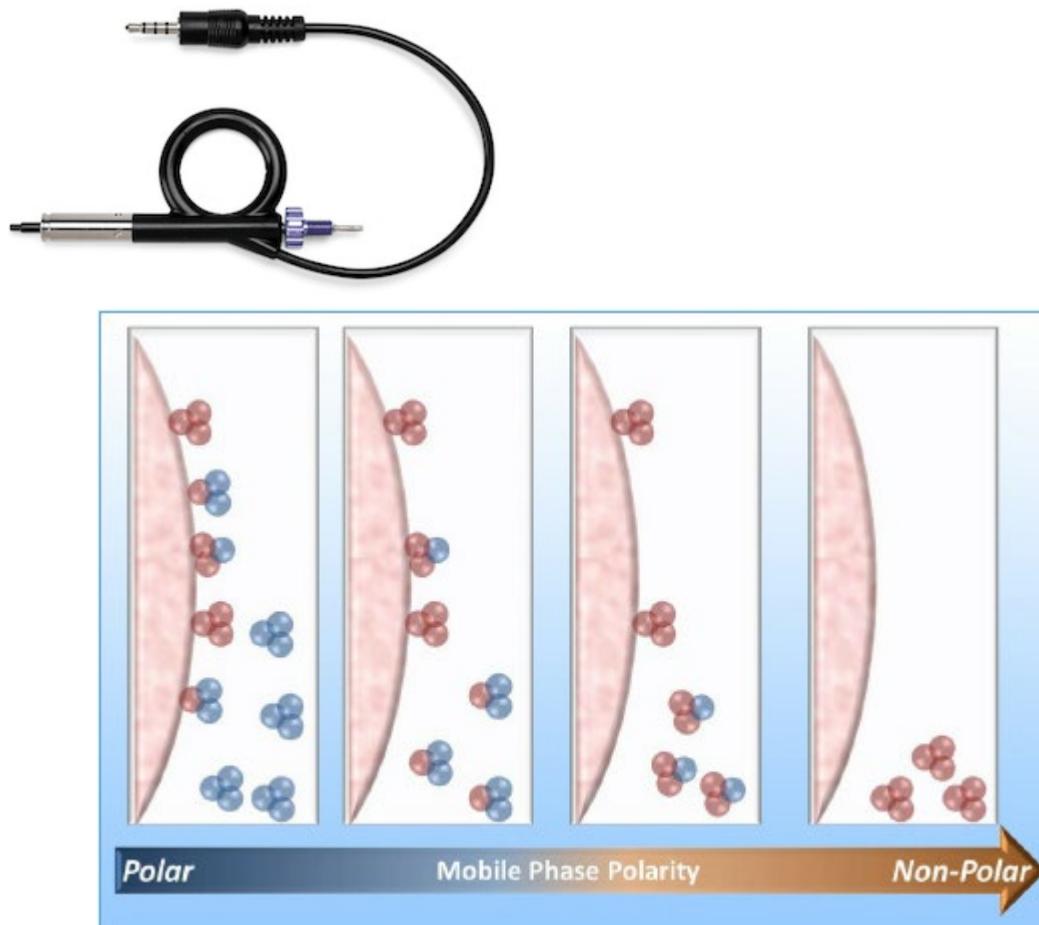
LC-MS/MS Workflow



Reversed-Phase LC Basics

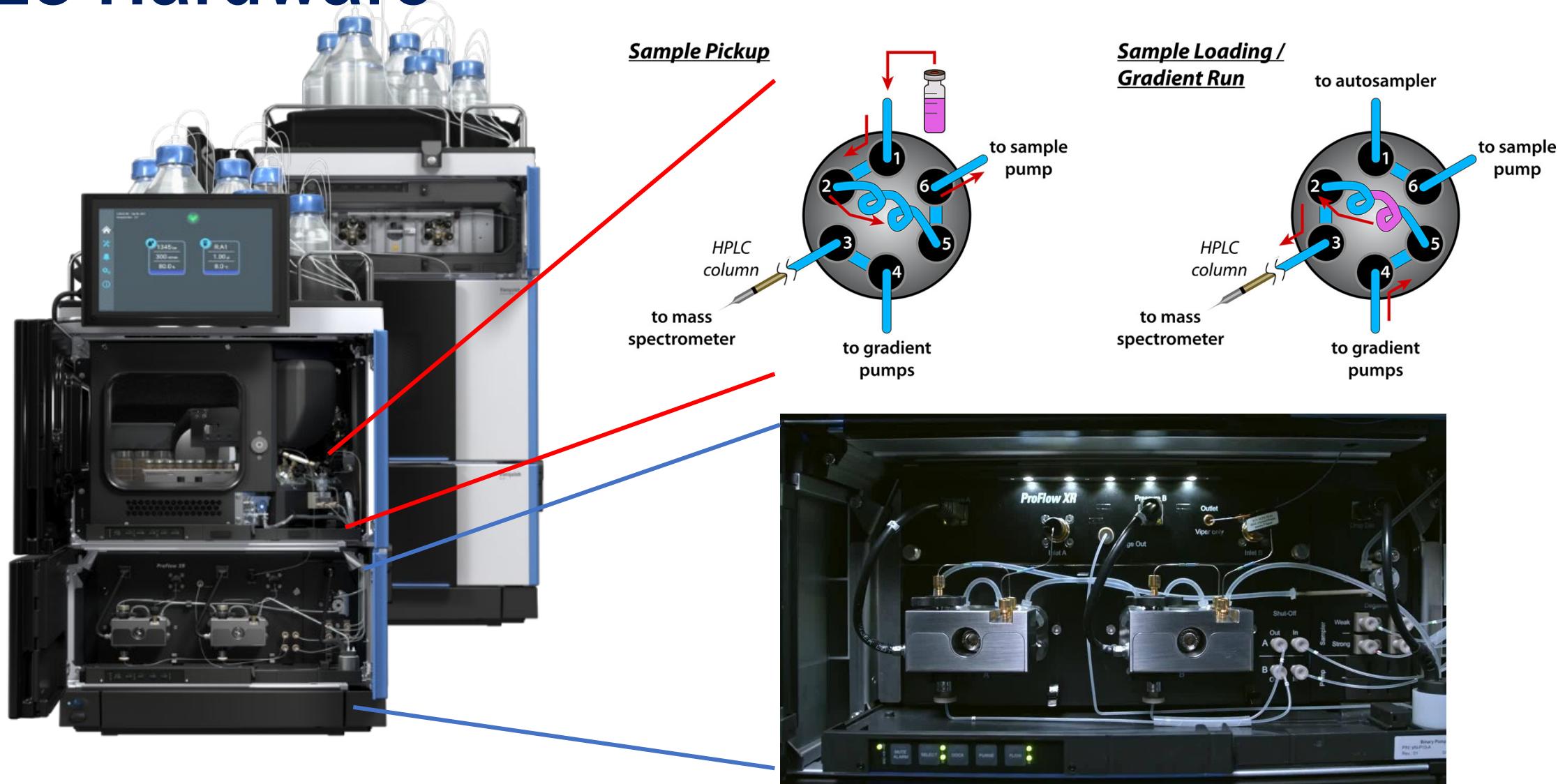
- Separation by hydrophobicity
- Longer peptides = more hydrophobic
- Gradient elution: increasing % organic → elute peptides by hydrophobicity
- Typical mobile phases (A: water + 0.1% FA; B: ACN + 0.1% FA))

C18 Column Chemistry



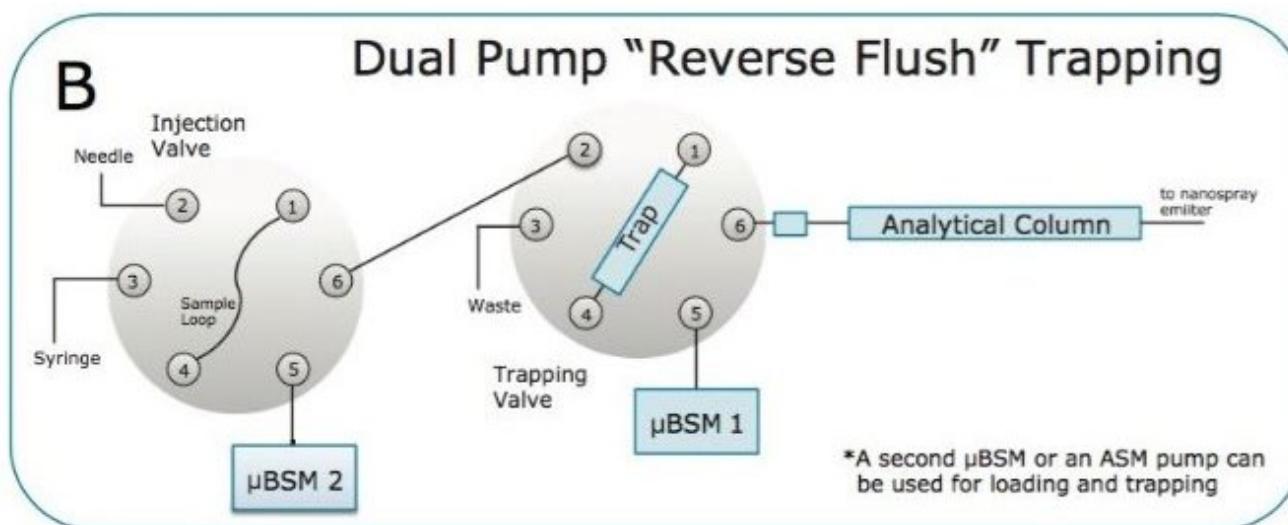
- Hydrophobic interactions retain peptides
- Column is washed with increasing acetonitrile
- Formic acid improves ionization downstream (acidic environment)

