Enhancing limited sample- and single-cell proteomics through tailored Data Independent Acquisition

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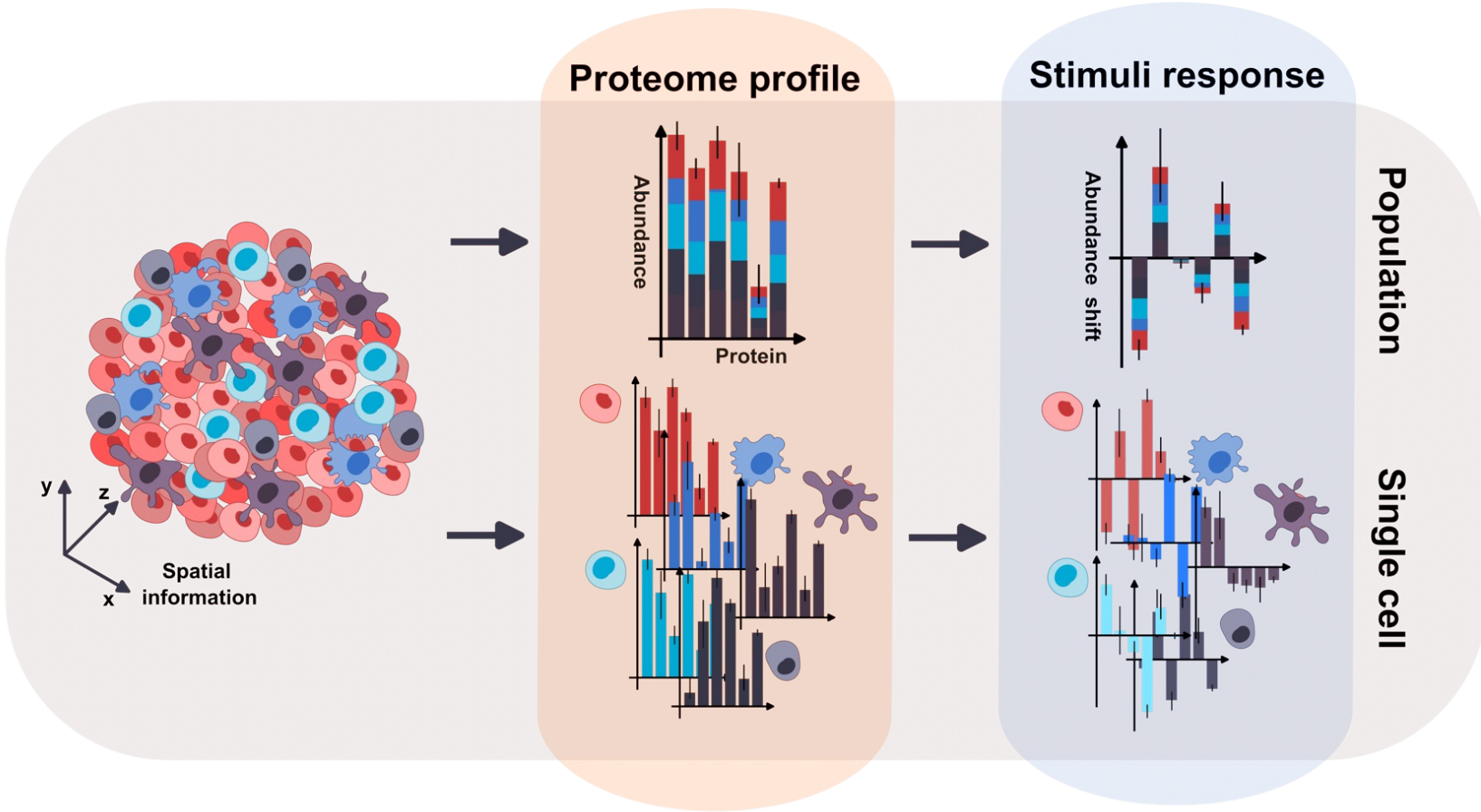
Why do we need single-cell proteomics?

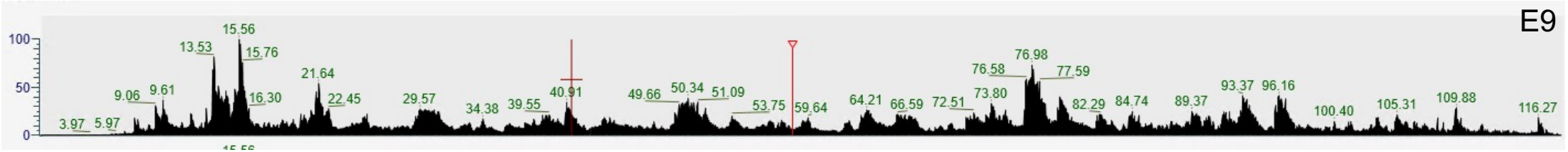
- Cell-state heterogeneity remains opaque with population-based techniques.
- Single-cell resolution can provide deeper insight into developmental programs and disease pathology.
- Single-cell proteomics (scp-MS) is/has reached a stage where it can be leverage for biological application



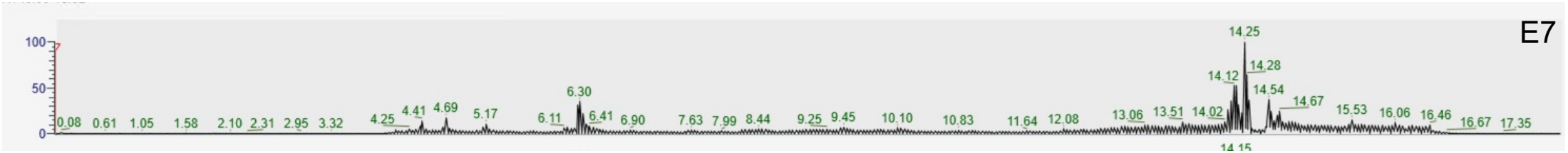
The pivotal challenge in scp-MS

Obtaining deep proteome coverage from the amount of protein encapsulated in a single cell (50-250pg)

Standard sample (250ng)

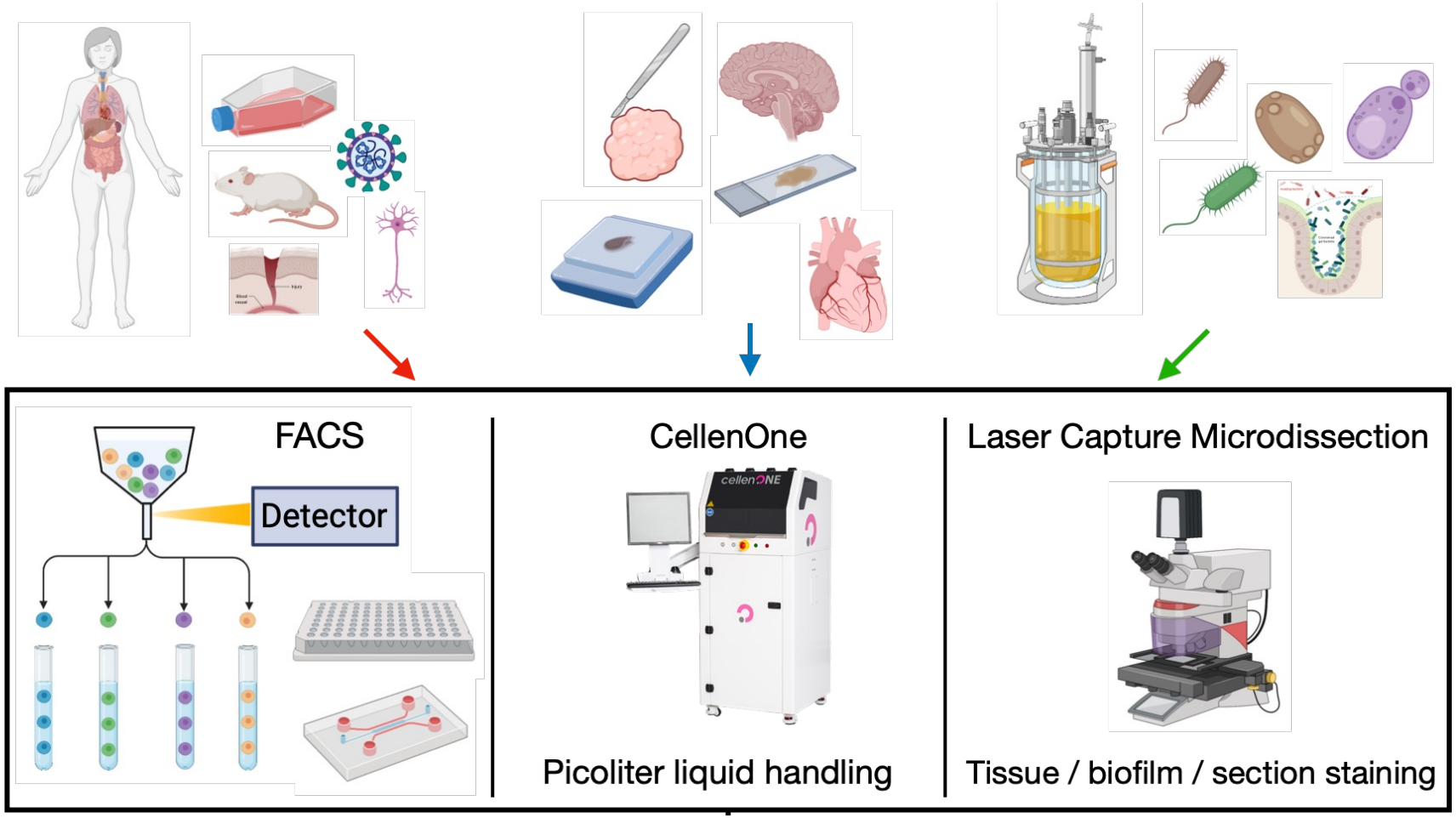


Single HEK293 cell (-- pg)

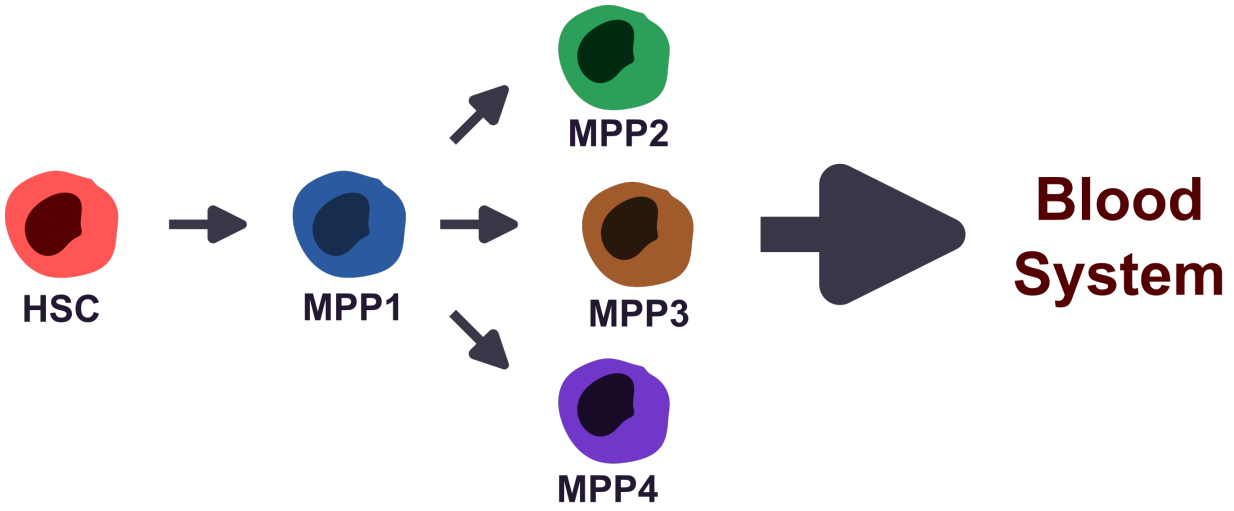


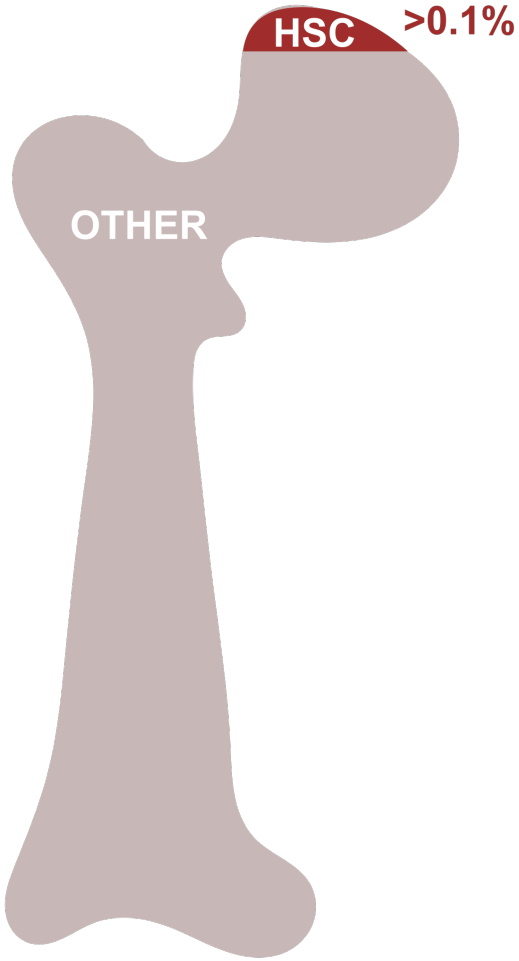
This challenge is slowly being overcome by sample preparation and tailored data acquisition approaches

