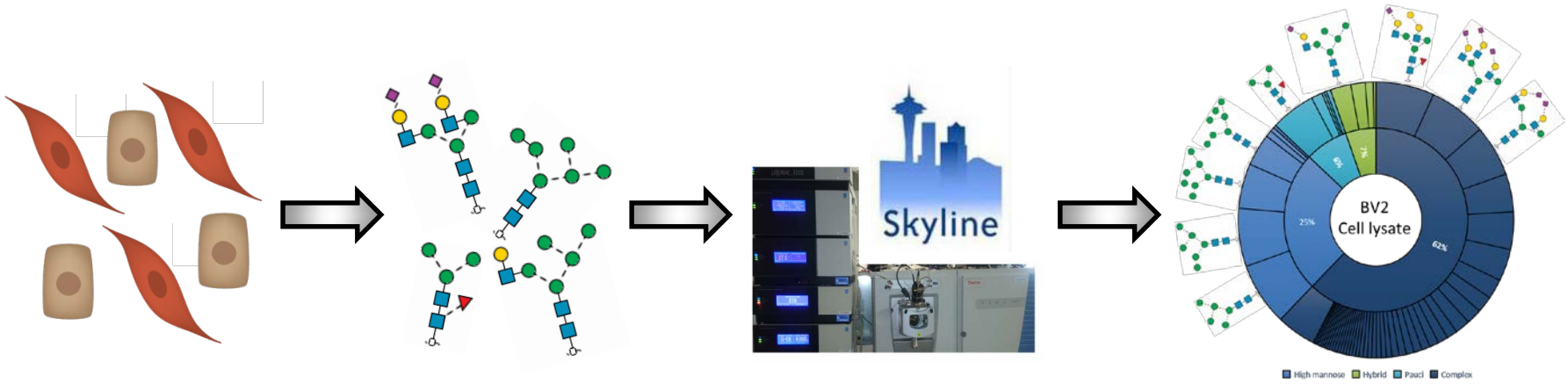
Applications of Skyline for automated profiling of protein glycosylation



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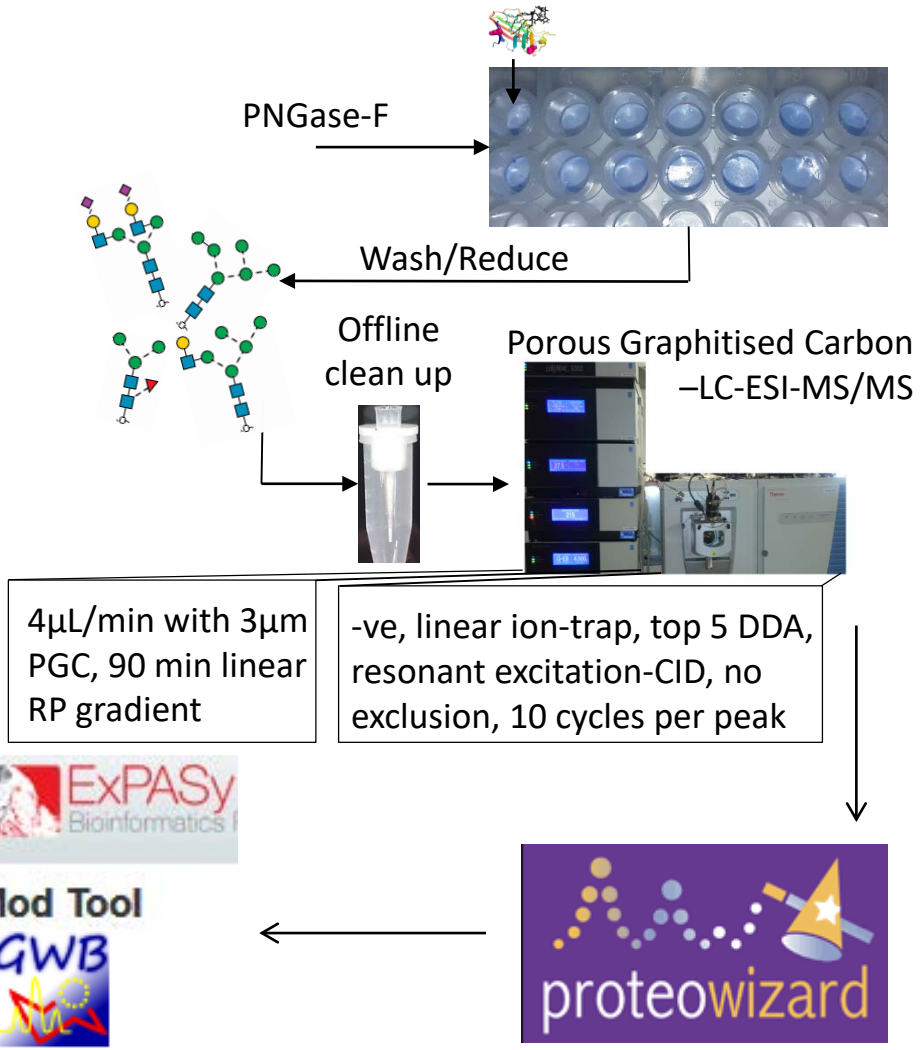
Why is glycan structure important?