LC Hardware





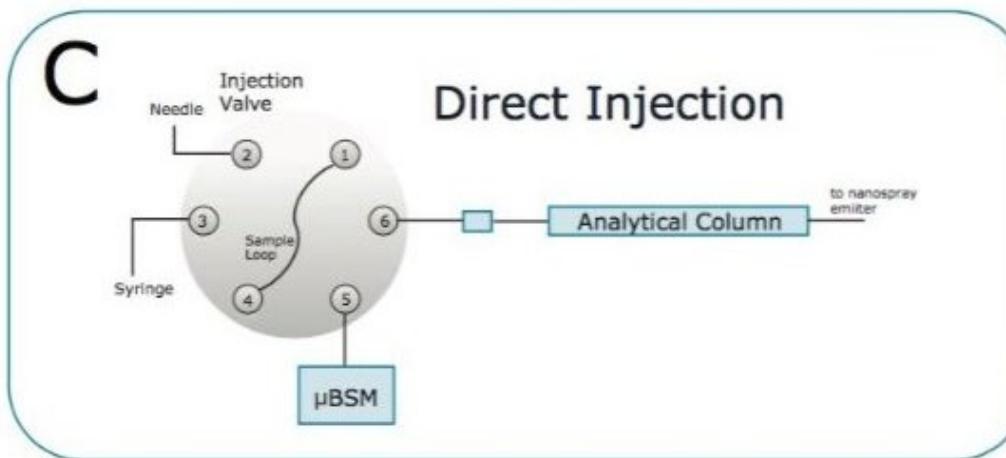
Trap and Elute



- High-flow trapping
- Peak focusing
- Shorter gradients
- Parallelization

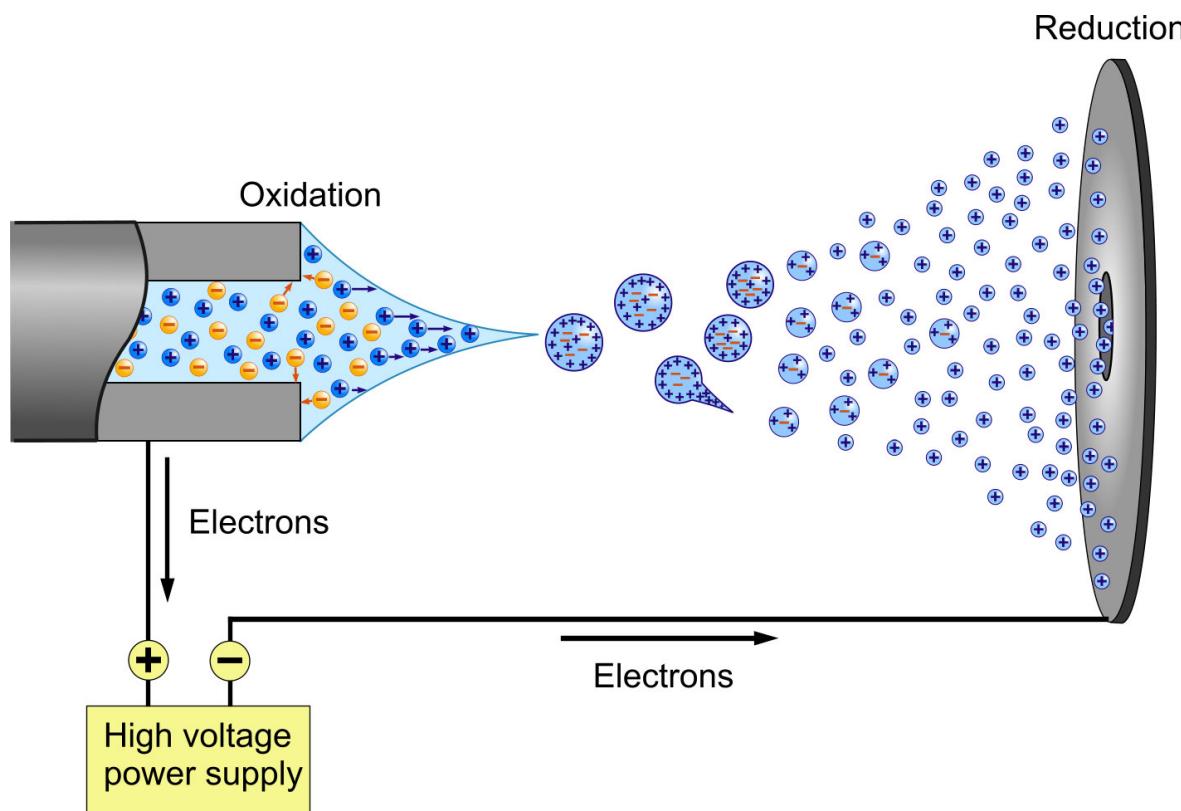


Direct Injection Method



- Simple configuration
- Minimal Sample Volume (“no loss” of sample)
- Can be good for very low abundance or problematic samples with trap and elute

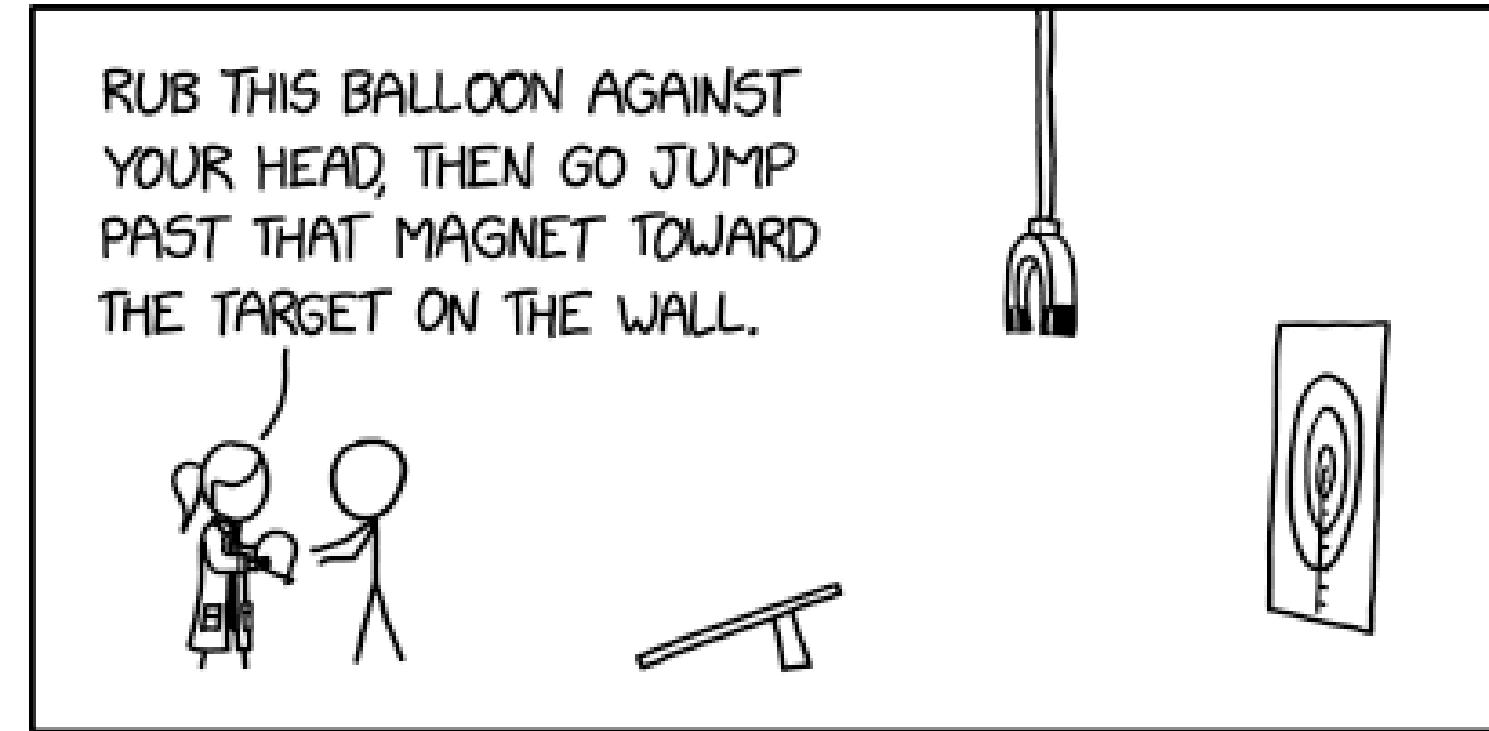
Ionization



- Peptide must be charged and in gas phase
- Nano ESI: **high voltage applied to a liquid at the tip of a capillary, producing a fine spray of charged droplets.**
- As solvent evaporates, **Coulombic repulsion** causes the droplets to shrink and eventually release **ionized peptides** into the gas phase



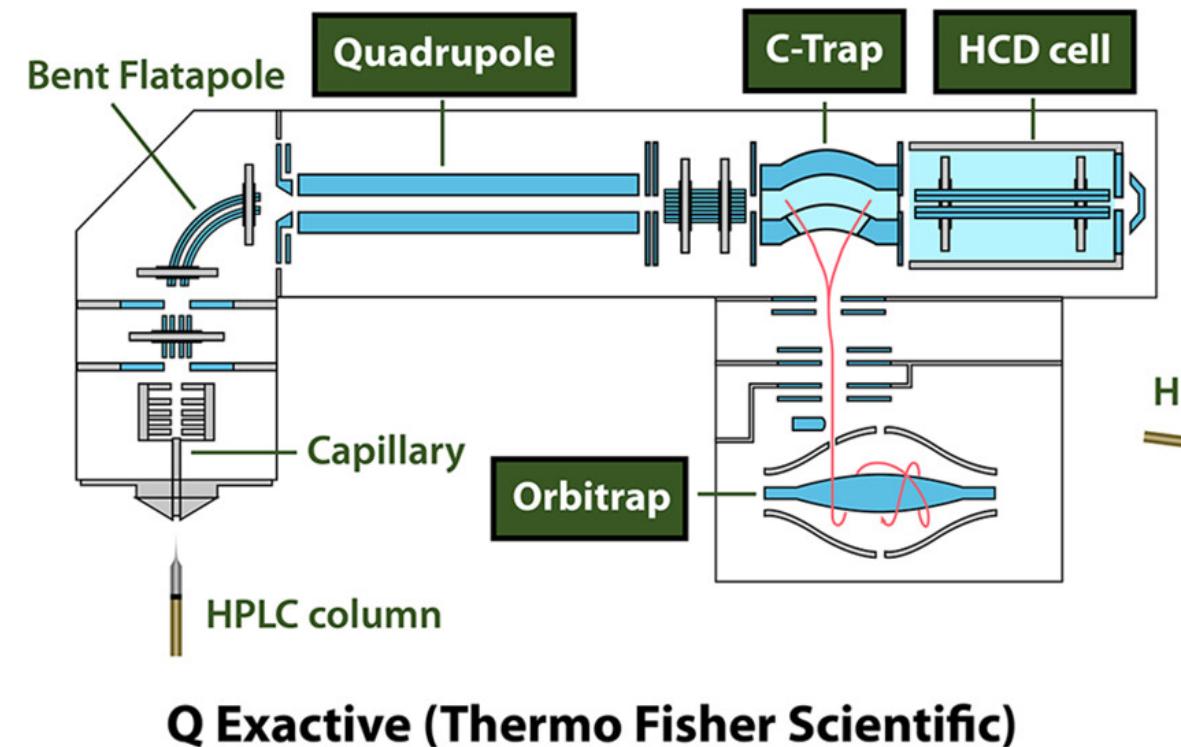
Mass Spectrometer



BEFORE THE BATHROOM SCALE WAS INVENTED, THE ONLY WAY TO WEIGH PEOPLE WAS MASS SPECTROMETRY.

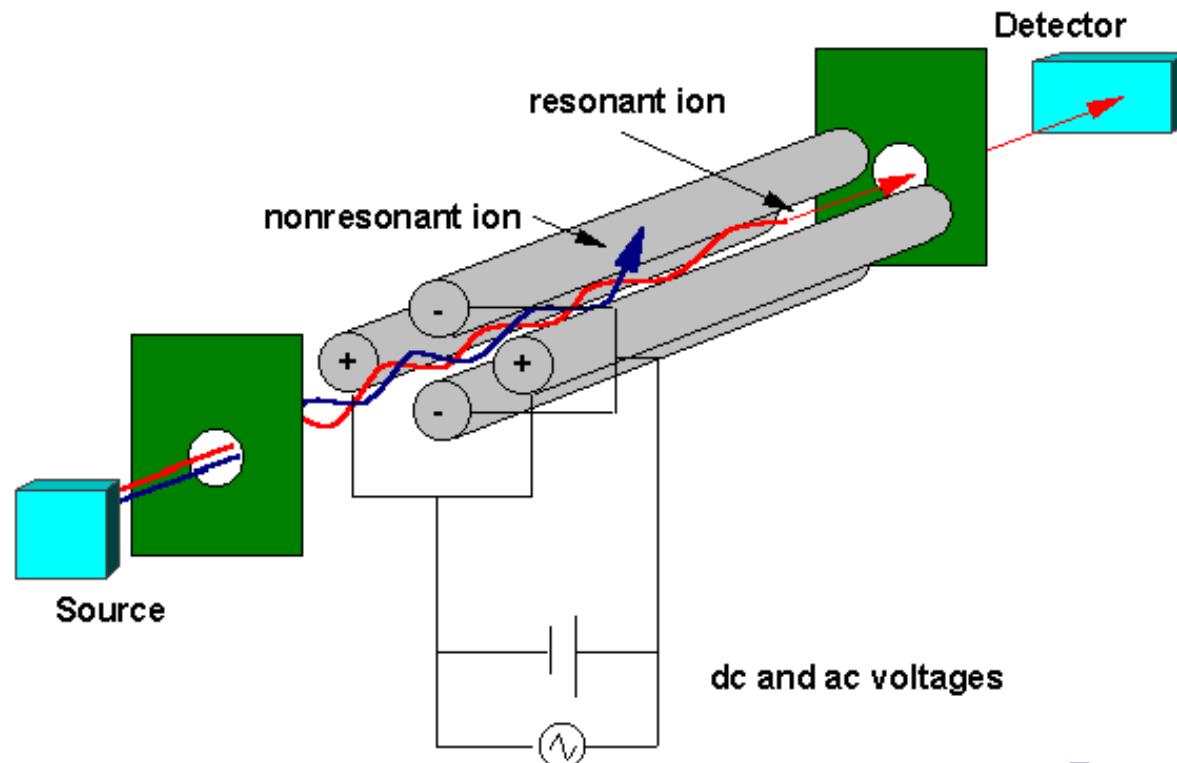
Mass Spectrometer

- Analyze Molecules by moving them through electric fields
- Actually measuring m/z , not mass
- Need to be in gas phase (can't collide with other molecules)
- Many different ways to move ions around



Basic principle of quadrupoles

- Quadrupoles are m/z filters
 - For a given DC and RF voltage only ions with a particular m/z are stable