Single-cell isolation

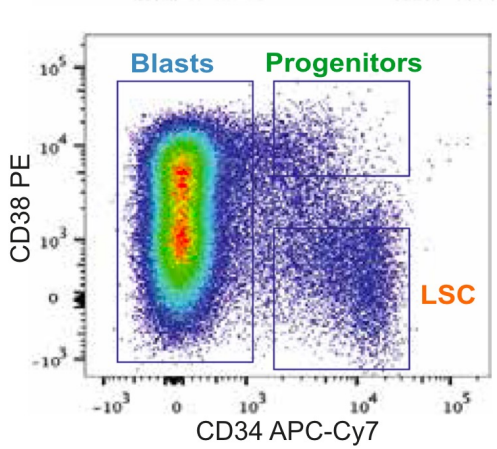


Reaching your population of interest





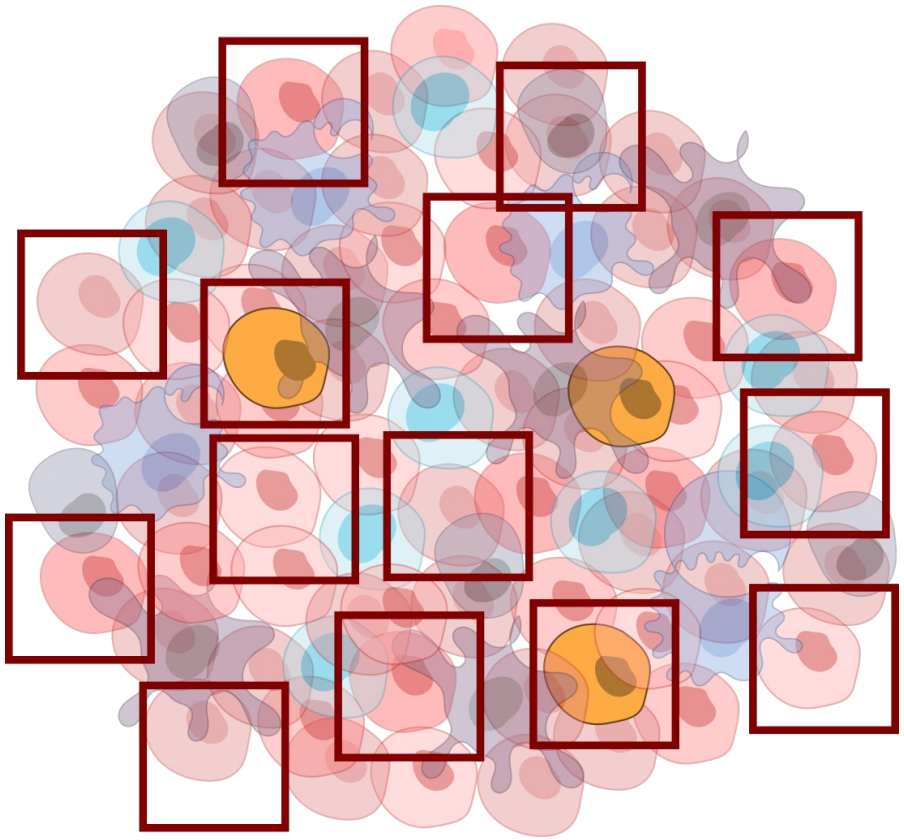
In a 5000 cell dataset only 5 stem cell will be capture!



Cell population specific markers can be used to isolate your population of interest!

What encompasses a good scp-MS acquisition method

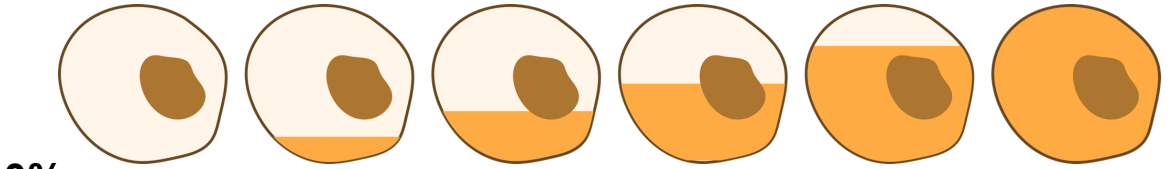
High throughput!



5 000 cell dataset cost:

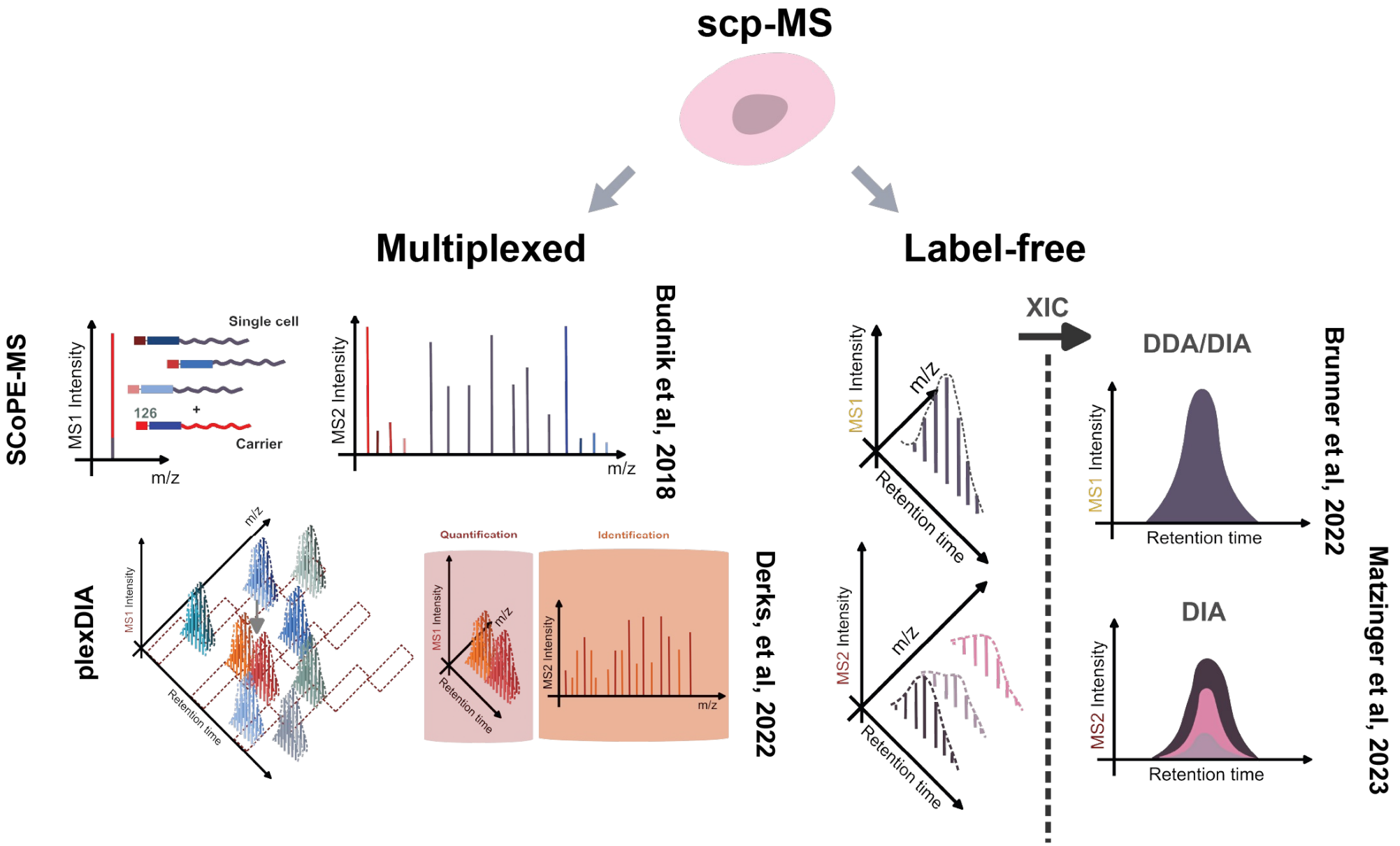
- 20 sample per day (SPD) methods ~ 8 months
- 40 SPD > 4 months
- 80 SPD ~ 2 months
- 160 SPD ~ 1 month

Proteome coverage



- The total number of protein groups expressed in a single-cell is unknown
- In a cell population, 14 000 – 18 000 proteins can be detected (Bekker-Jensen et al, 2017, Sinitcyn et al, 2023)
- In a single cell there are probably ~5000-8000 expressed proteins (SPECULATION!)

Multiplexed vs label-free single-cell proteomics approaches



Principles behind multiplexed scp-MS

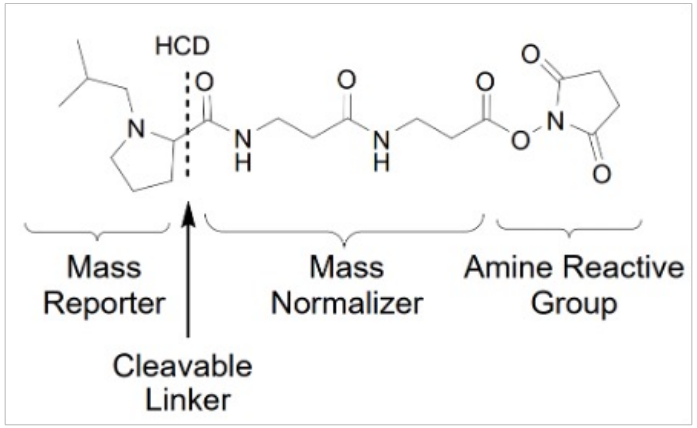
Multiplexed single-cell proteomics enabled through SCoPE-MS

SCoPE-MS – Budnik, ..., Slavov 2018

SCoPE2 – Specht, ..., Slavov 2021

SCoPE2 – Petelski, ..., Slavov 2021

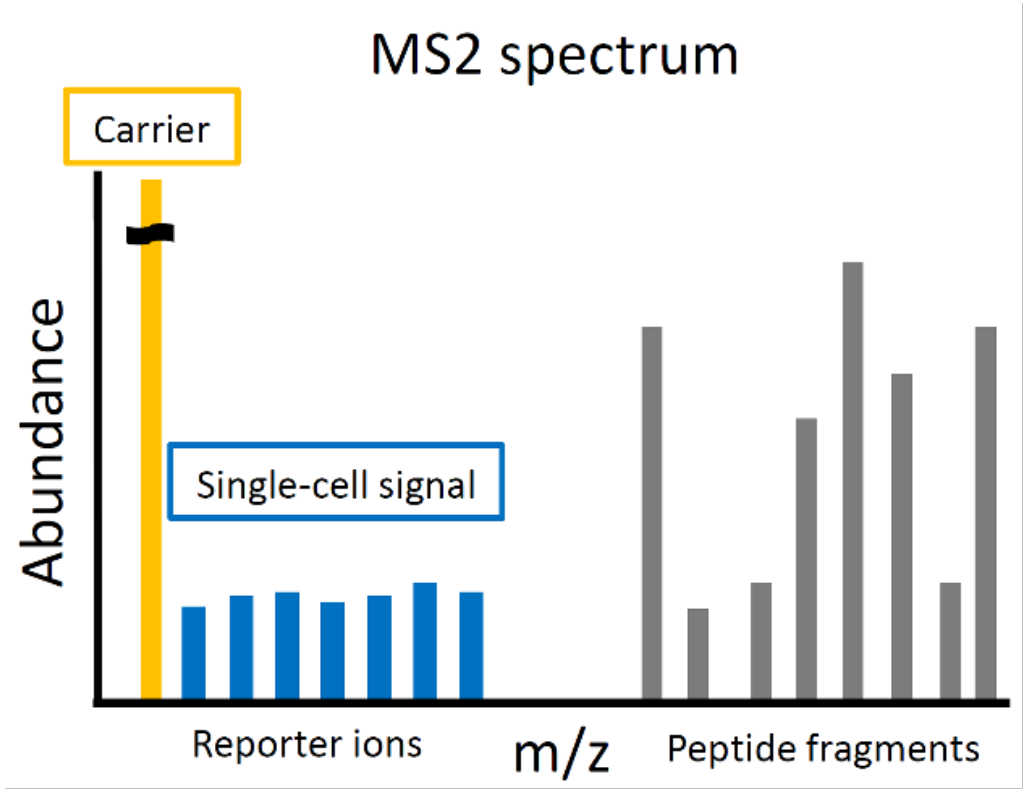
1.Isobaric multiplexing using tandem mass tag (TMTpro)



Example TMTpro labelled peptide:

(N-Term TMTpro)-VSHVSTGGGASLELLEG**K**(**TMTpro**)

2.Addition of carrier channel to facilitate peptide identification (e.g. 200 cell equivalent)



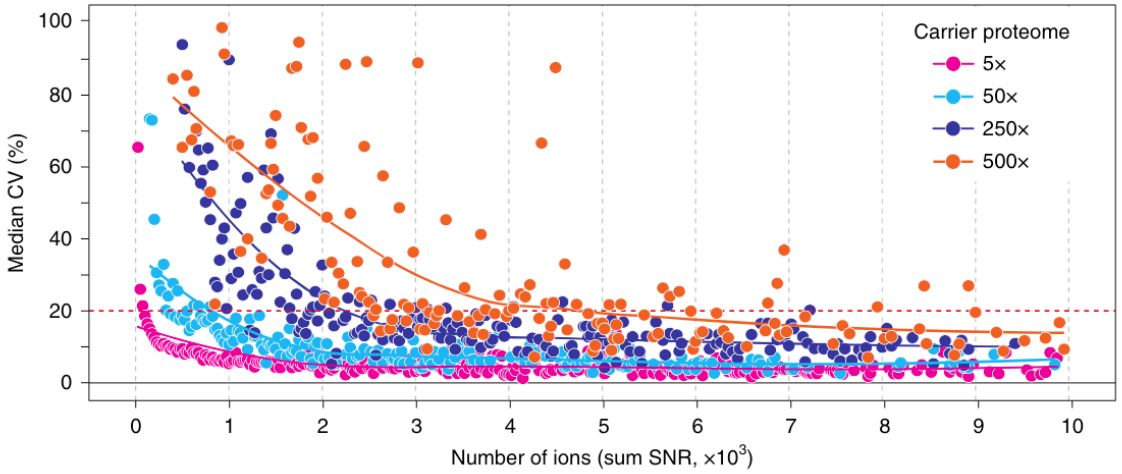
LFQ vs SCoPE-MS

Pros

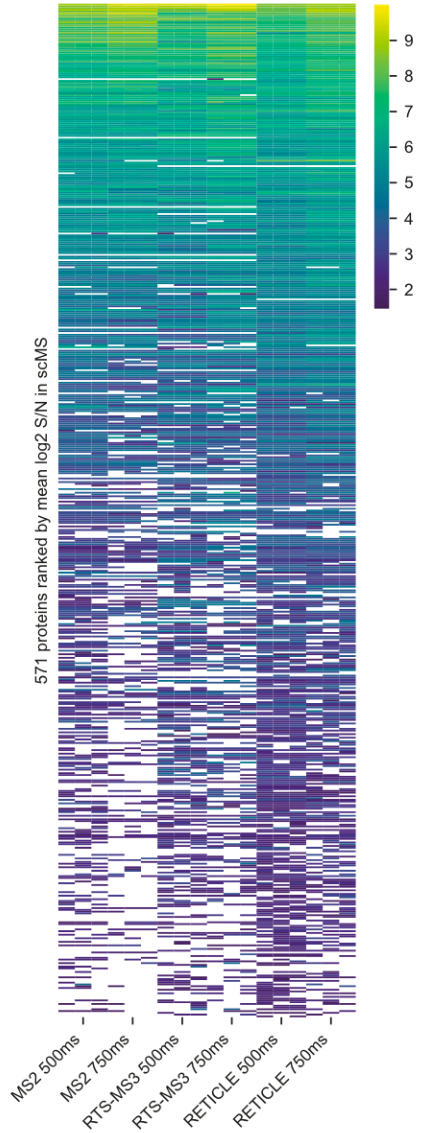
- Higher throughput then label-free methods (320 cells per day vs 40 cells per day)

