Skyline webinar #25

21st January 2025

Comparing Acquisition Methods with Skyline

Overview

A) Datatypes – PRM, DIA, DDA

B) Description of tutorial dataset

C) Skyline: “Targeted data evaluation”

Overview

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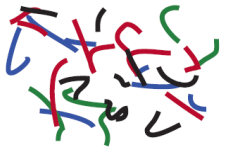
The standard bottom-up proteomic workflow

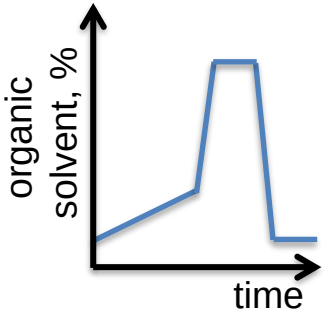
Protein extraction

Proteins

Protein digestion

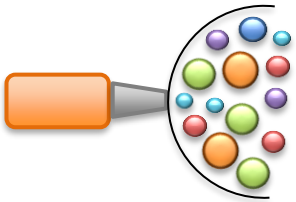


Peptides



HPLC
Peptide separation

+



ESI
Ionization

+

Q-Orbi
Q-TOF

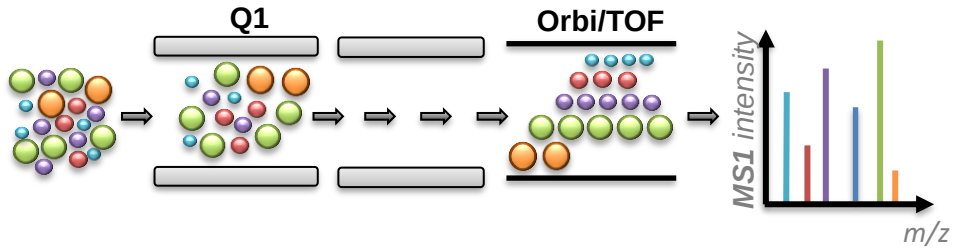
PRM
DIA
DDA

Mass spectrometer
Identification & Quantification

Slide adapted from Christina Ludwig

Principle of targeted data acquisition - PRM

1. Optional (MS1) full scan:



Slide adapted from Christina Ludwig

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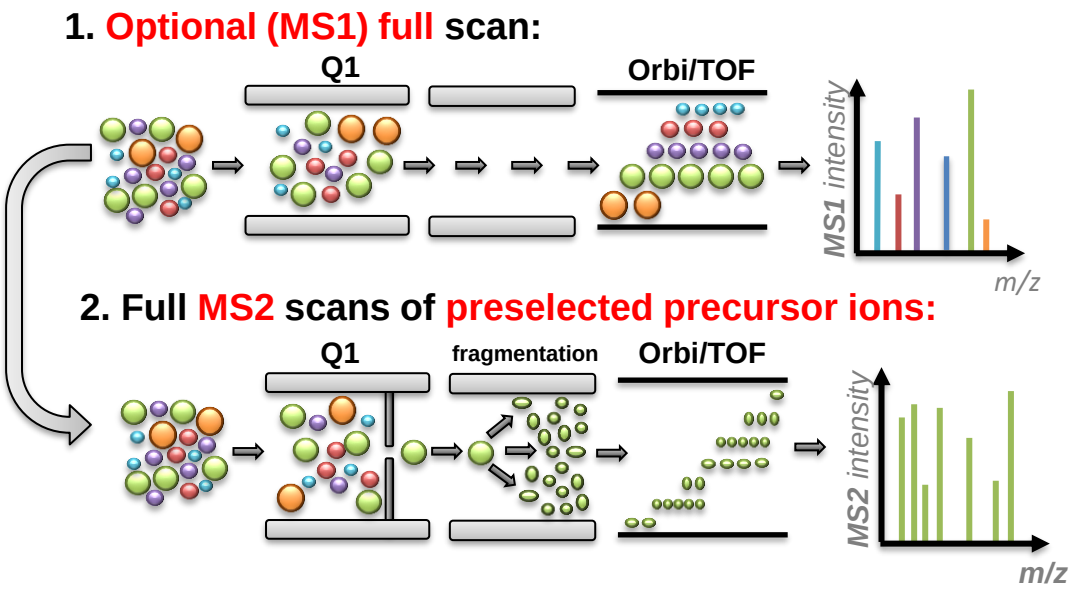

