THE OHIO STATE UNIVERSITY
COLLEGE OF MEDICINE

Designing DIA Experiments for Phosphoproteomics

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@briansearle

Speaker Disclosures
Brian C. Searle

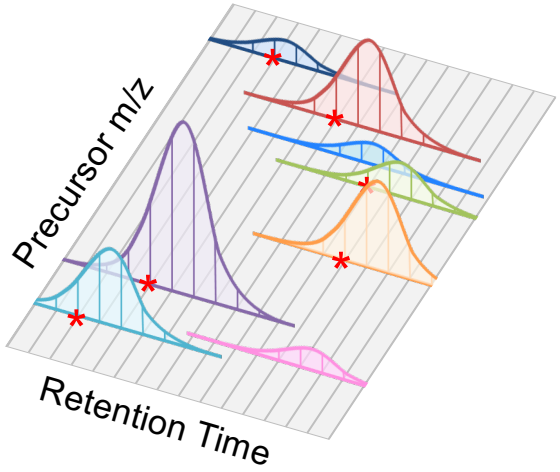
Relevant financial relationships:

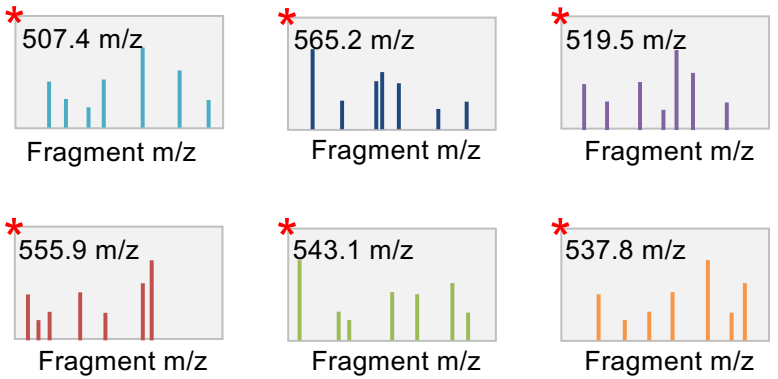
- Employed as an Assistant Professor at the Ohio State University

- Founder and shareholder at Proteome Software

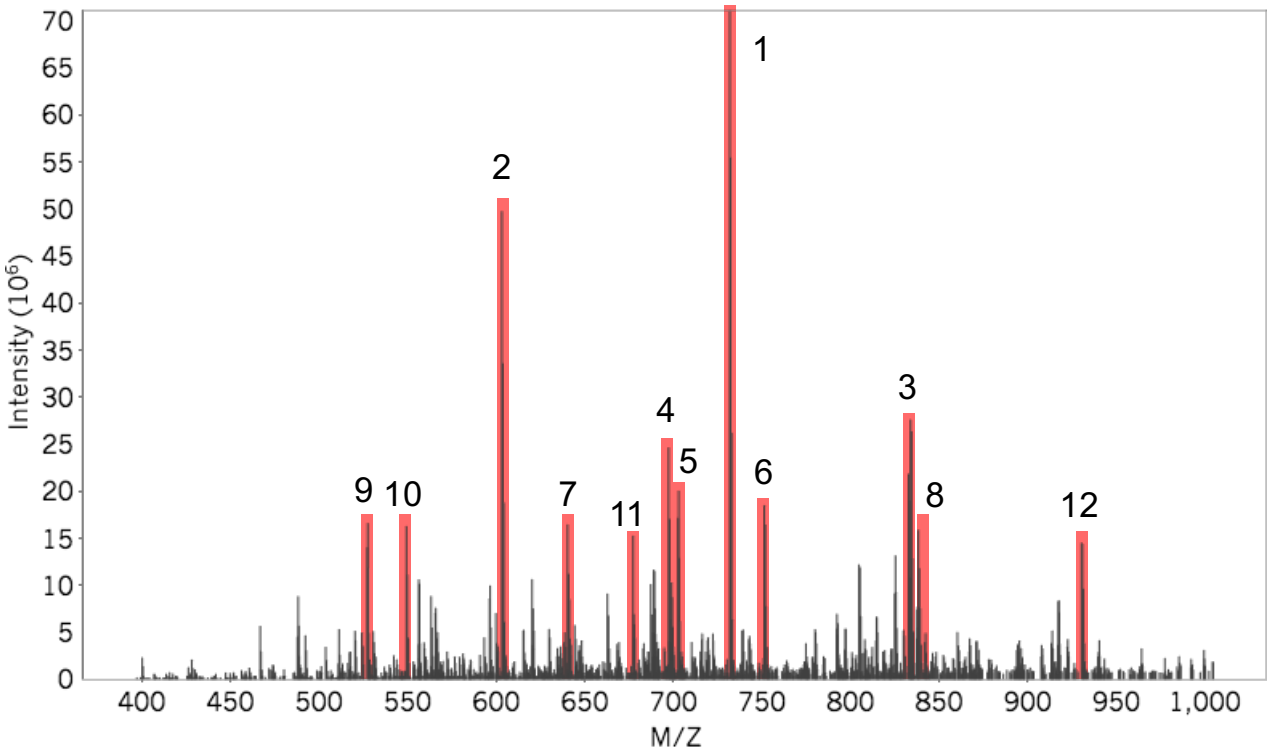
Research funding sources for this work:

DDA:

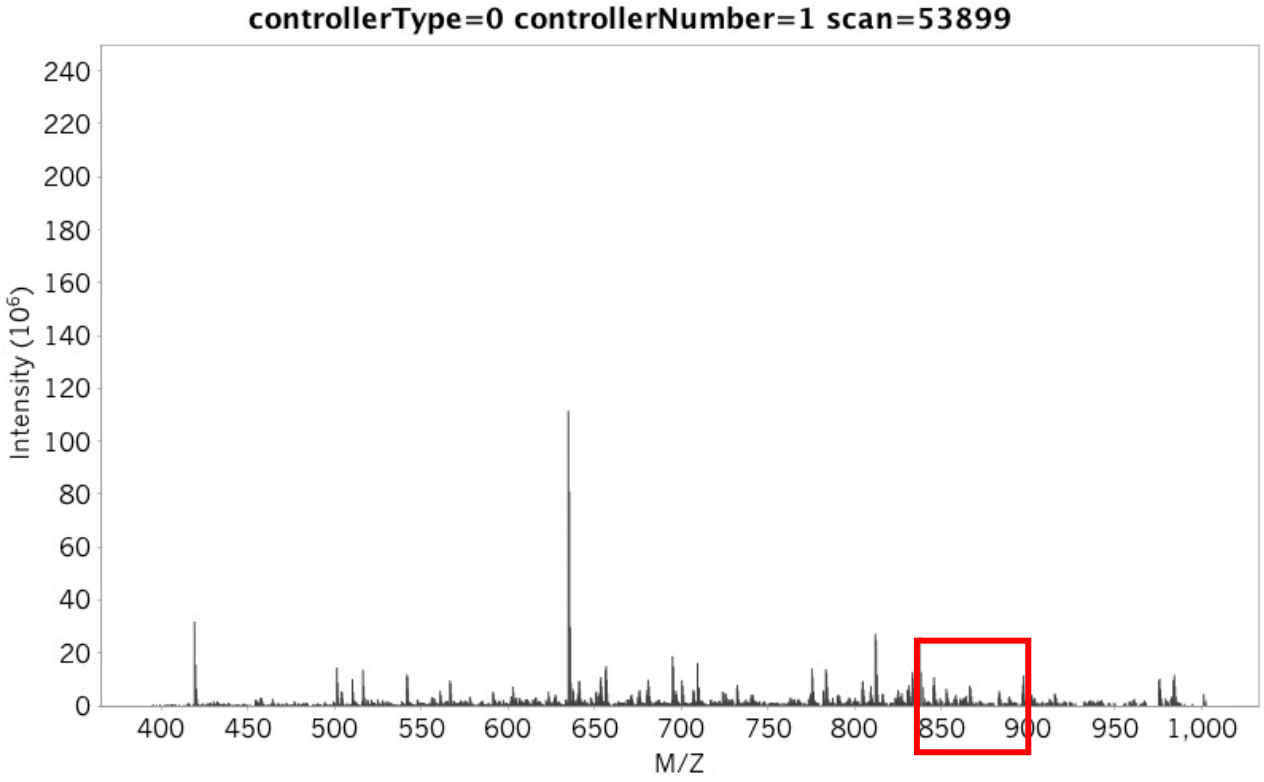

Precursor m/z
Retention Time


507.4 m/z
Fragment m/z
565.2 m/z
Fragment m/z
519.5 m/z
Fragment m/z
555.9 m/z
Fragment m/z
543.1 m/z
Fragment m/z
537.8 m/z
Fragment m/z

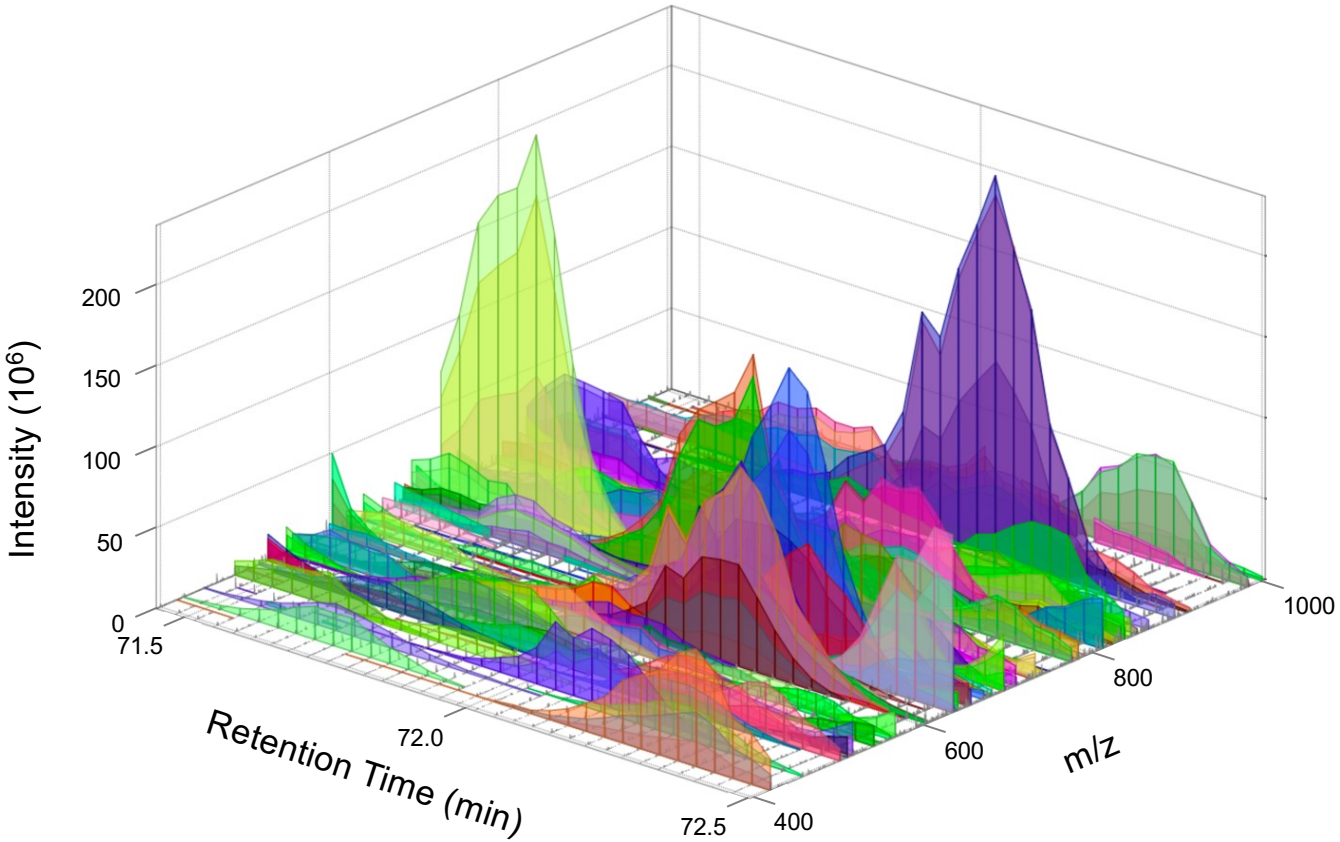
The instrument only has so much time before the next batch of peptides