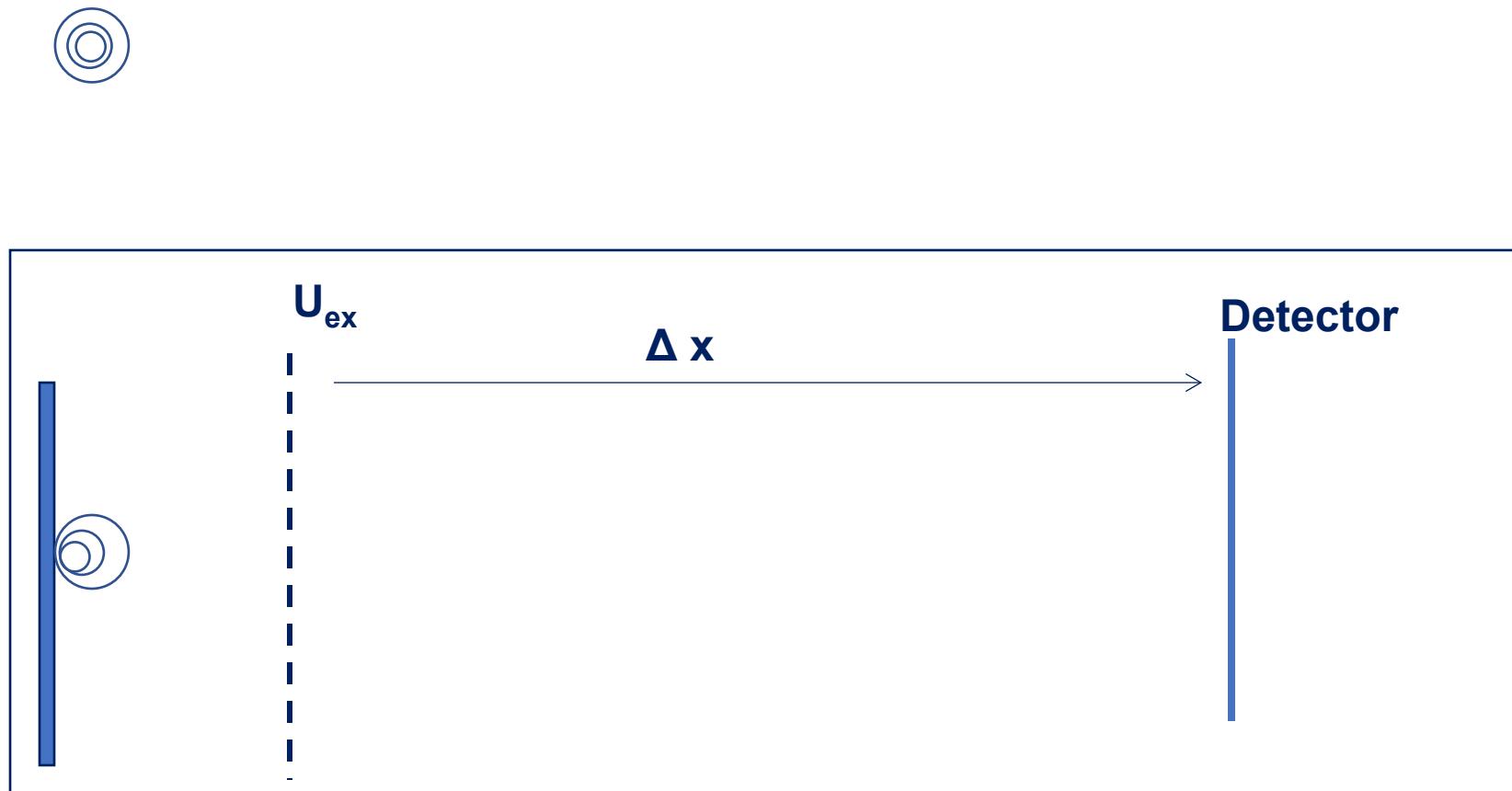
From Dr. Kenny Lee's Mass Spec course

Ion Trap

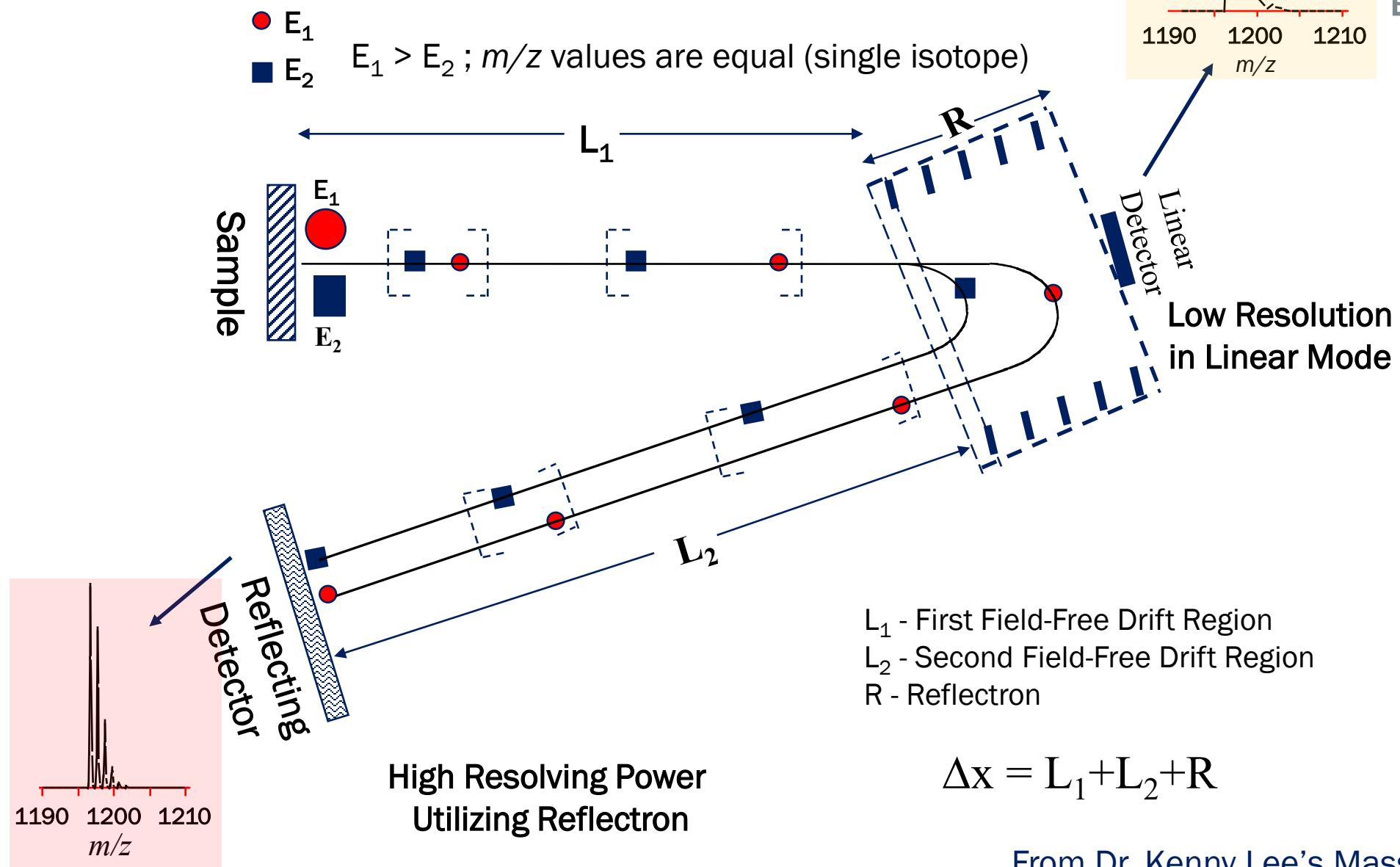


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Time of Flight (TOF)