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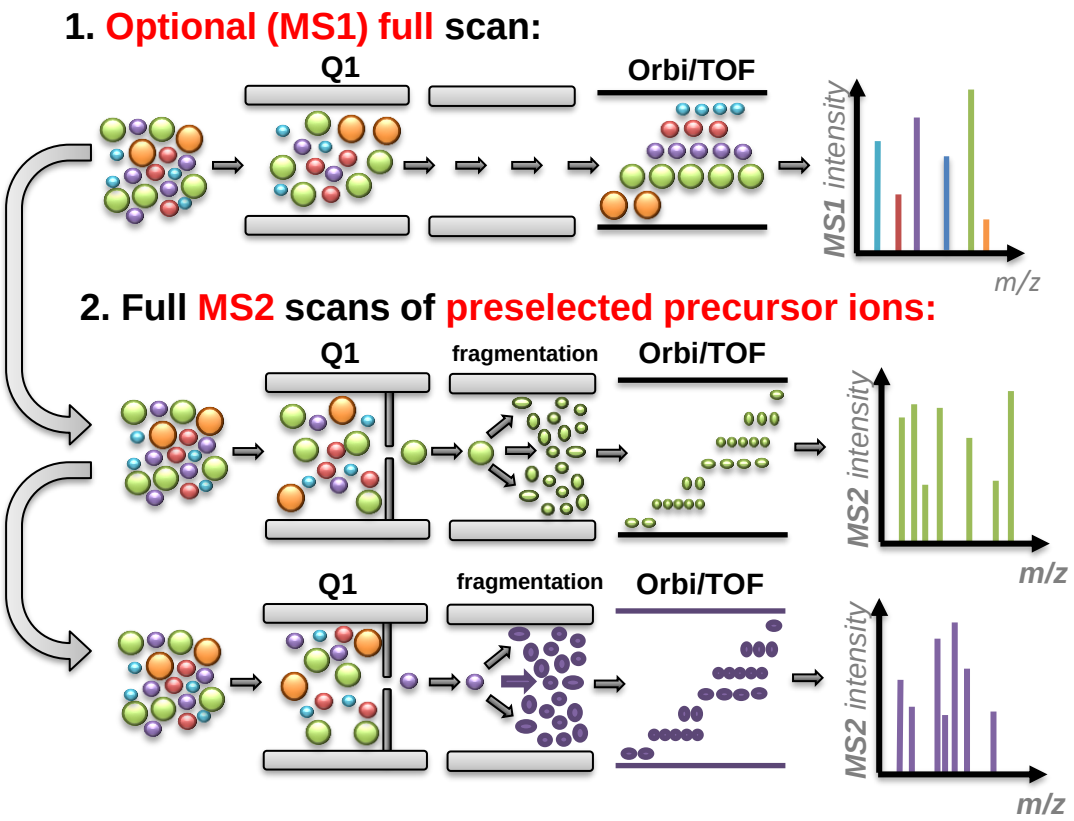