- Glycan structure is highly regulated in human disease and development
  - Mediated by enzymes
- Glycosyltransferases build and glycosidases trim
- Glycan biosynthesis generates isomers
    - GlcNAc
    - Fucose
    - Galactose
    - Sialic acid
- LC-MS is one method to characterize and quantify structures

Obtaining glycans and isomer discrimination

- Our method assesses protein *N*-glycosylation by enzymatically releasing glycans

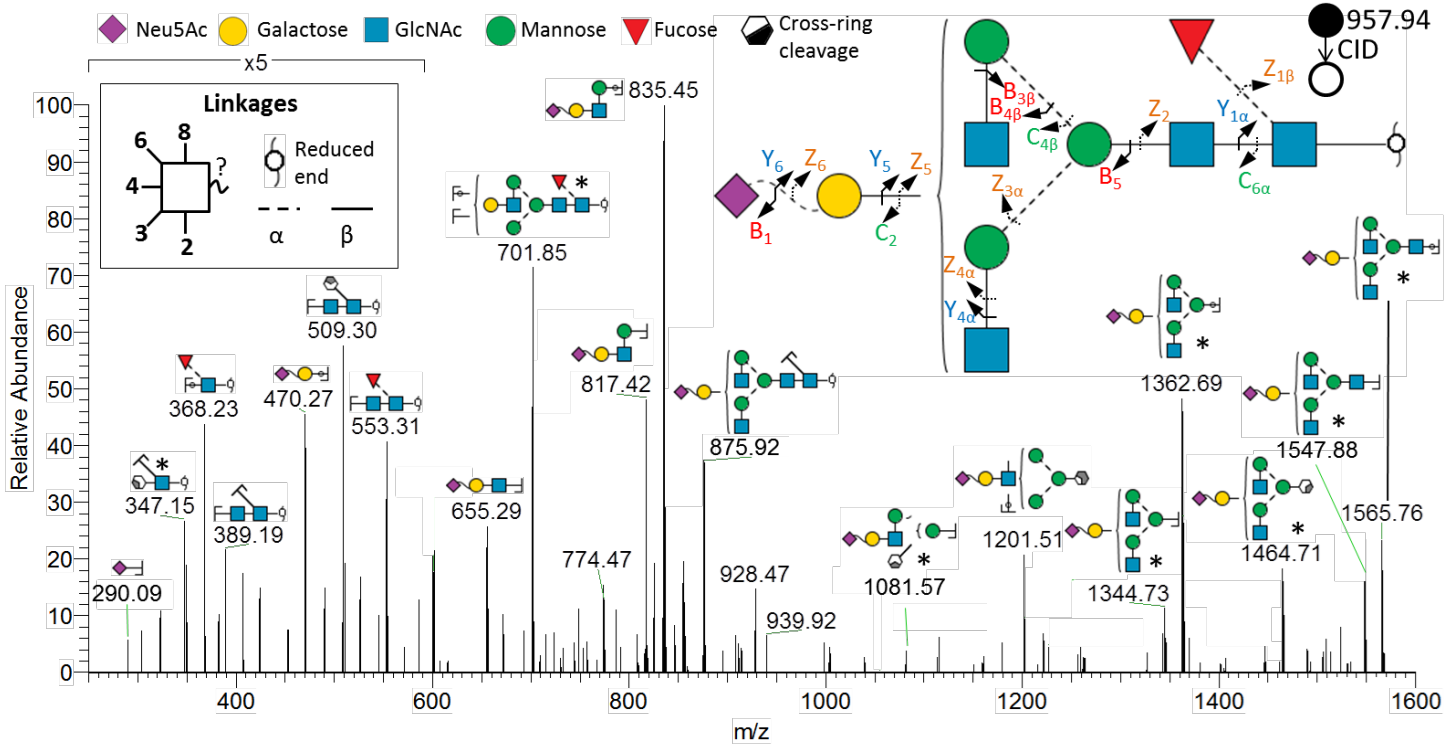
- Aim: Develop isomer discrimination strategies using Skyline
  1. Diagnostic product ions
  2. System-independent retention values
  3. Combined approach for robust peak picking



Jensen, P.H., Karlsson, N.G., Kolarich, D., Packer, N.H.: Structural analysis of *N*- and *O*-glycans released from glycoproteins, (2012).

Structural characterisation (-ve MS/MS)

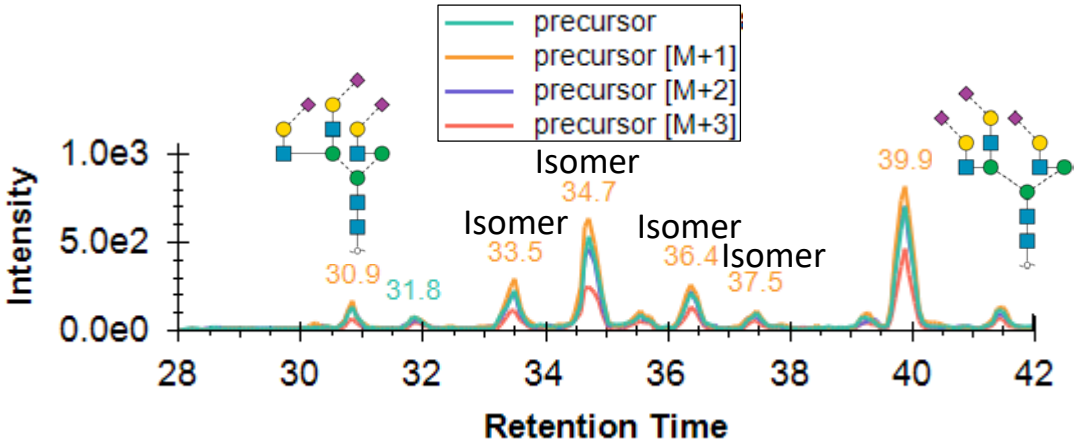
- **Solve:**
  - Composition
- **Solve:**
  - Sequence
- We can discriminate isomers using these ions



Structural characterisation (PGC-LC-ESI-MS)