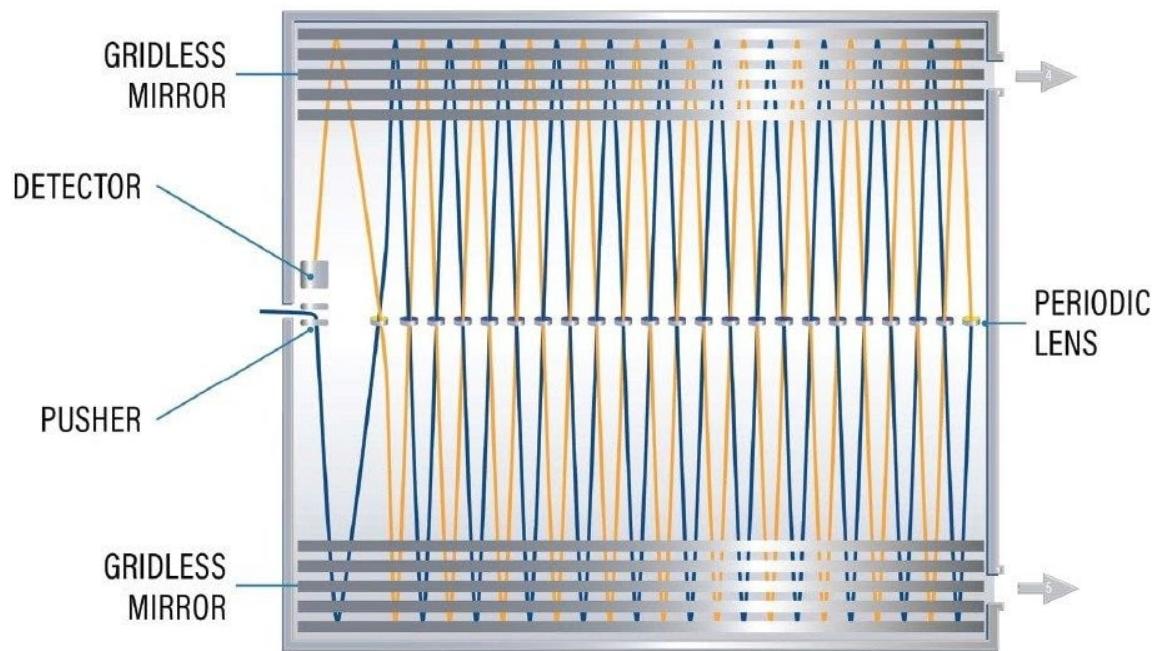
Reflectron or ion mirror



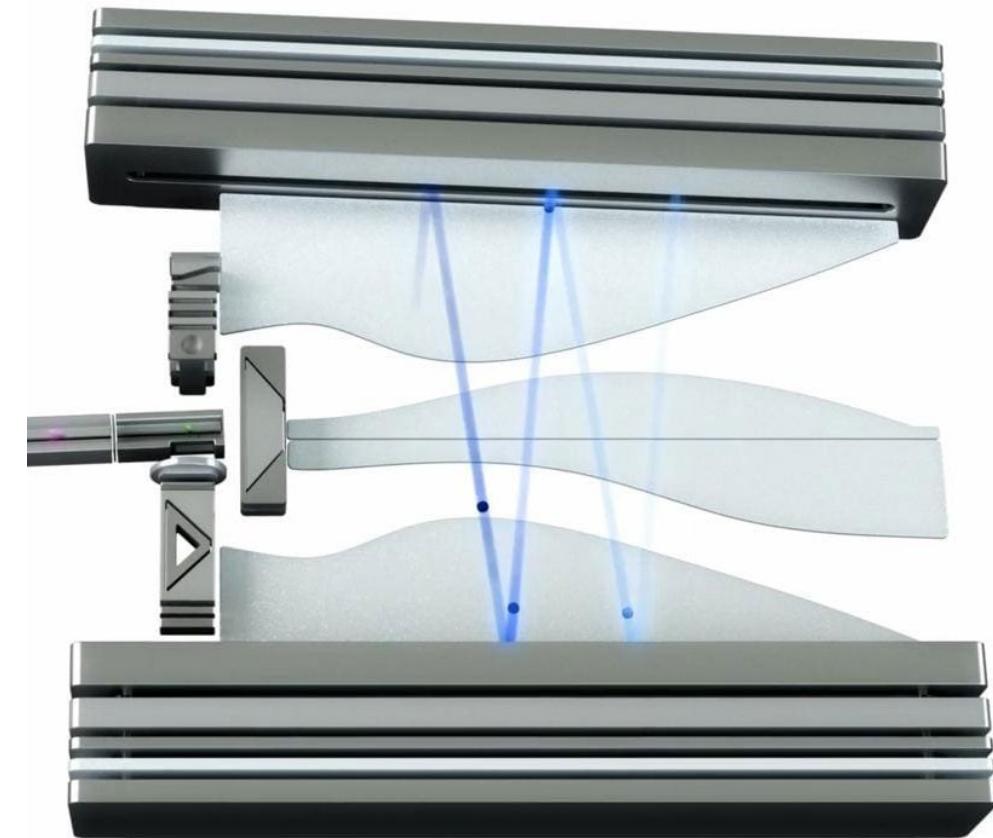
$$\Delta x = L_1 + L_2 + R$$

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Multi-Reflectron Designs



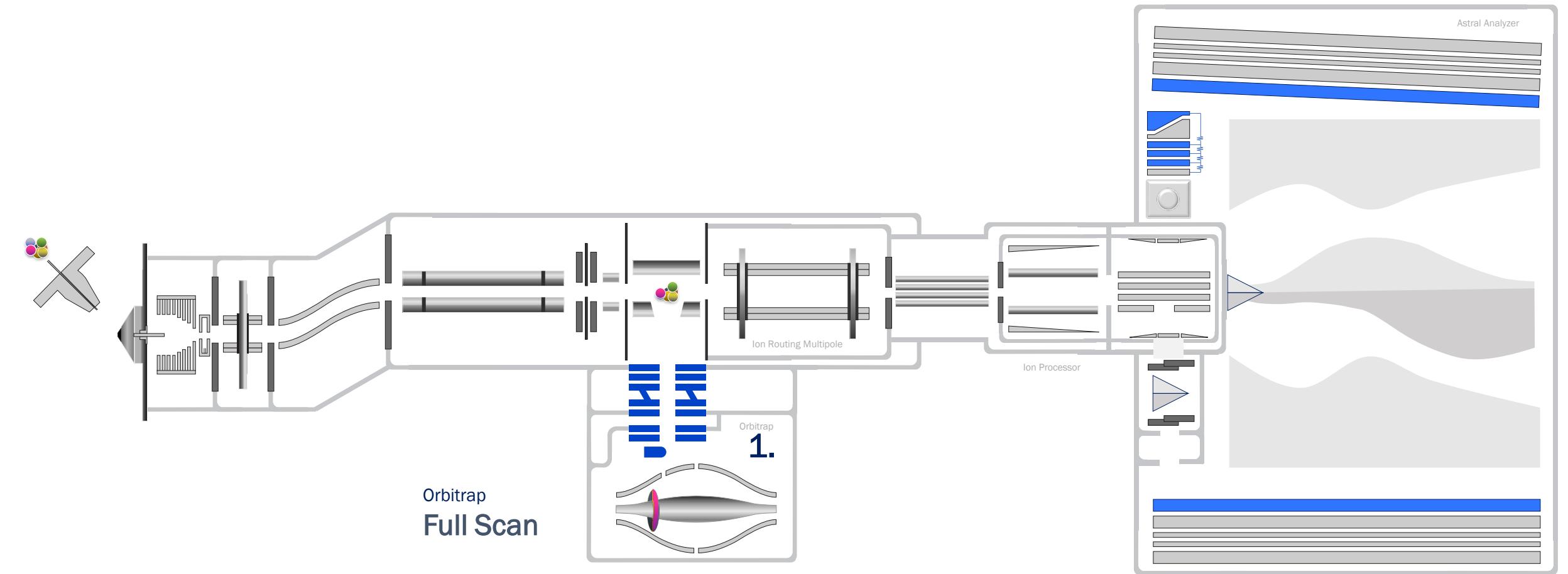
Waters MRT



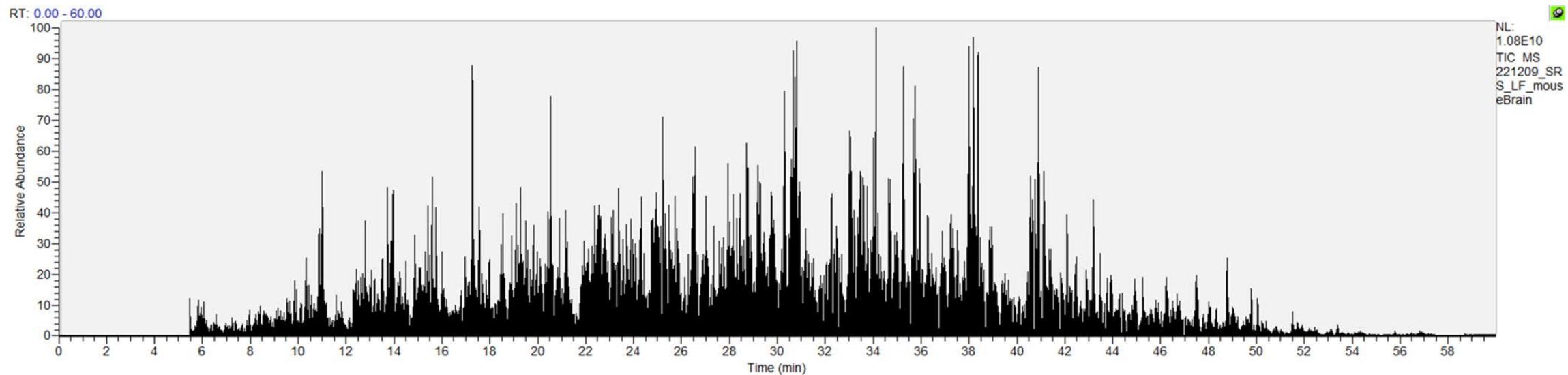
Thermo Astral

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Orbitrap

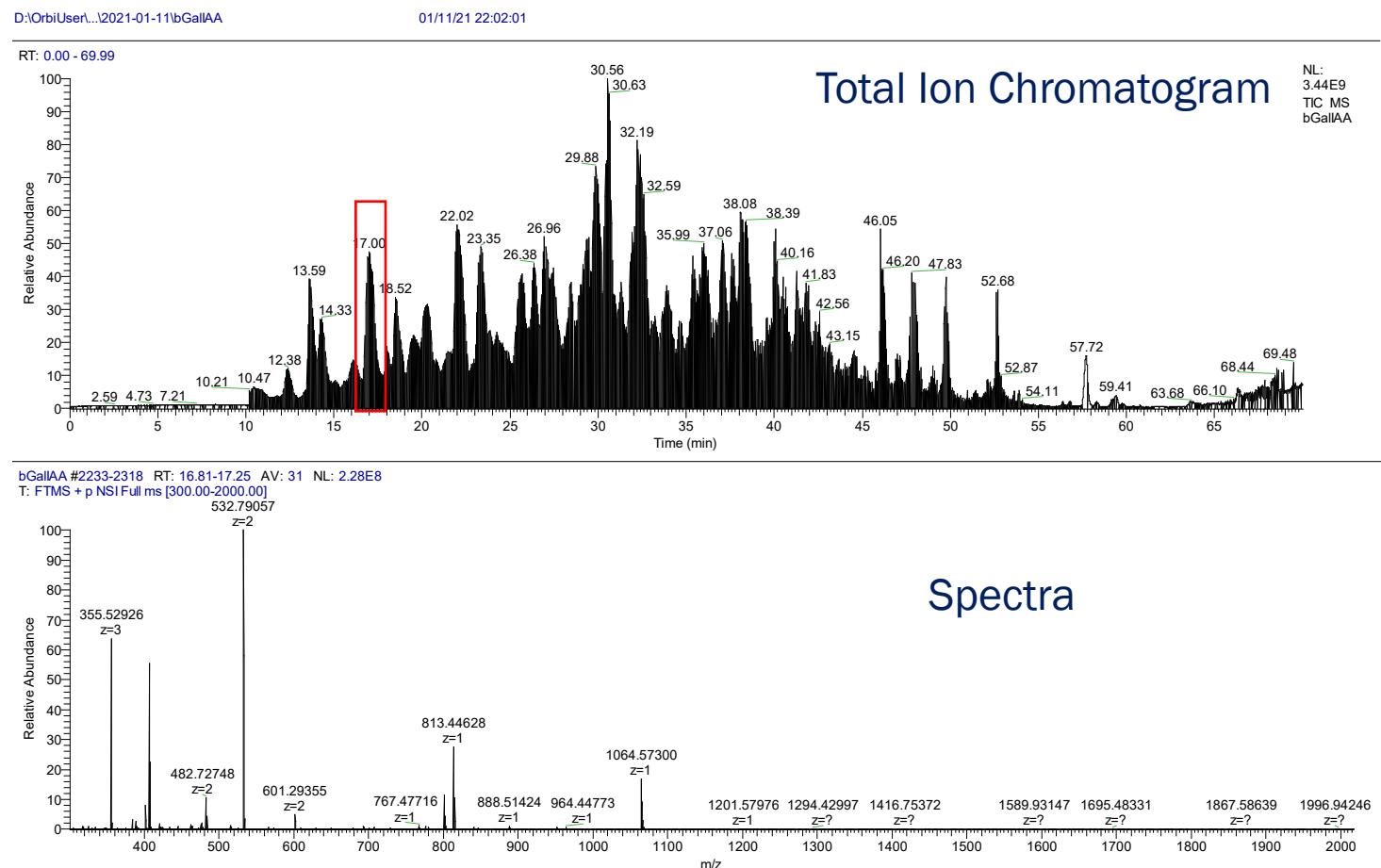


MS1 Scan

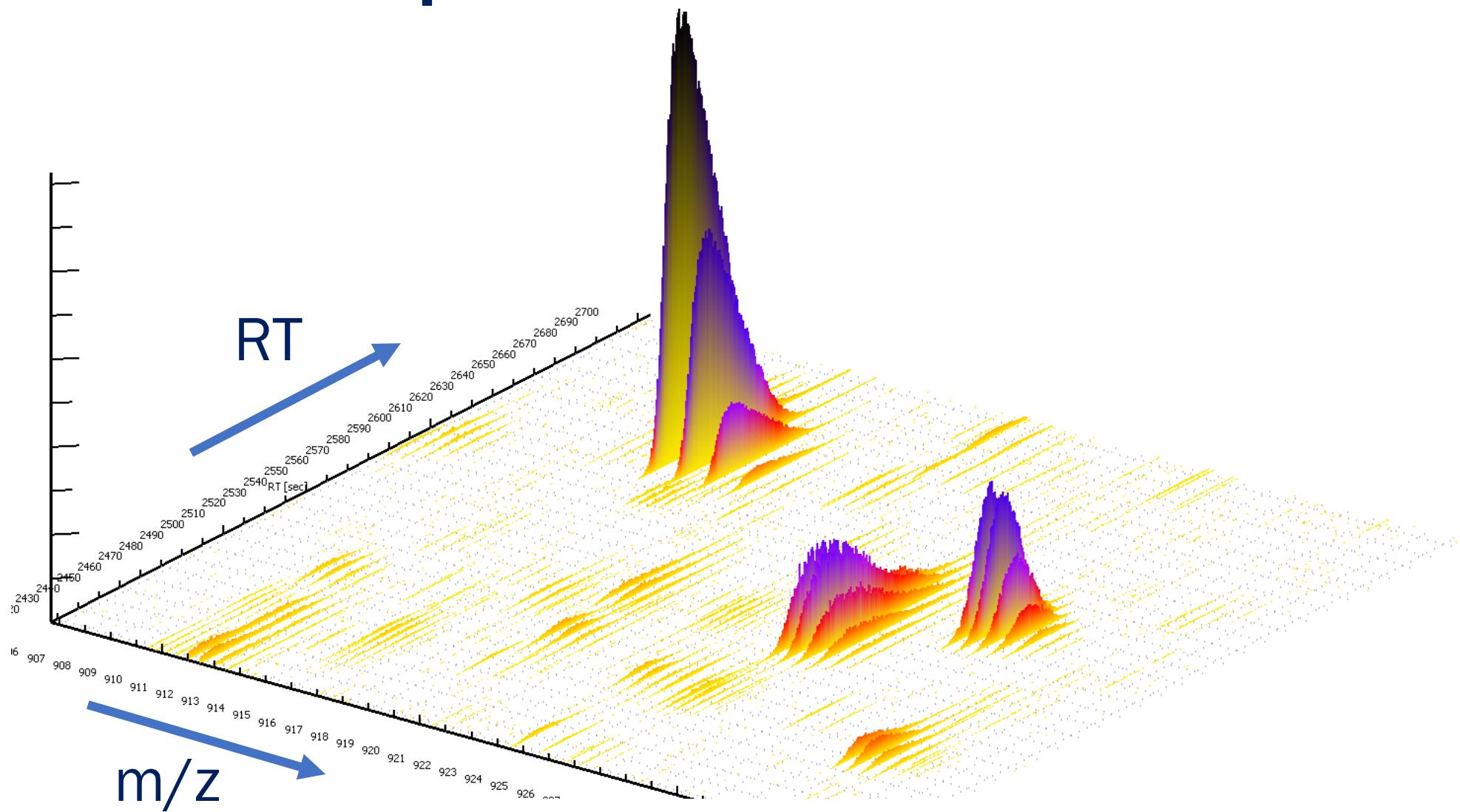


Chromatogram and Spectra

- Chromatogram
 - Sum of intensities vs Elution time
- Spectra
 - m/z present at a given time