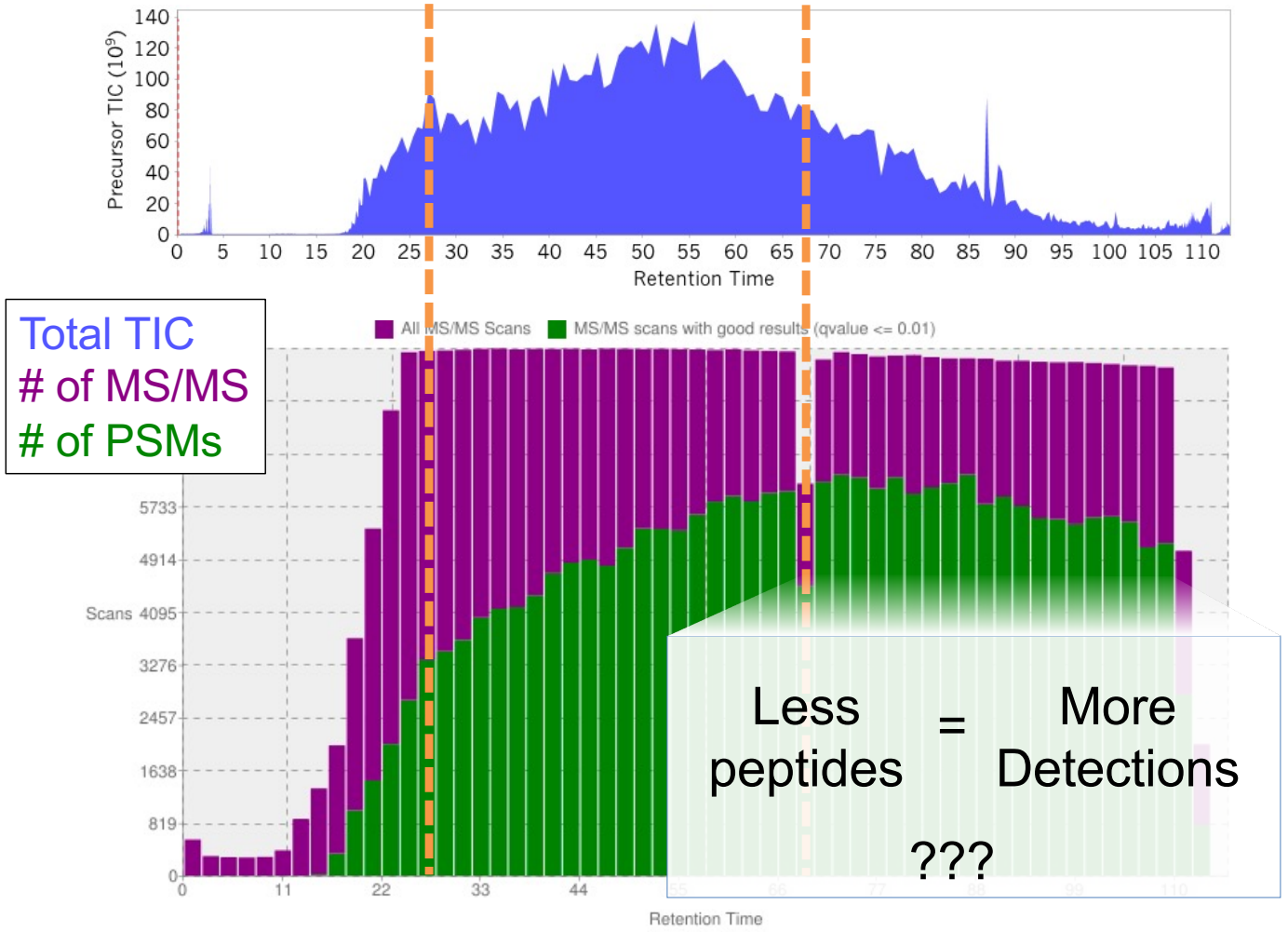


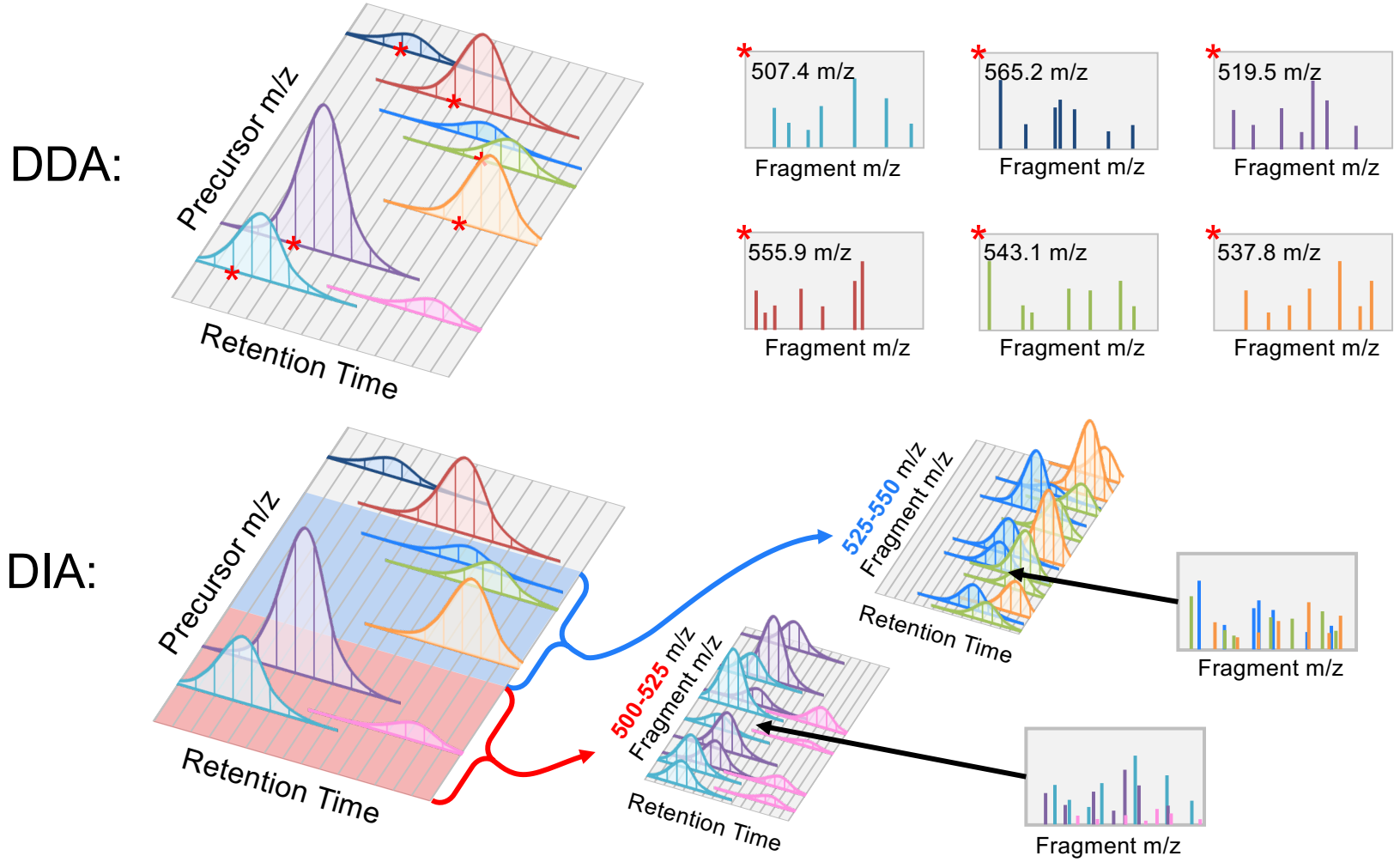
60 seconds of peptides (~1% of the gradient)

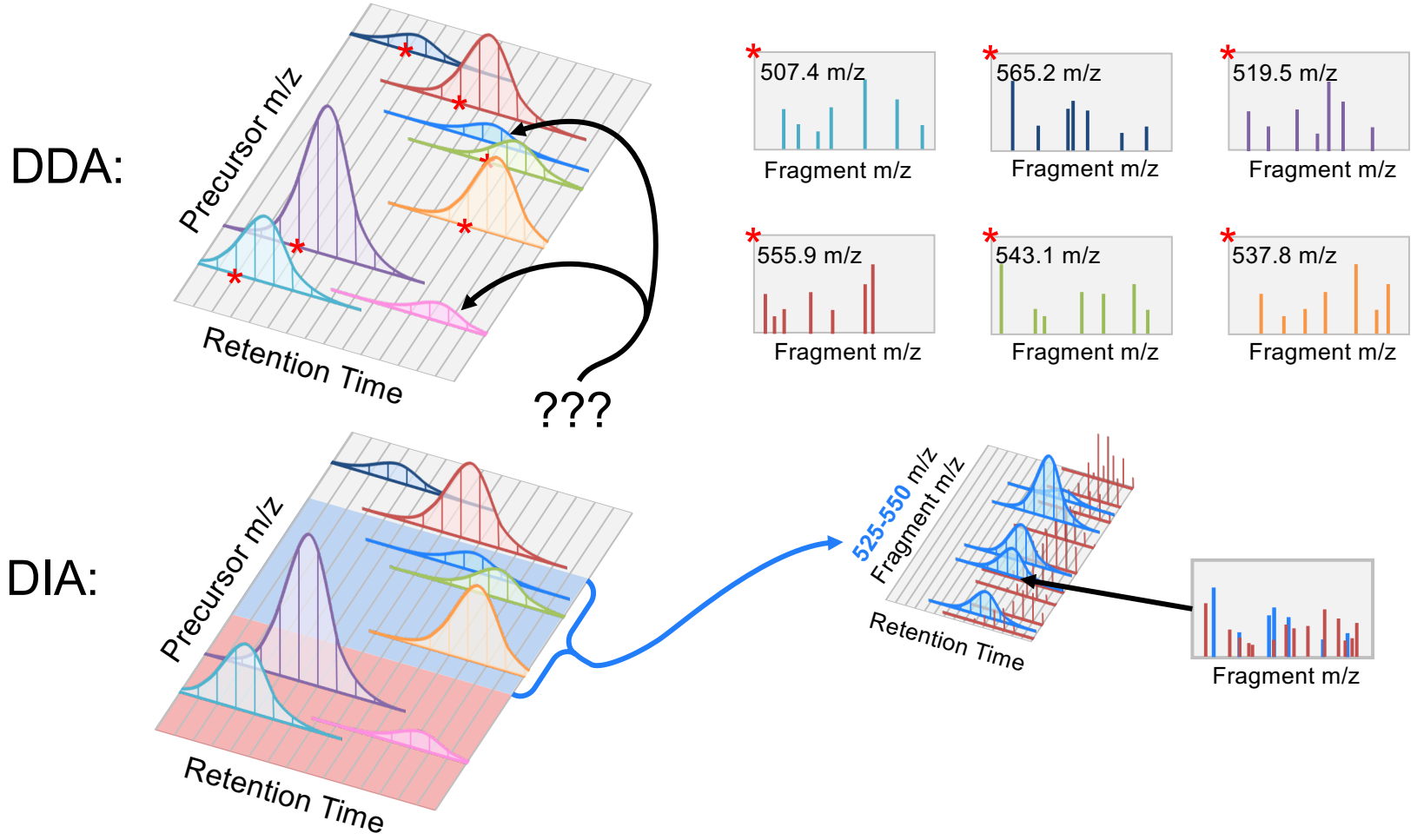


60 seconds of peptides (~1% of the gradient)



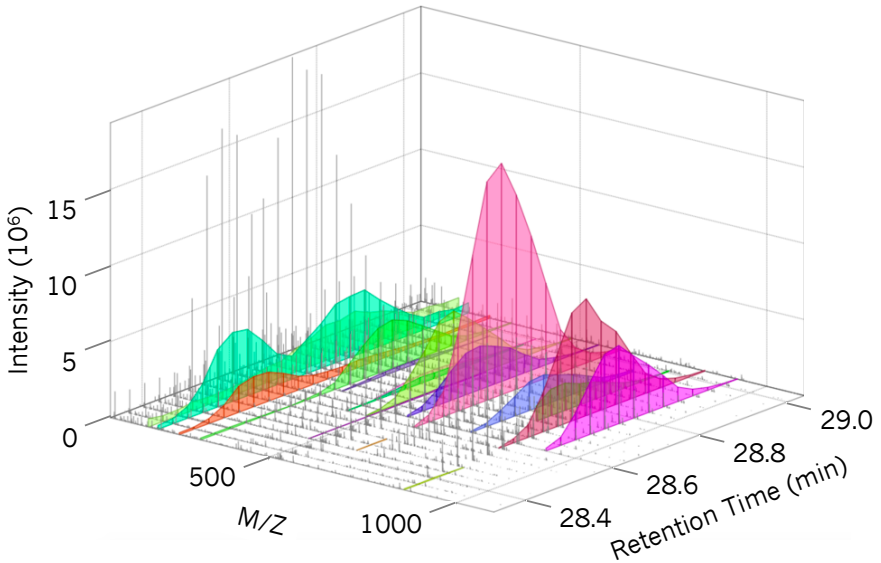

Precursor TIC (10^9)
Retention Time
Total TIC
# of MS/MS
# of PSMs
All MS/MS Scans
MS/MS scans with good results (qvalue <= 0.01)
Scans
Retention Time
Less peptides = More Detections
???


DDA:
Precursor m/z
Retention Time
507.4 m/z
565.2 m/z
519.5 m/z
555.9 m/z
543.1 m/z
537.8 m/z
Fragment m/z
DIA:
Precursor m/z
Retention Time
525-550 m/z
Fragment m/z
Retention Time
Fragment m/z
500-525 m/z
Fragment m/z
Retention Time
Fragment m/z

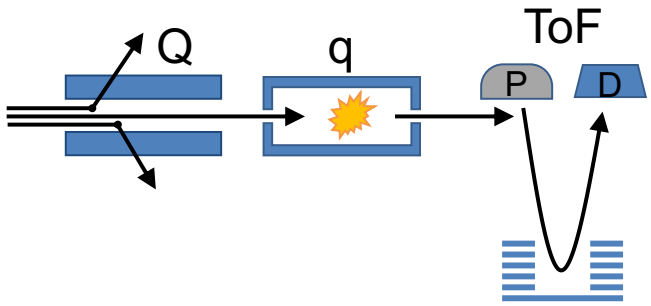

DDA:
Precursor m/z
Retention Time
???
507.4 m/z
565.2 m/z
519.5 m/z
555.9 m/z
543.1 m/z
537.8 m/z
Fragment m/z
DIA:
Precursor m/z
Retention Time
525-550 m/z
Fragment m/z
Retention Time
Fragment m/z

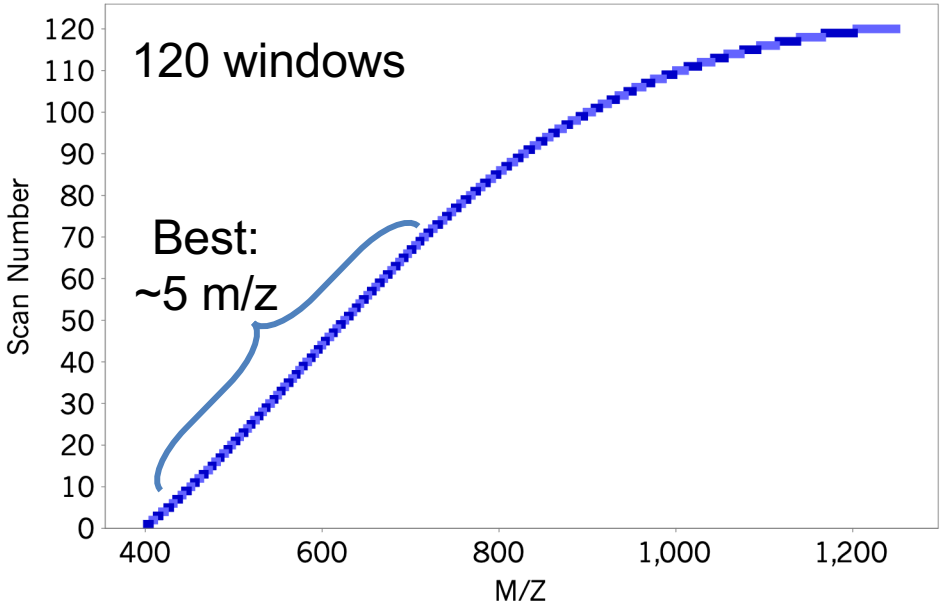
Contents

- Considerations when designing DIA experiments for phosphoproteomics
- Peptide-centric searching for peptides in DIA data and generating PTM libraries
- Complications when interpreting PTMs with DIA

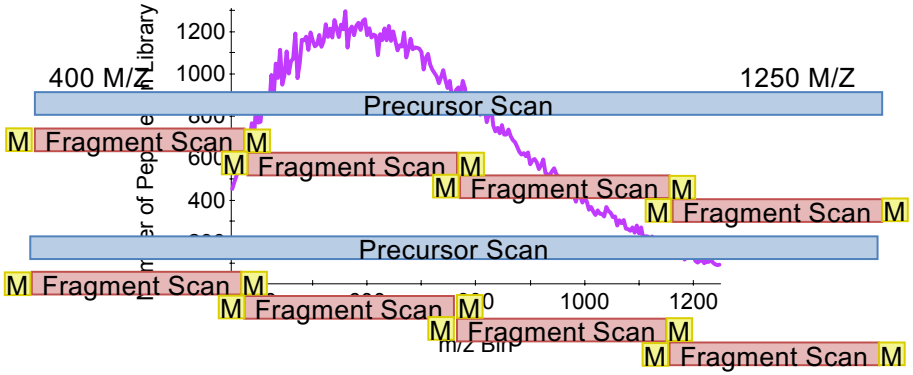


ToF acquisition

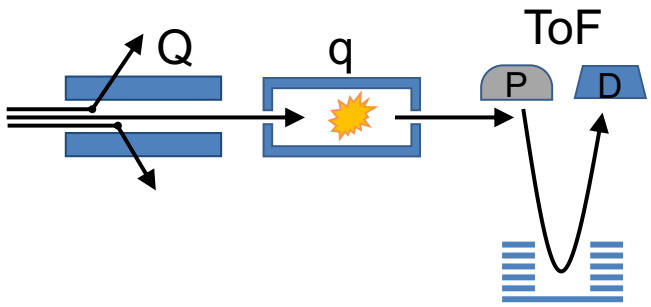




- Very fast! (1000s of pushes / sec)
- True profile scans
- Always see “signal” after averaging sufficient scans