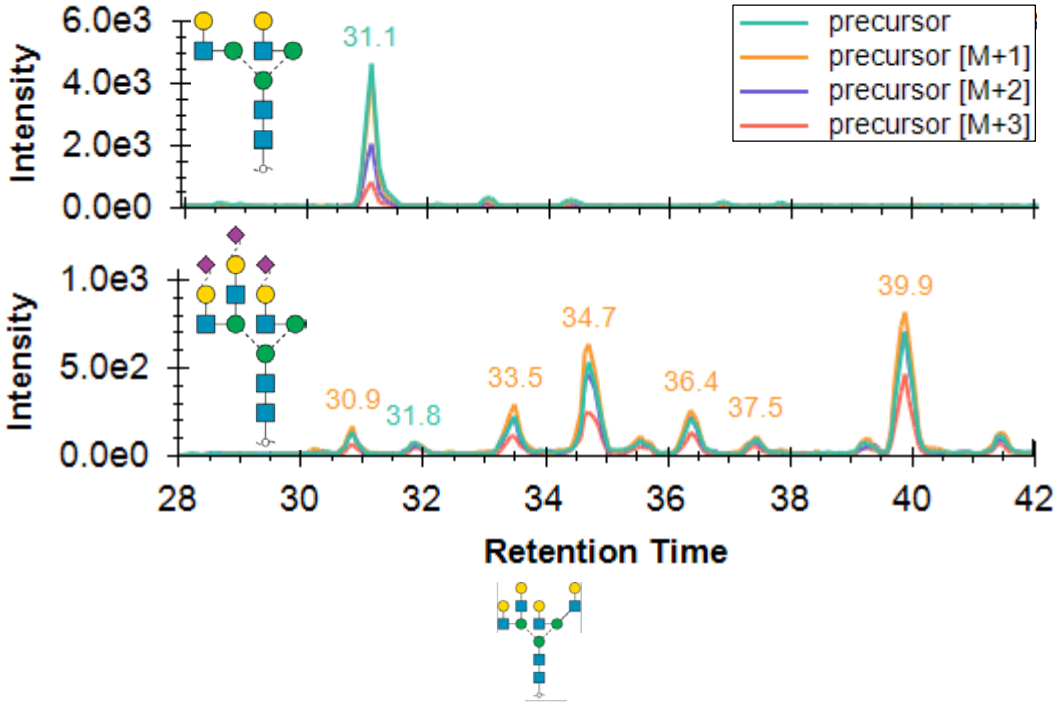
- Porous graphitised carbon (PGC) separates isomeric glycan structures
  - Provides structural separation of glycans prior to MS

- PGC-LC-ESI-MS/MS can characterise and quantify glycans but it has limitations
  - Isomers have similar MS2 spectra
  - Negative mode spectra is complex
  - Retention time is system-specific at best



Complexity of structure-based analysis

- Glycan structure elucidation is robust but manual
  - Skyline was a good automation option
- Automated peak picking with Skyline works well for 1 peak in an EIC
- Manual peak picking required for >1 isomer
- Diagnostic ions are one automated peak picking solution



Applying diagnostic ions in Skyline

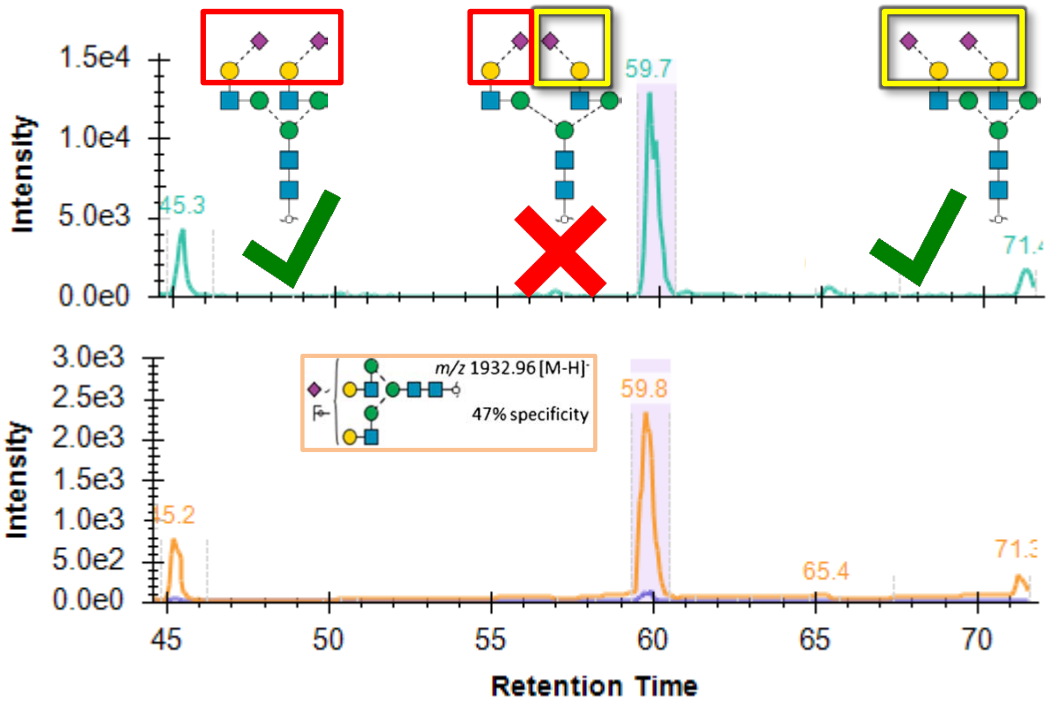
- Two glycan isomers with a slight difference in topology
- Core-fucose not at equal intensity
  - Structure matters for RE-CID!
- Published diagnostic ions work well
  - Complementary set of diagnostic ions not identified
- We expanded this repertoire using newly identified diagnostic ions
  - By subtracting their MS2 spectra

Harvey, D.J.: Fragmentation of negative ions from carbohydrates: Part 3. (2005).

Ashwood, C et al. Discrimination of Isomers of Released N- and O-Glycans Using Diagnostic Product Ions in Negative Ion PGC-LC-ESI-MS/MS (2018).