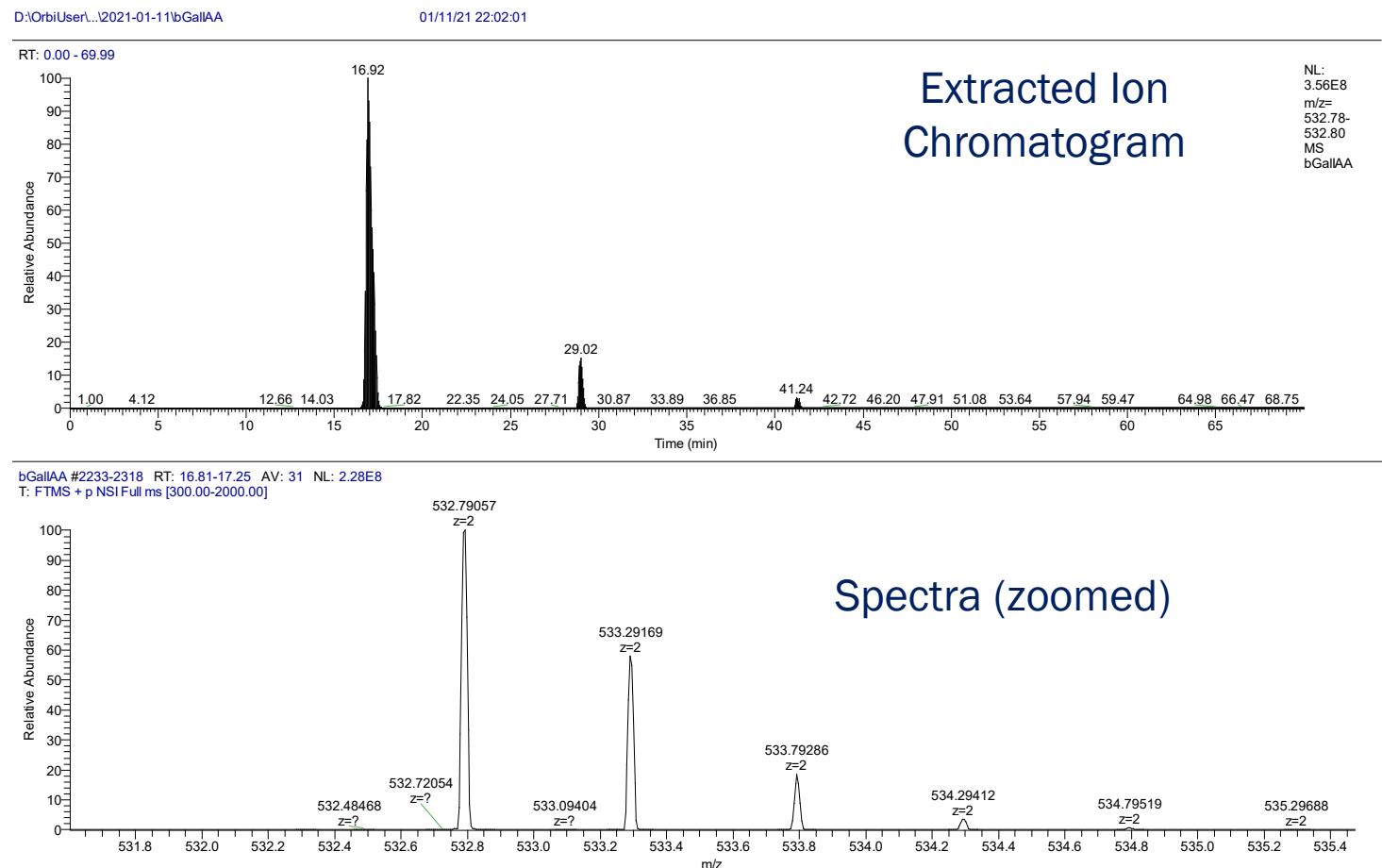


Data is 3-D plot

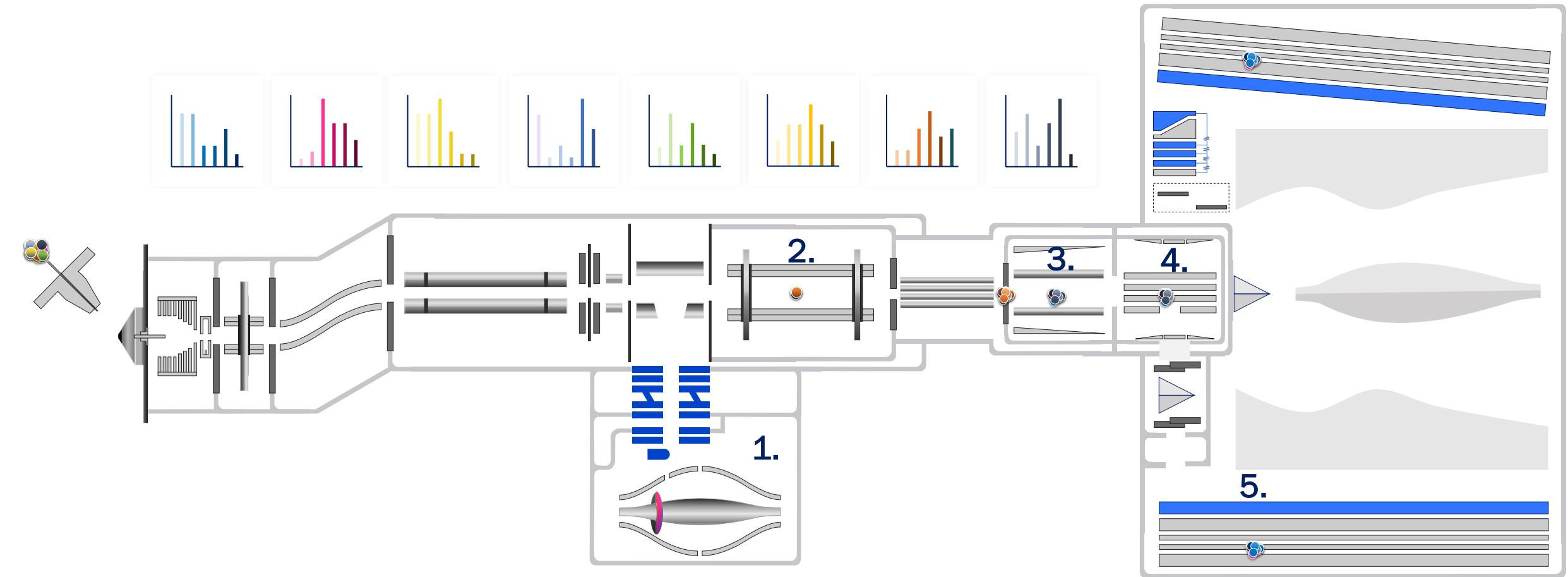


Chromatogram and Spectra

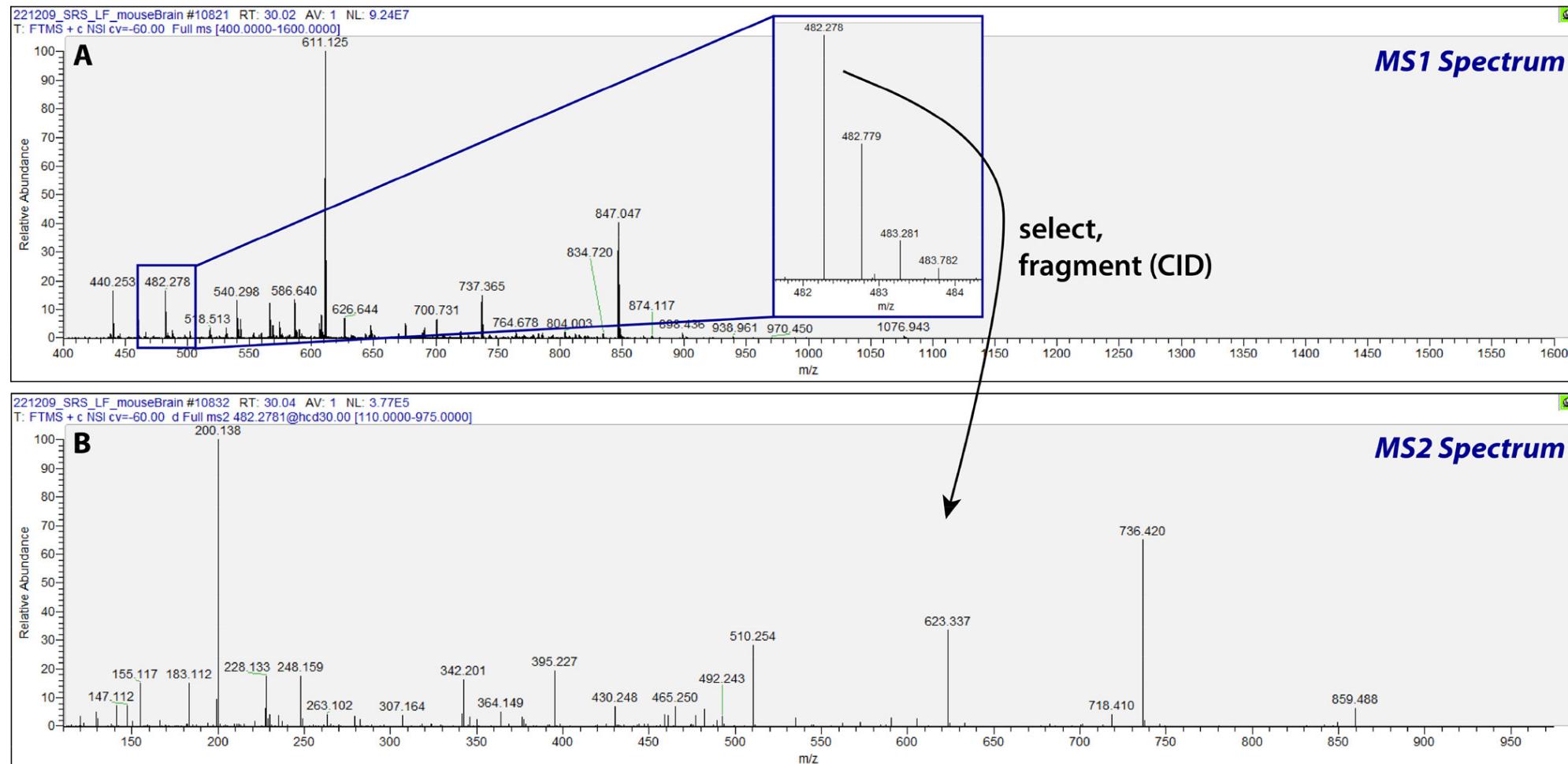
- Chromatogram
 - Sum of intensities vs Elution time
- Spectra
 - m/z present at a given time
 - Isotopic Peaks
 - Tryptic peptides are multiply charged species