ToF acquisition



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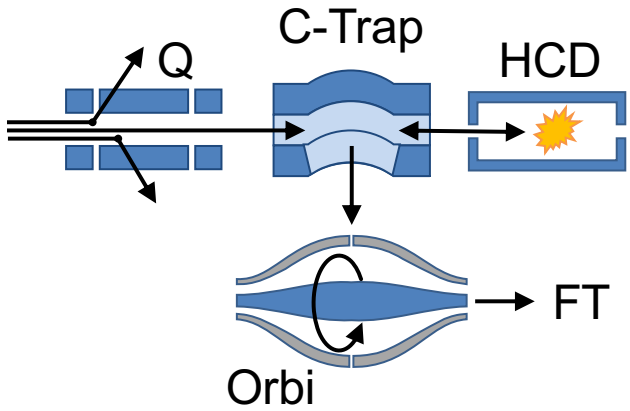
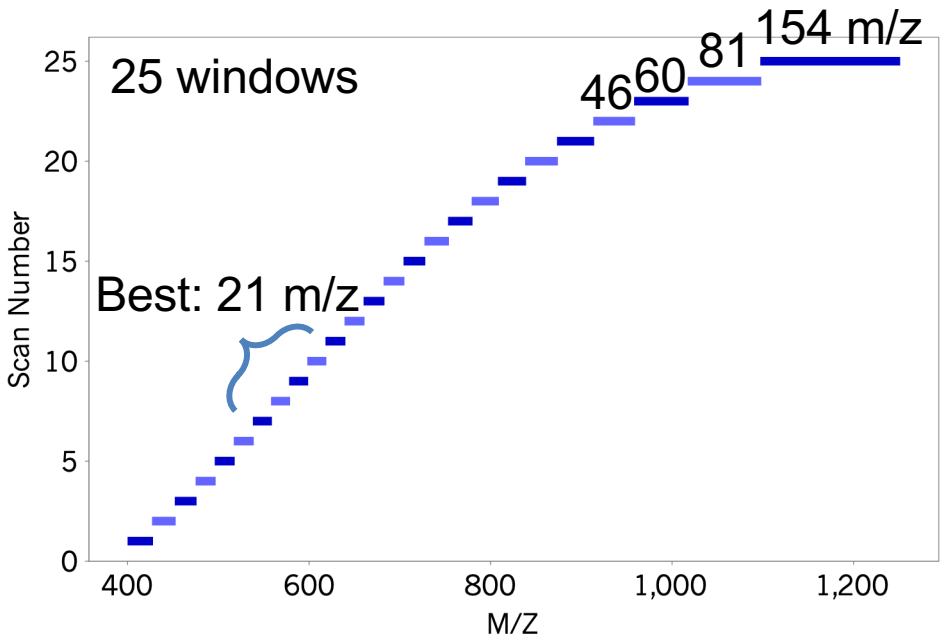
- True profile scans
- Always see “signal” after averaging sufficient scans

Orbitrap acquisition

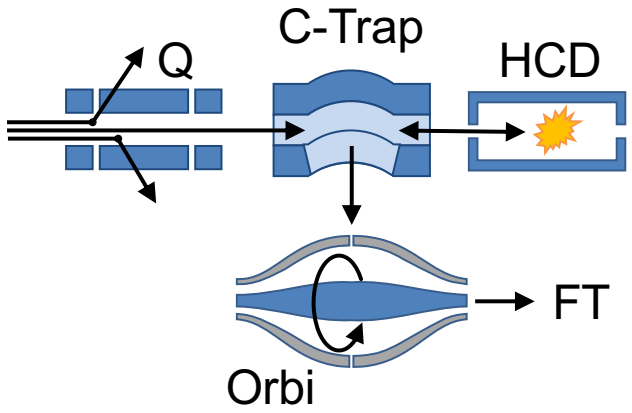


- Relatively slow (10-20 MSMS/sec)

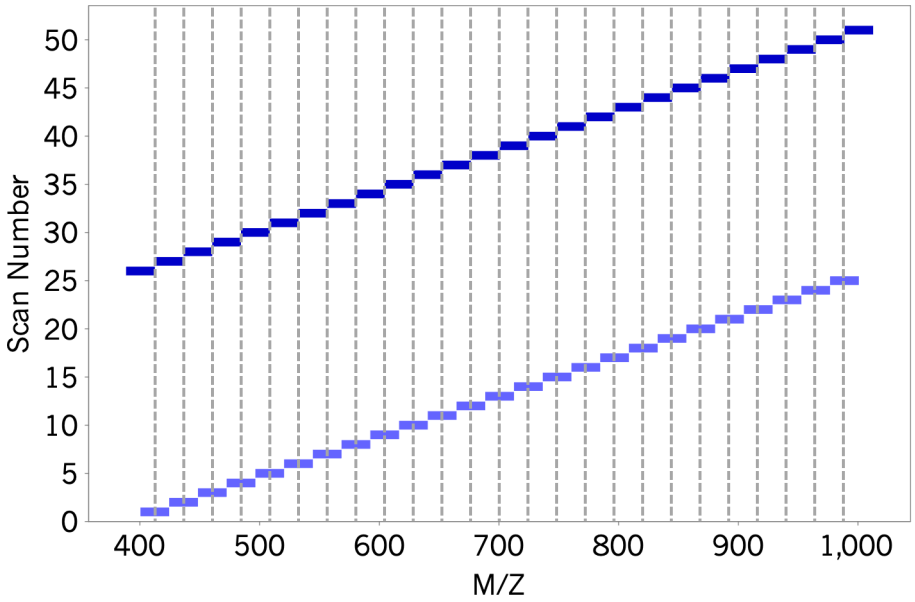


10 Hz * 25 windows = 2.5 sec

Orbitrap acquisition



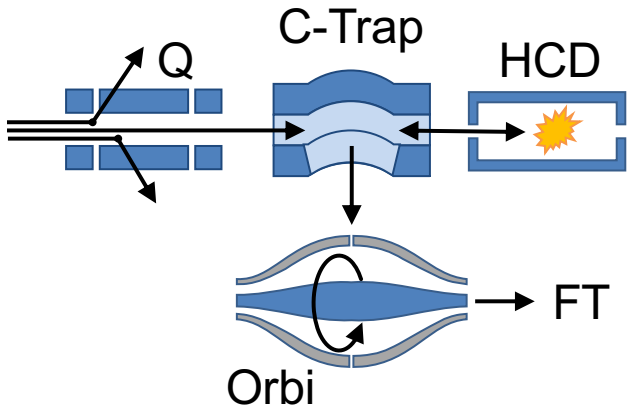
- Relatively slow (10-20 MSMS/sec)
- Pseudo-profile from FT (built-in denoising)
- Segmented quad: flat(ish) transmission



$$10\ \text{Hz} * \frac{50\ \text{windows (24 m/z)}}{2\ \text{cycles}} = \frac{2.5\ \text{sec}}{(12\ \text{m/z})}$$

Amodei et al, J Am Soc Mass Spectrom. 2019 Apr;30(4):669-684.

Orbitrap acquisition



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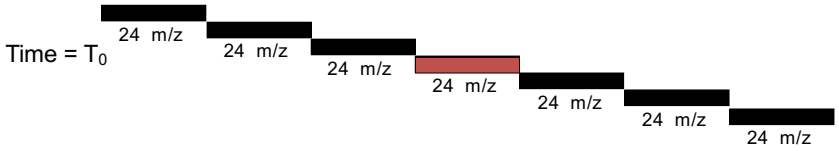
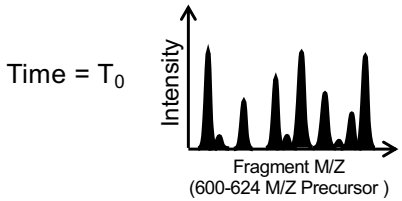
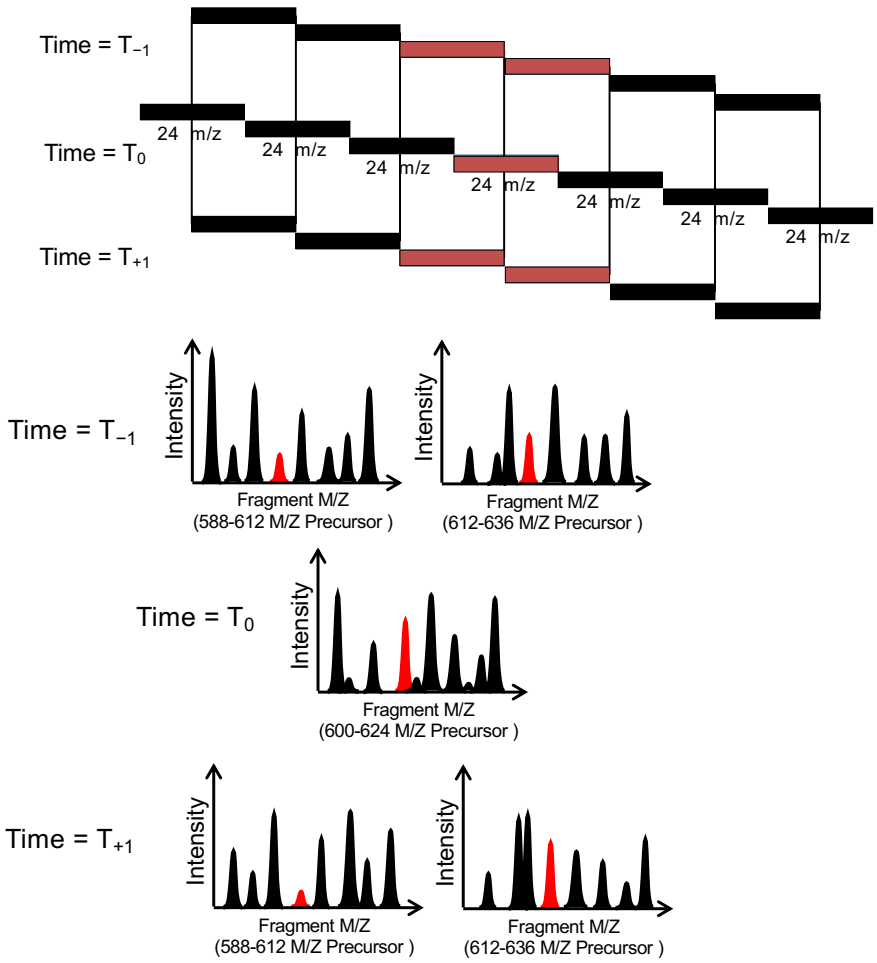
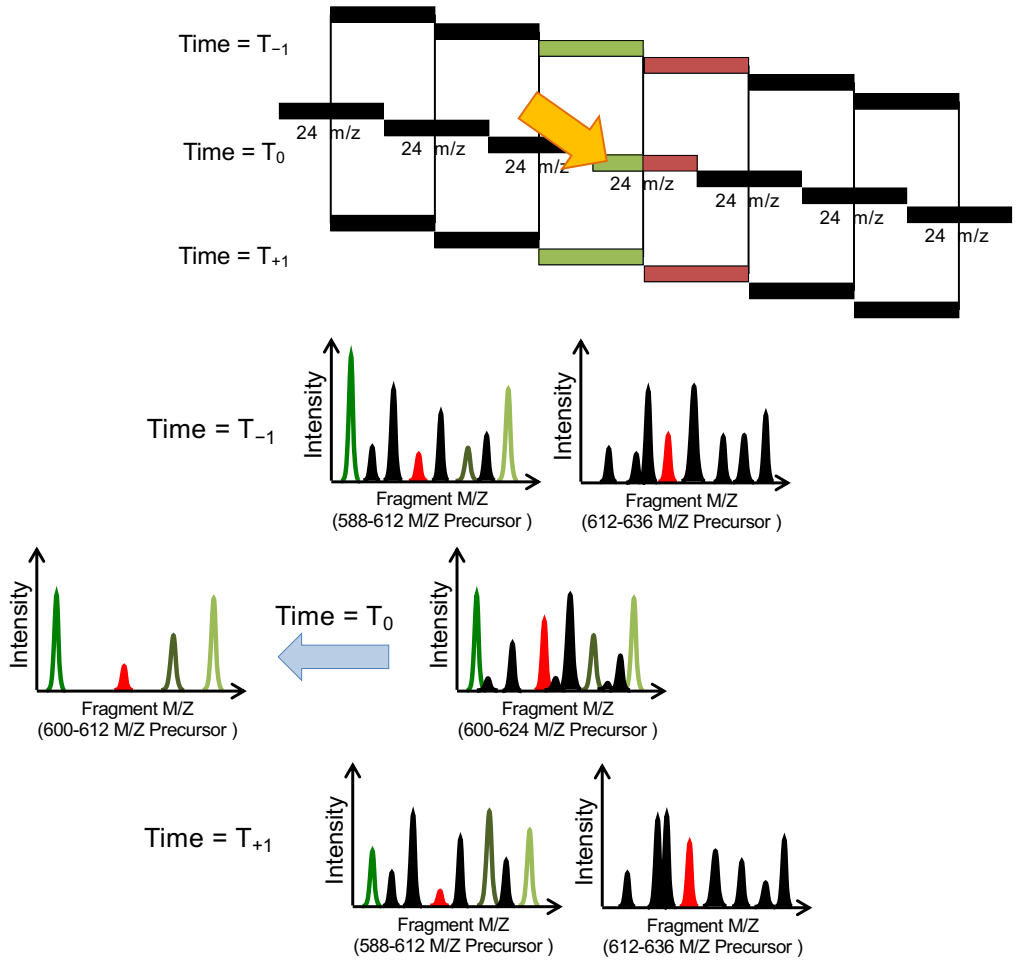
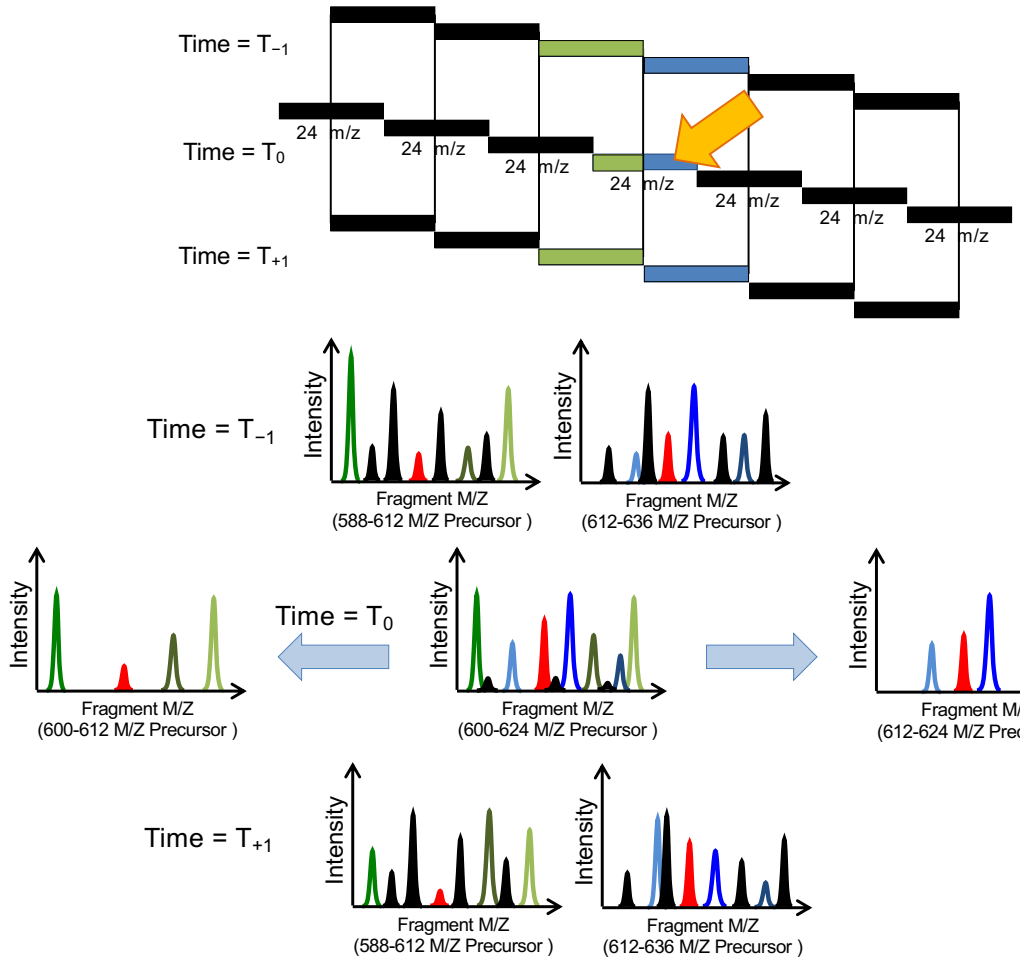