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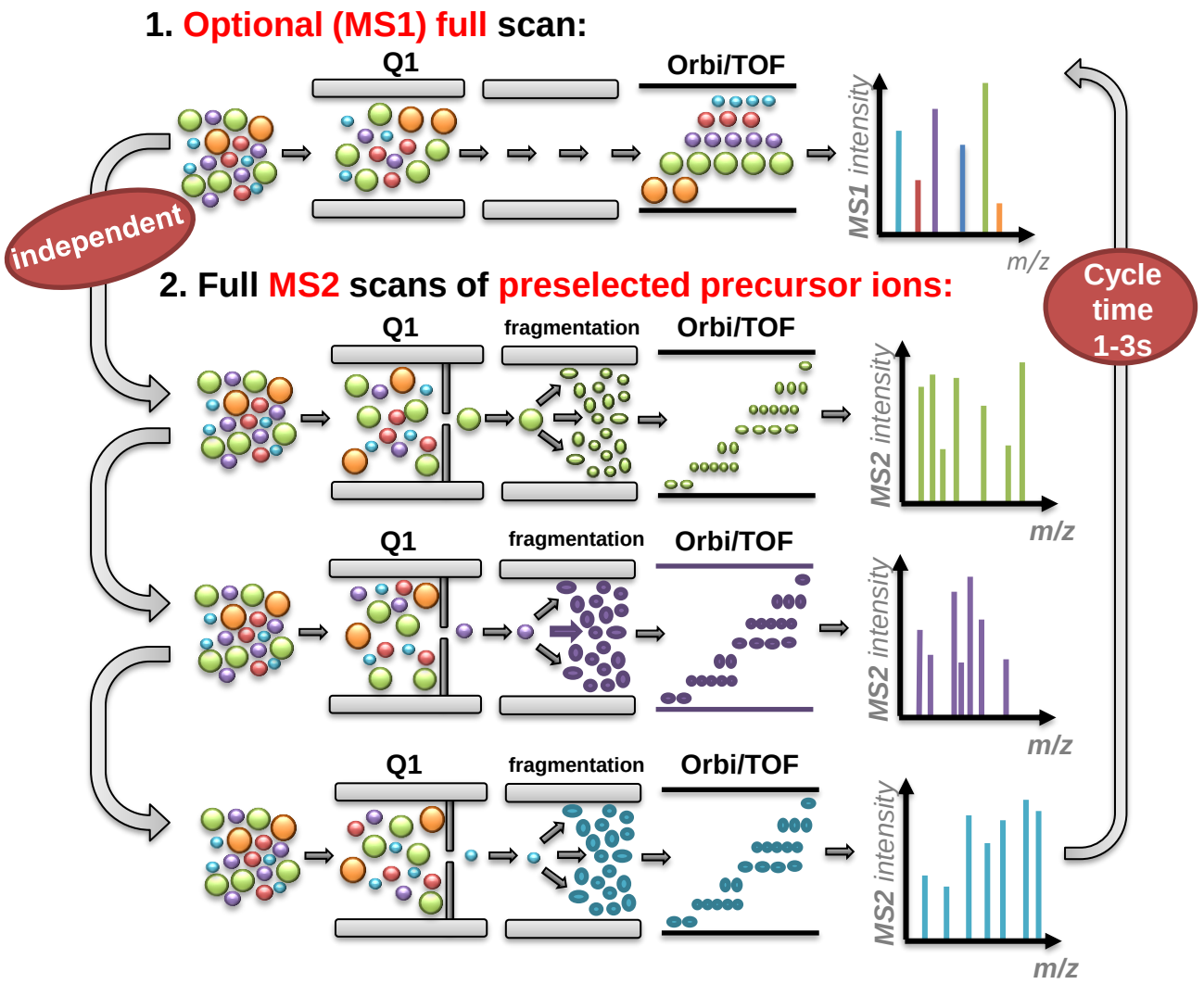