Cons

- The carrier channel introduces a bias in the identification and quantification
- High degree of missing values (~40%)
- Lower proteome coverage compared to LFQ (~1000 vs 2000 protein groups)
- Collecting enough cells for the carrier channel can be challenging when primary patient material is used



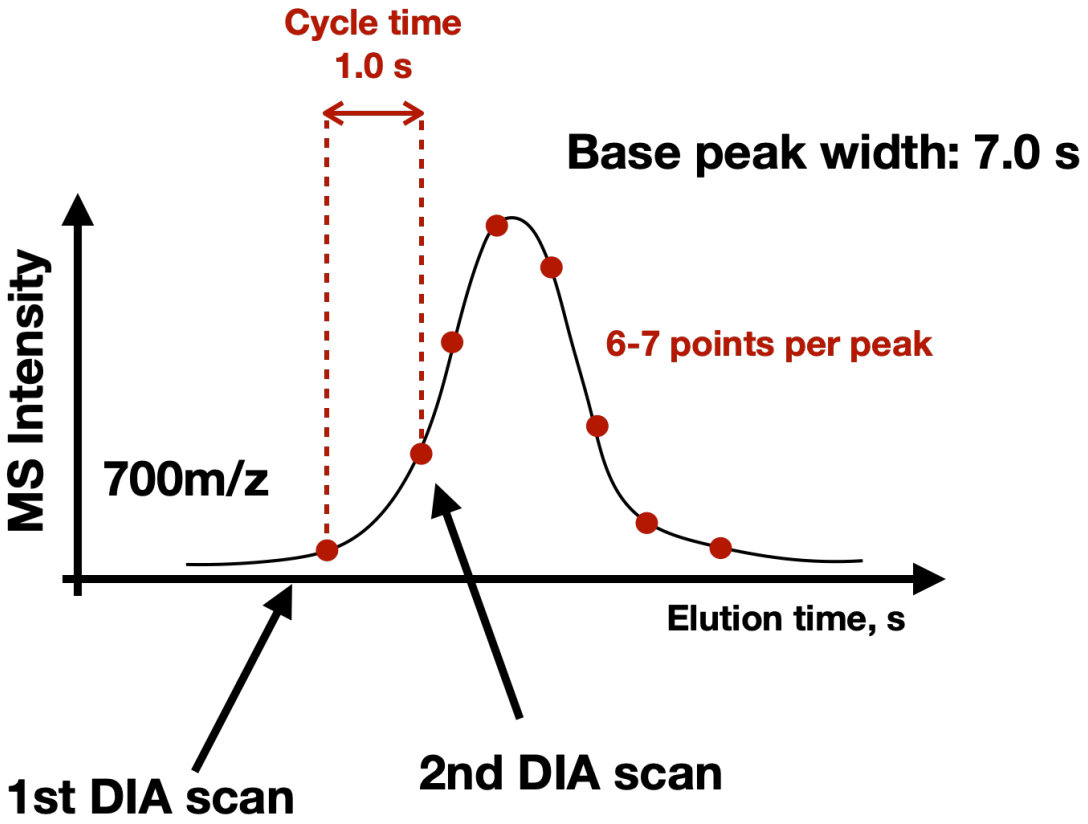
Cheung et al, 2021

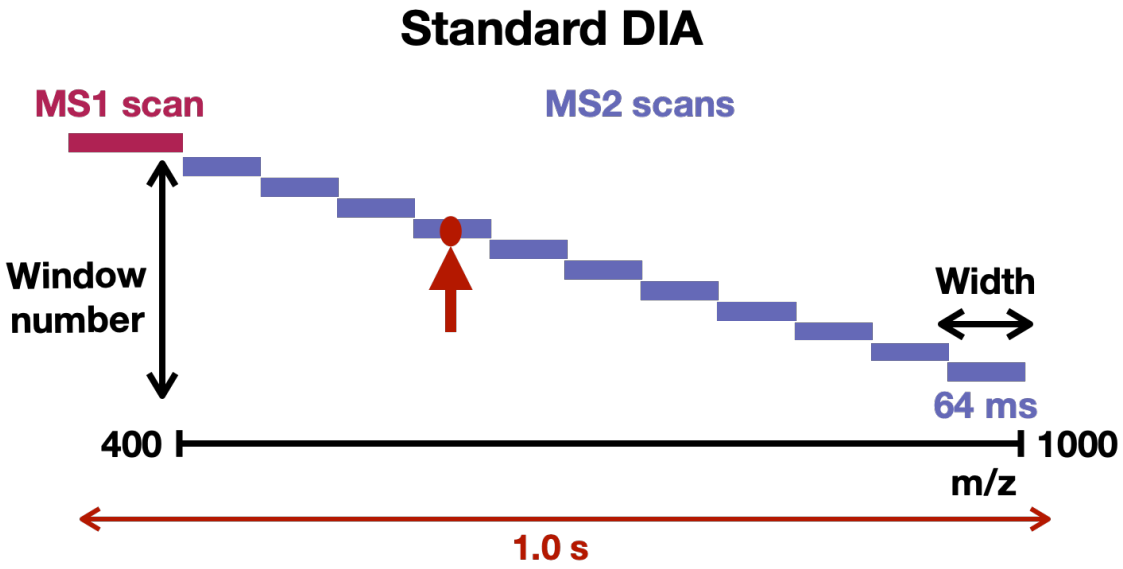


Furtwängler et al, 2022

DIA for single-cell proteomics

The scan cycle time dictates the method optimization space



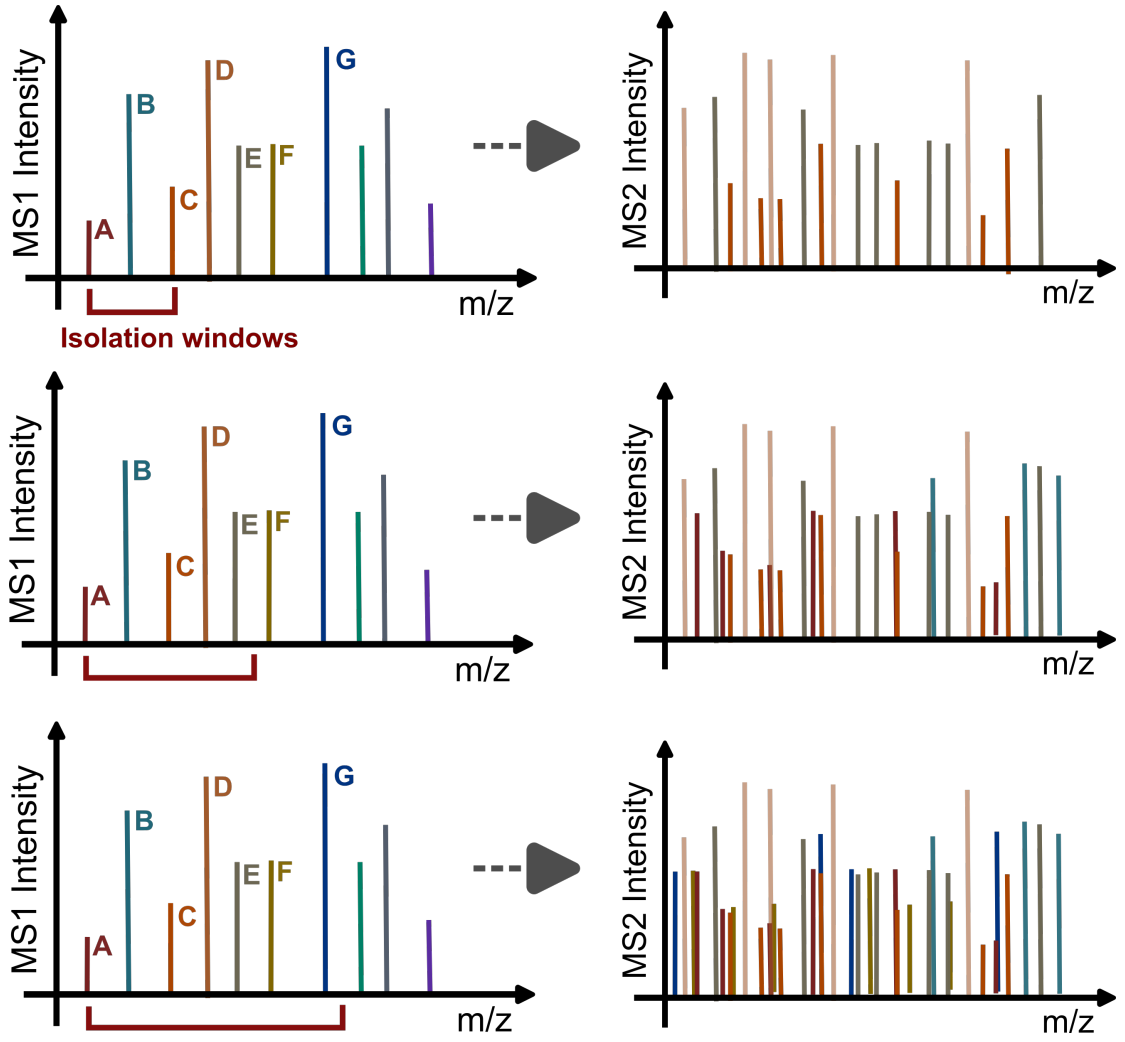


To compensate for increased resolution e.g. 30K -> 60K, the DIA isolation window with has to be accordingly increased to maintain cycle time

What DIA isolation window should I use?

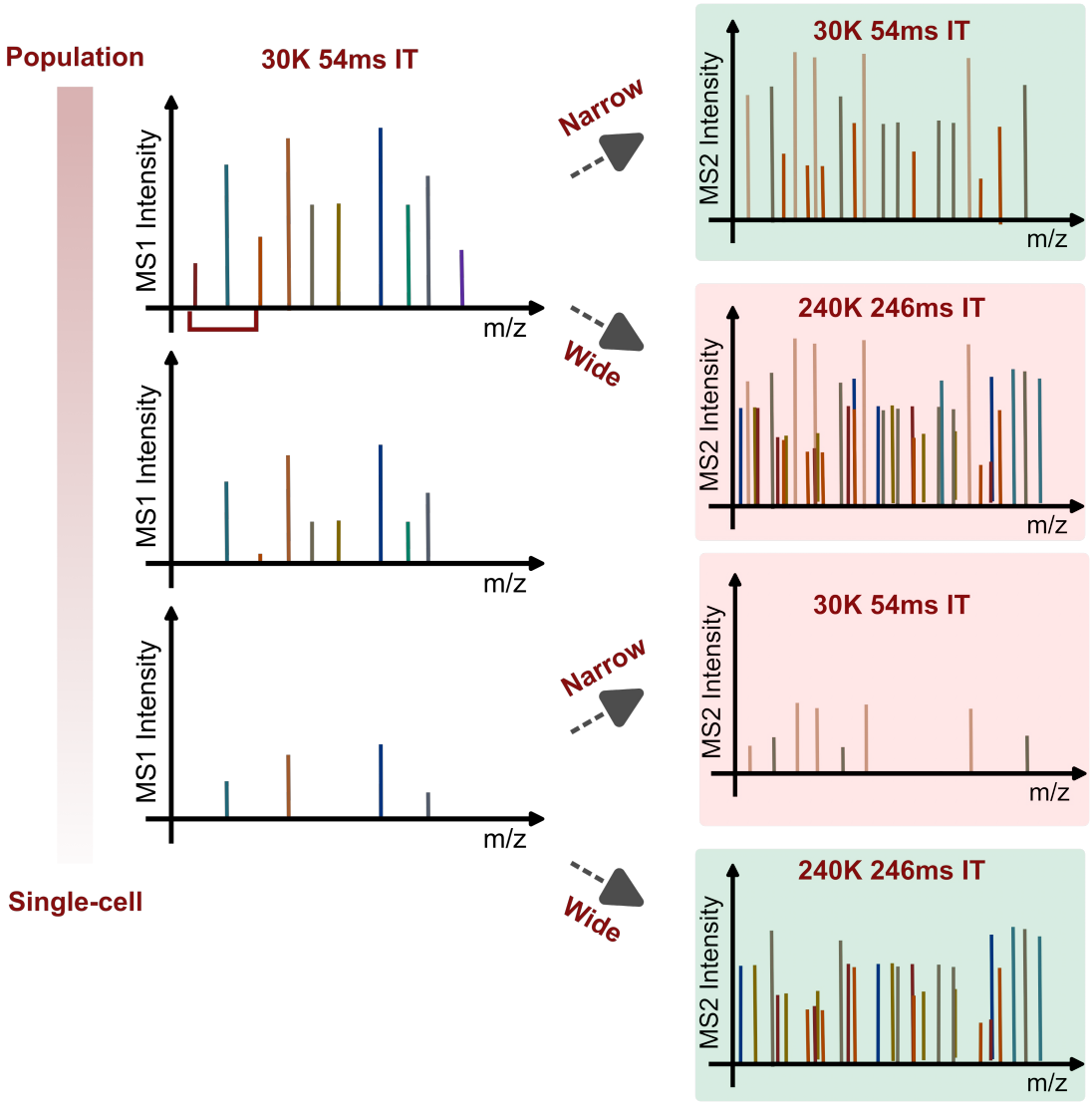
- **Increasing the isolation window size during DIA based acquisition should in theory hamper peptide identification due to more extensive precursor co-isolation.**

- **In high load samples (>10ng) narrow isolation windows with relatively low resolution generally give best performance.**



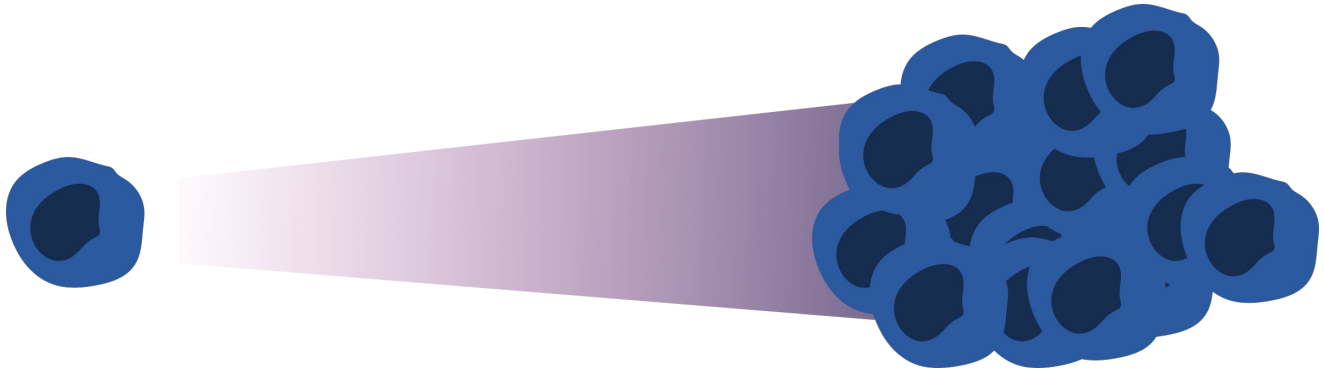
DIA isolation window size for limited input

- DIA method design for limited input samples (< 10ng) has not been comprehensively explored.