Time = T_0
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z


Time = T_0
Intensity
Fragment M/Z
(600-624 M/Z Precursor)


Time = T−1
Time = T0
Time = T+1
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
Time = T−1
Intensity
Fragment M/Z
(588-612 M/Z Precursor)
Intensity
Fragment M/Z
(612-636 M/Z Precursor)
Time = T0
Intensity
Fragment M/Z
(600-624 M/Z Precursor)
Time = T+1
Intensity
Fragment M/Z
(588-612 M/Z Precursor)
Intensity
Fragment M/Z
(612-636 M/Z Precursor)


Time = T−1
Time = T0
Time = T+1
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
Time = T−1
Intensity
Fragment M/Z
(588-612 M/Z Precursor)
Intensity
Fragment M/Z
(612-636 M/Z Precursor)
Intensity
Fragment M/Z
(600-612 M/Z Precursor)
Time = T0
Intensity
Fragment M/Z
(600-624 M/Z Precursor)
Time = T+1
Intensity
Fragment M/Z
(588-612 M/Z Precursor)
Intensity
Fragment M/Z
(612-636 M/Z Precursor)


Time = T−1
Time = T0
Time = T+1
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
24 m/z
Intensity
Fragment M/Z
(588-612 M/Z Precursor)
Intensity
Fragment M/Z
(612-636 M/Z Precursor)
Intensity
Fragment M/Z
(600-612 M/Z Precursor)
Time = T0
Intensity
Fragment M/Z
(600-624 M/Z Precursor)
Intensity
Fragment M/Z
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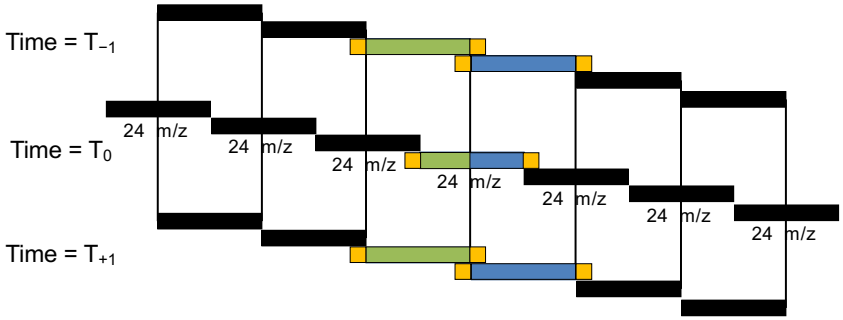
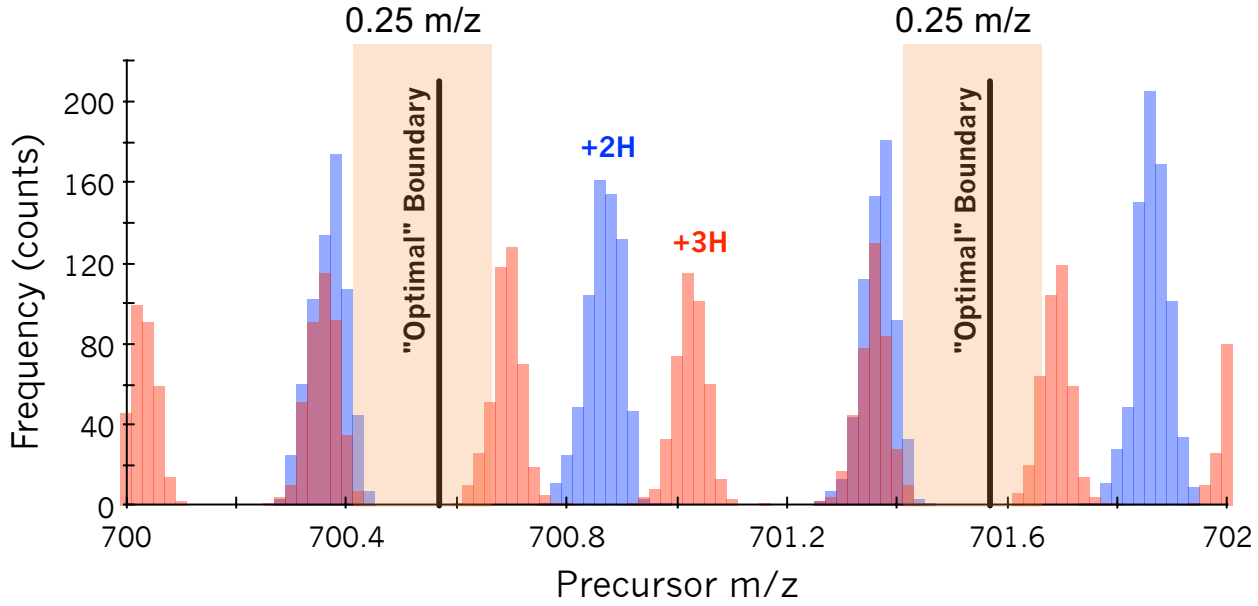
Intensity
Fragment M/Z
(588-612 M/Z Precursor)
Intensity
Fragment M/Z
(612-636 M/Z Precursor)



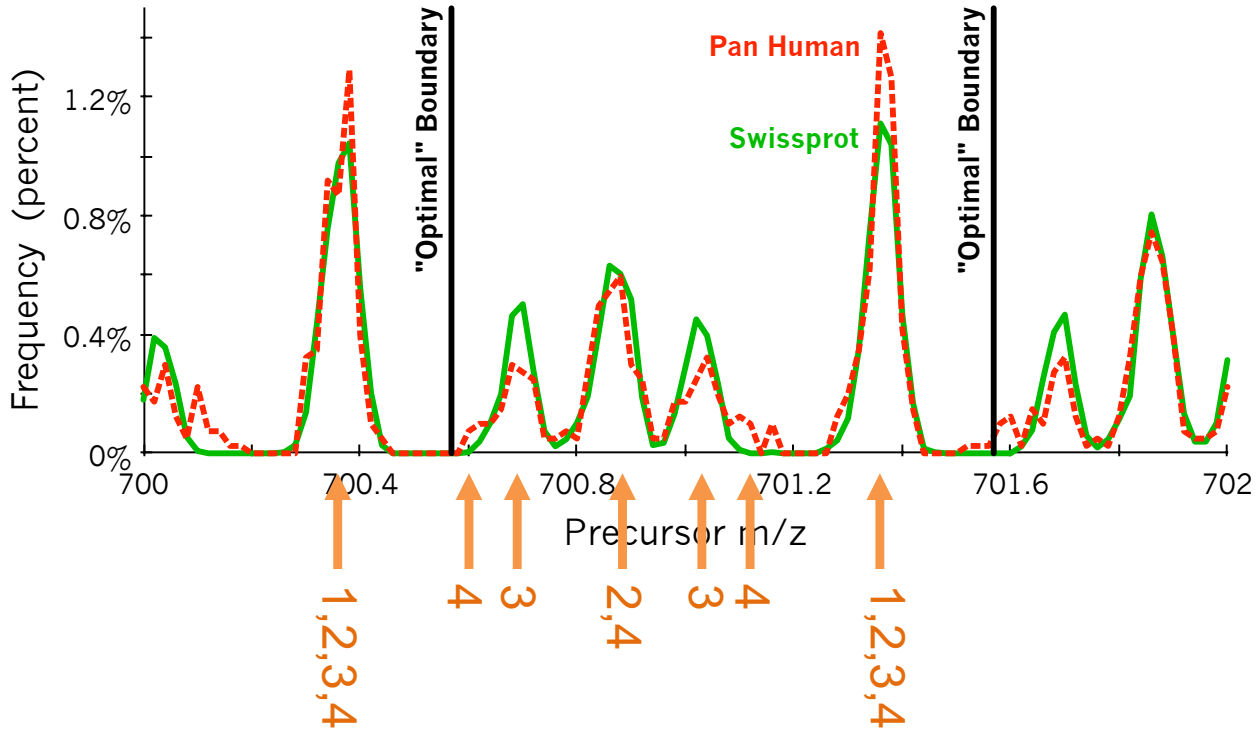
…staggering is incompatible with both variable width windows and margins

“Forbidden zones” take advantage of m/zs where peptides don’t exist

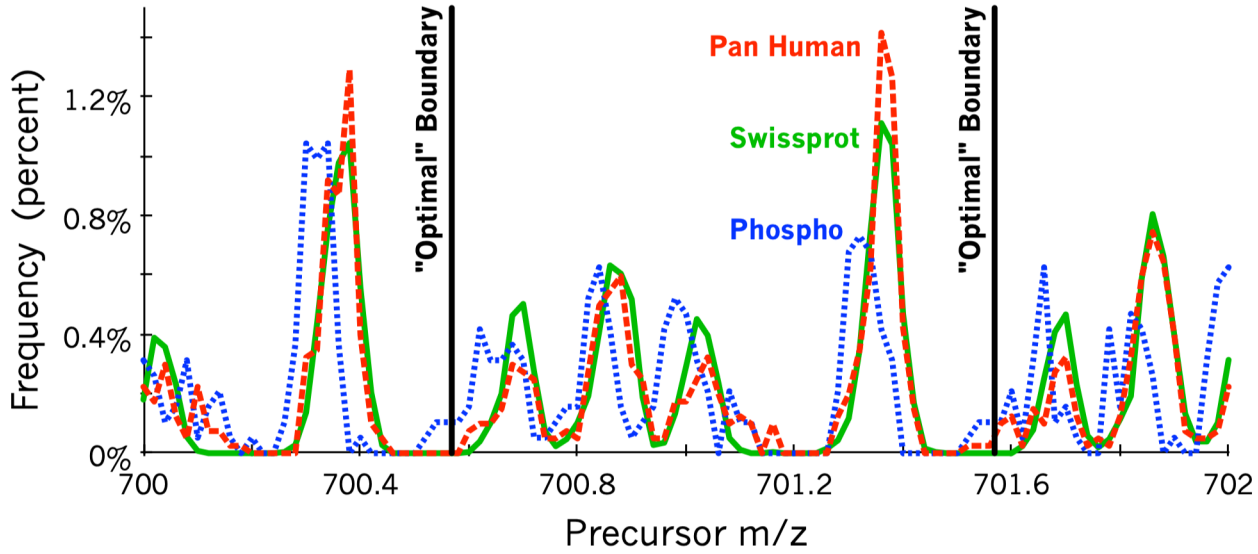


- Peptides are made of H C N O S

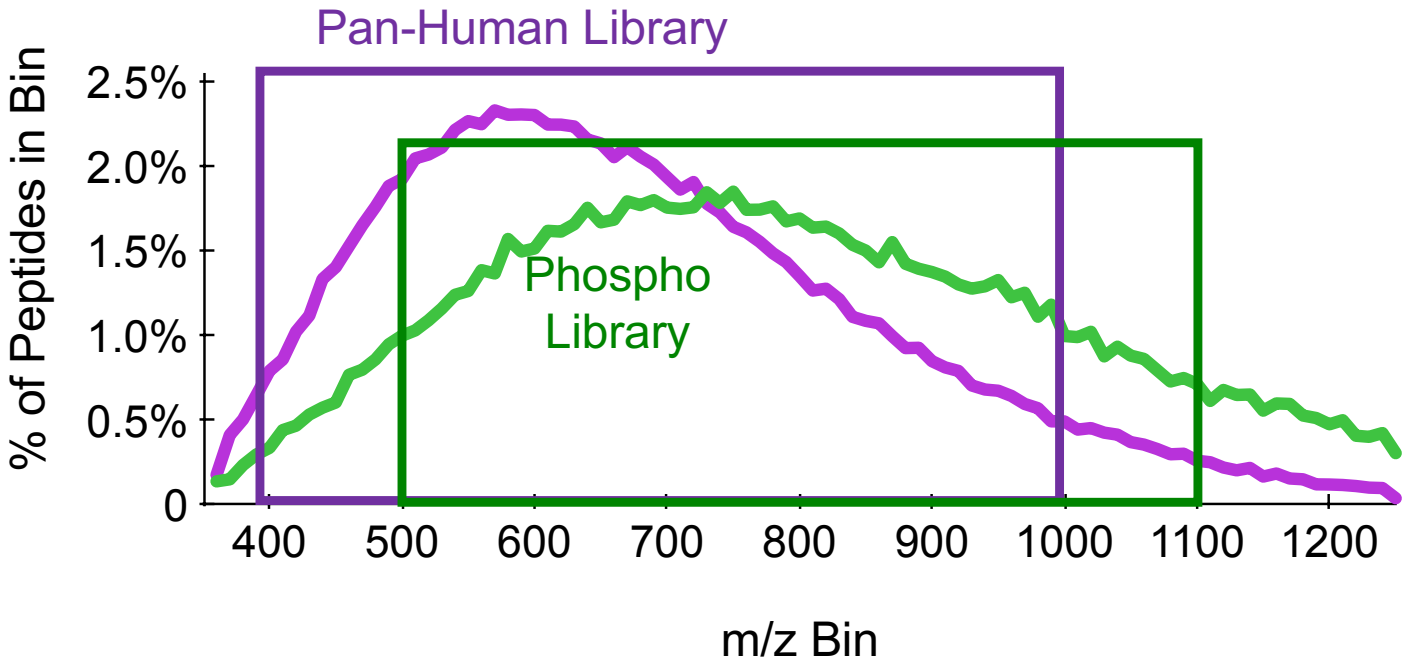
“Forbidden zones” take advantage of m/zs where peptides don’t exist