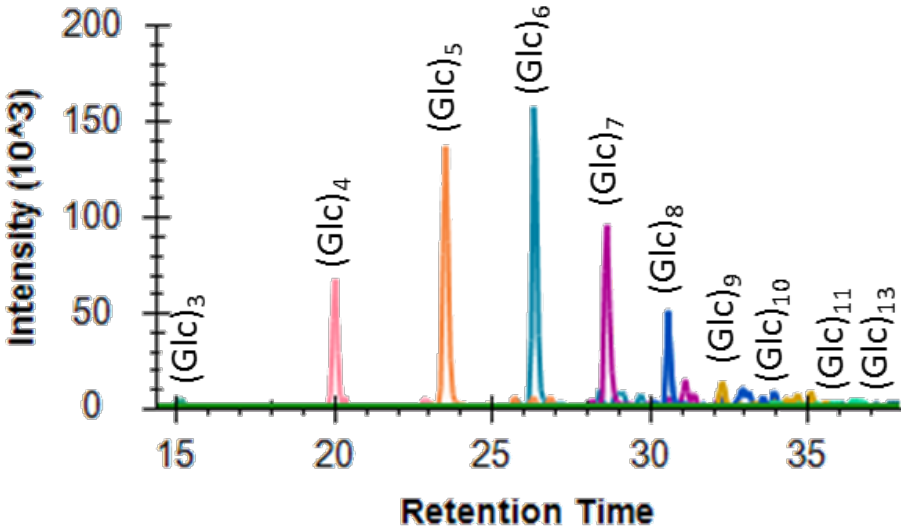
Discriminating between isomers

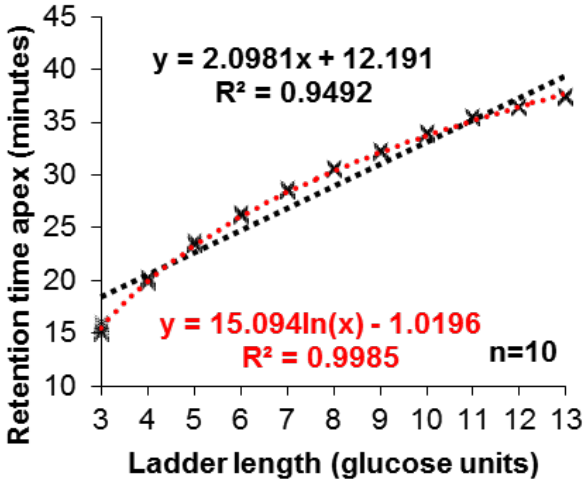
- Glycan structures can be automatically picked by their diagnostic ions
- Doesn't work well for glycans with mixtures of features
  - Sialic acid linkages ◆
  - No MS2, no isomer discrimination
- In these cases, retention time is more useful

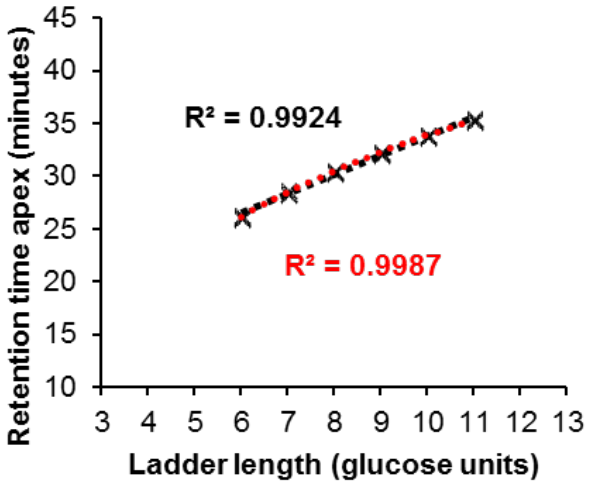


Normalizing retention time

- Underlying challenge of RT reproducibility
  - System-specific explicit RT
- Glucose polymer ladder can be used to normalise retention time
- Elutes logarithmically but can get a suitable linear equation
  - 6 point calibration