Aim of the project

Aim : Establish limited input tailored LFQ DIA methods for optimal proteome coverage while maintaining quantitative accuracy



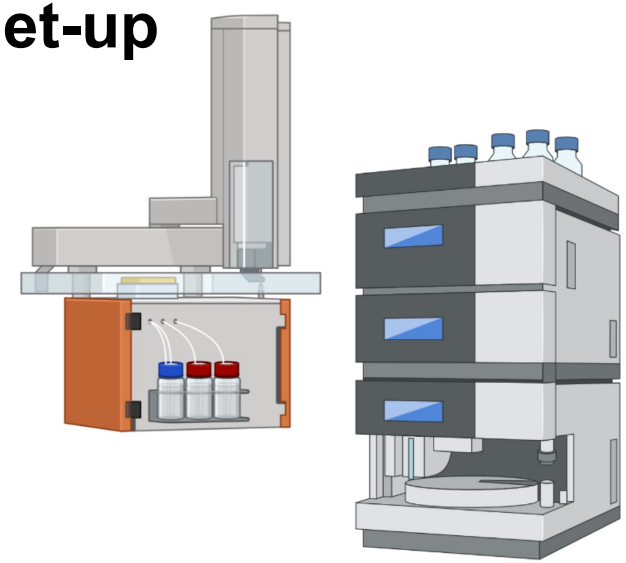
Single-cell

Rare cell population
Minimal-residual disease
FFPE tissue slices

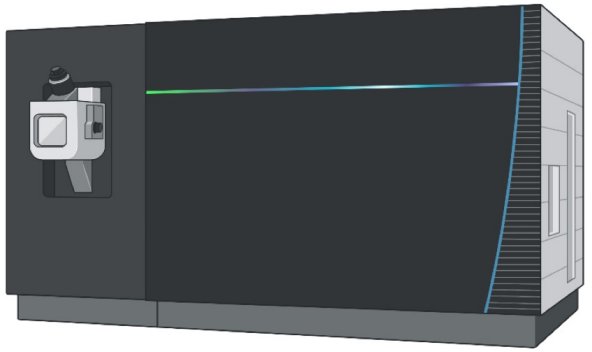
Results

Experimental set-up

Pierce Hela digest and HEK293 single-cells

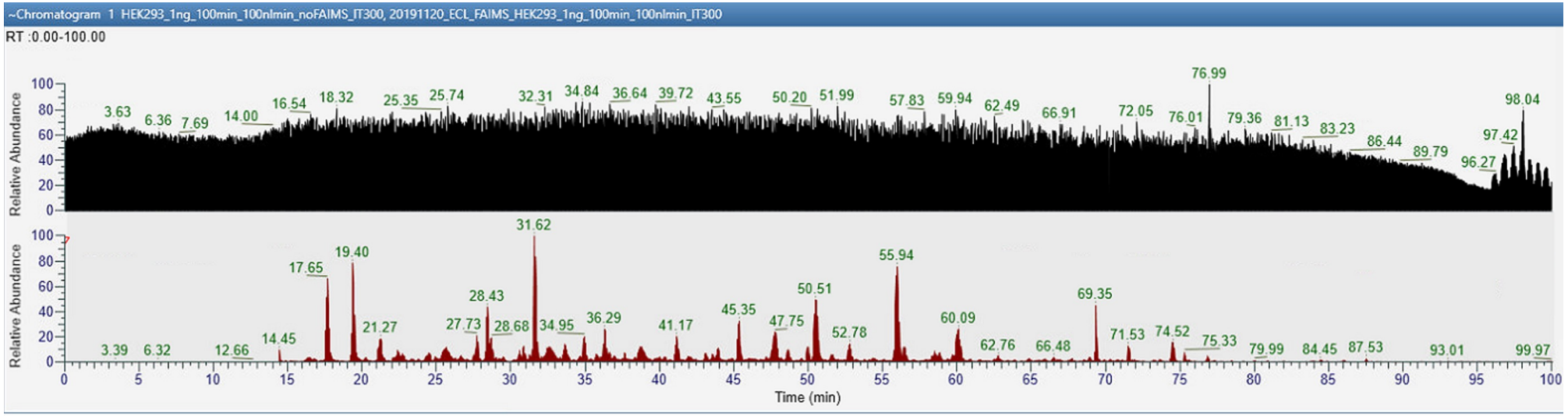


Evosep One or Dionex UltiMate 3000



Orbitrap Eclipse Tribrid + FAIMSPro

Spectronaut™
powered by Pulsar

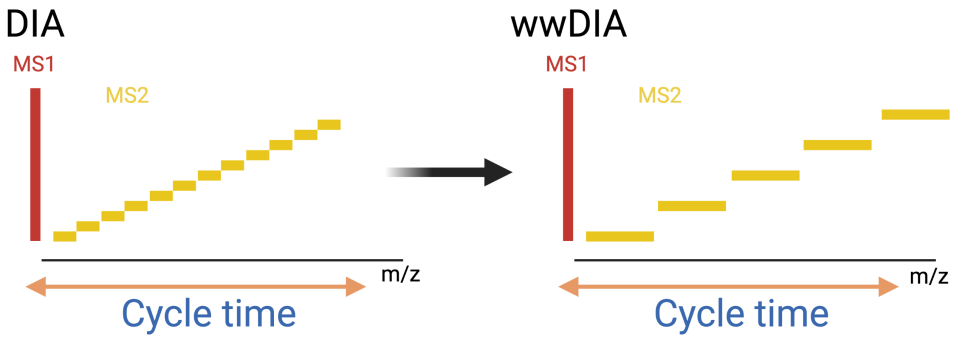


Without FAIMS

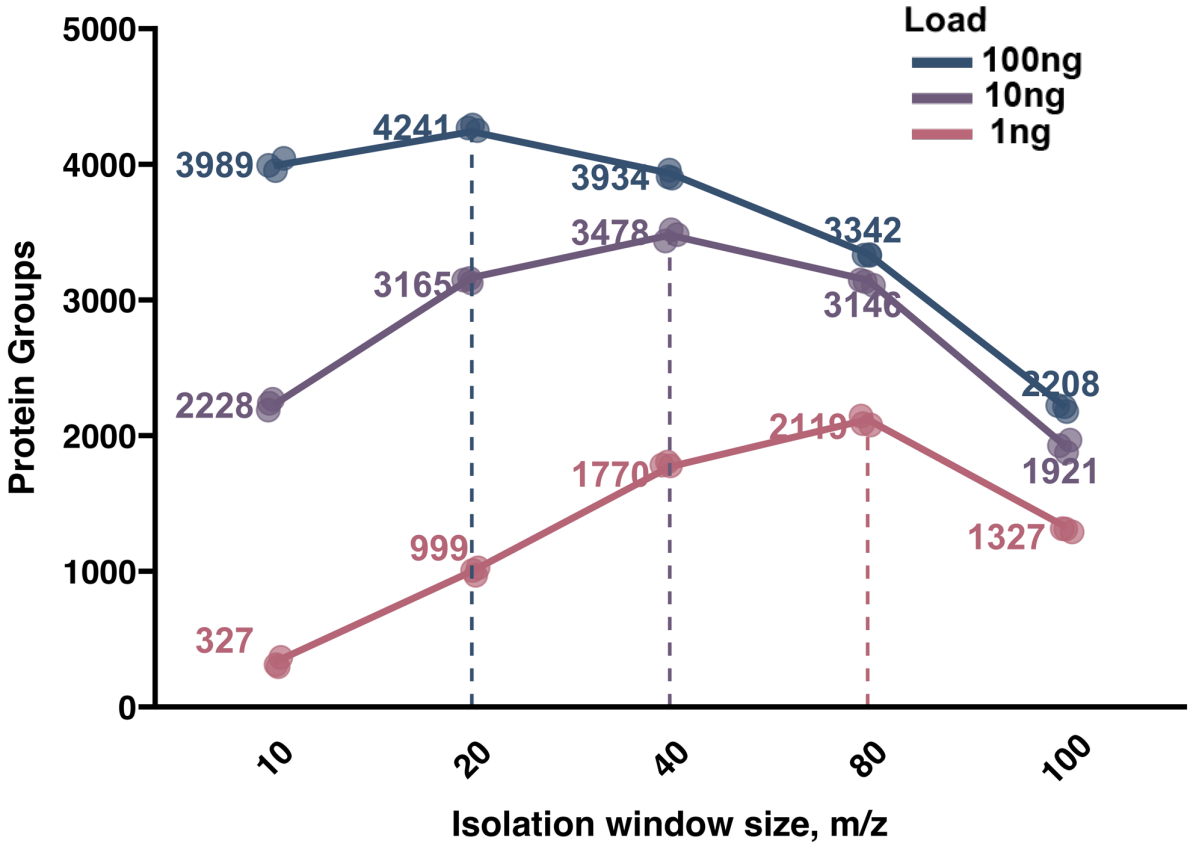
With FAIMS

Inspired by: Karl Mechtler, IMP

Evaluation of wide-windowed DIA for limited sample -and scpMS

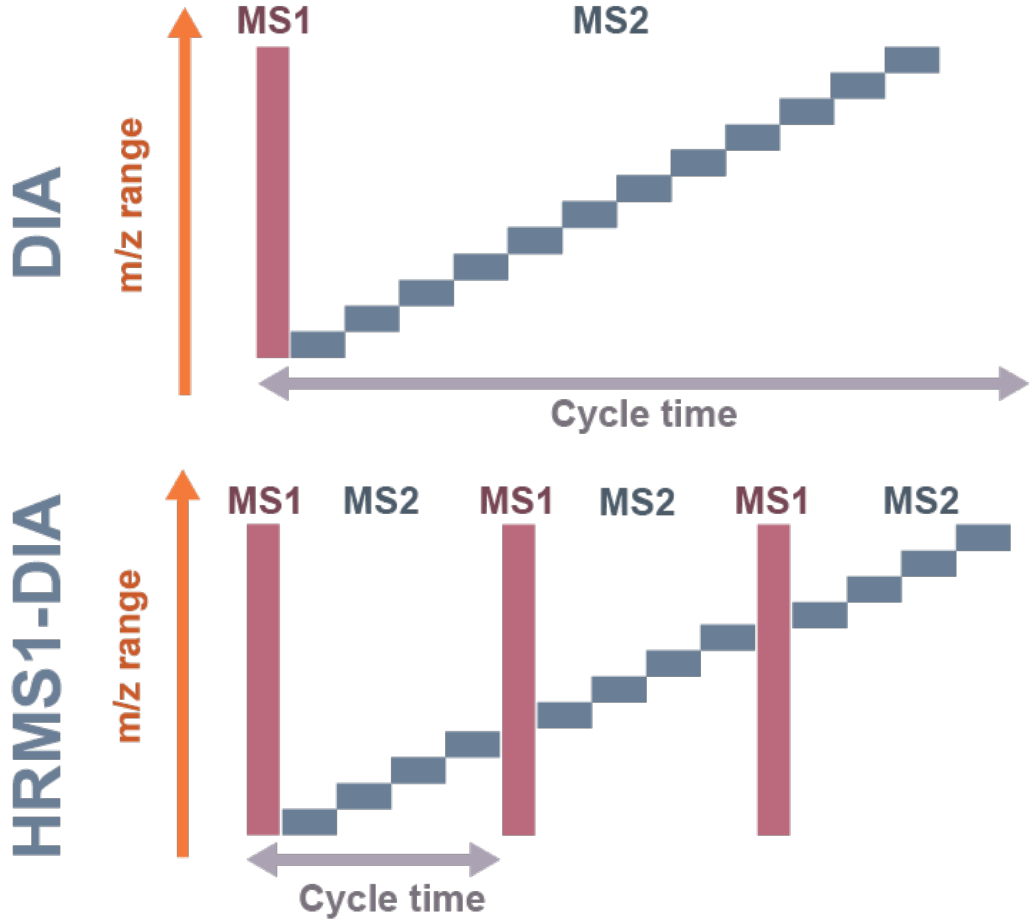


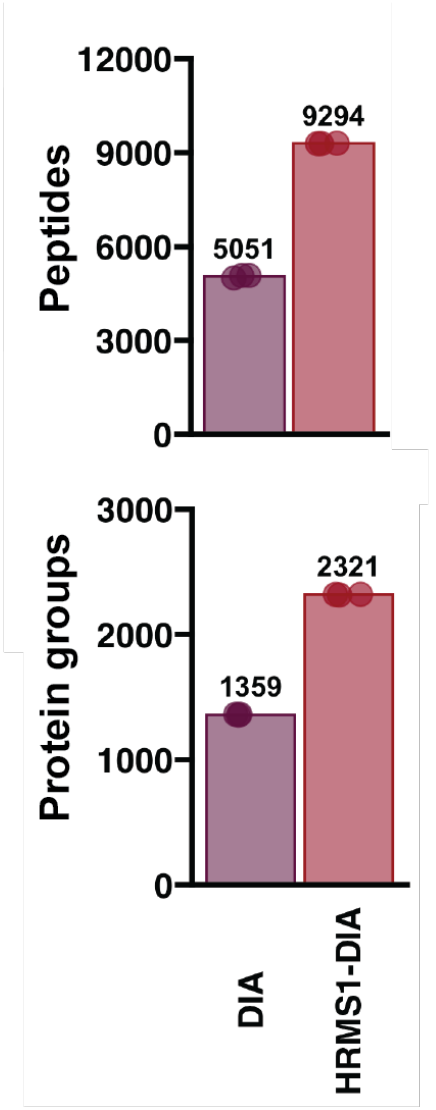
Resolution		Window		
MS1	MS2	Size	Number	Cycle-time
120K	15K	10mz	40	1.536s
120K	30K	20mz	20	1.536s
120K	60K	40mz	10	1.536s
120K	120K	80mz	5	1.536s
120K	120K	100mz	4	1.280s