Phosphopeptides have different forbidden zones (-0.18 m/z)



Optimize M/Z ranges to a specific proteome



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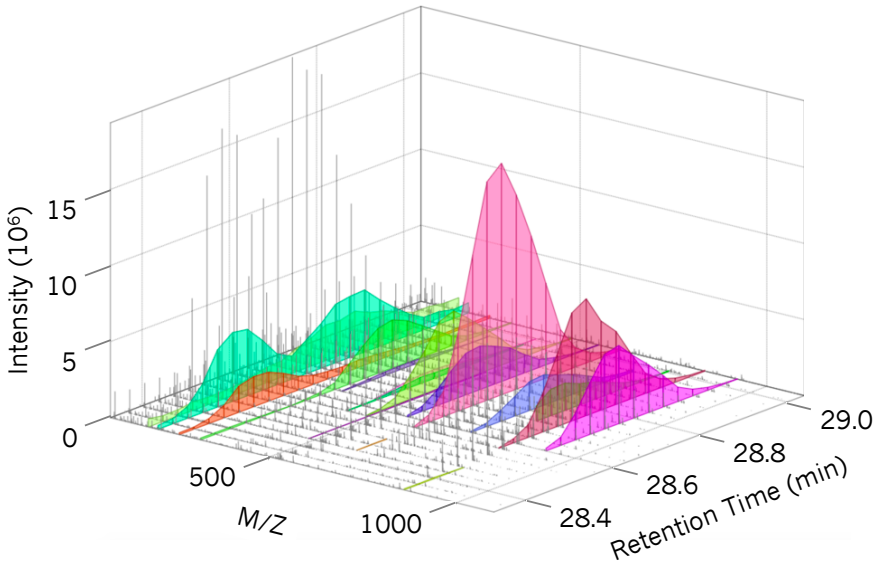
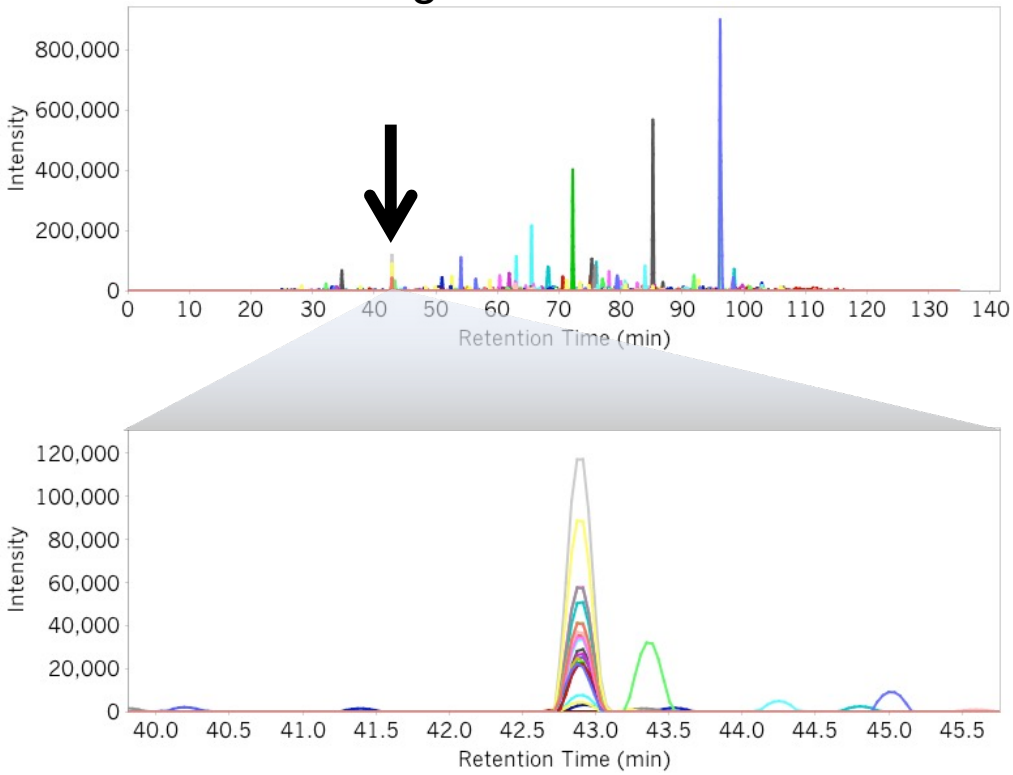


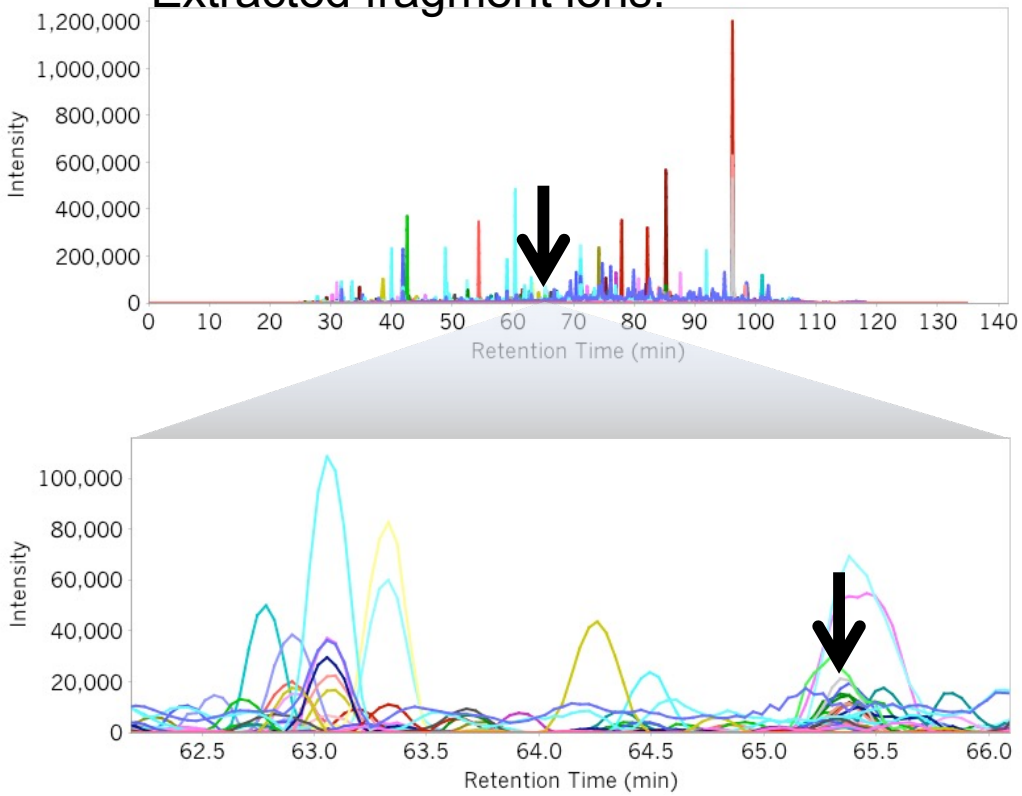
HHAAYVNNLNVTEEK (+2H)
SODM_HUMAN

Extracted fragment ions:

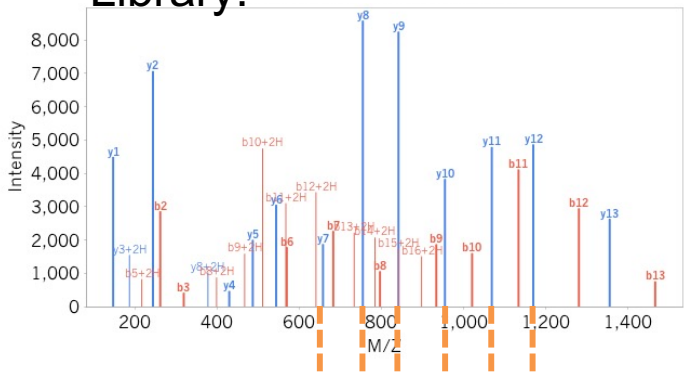


FNGGGHINHSIFWTNLSPNGGGEPK (+3H)
SODM_HUMAN

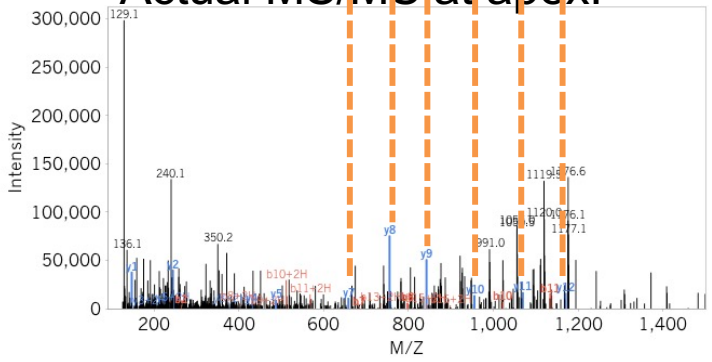
Extracted fragment ions:



Library:

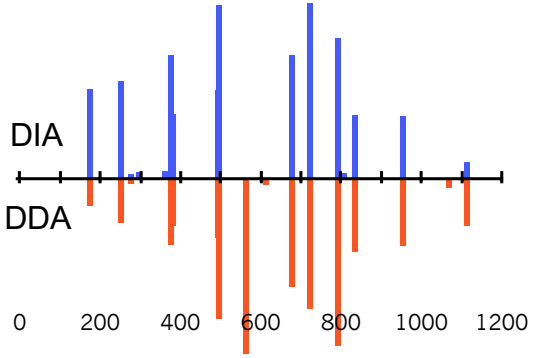


Actual MS/MS at apex:

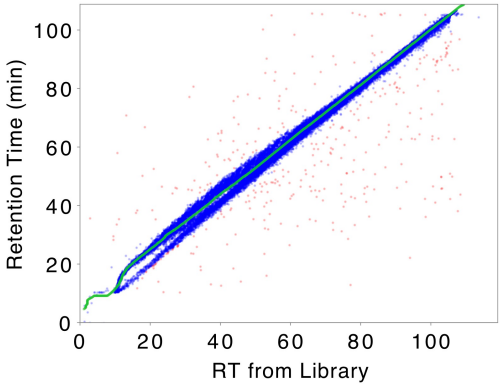


What's in a library?