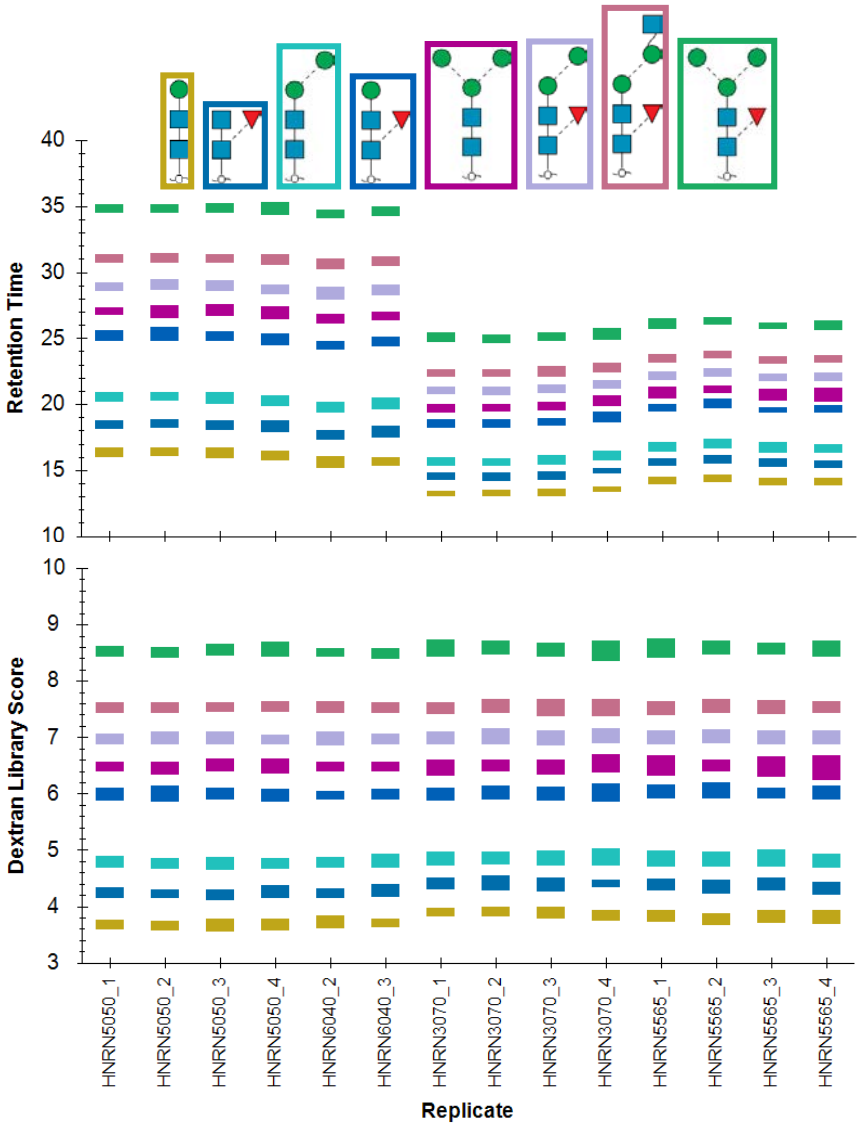






RT normalization with dextran ladder

- Spike dextran ladder in glycan mixture and analyse with LC-MS
- 6-point RT normalisation using Skyline (Peptide side)
- Log RT normalisation can be used to better describe glycan structures
  - More on that later



Glycan iRT library

- Wide range of glycans released from mammalian glycoprotein standards
- Good coverage when applied to Human glioblastoma cell line *N*-glycans
- Filled in gaps for full coverage as ladder is spiked into cell lysate glycans
  - Tri- and Tetra-antennary *N*-glycans
- Manual conversion of retention times to iRT after Skyline manual peak picking

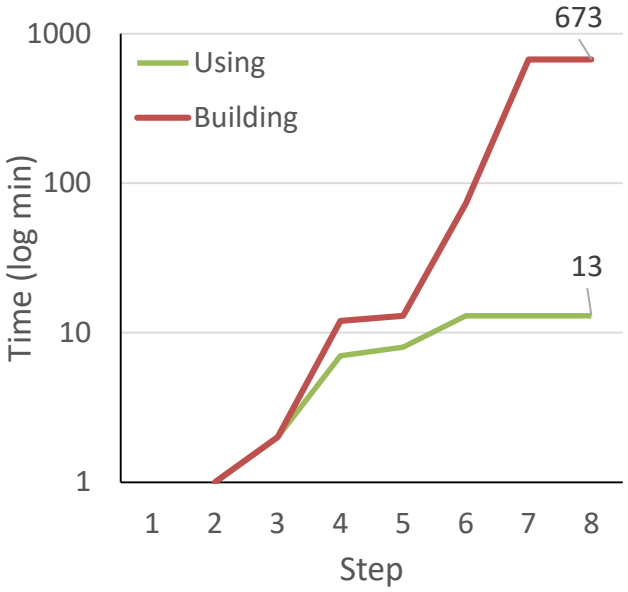
No one method is comprehensive

- Closely eluting isomers still a challenge
  - iRT variation > difference between isomers
- We can use a linear normalisation method but we know the elution is logarithmic
  - Made a script to convert RT to iRT (log) before Skyline import
- Now let's combine diagnostic ions with iRT for robust peak picking

https://github.com/chrashwood/mzML-RT-to-iRT-Exponential

Workflow for generating a spectral library

PGC-LC-ESI-MS/MS
Data acquisition



Using the library

1. Raw file
2. Skyline ladder spectral library
3. RT Eqn determination
4. Proteowizard
5. iRT conversion
6. Skyline import

4. Proteowizard/ OpenMS
5. iRT conversion
6. GlycoMod (Composition ID)
7. GlycoWorkBench (Structure ID and ion annotation)