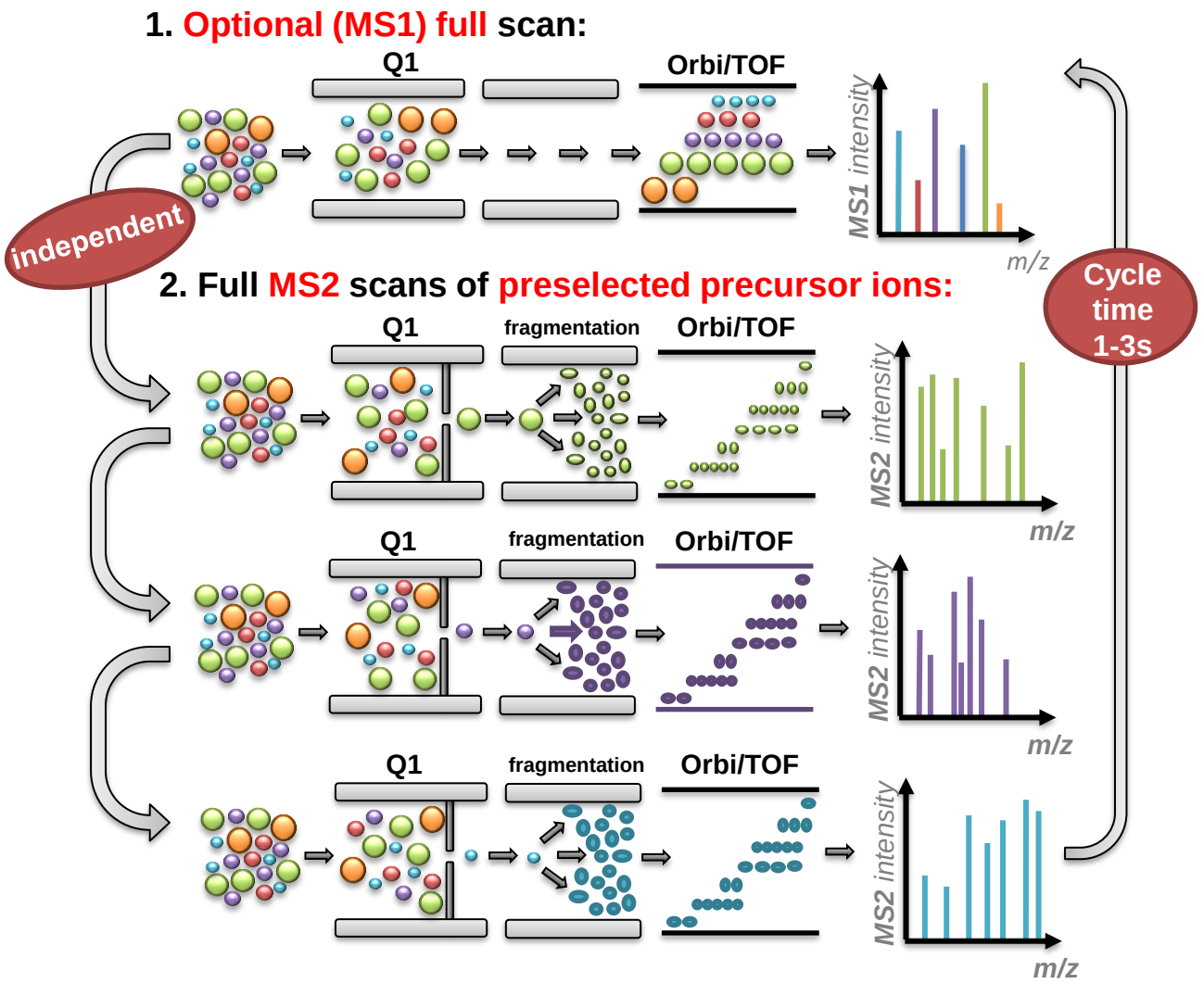
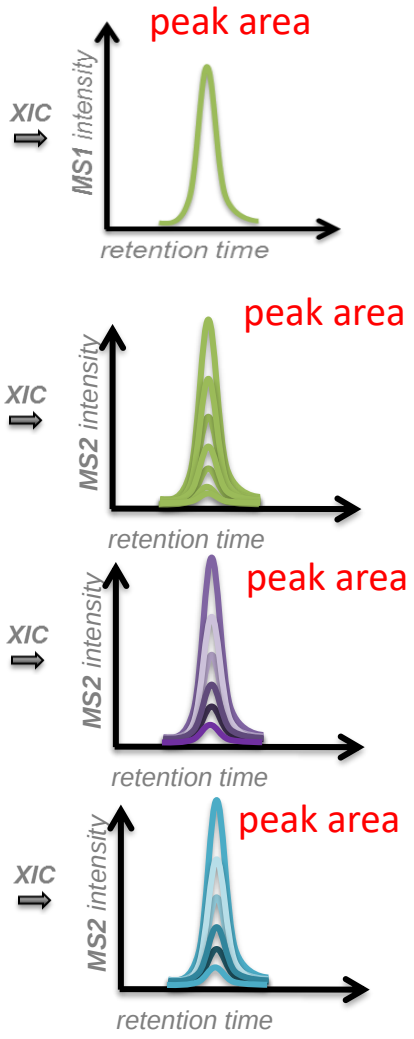
Principle of targeted data acquisition - PRM