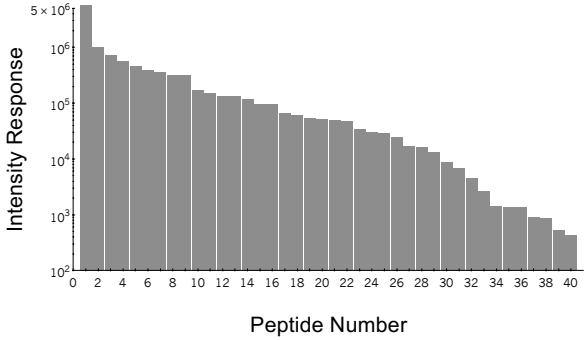
Fragmentation Pattern



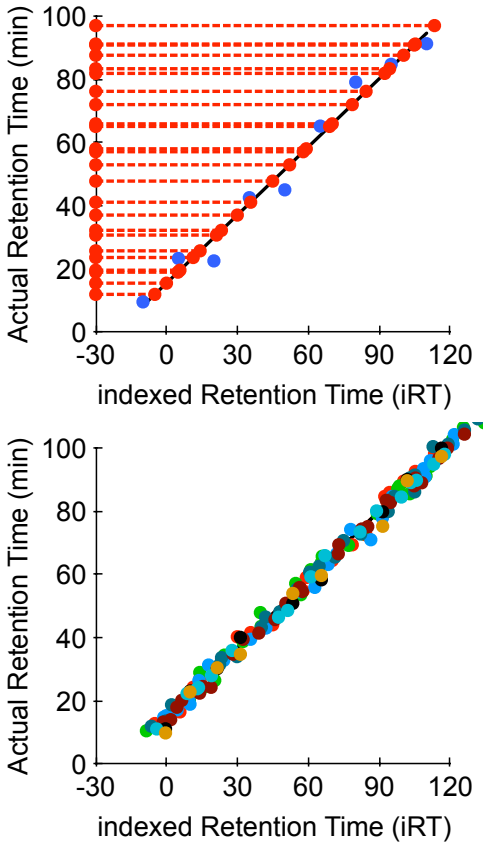
Retention Time

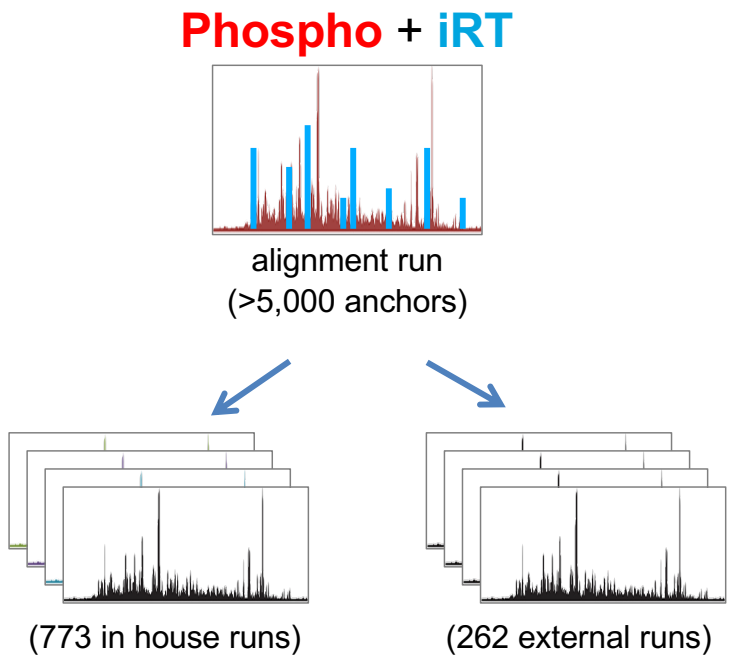


Limited # Peptides



Constructing a massive phosphopeptide library

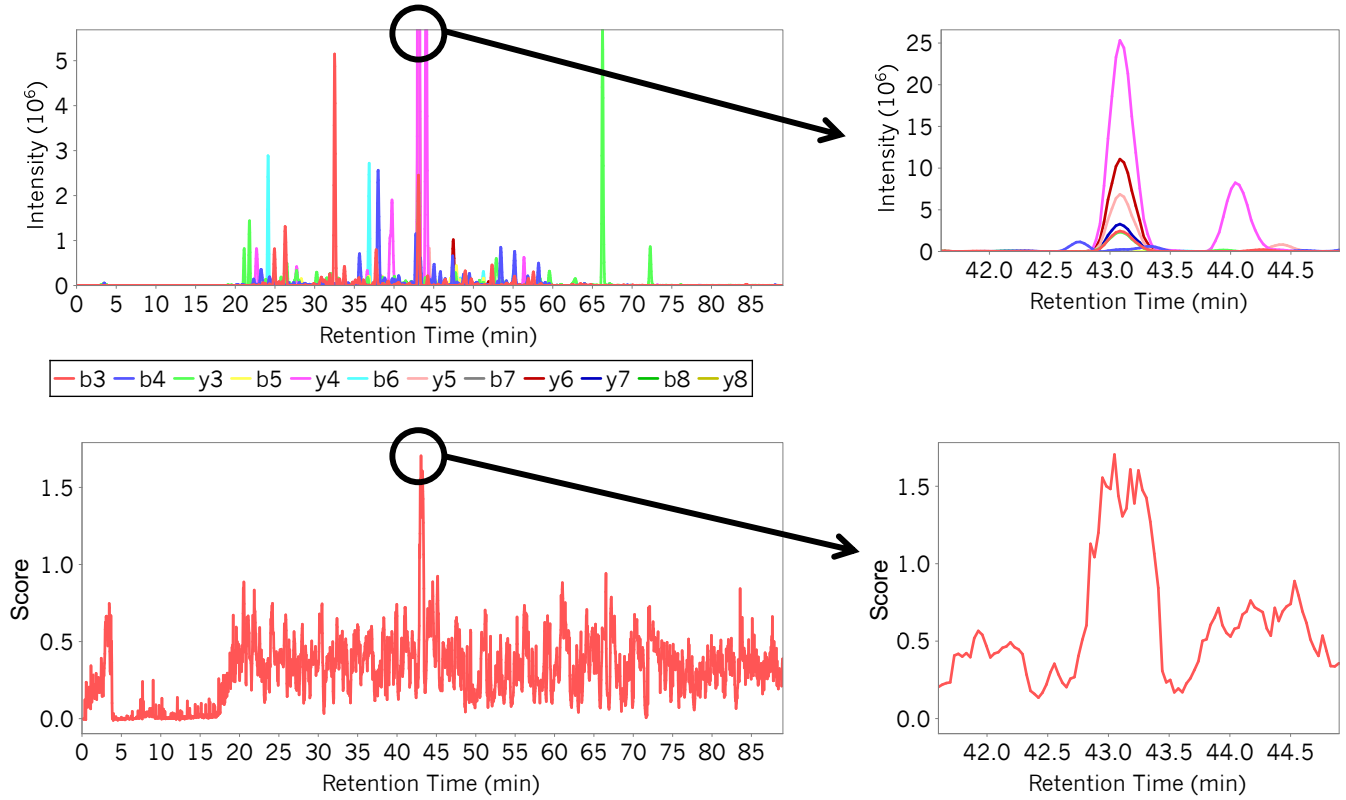




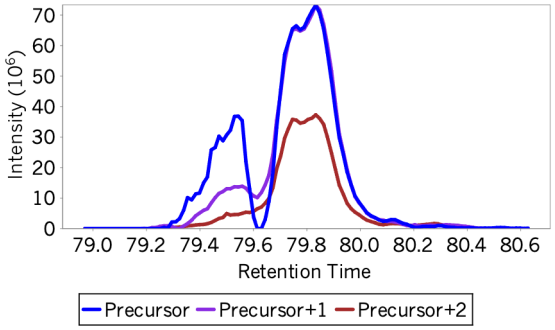
- https://phosphopedia.gs.washington.edu
- https://bitbucket.org/searleb/encyclopedia

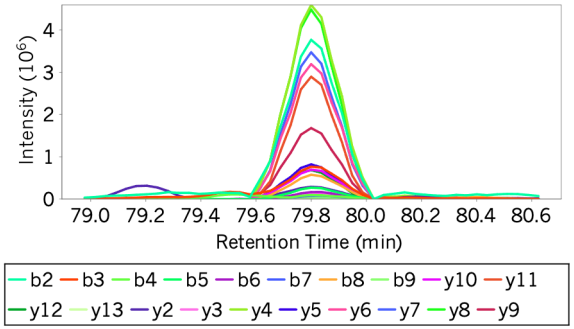
Lawrence, Searle et al, Nat Methods. 2016; 13, 431–434.

Peptide-centric searching with PECAN scores peptides across retention time

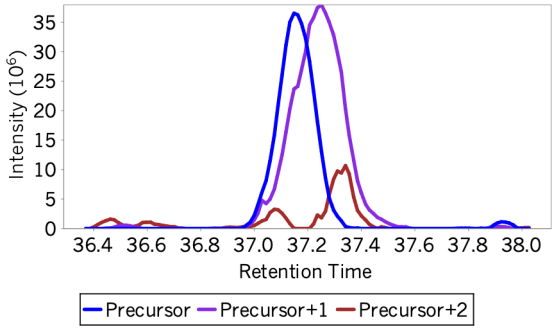


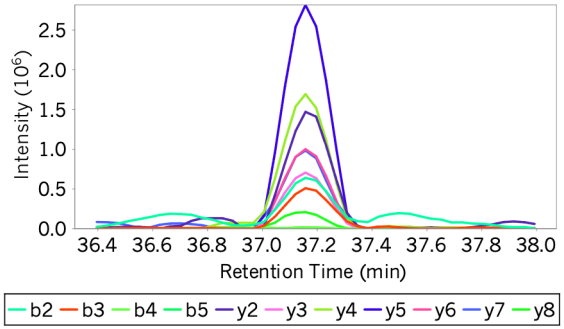
Early DDA triggers



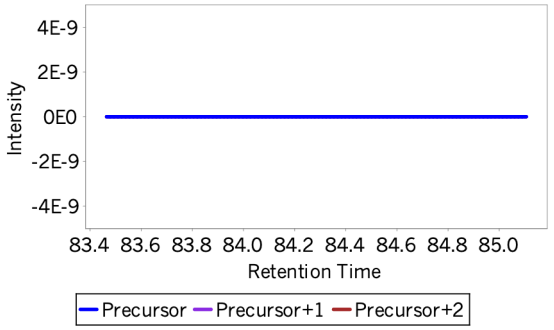


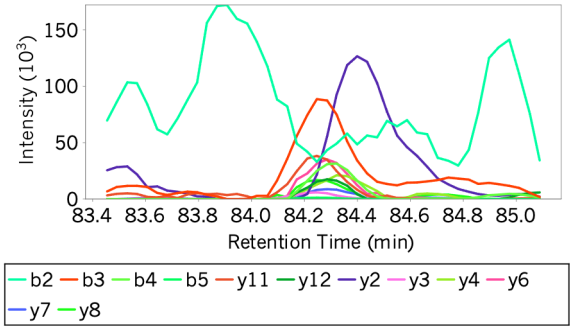
Coeluting interference





No precursor signal





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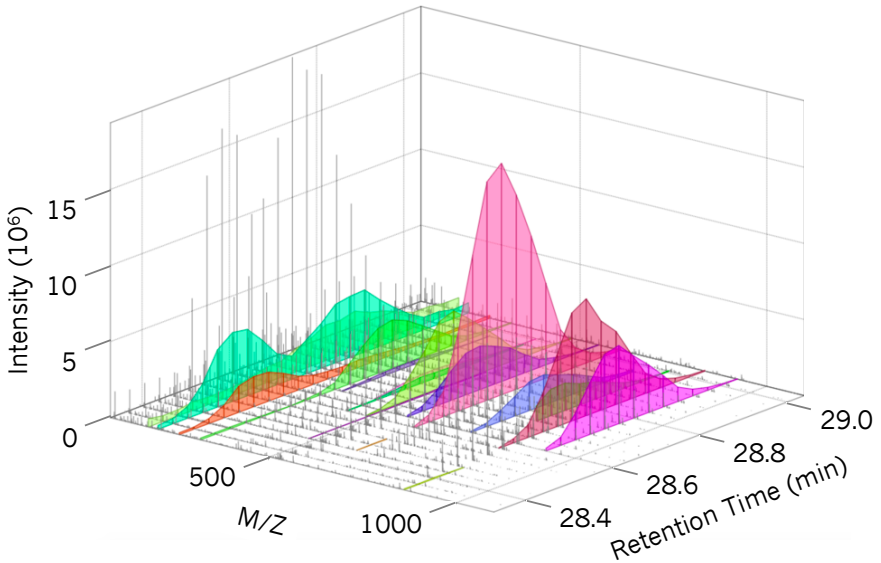
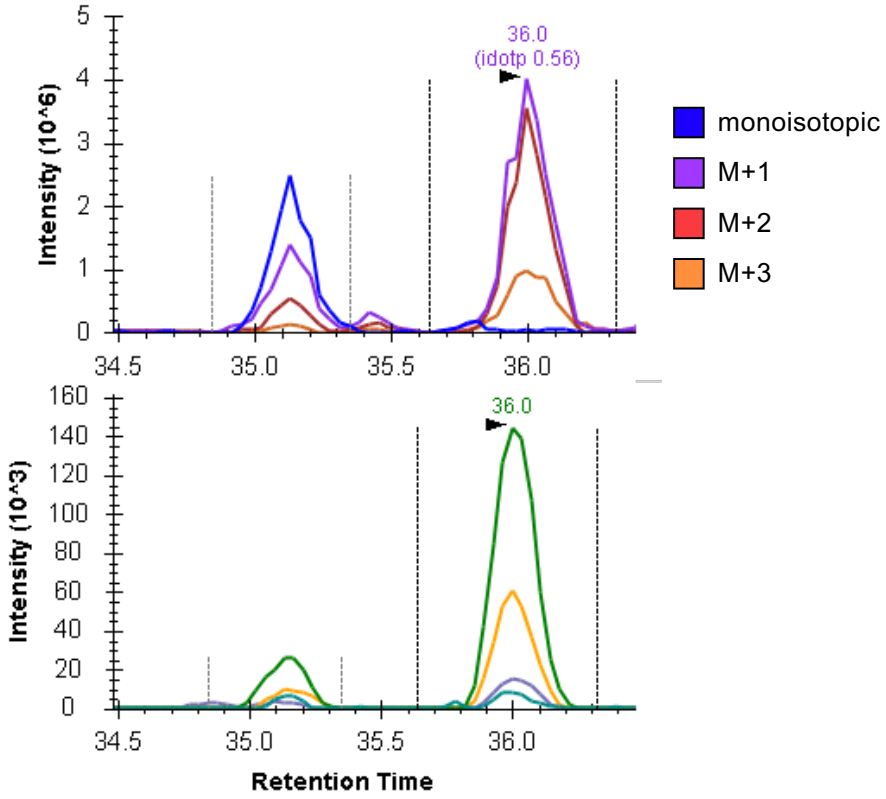
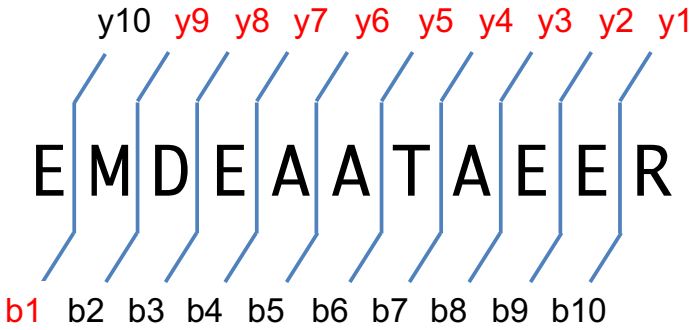
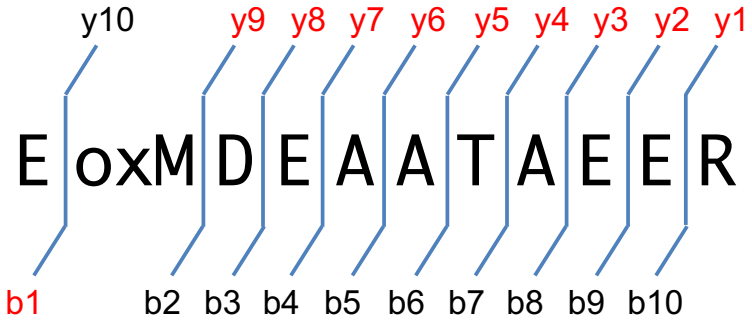


What's going on here?



Modified peptides share fragment ions



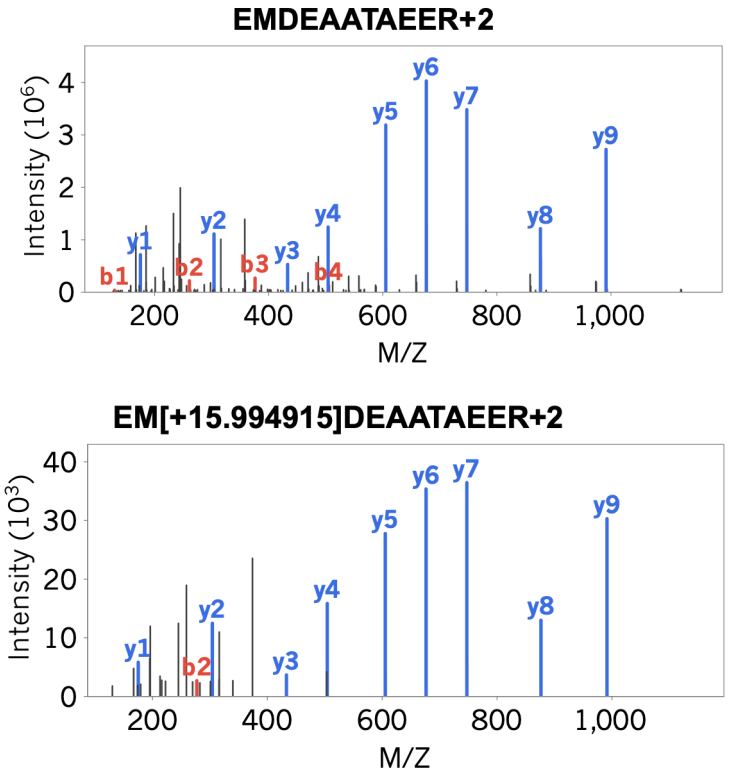


Precursor = 626.26 m/z

Same 24 m/z isolation window!

Precursor = 634.26 m/z

Modified peptides share fragment ions



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Same 24 m/z isolation window!