Legend

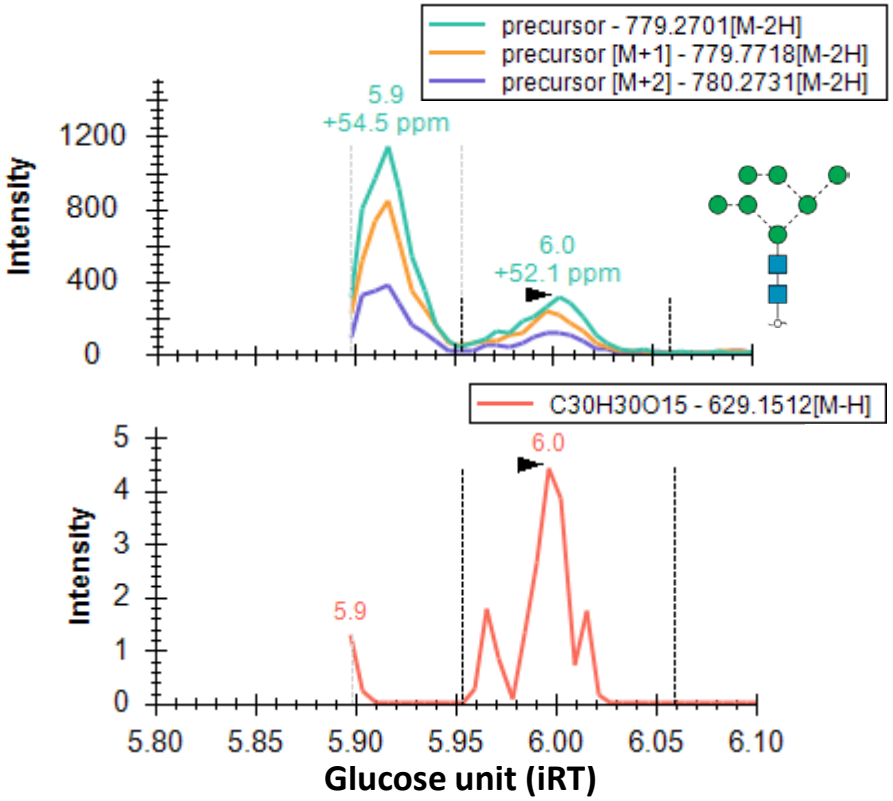
Automated

Manual but software assisted

Robust peak picking from a combined approach

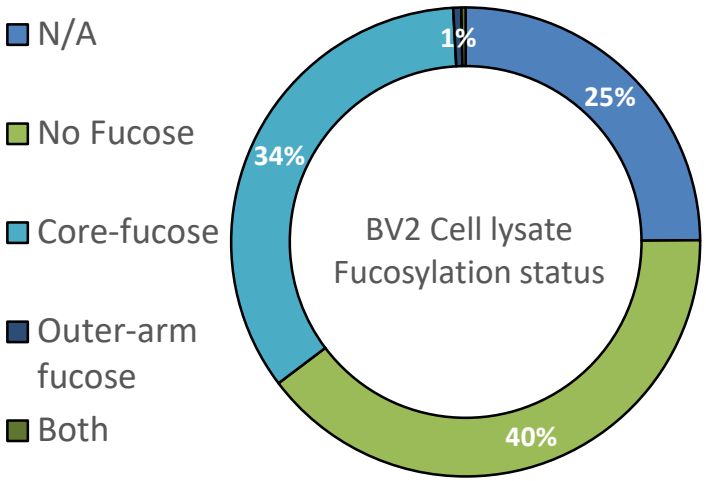
- Glycan library with diagnostic ions and explicit iRTs created for 309 *N*-glycan structures using:
  - 7 purified glycoproteins
  - 7 mammalian cell lysate and secreted protein mixtures
  - 5 regions of the mouse CNS

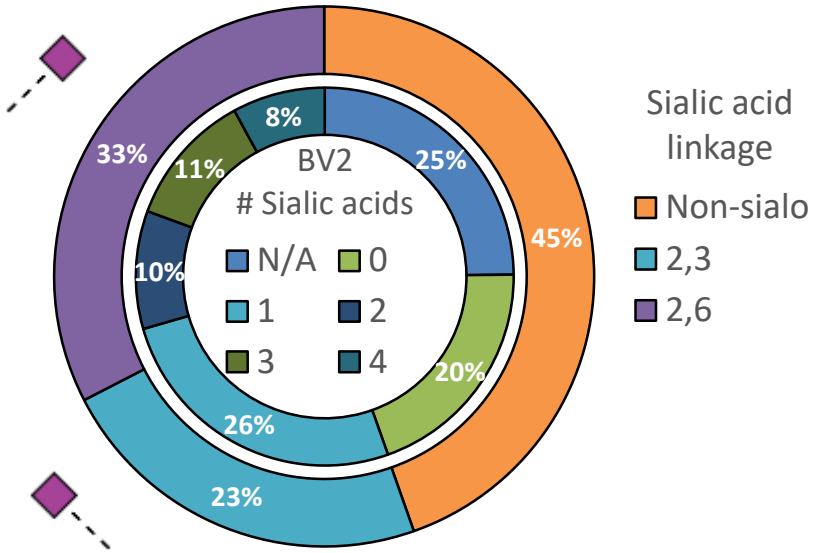
- Explicit iRT is generally doing most of the peak picking
  - Diagnostic ions/spectral libraries are still useful