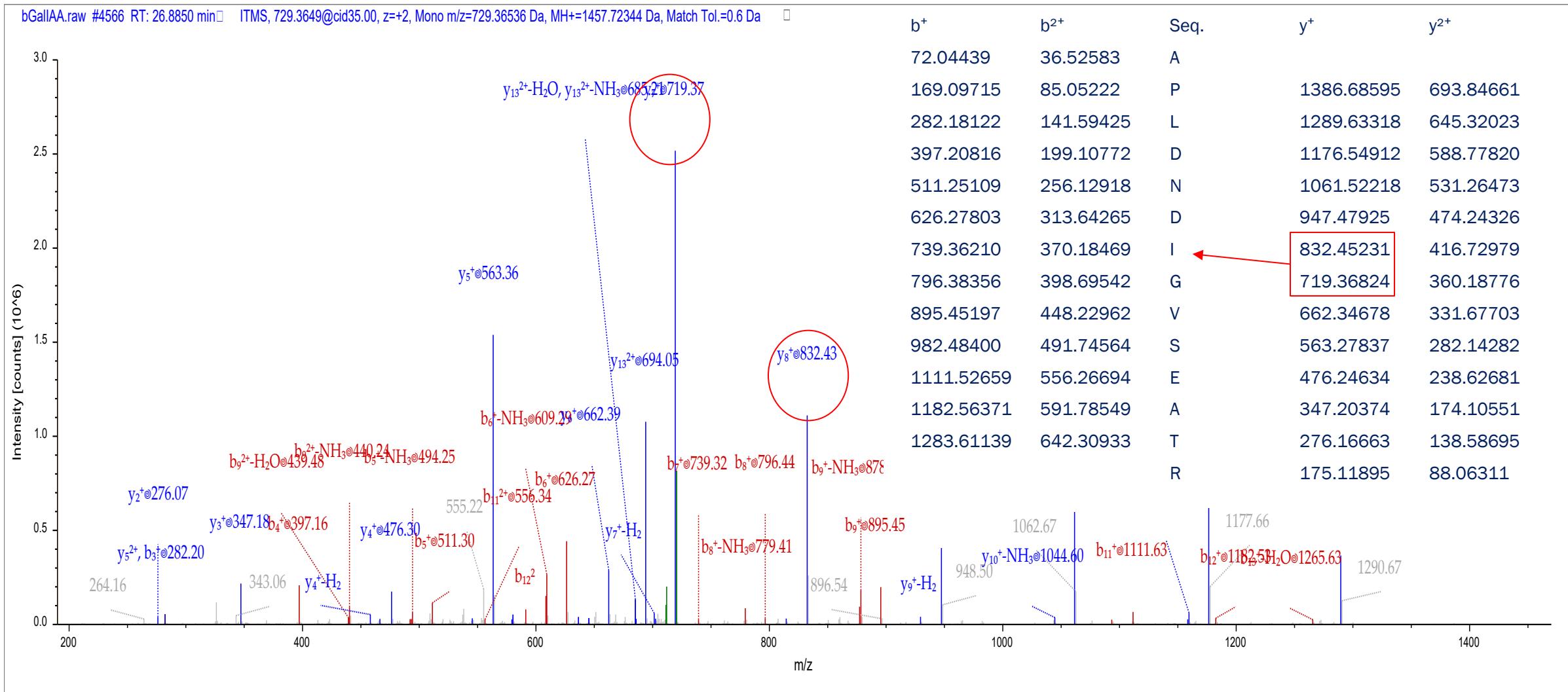
MS2 Scan



Example of MS2 spectrum



MS2: Sequencing Information



Collision-Induced Dissociation (CID)

- Ions are accelerated and collide with inert gas molecules (He or N₂) causing them to fragment
- Peptides fragment at peptide bonds generating b-ions (N-terminal half) and y-ions (C-terminal half)
- Usually performed in linear ion trap or quadrupole ion trap

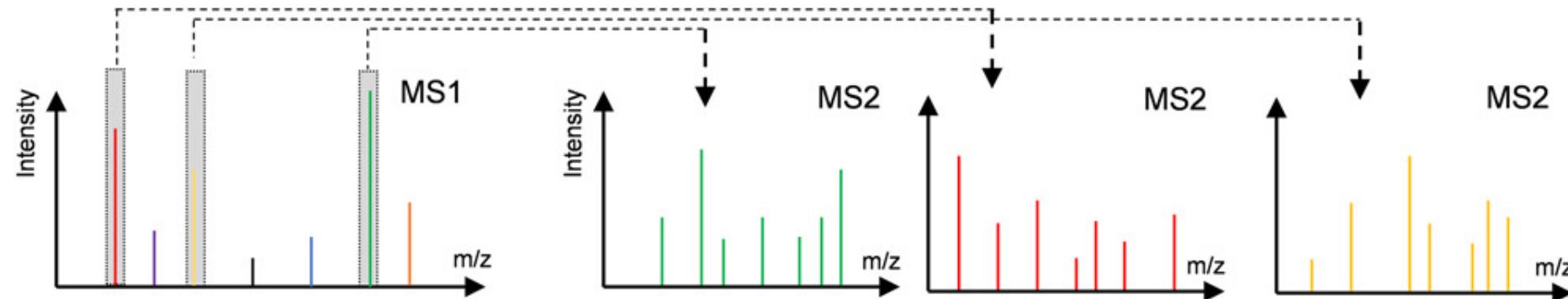
Higher-Energy Collisional Dissociation

- Orbitrap specific
- Beam-type collision cell rather than trapped-ion collision cell
- Produces b and y ions but with more extensive fragmentation

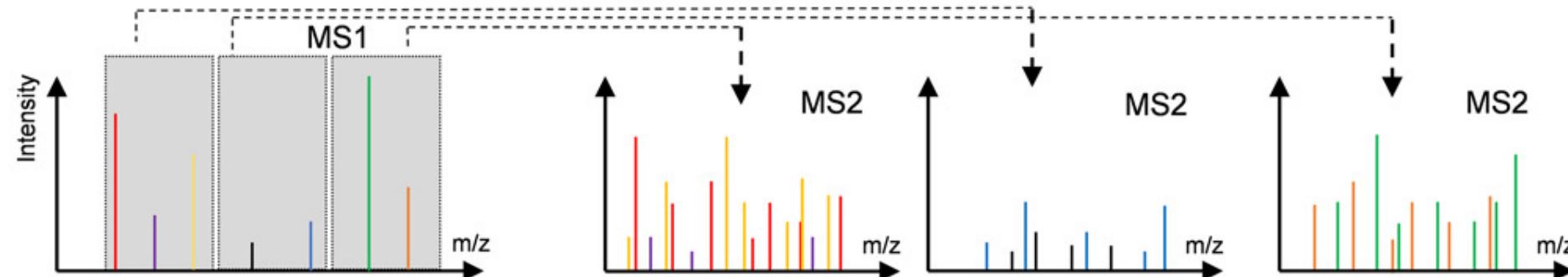
Feature	CID	HCD
Energy	Low to moderate	High
Location	Ion trap	Beam-type cell (Orbitrap)
Ion Types	b- and y-ions	b- and y-ions (more extensive)
Resolution	Often low	High (Orbitrap)
Best For	General peptide ID	Quantitative workflows, PTMs

Data-Dependent vs Data-Independent

DDA (isolation of top 3 ions)



DIA (isolation of 3 wide mass windows)



DDA Overview

- MS1 data determines what is fragmented
- Top N protein, so probe most abundant protein first
- MS2 spectra are only from one peptide (“easy” to interpret)
- Quant happens on the MS1

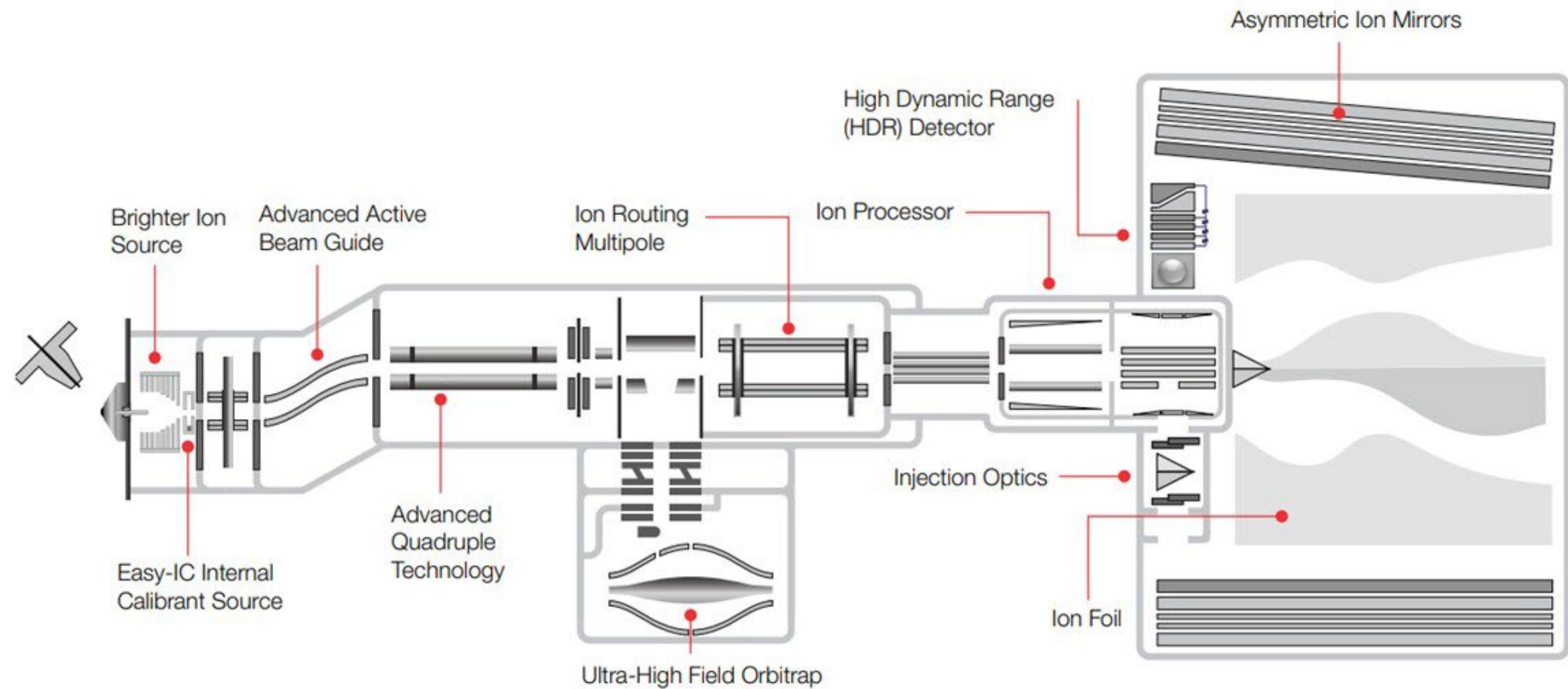
DIA Overview

- m/z windows defined and instrument fragments at systematic windows, number of windows determined by instrument speed
- Need a fast instrument so you can have narrow enough windows to keep MS2 spectra “deconvolutable”
- Quant happens on the MS2
- Chromatography and cycle time need to be correlated to get good quant

DDA vs DIA

Feature	DDA (Data-Dependent Acquisition)	DIA (Data-Independent Acquisition)
MS2 Trigger	Based on MS1 intensity (top N peaks)	Systematically fragments all ions in wide m/z windows
Selection	Stochastic (varies run to run)	Deterministic (same ions always fragmented)
Coverage	Biased toward abundant peptides	More complete, including low-abundance peptides
Quantification	Less consistent across runs	High reproducibility and data completeness
Spectra	Clean, easy to interpret	Complex, requires deconvolution
Feature	DDA (Data-Dependent Acquisition)	DIA (Data-Independent Acquisition)
MS2 Trigger	Based on MS1 intensity (top N peaks)	Systematically fragments all ions in wide m/z windows