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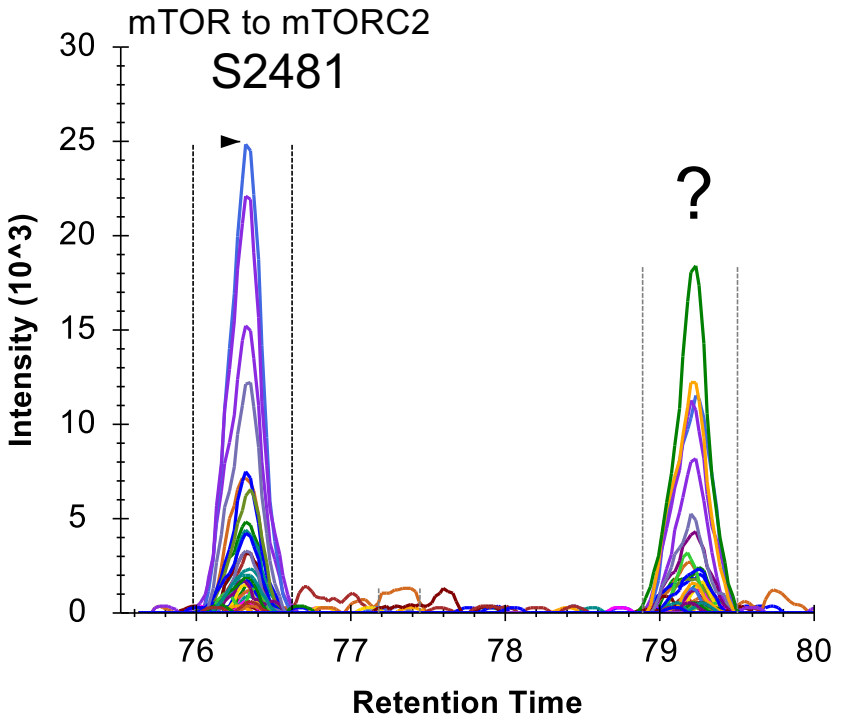
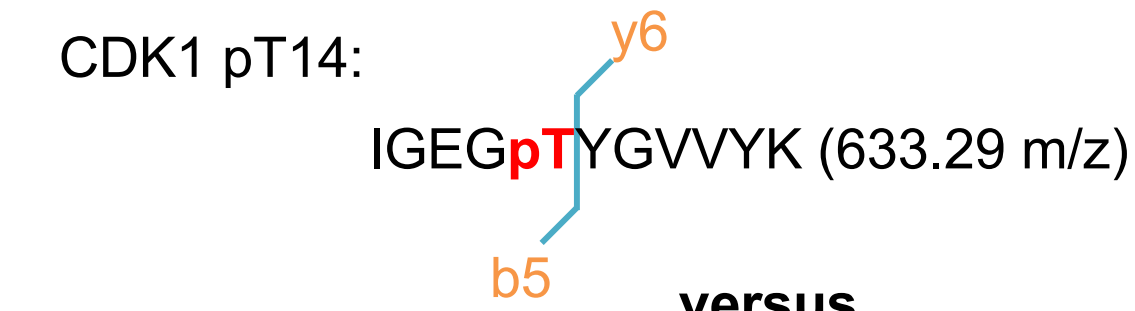


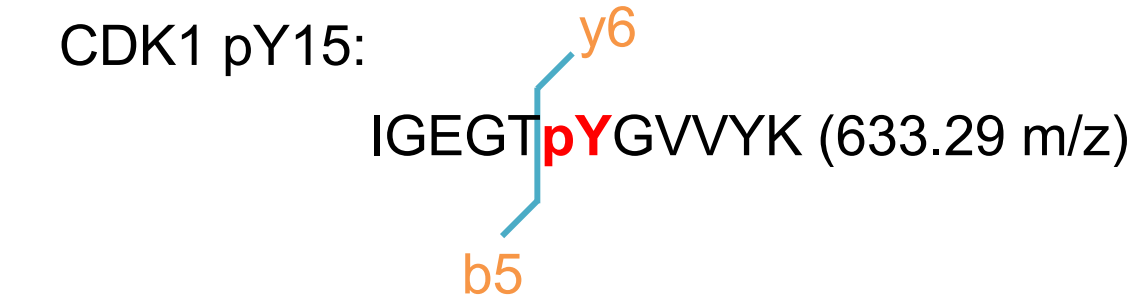
What is a positional isomer?

CDK1 pT14:



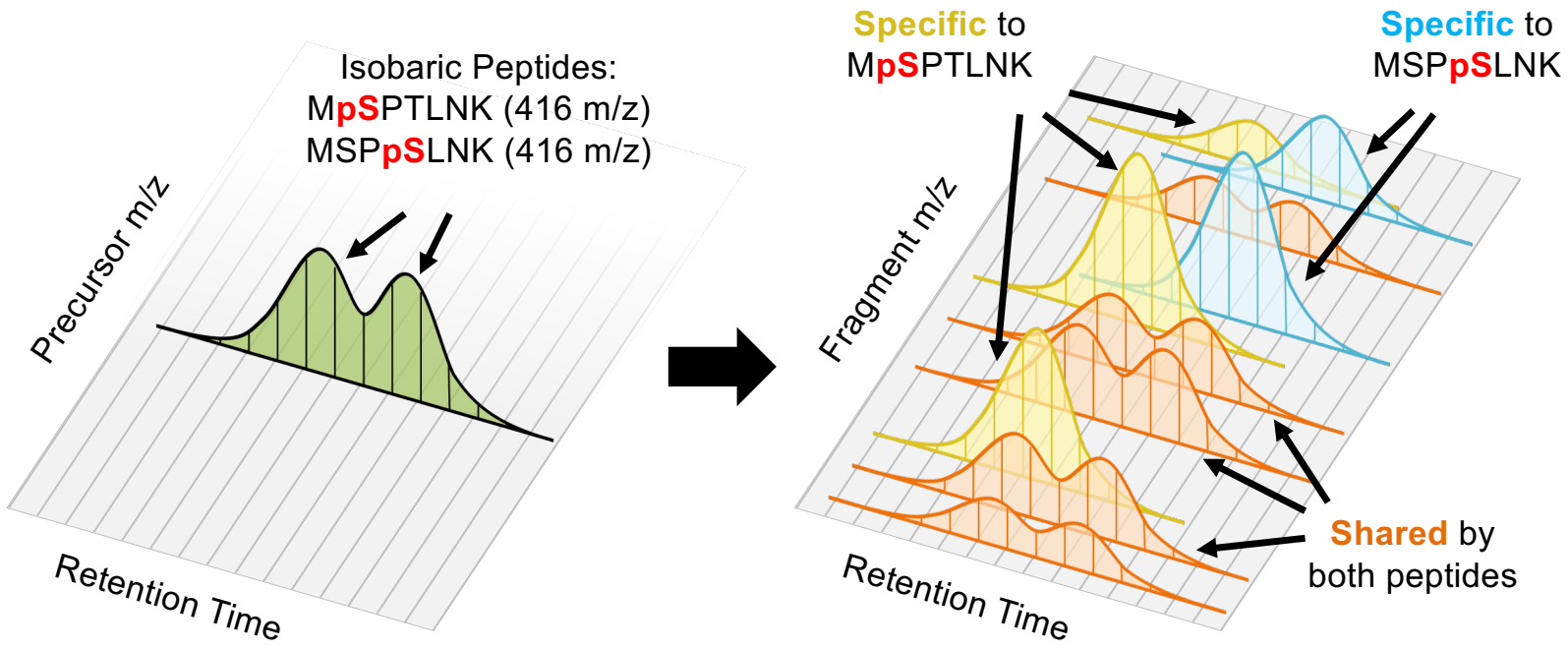
versus

CDK1 pY15:

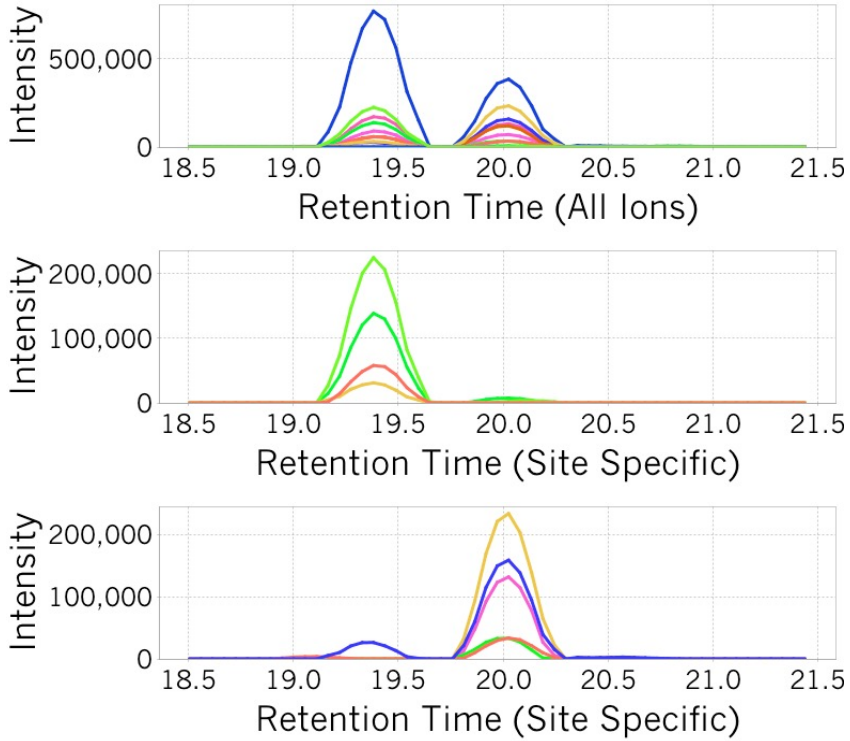


- Parent mass SAME
- A FEW fragmentation masses differ
- HPLC retention times MAY differ

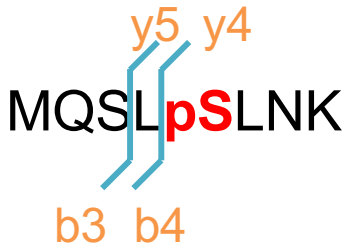
Using site specific ions to identify phosphopeptides

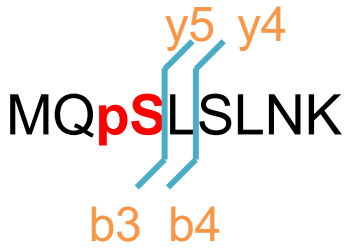


Localizing using site specific ions for phosphopeptides



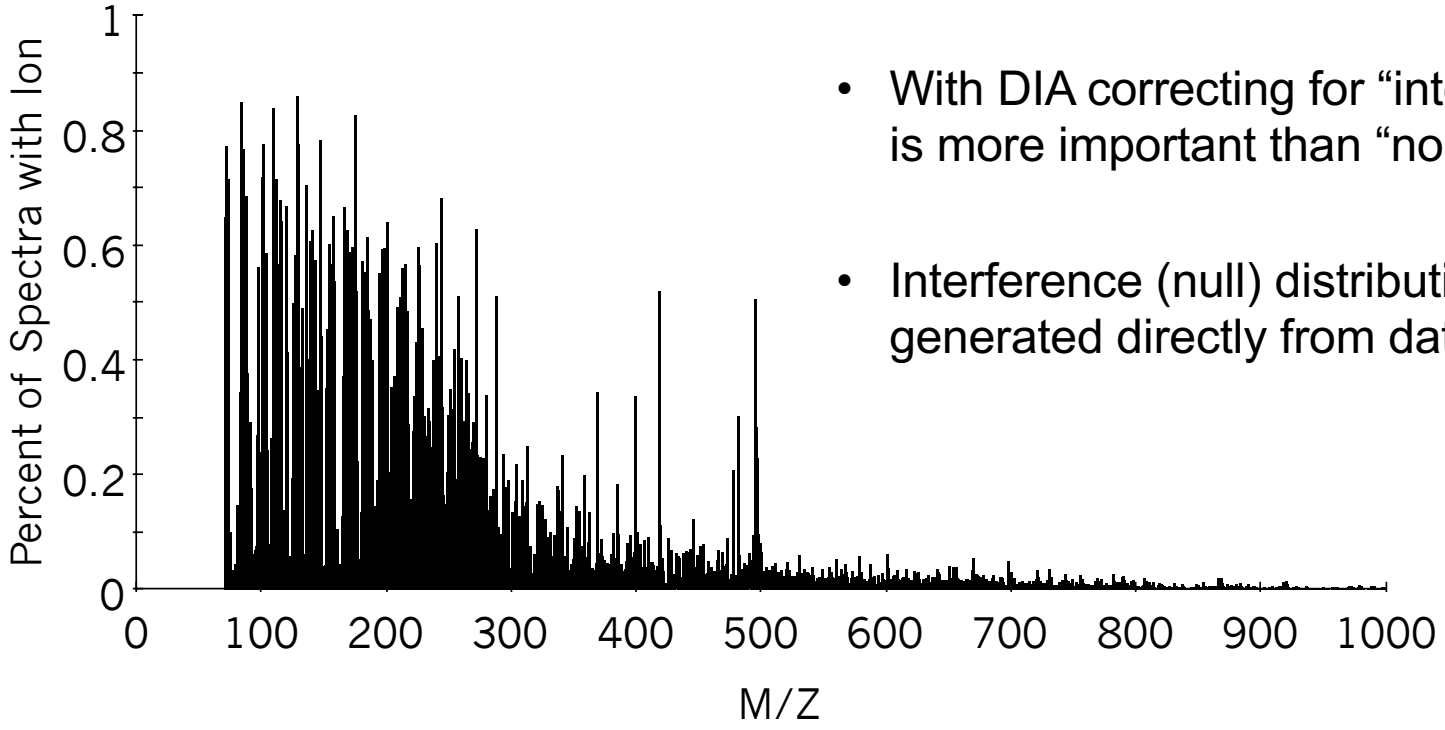
All ions for MQSL**pS**LNK
or MQ**pS**LSLNK





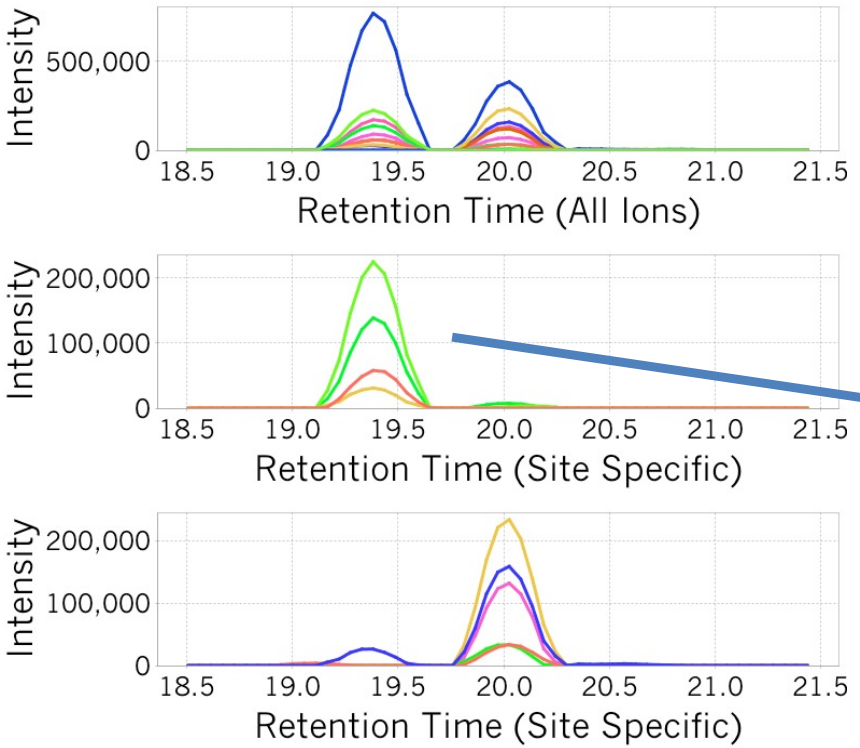
Ascore (and Maxquant, PhosphoRS…) assumes a random likelihood of seeing every ion