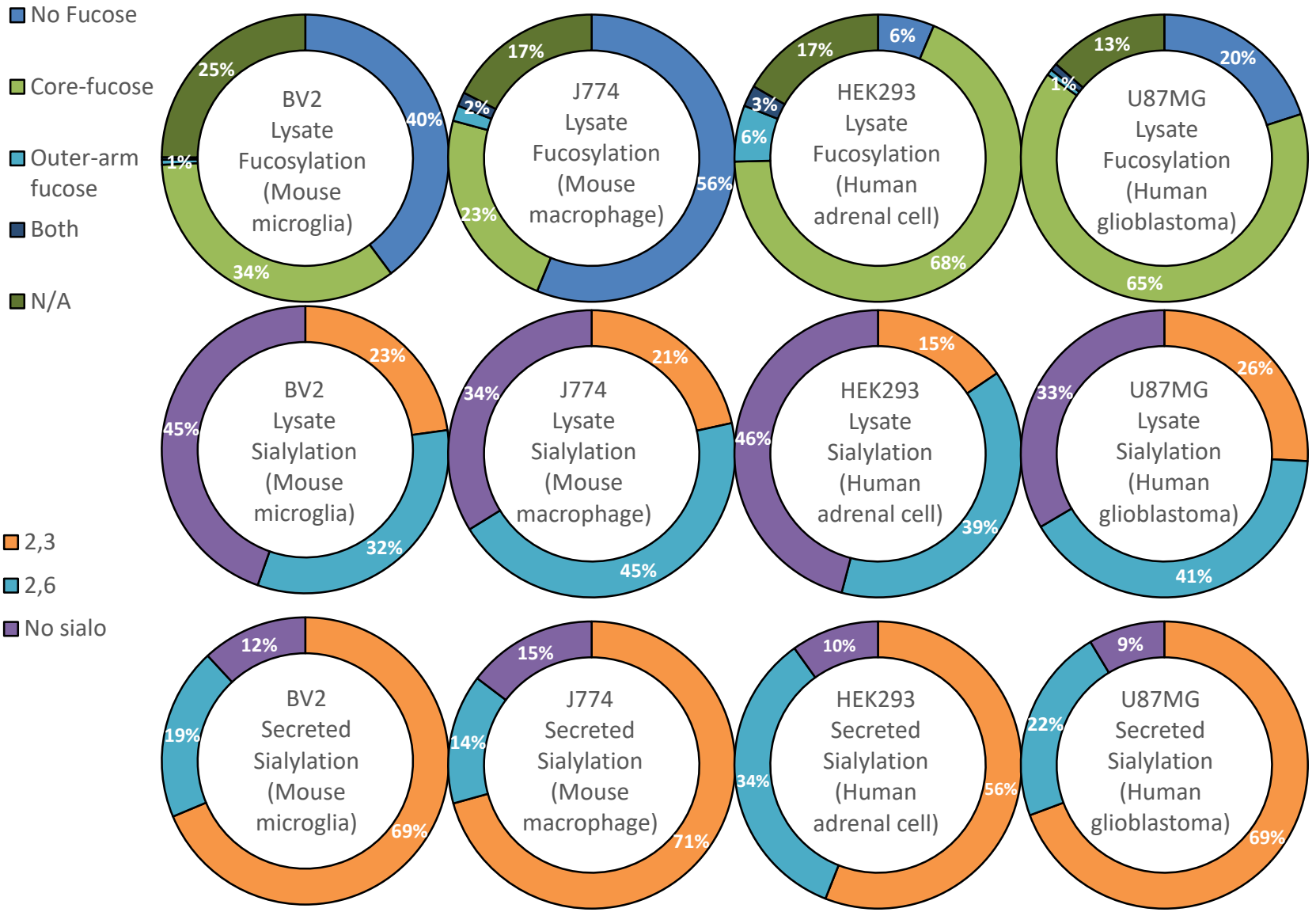
Mapping a cellular *N*-glycome

- Class level
- Structure level
- Fucosylation ▼
- Sialylation ◆



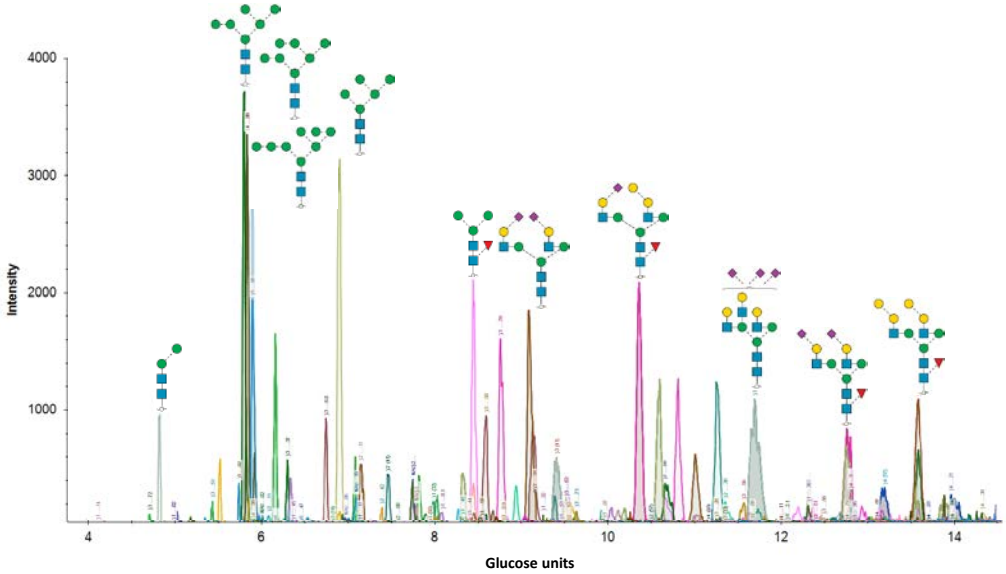


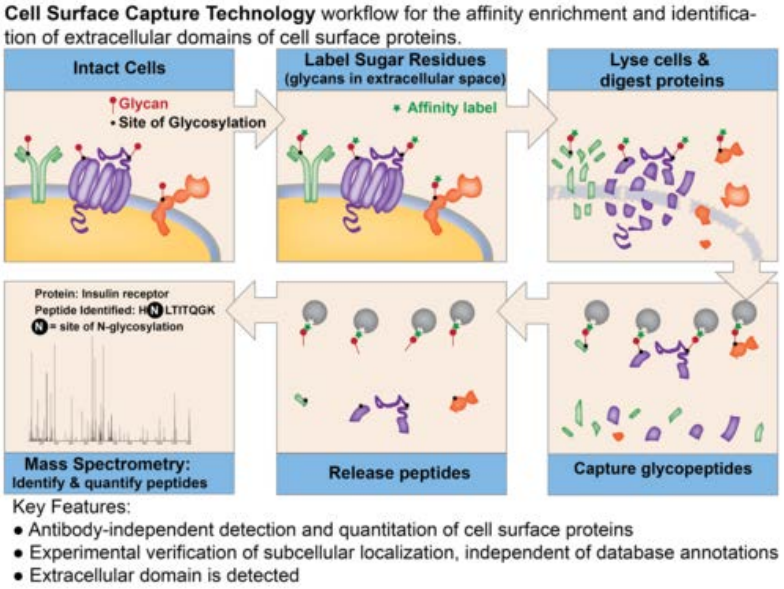
Comparing cellular *N*-glycomes



Conclusions and future directions

- Automated data analysis, reduced complexity
  - Diagnostic ions and explicit iRT
  - Open-source and data on Panorama once published
- High throughput using glycan library with Skyline
  - Library can be tailor-made
- Supervision of peak picking still required
  - Optimization of peak scoring model
- Working with cell lysate, not representative of the cell surface
  - New position as post-doc in the Gundry lab specializing in cell surface glycoprotein capture





Acknowledgements

- Glyco@MQ
  - Packer Lab
- Thermo Fisher Scientific
  - Method development assistance
- Gundry Lab
- Skyline team
  - Particularly Brian!