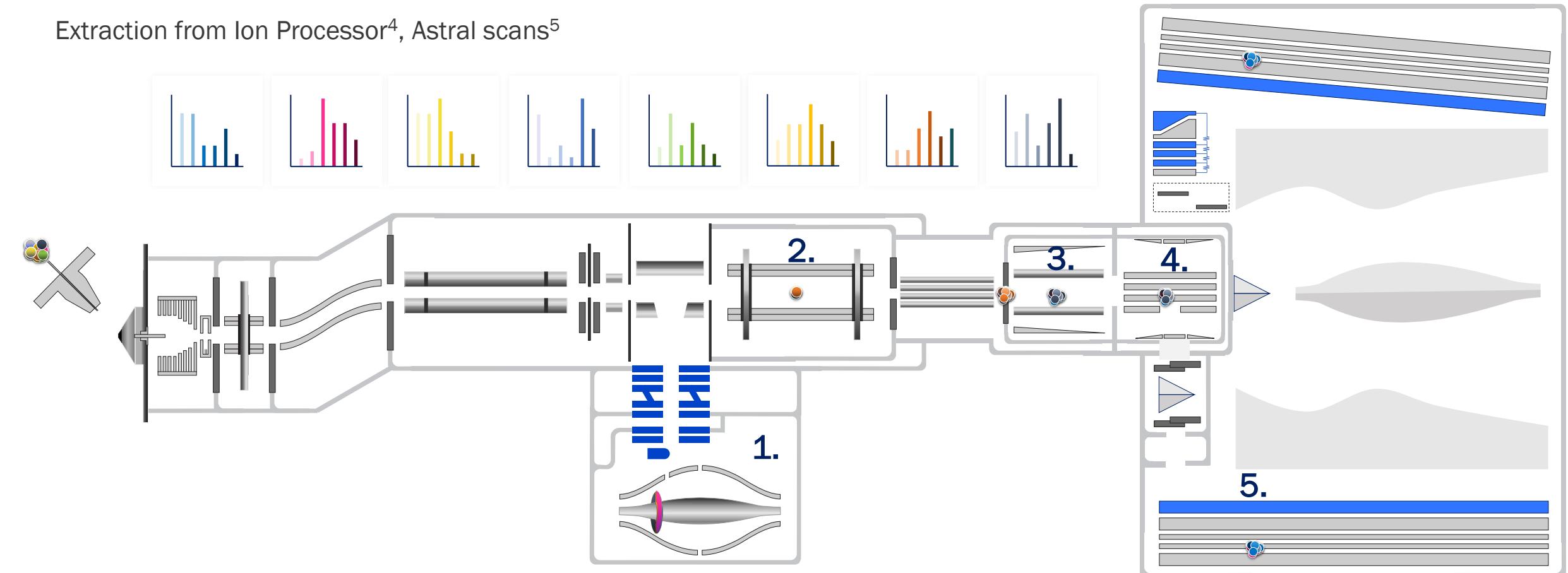
Orbitrap Astral Overview



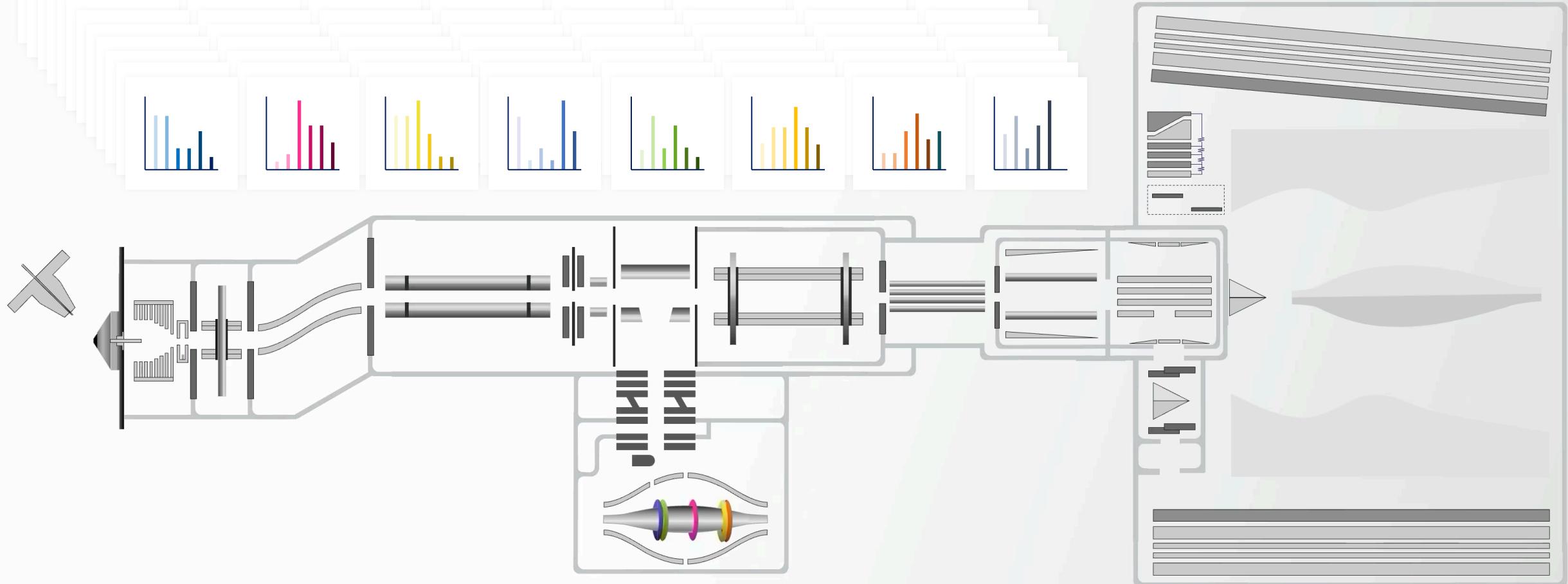
Data Independent Acquisition (DIA) or Data-dependent Acquisition (DDA)

Orbitrap scan¹, Quad isolation → IRM concentration², Trap/fragment in Ion Processor³,

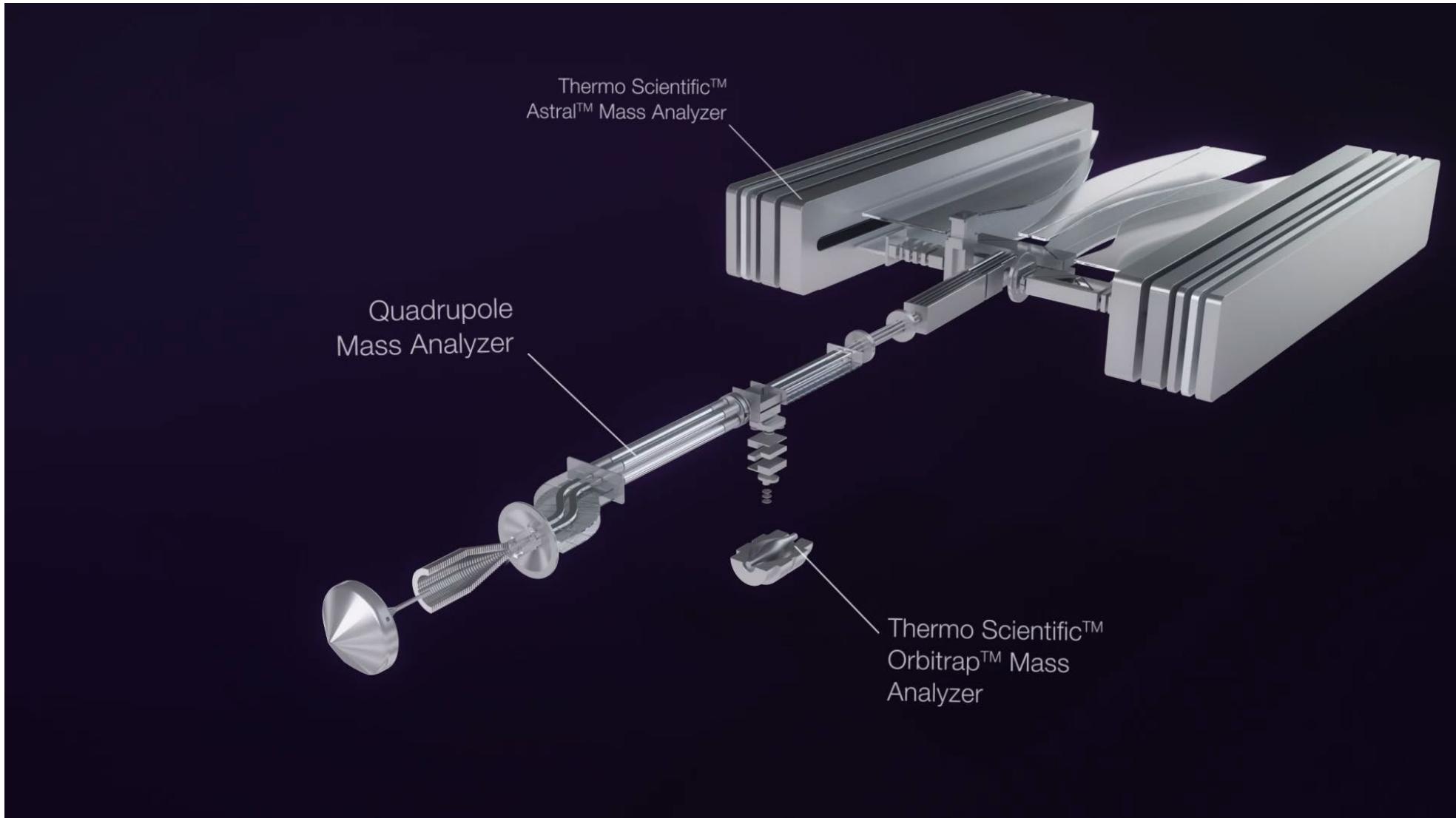
Extraction from Ion Processor⁴, Astral scans⁵



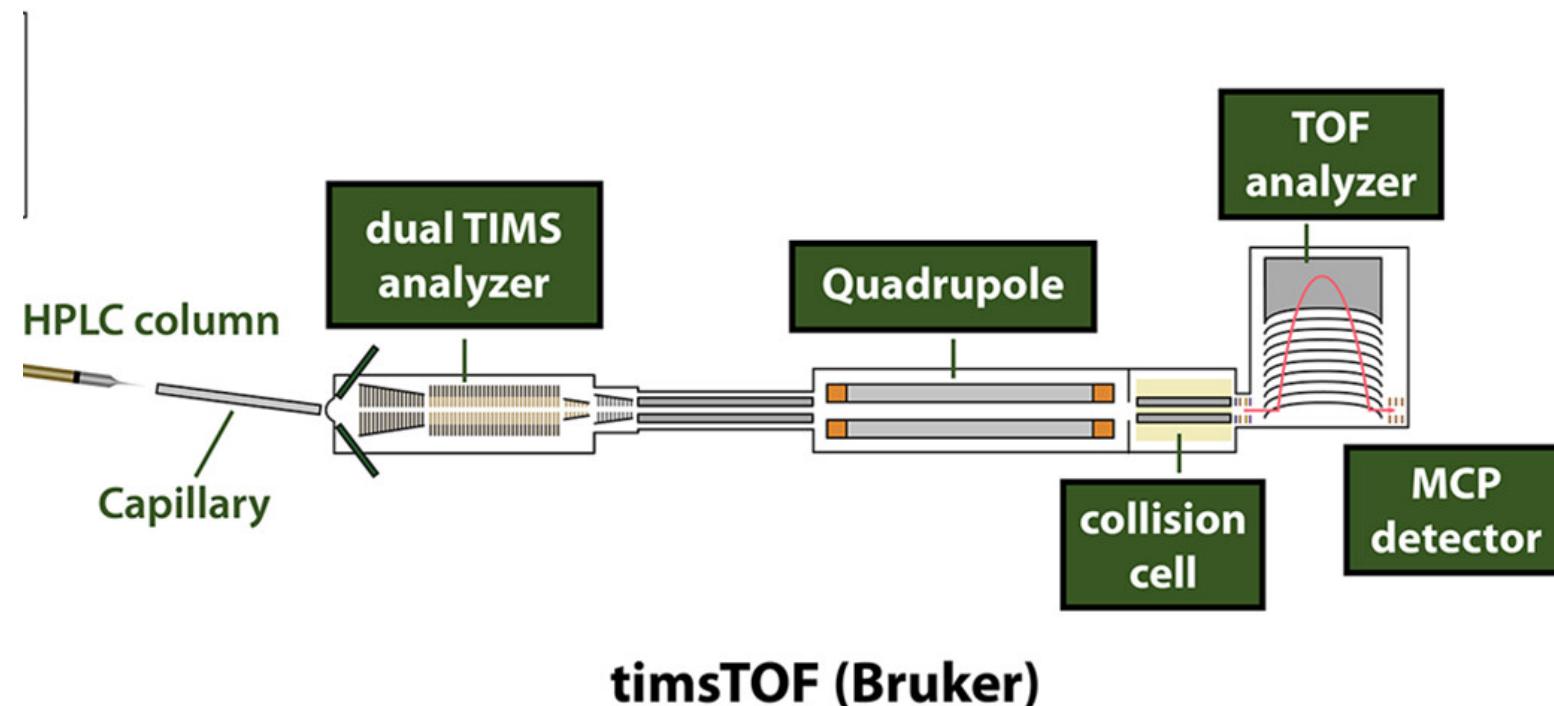
Astral acquisition at scan rates up to **200 Hz**