- Wider isolation windows allow for the accommodation of higher resolution/IT.
- Detrimental chimeric spectra effects are overcome by higher resolution/IT

Evaluation of high resolution MS1 (HRMS1) DIA for limited sample -and scpMS

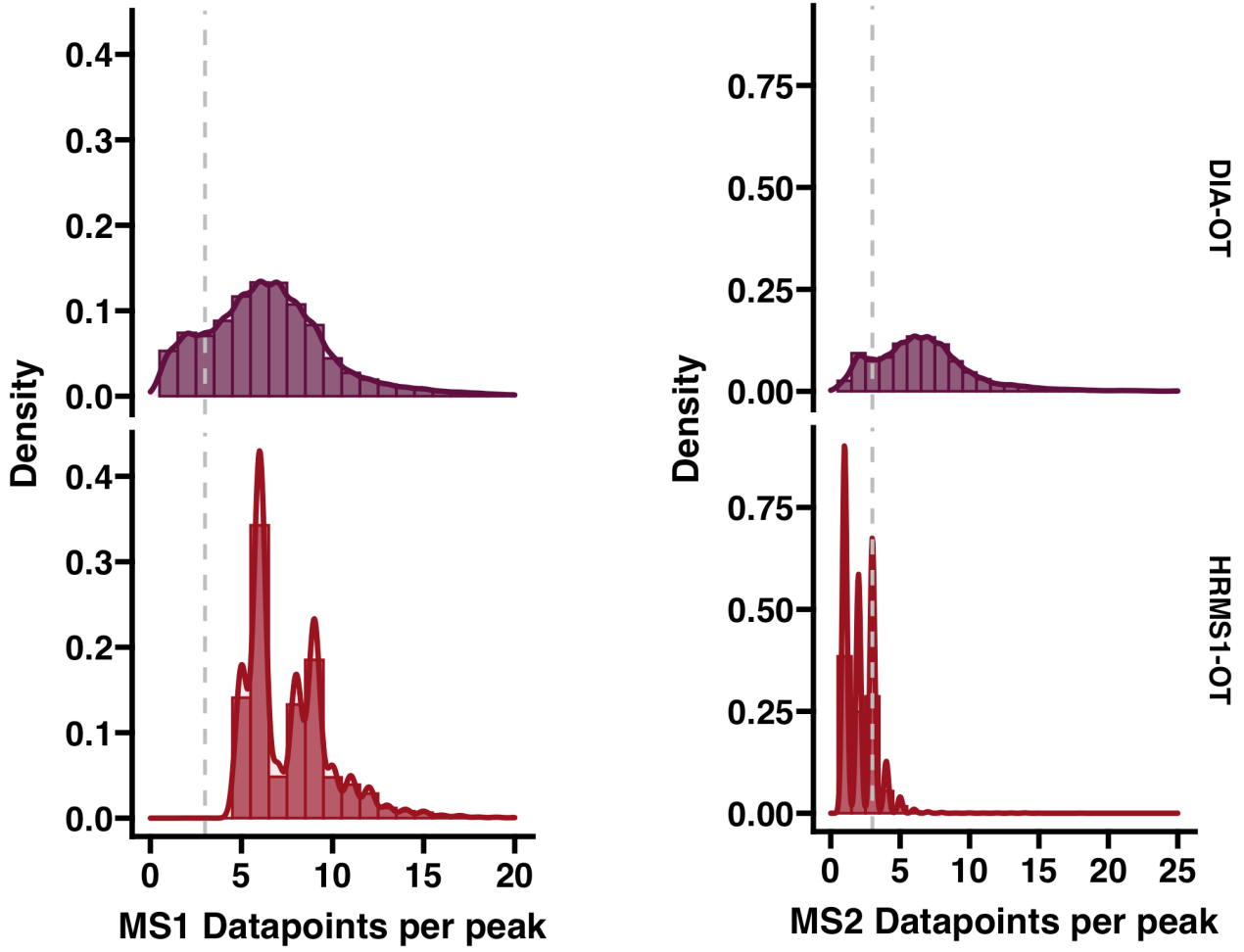


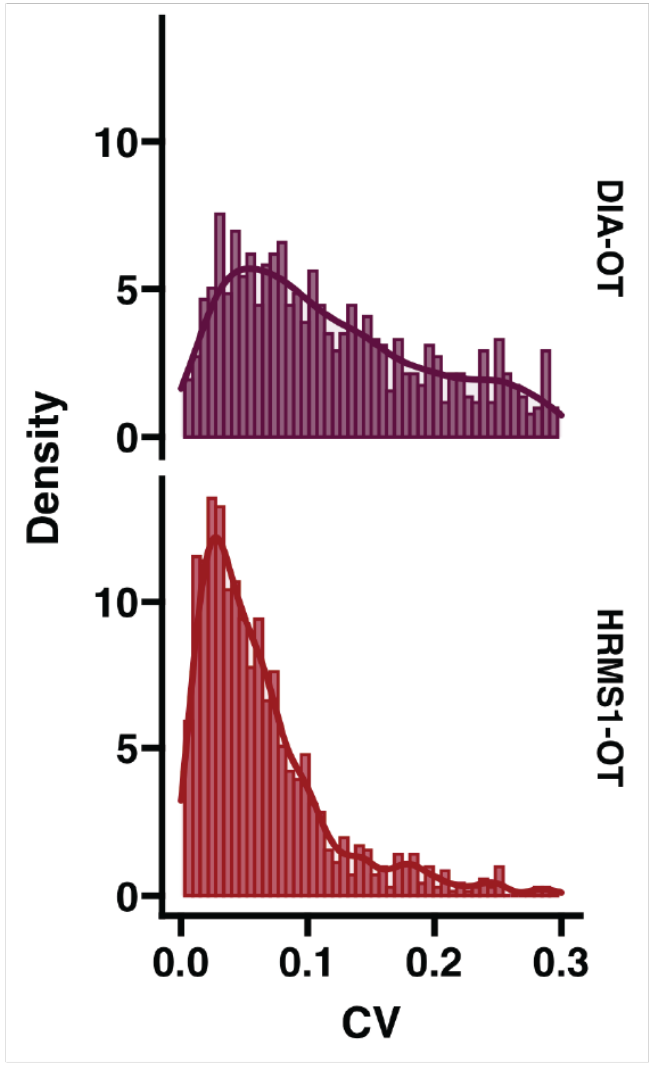


Xuan, Y *et al.* 2020

1 ng injections at 58min gradient (20 sample per day)

Evaluation of high resolution MS1 (HRMS1) DIA for limited sample -and scpMS





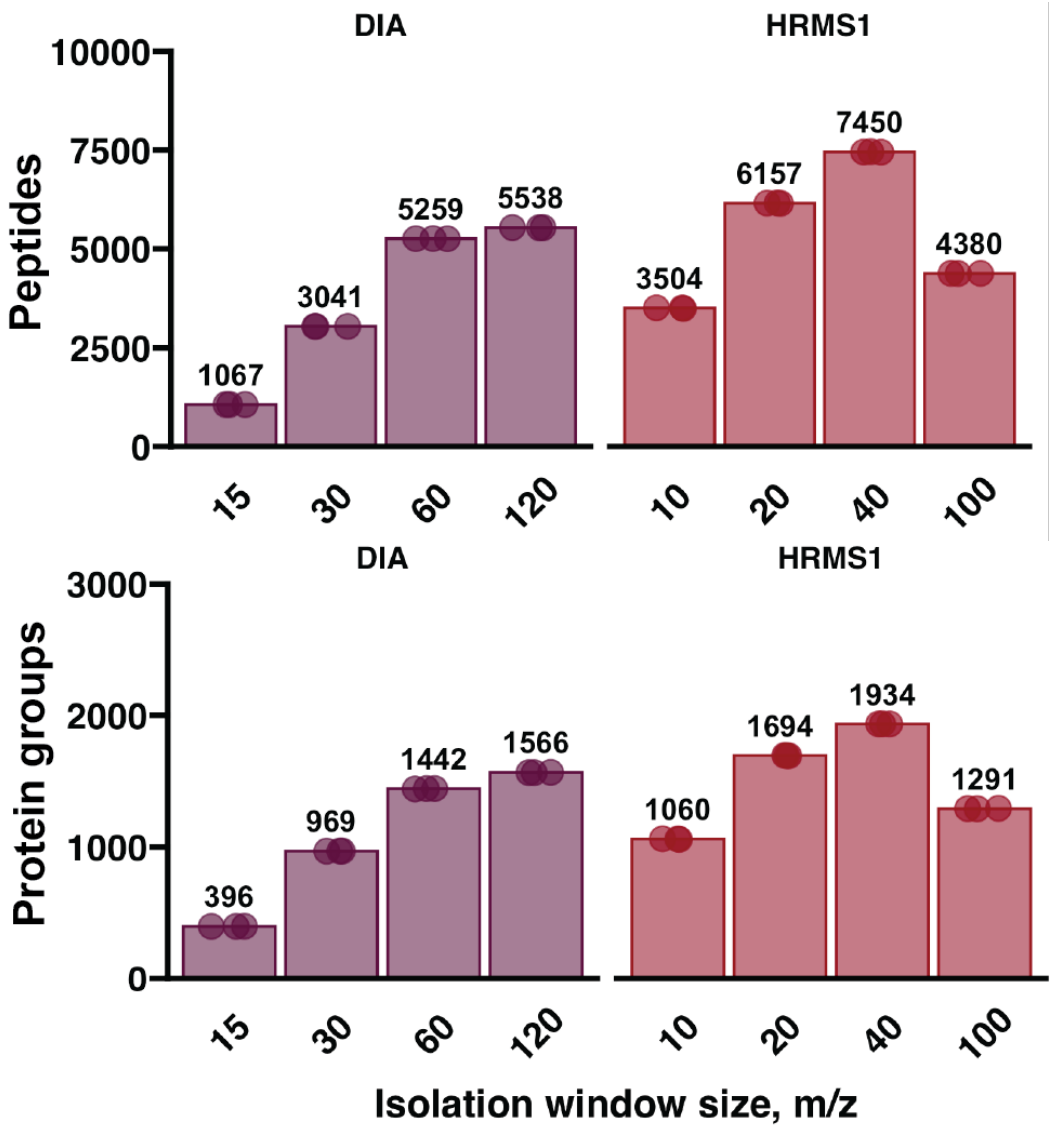
Xuan, Y *et al.* 2020

1 ng injections at 58min gradient (20 sample per day)

Wise-isolation window HRMS1 (WISH) DIA enhanced quantified proteome depth

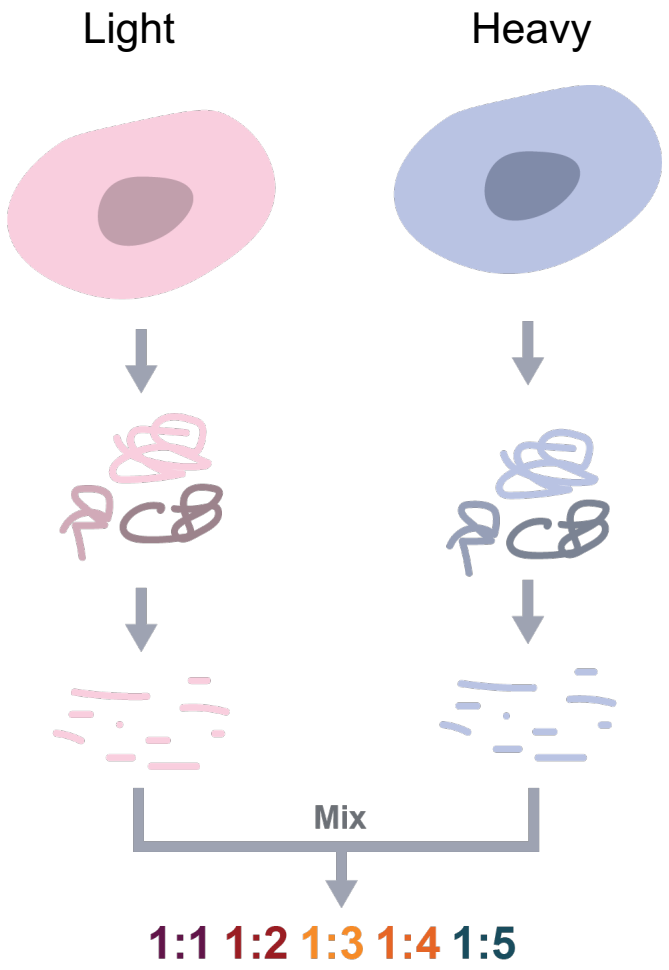
Resolution		Window		DIA
MS1	MS2	Size	Number	Cycle-time
120K	15K	15mz	40	1.536s
120K	30K	30mz	20	1.536s
120K	60K	60mz	10	1.536s
240K	120K	120mz	5	1.536s