Estimating a null distribution for interference

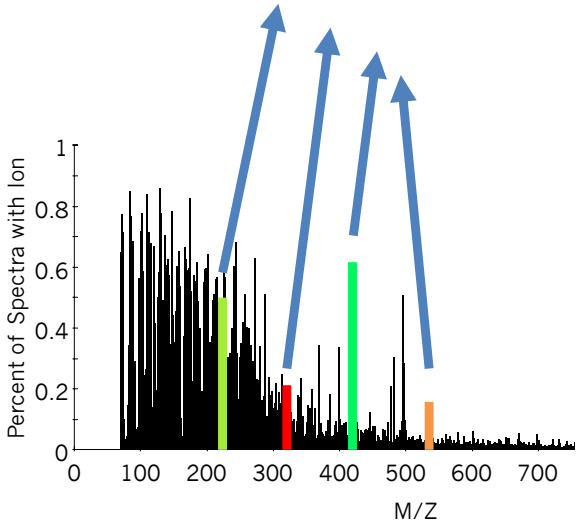


- With DIA correcting for “interference” is more important than “noise”
- Interference (null) distribution generated directly from data

Localizing using site specific ions

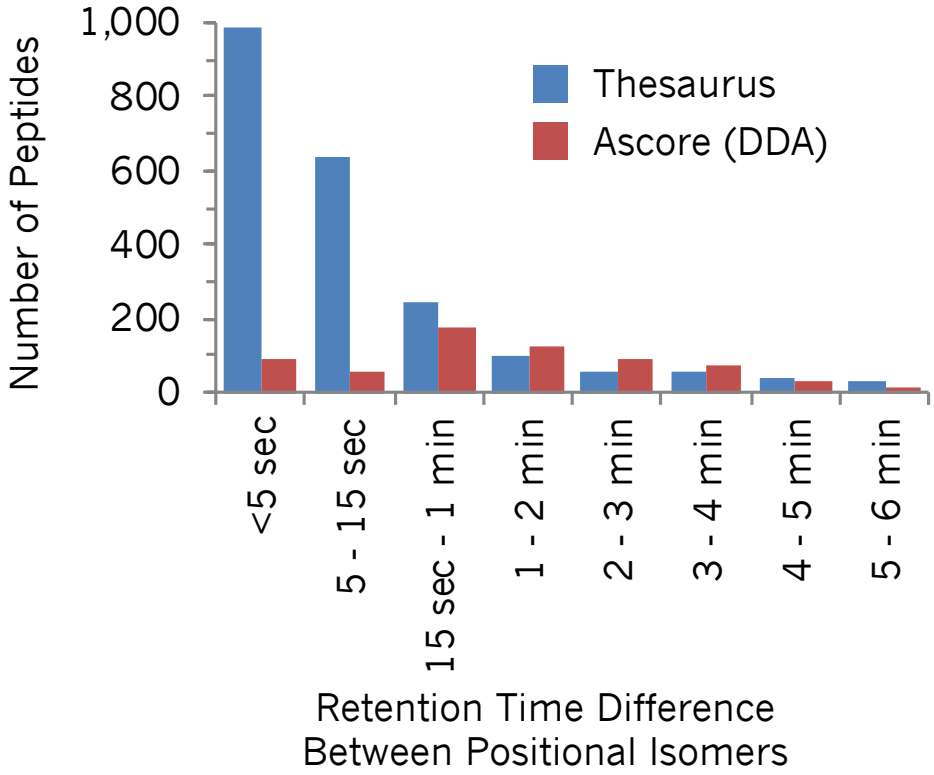


$$Localization\ p(null|m) = \frac{\prod_{\substack{i\ in \\ present \\ peaks}} p(null|m_i)}{N\ considered\ masses}$$

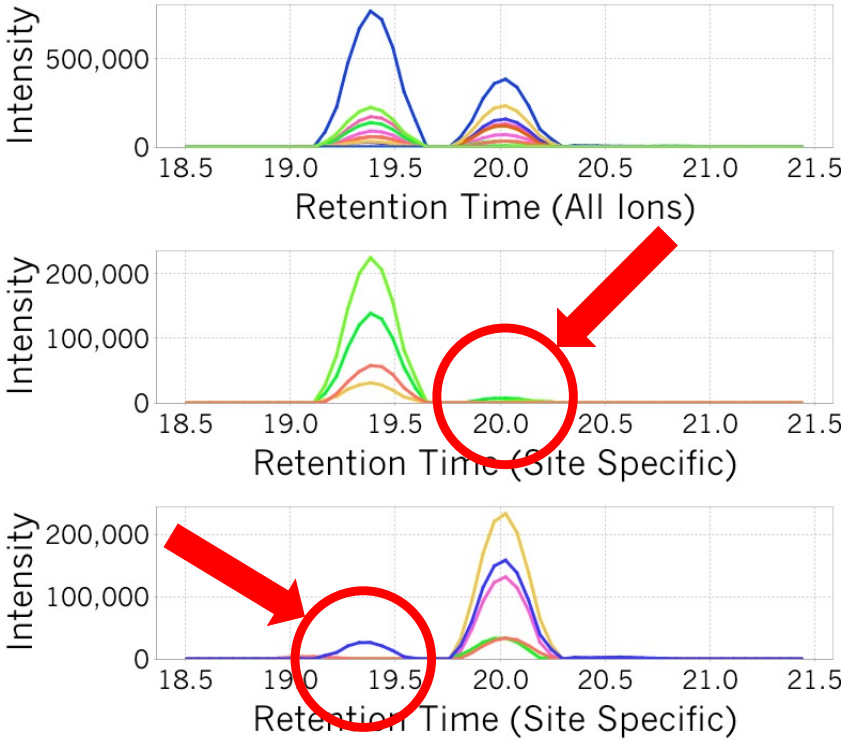


Searle et al, Nat Methods. 2019; 16, 703–706.

Many positional isomers elute within 60 seconds of each other



Localizing using site specific ions for phosphopeptides



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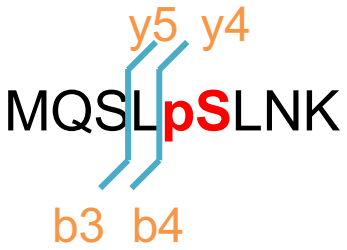
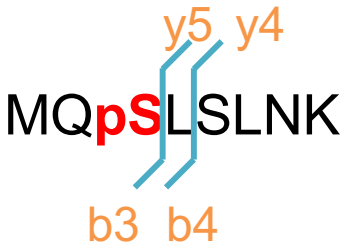
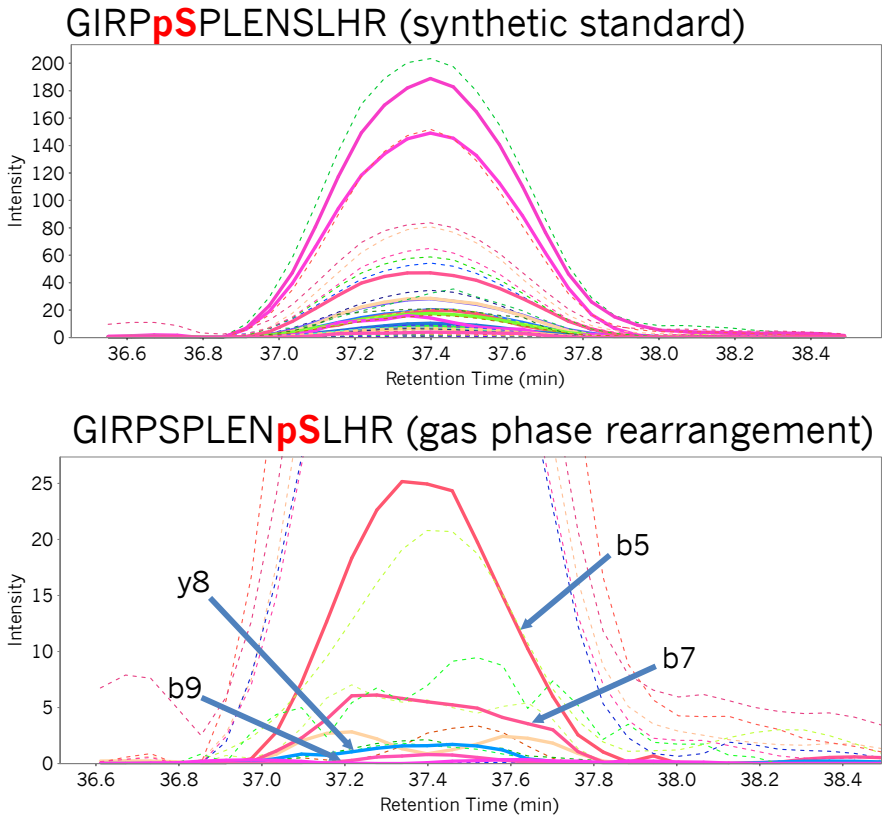
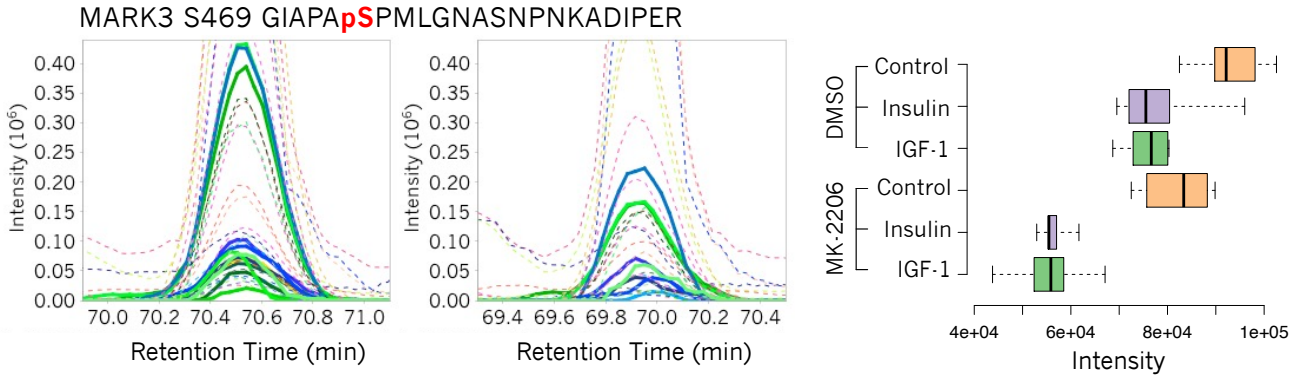


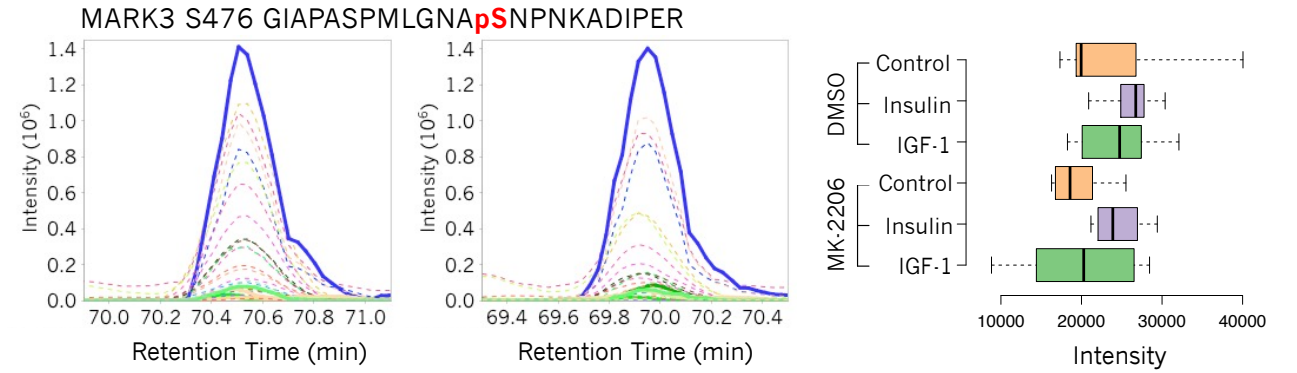


Some peptides rearrange in the gas phase



Some phosphopeptides don't resolve chromatographically





Resources for DIA best practices

- DIA Perspective: “Acquiring and Analyzing Data Independent Acquisition Proteomics Experiments without Spectrum Libraries”
  https://www.mcponline.org/article/S1535-9476(20)34974-4/fulltext

- Quickstart guide for setting up DIA:
  https://bitbucket.org/searleb/encyclopedia/downloads/dia_methods_setup_v1.4.pdf

- Recommended settings for DIA on Orbitraps:
  https://docs.google.com/spreadsheets/d/1A8AQlmLroAkQcAcsiGTNvnGBE2lGpkMwhh0YLTBHXKA

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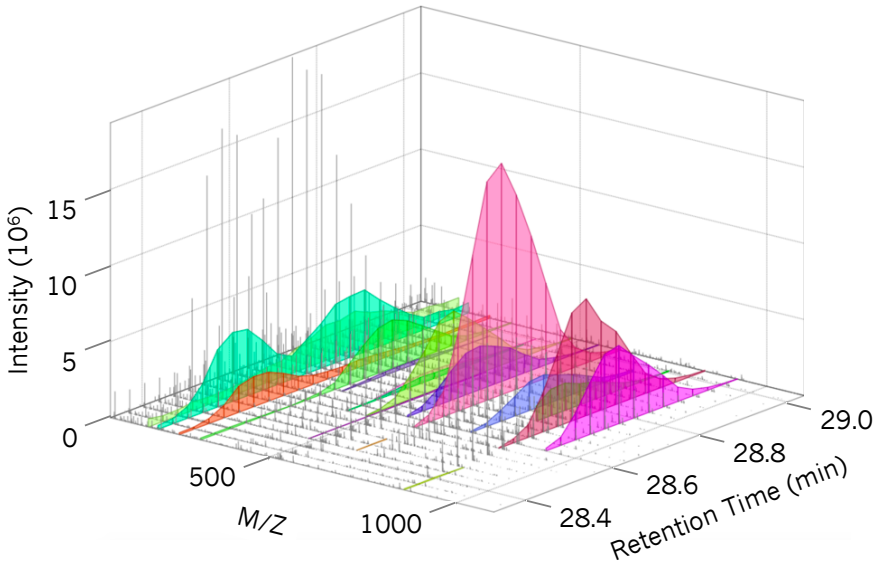

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- Rob Lawrence

Penn State University
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