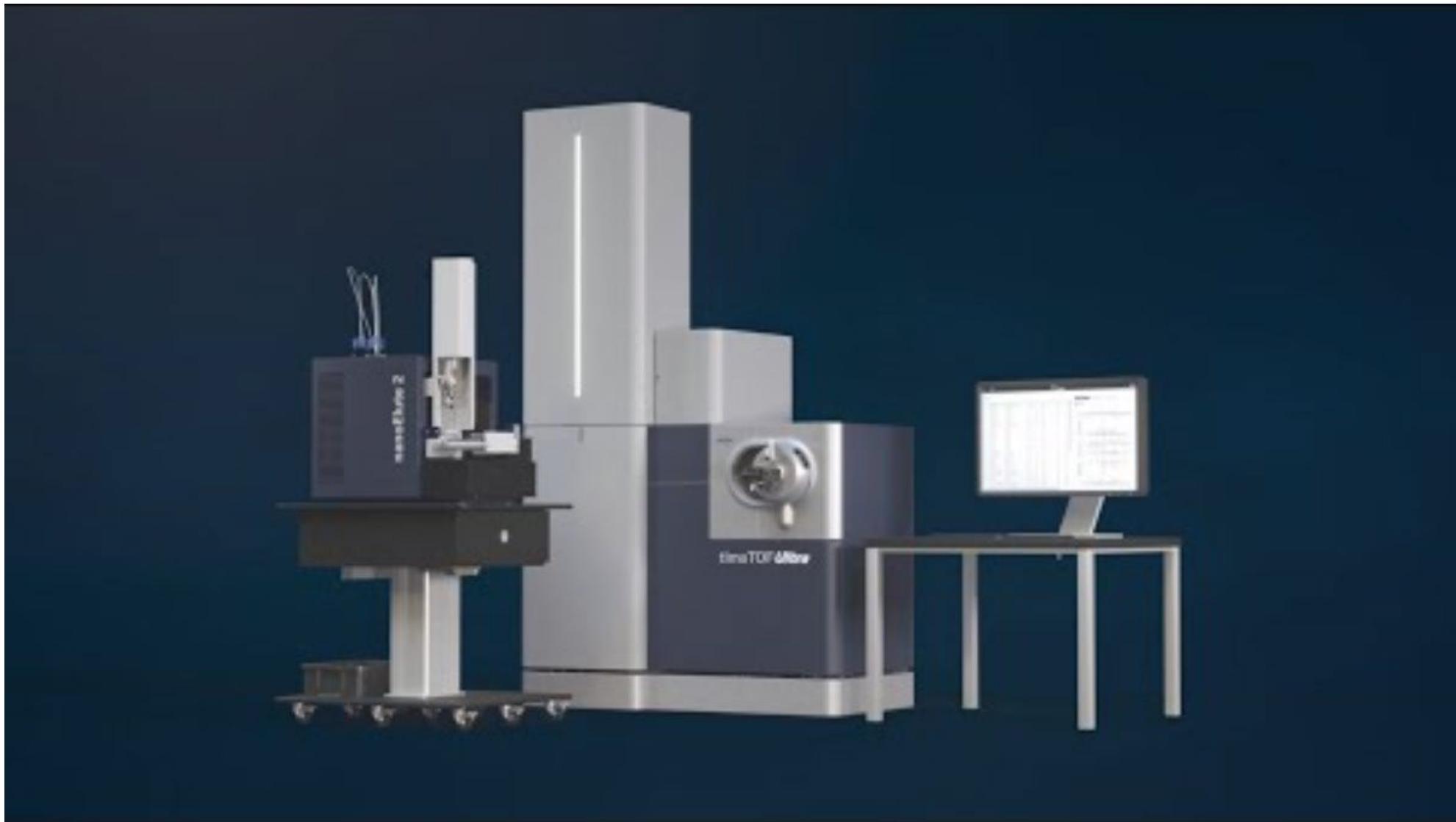
Orbitrap Astral Overview



Bruker TIMS TOF Overview



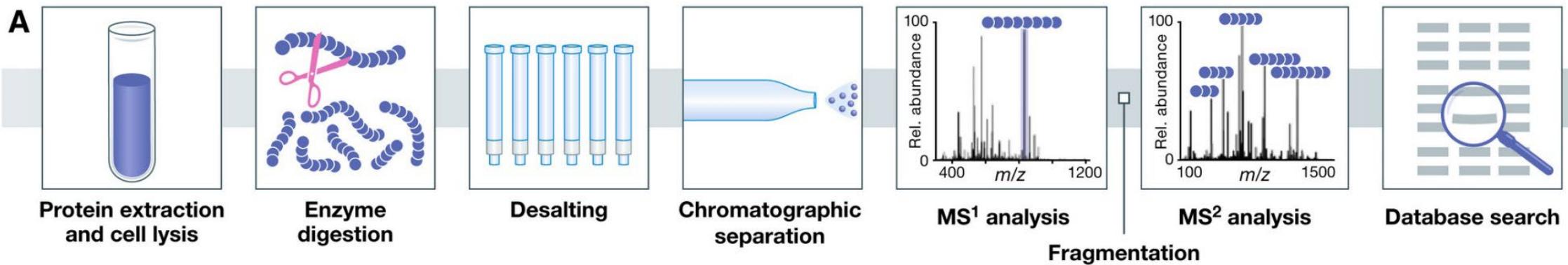
Bruker TIMS TOF Overview



Orbitrap Astral vs Bruker timsTOF: Instrument Comparison

Feature	Orbitrap Astral	Bruker timsTOF
Mass Analyzers	Orbitrap + Astral (hybrid)	Trapped Ion Mobility + TOF
Scan Speed	Up to 200 Hz (270 Hz Zoom)	Up to 100 Hz (PASEF)
Ion Mobility	No (FAIMS optional)	Yes (TIMS)
Resolution	Up to 1M	High resolution + ion mobility
Sensitivity	Single ion detection	High sensitivity with TIMS
Dynamic Range	>5 orders of magnitude	~4–5 orders of magnitude
DIA Capability	HR-DIA with high scan rate	diaPASEF with ion mobility
Throughput	100–300 samples/day	100–200 samples/day
Best For	Deep coverage, single-cell, TMT	High-speed DDA/DIA, PTMs

Putting it all Together



Key takeaways

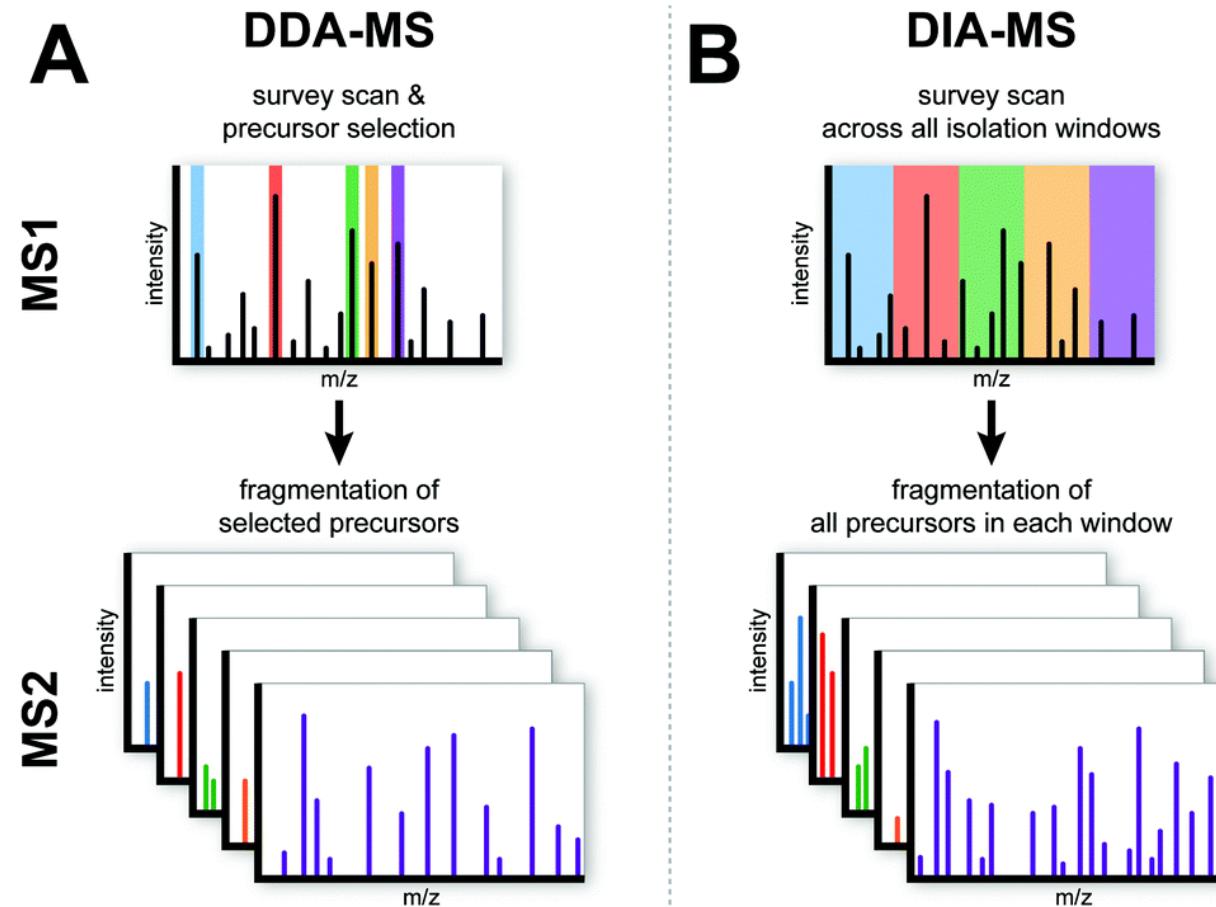
- LC separates peptides by hydrophobicity using C18 columns and gradient elution.
- Electrospray ionization (ESI) is essential to transfer peptides into the gas phase for MS analysis.
- MS1 scans detect intact peptide ions; MS2 scans fragment them to reveal sequence information.
- Fragmentation methods like CID and HCD generate b/y ions for peptide identification.
- Orbitrap Astral offers high resolution and scan speed; timsTOF adds ion mobility for enhanced separation.
- DDA selects top N precursors for MS2; DIA fragments all ions in wide m/z windows for comprehensive coverage.
- Understanding these principles enables confident peptide identification and quantification in bottom-up proteomics.

Acknowledgements and Resources

- Steven R. Shuker. *Journal of Proteome Research* 2023 22 (7), 2151-2171. DOI: 10.1021/acs.jproteome.2c00838
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- Bruker Corporation: <https://www.bruker.com/en/products-and-solutions/mass-spectrometry/tims-tof/timsultra-aip.html>
- Matthew A. Lauber, Stephan M. Koza, Kenneth J. Fountain. Waters Application note: 720005047, May 2014
- Dr Kenny Lee Mass Spec Course (CHEM 629R BYU)

Questions?

Data-Dependent vs Data-Independent





PASEF

1. Ion Accumulation:

- Ions are accumulated in the **first region** of the dual TIMS analyzer.
- This happens in parallel with the analysis of previously accumulated ions.

2. Ion Mobility Separation:

- Ions are separated based on their **mobility** (size, shape, and charge) in the **second region** of the TIMS device.
- This adds an **orthogonal dimension** of separation to m/z.

3. Serial Fragmentation:

- As ions elute from the TIMS device, they are **synchronously selected** and fragmented in rapid succession.
- Multiple precursors can be fragmented **within a single TIMS scan**



MS2 Scan

- MS2 (tandem mass spectrometry) involves selecting a specific precursor ion from the MS1 scan and fragmenting it to generate product ions
- Provides sequence-specific fragment ions that are used to identify amino acid sequence of peptides
- Fragment ion intensities can be used for quantification in methods like TMT or DIA
- Quadrupole mass analyzer used for Isolation of precursor ions
- Fragmentation occurs in a collision cell and then measured in downstream mass analyzer