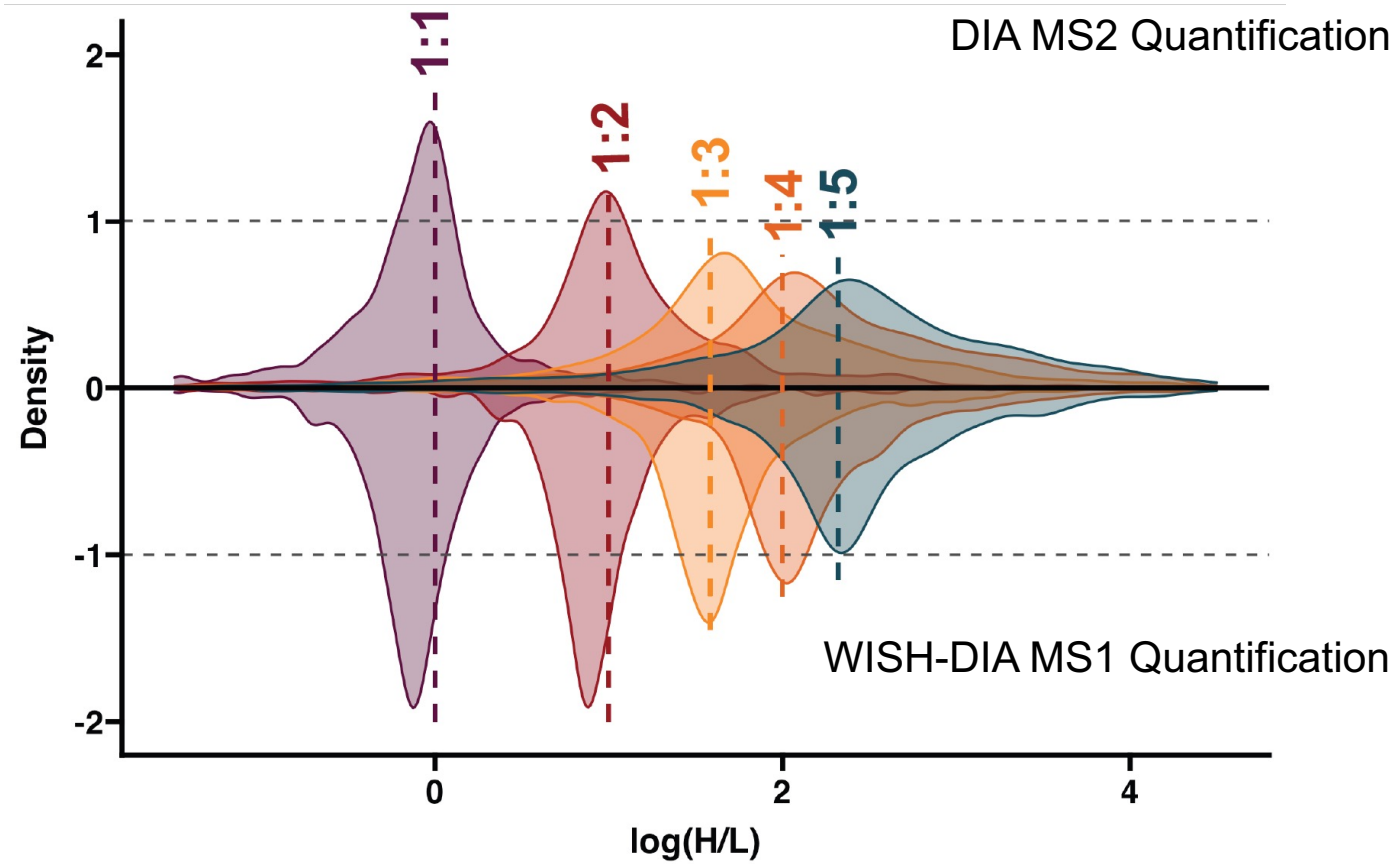
Resolution		Window		HRMS1
MS1	MS2	Size	Number	Cycle-time
120K	30K	10mz	60	1.536s
120K	60K	20mz	30	1.536s
120K	120K	40mz	15	1.536s
240K	240K	100mz	6	1.280s



1 ng injections at 31min gradient (40 sample per day)

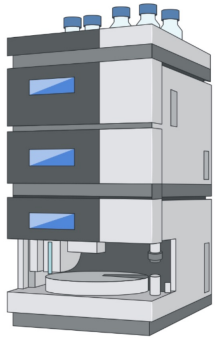
Wise-isolation window HRMS1 (WISH) DIA enhanced quantified proteome depth



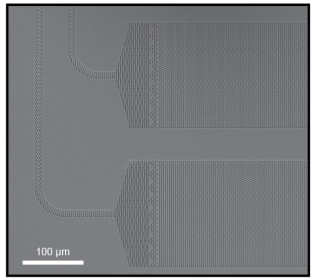


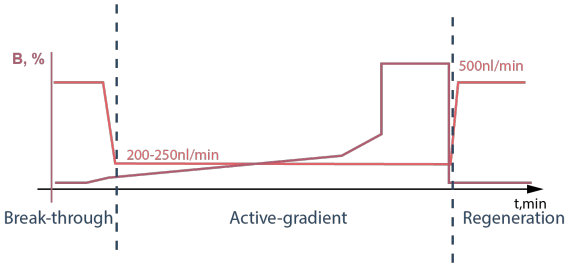
1 ng injections at 31min gradient (40 sample per day)

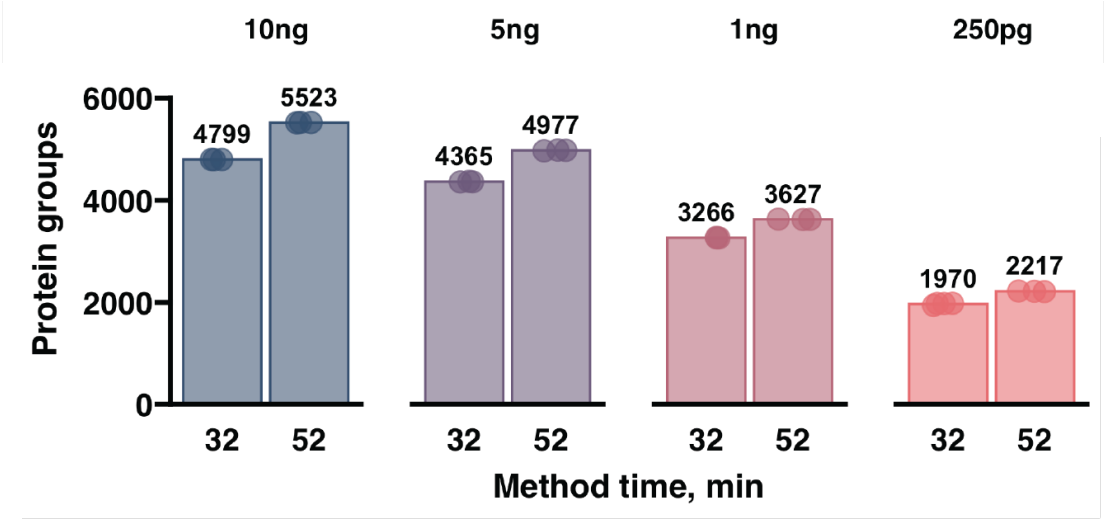
Utilizing the synergy between *µ*PAC Neo Low Load and wide isolation window HRMS1-DIA for low-input proteomics



*µ*PAC Neo Low Load

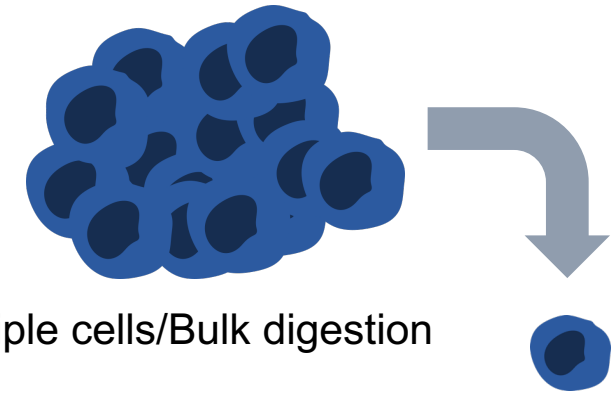






Increasing proteome coverage with the use of spectral libraries

High-load library search

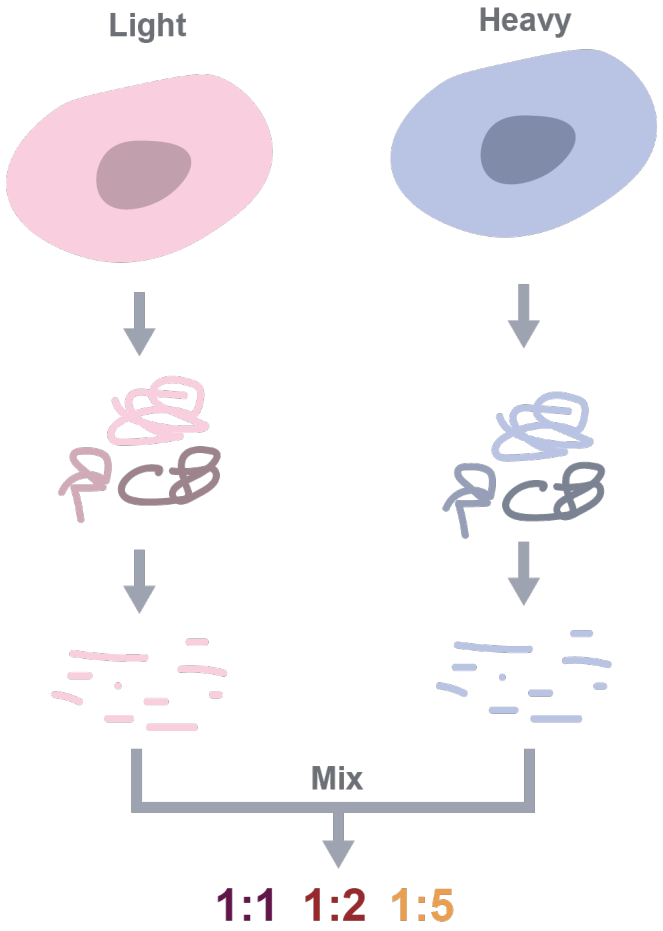


Multiple cells/Bulk digestion

Gas-phase fractionated library search

What is the quantification quality of the additionally identified proteins?

Evaluating the use of libraries to increase proteome coverage of limited input samples



Light + Heavy = 10ng High-Load (HL)

Dilute 10x = 1ng Samples (direct + GPF)

For HL libraries the 10ng sample of each mix was run once

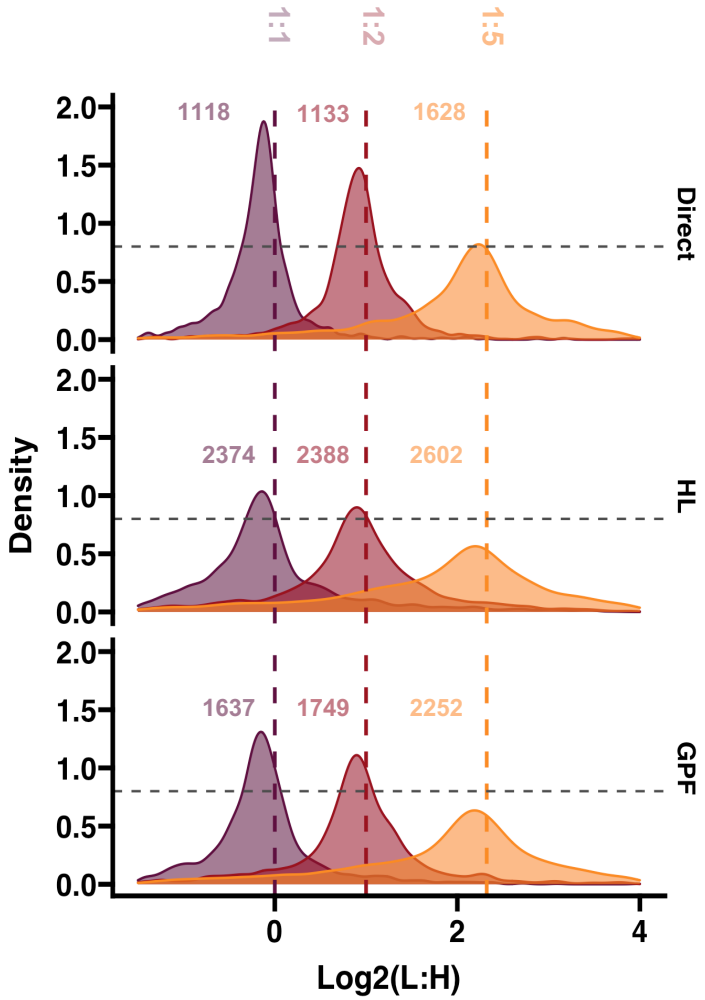
For GPF each mix was run 6 times (100mz each)

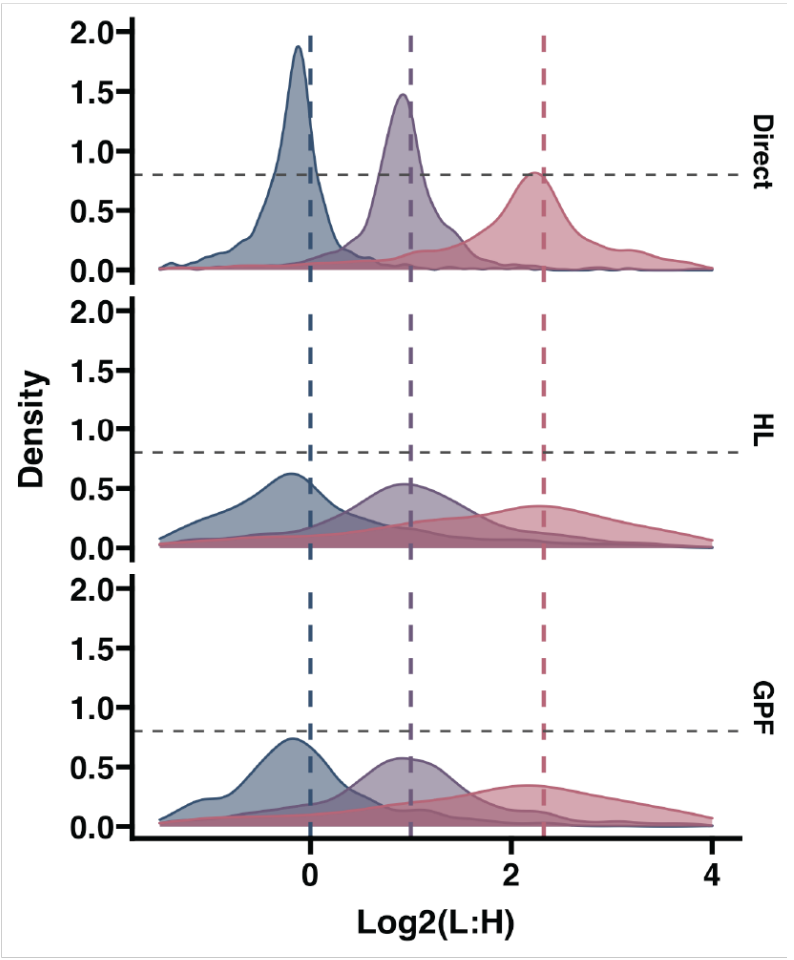
For direct each mix was run in technical triplicate (full mz)

Evaluating the use of libraries to increase proteome coverage of limited input samples