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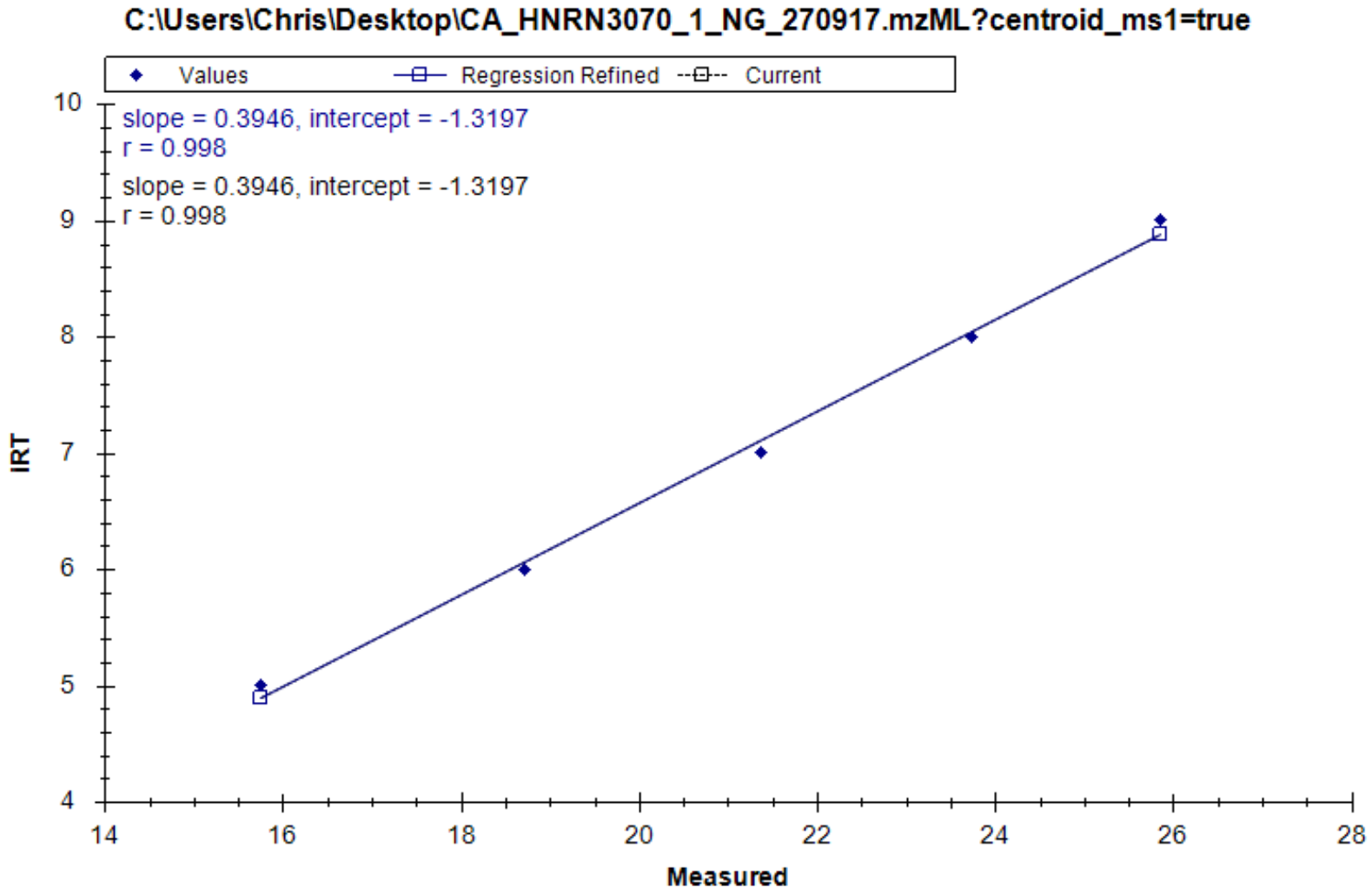
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Regression Refined
Current
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r = 0.998
slope = 0.3946, intercept = -1.3197
r = 0.998
IRT
Measured
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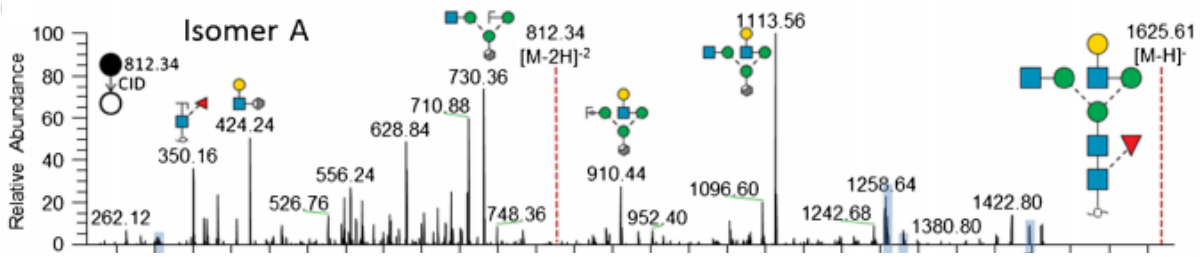
28

Identifying diagnostic ions

- Glycan isomers are separated on PGC before MS
  - Rarely chimeric MS2 spectra
- Averaged isomer MS2 spectra is very similar
- Subtracting the MS/MS spectra from two isomers
  - Product ions specific for each isomer appear



Spectral library application

- Peak picking of the ladder is manual when dealing with isomers
- Small molecule spectral matching feature for assigning glycan structures
- Diagnostic ions essential for picking the right peaks
  - And they’re annotated too!

Low resolution ion-traps are not an issue for discovery glycomics