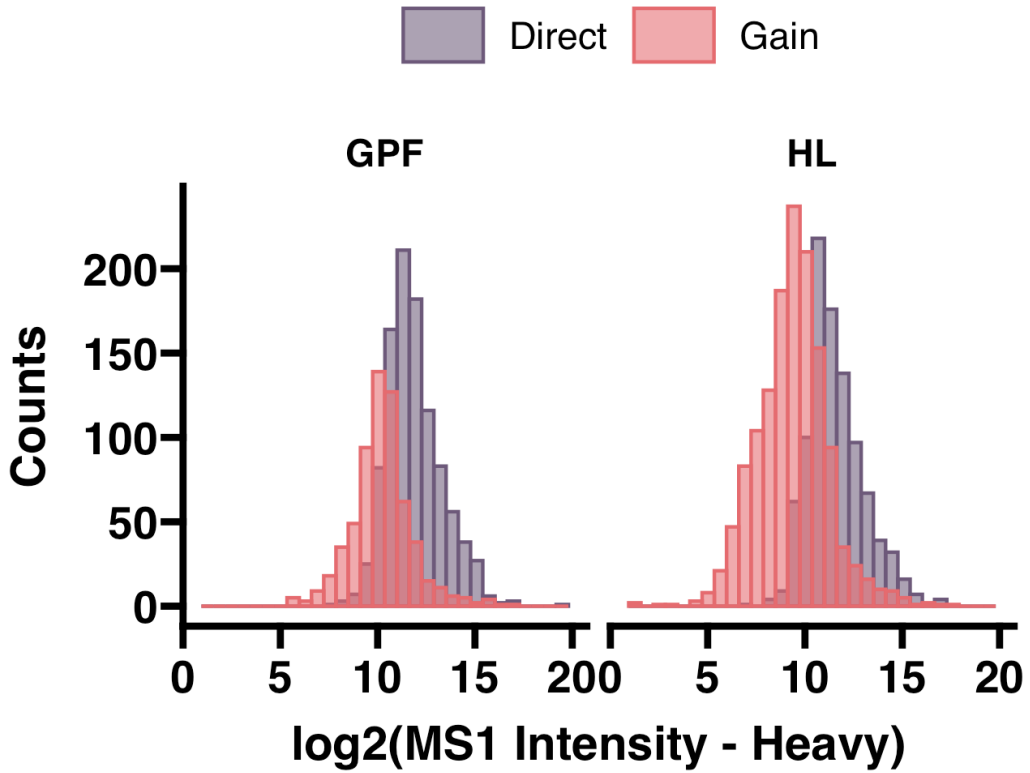
- **Direct** – 1ng searched as directDIA
- **HL** – 1ng searched with 10ng library
- **GPF** – 1ng + GPF runs searches as directDIA

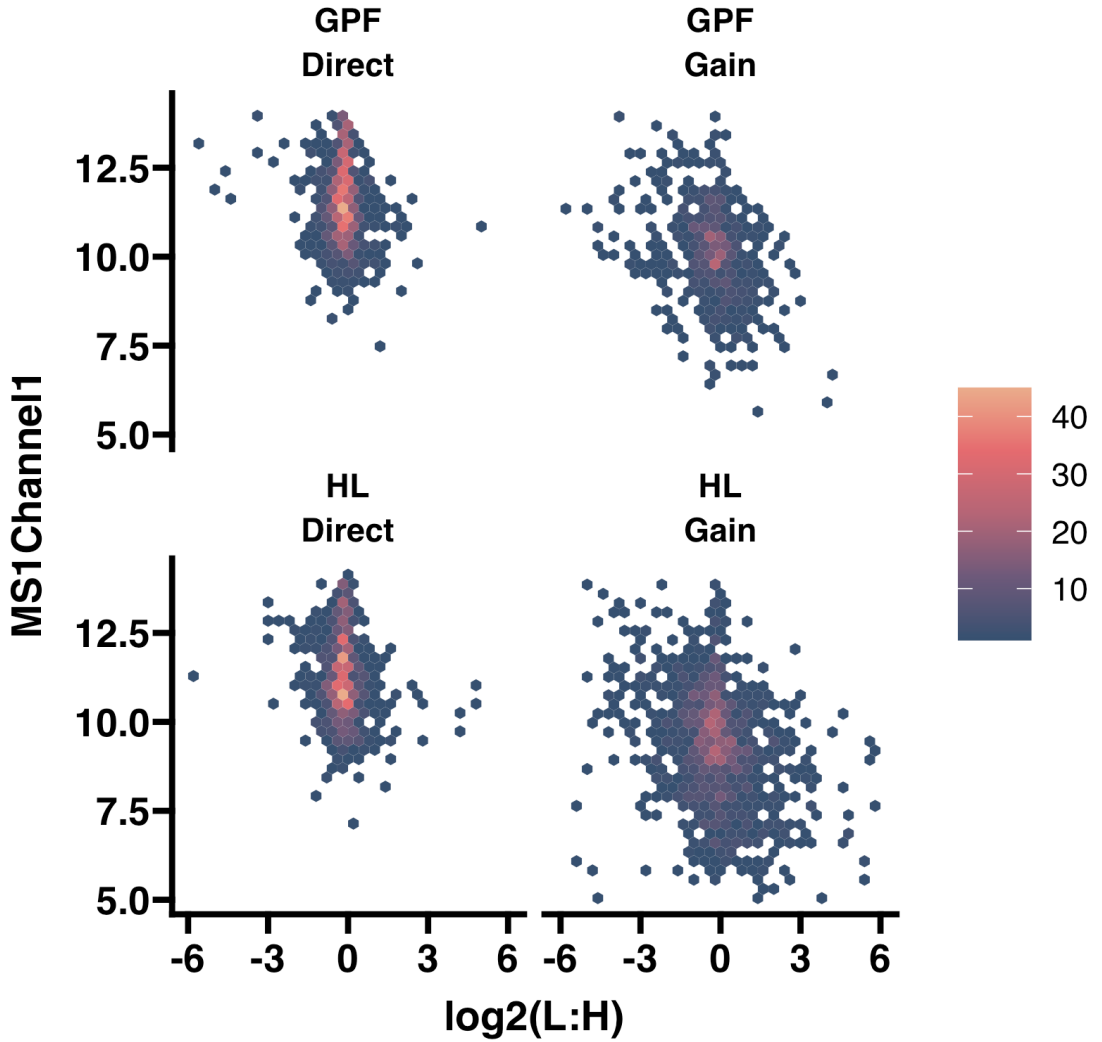
What is the accuracy of just the additional IDs?



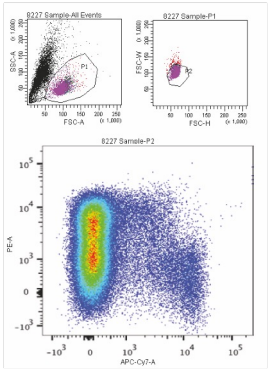


Evaluating the use of libraries to increase proteome coverage of limited input samples

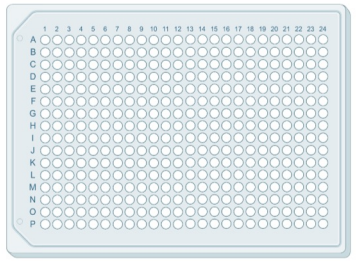




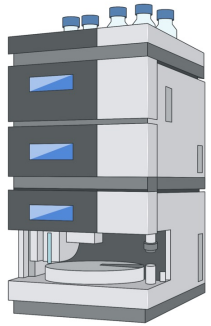
Single-cell DIA method optimization

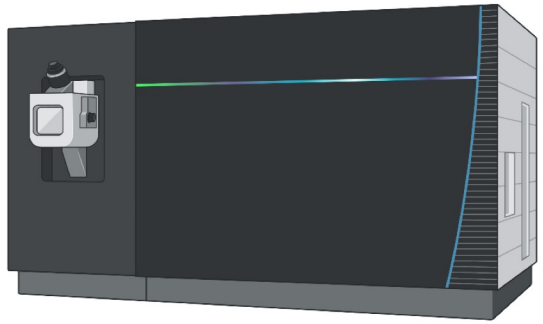


384-well plate



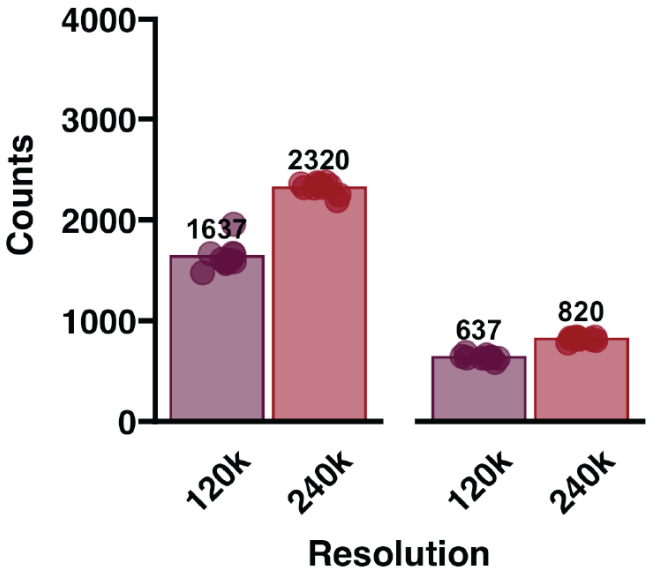
16hr digest (37° C)



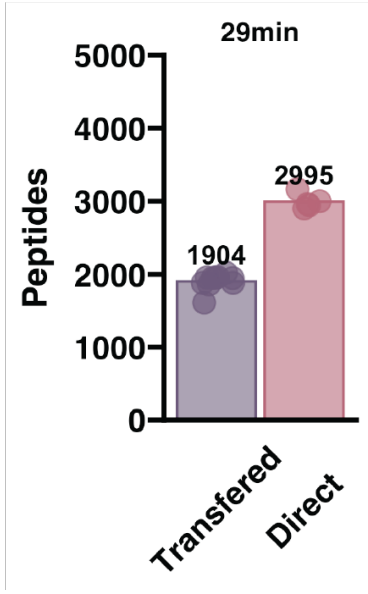


LC-MS analysis of single cells

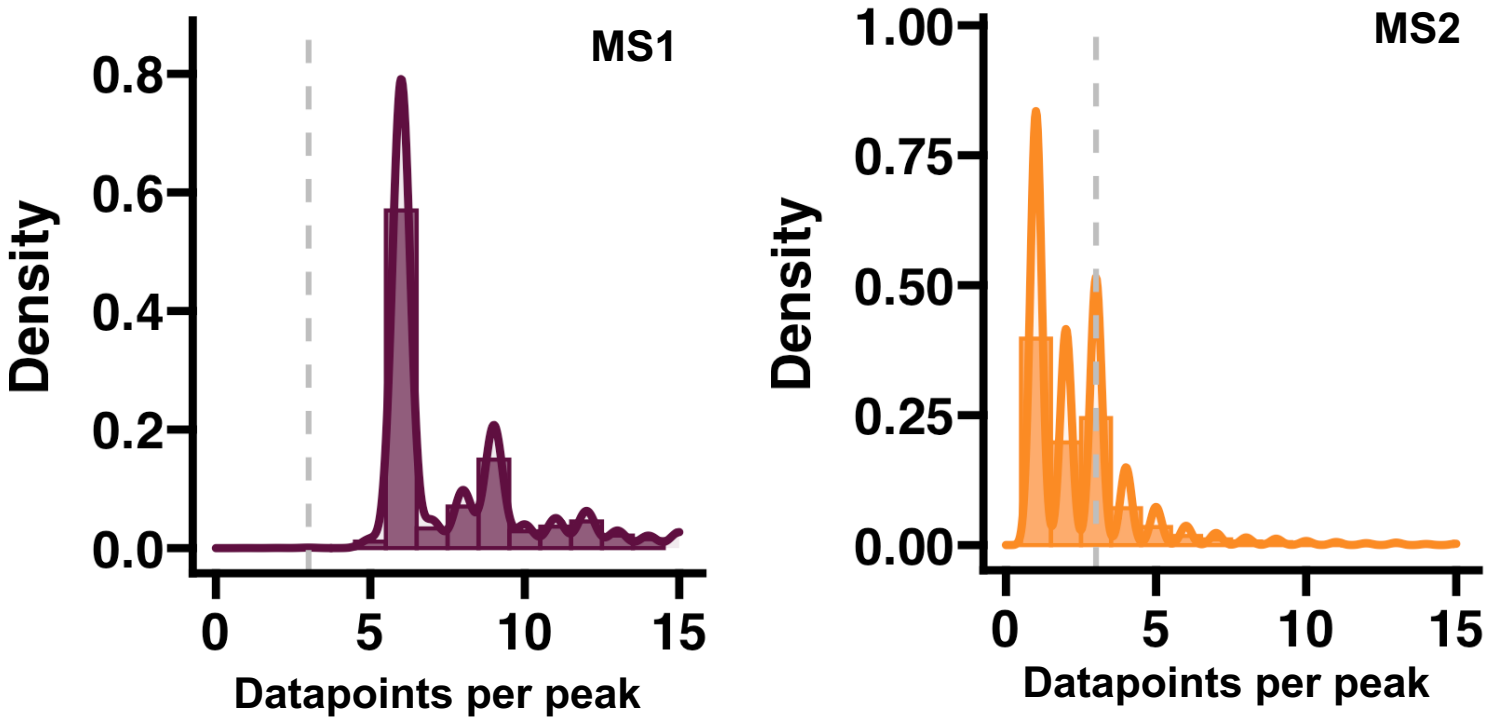
Peptides Protein.Groups



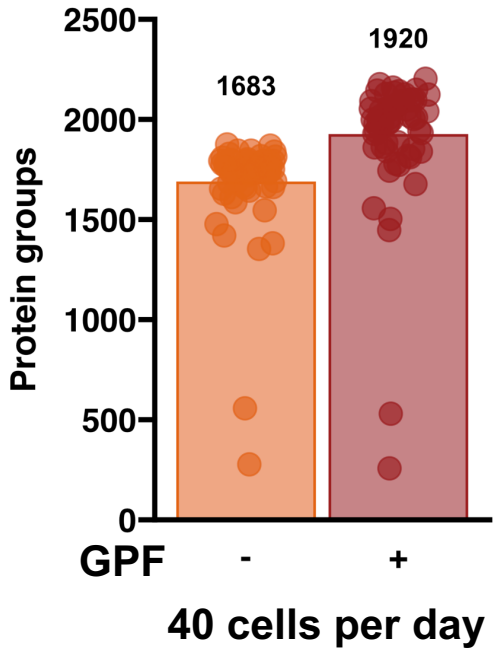
Resolution		Window		HRMS1
MS1	MS2	Size	Number	Cycle-time
120K	120K	40mz	15	1.536s
240K	240K	100mz	6	1.280s

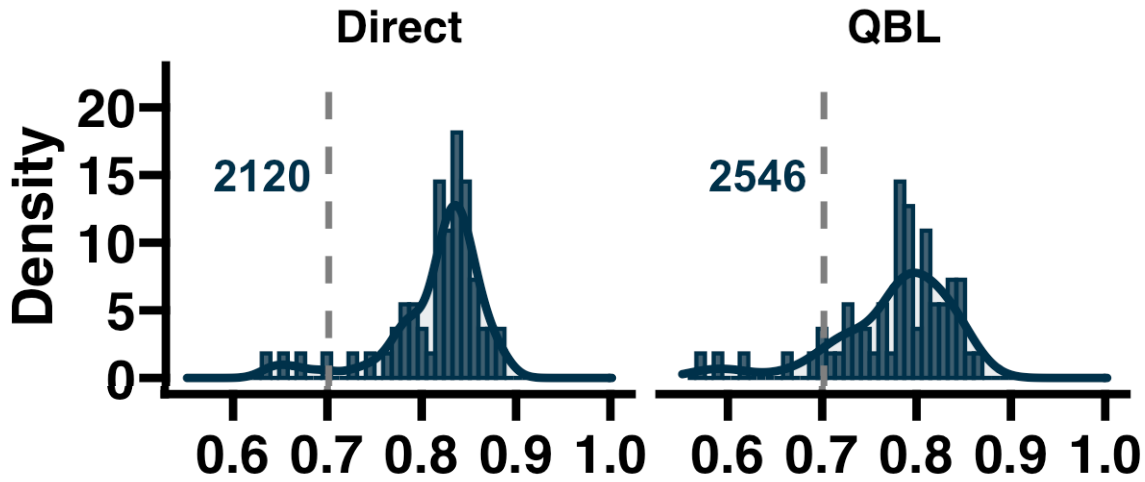


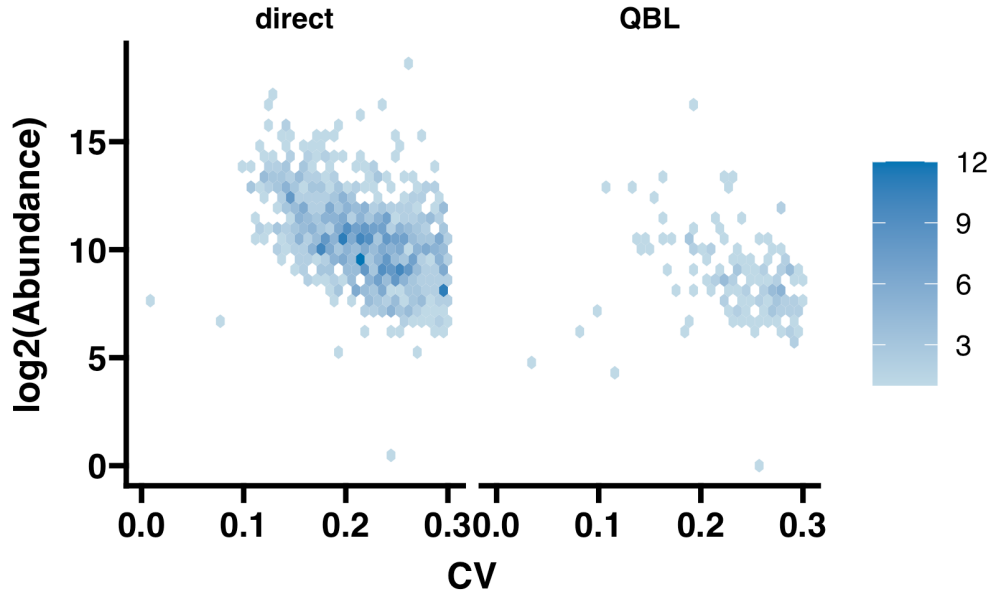
Single-cell DIA method optimization



Showcasing the established workflow for single cell analysis

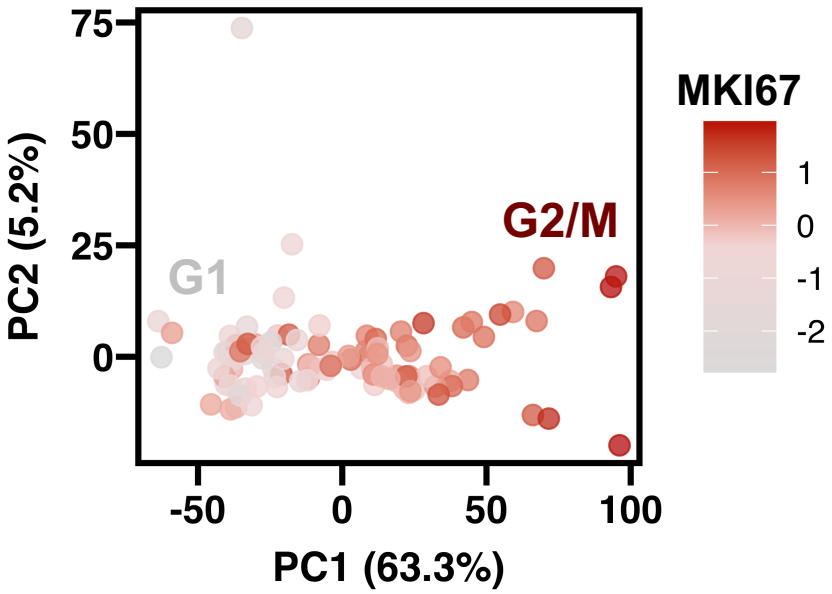


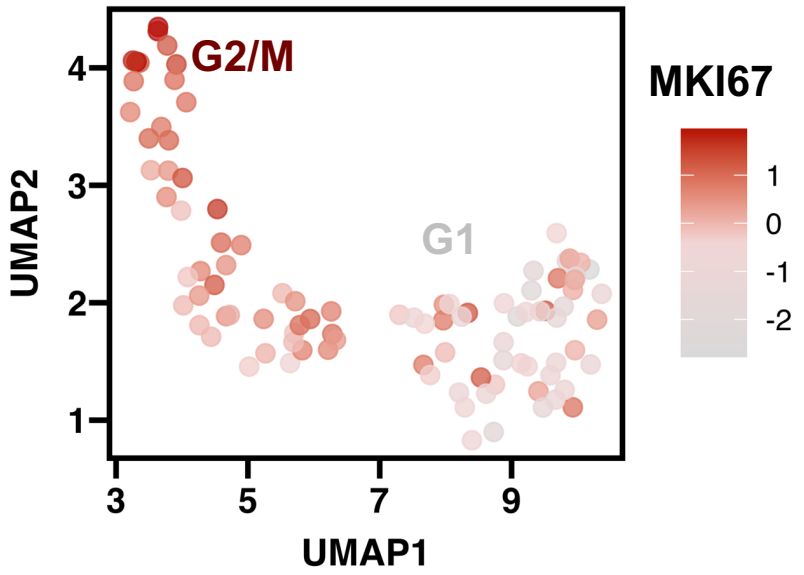


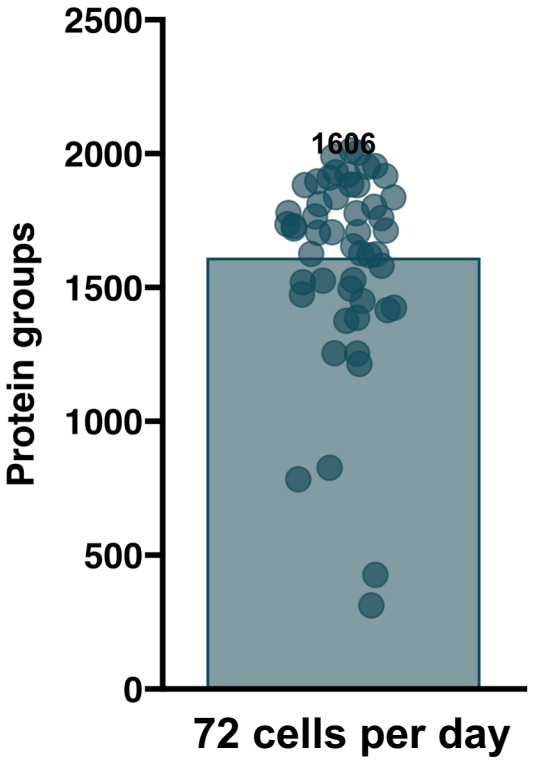


Single-cells were used as input for GPF

Showcasing the established workflow for single cell analysis



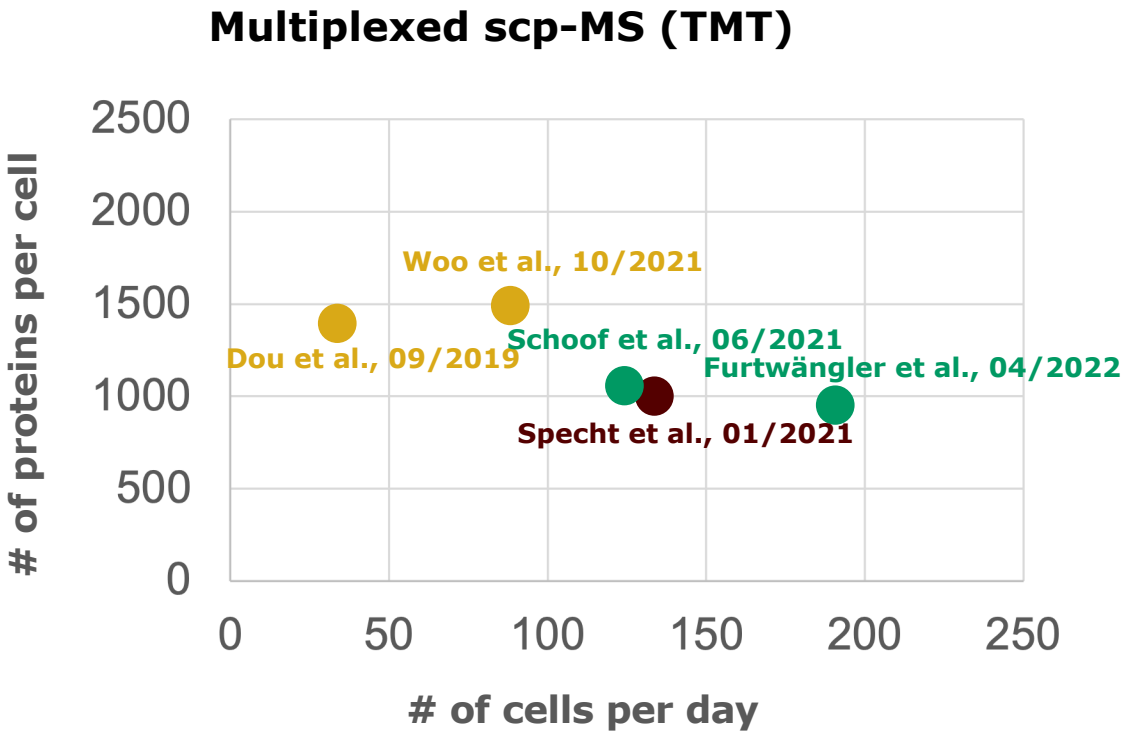


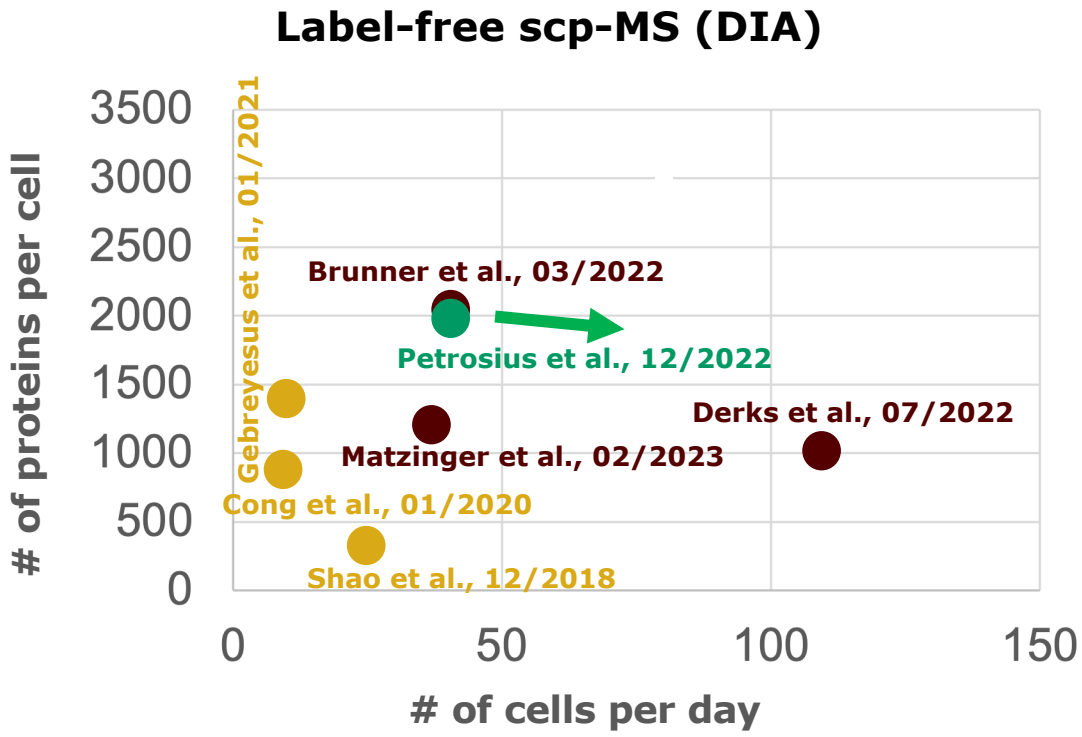


How do we compare to the state-of-the-art?

Low-throughput microfluidics

Our lab contributions





Conclusions

- **DIA method design should be adjusted accordingly to sample load for optimal performance.**
  - Wider windows and higher resolutions is favorable for low input samples
- **Precursor level quantification can be used to enhance analytical sensitivity**
  - WISH-DIA is quantification accuracy similar to standard DIA
- **GPF libraries are a promising alternative to high pH approaches for samples where fractionation is prohibitive**
- **Workflow uses standardized lab equipment and does not require single-cell proteomics designated liquid handling systems**
  - The same proteomic depth should be achievable on on hybrid instruments (e.g. Exploris 480)

Enhancing single-cell proteomics through tailored Data-Independent Acquisition and micropillar array-based chromatography

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