Slide adapted from Christina Ludwig

Principle of targeted data acquisition - PRM

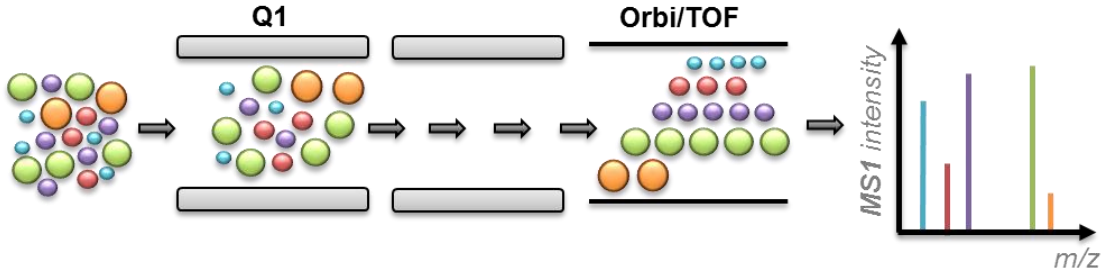




Slide adapted from Christina Ludwig

Principle of data independent acquisition - DIA

1. **Optional MS1** full scan:



Slide adapted from Christina Ludwig

Principle of data independent acquisition - DIA

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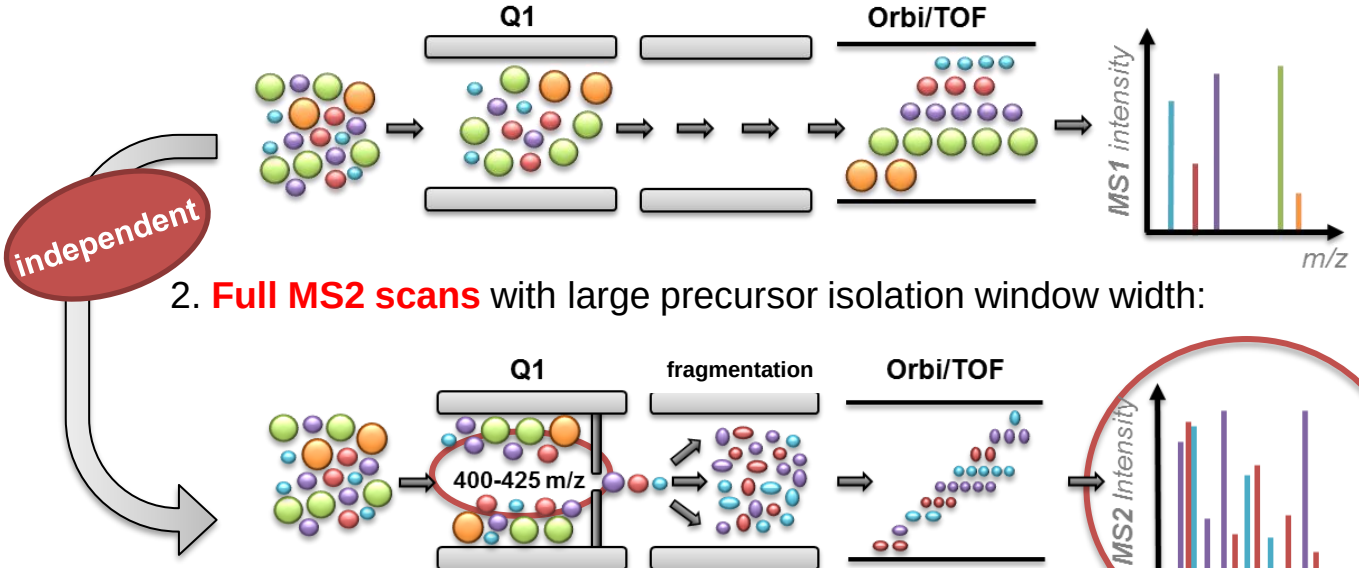
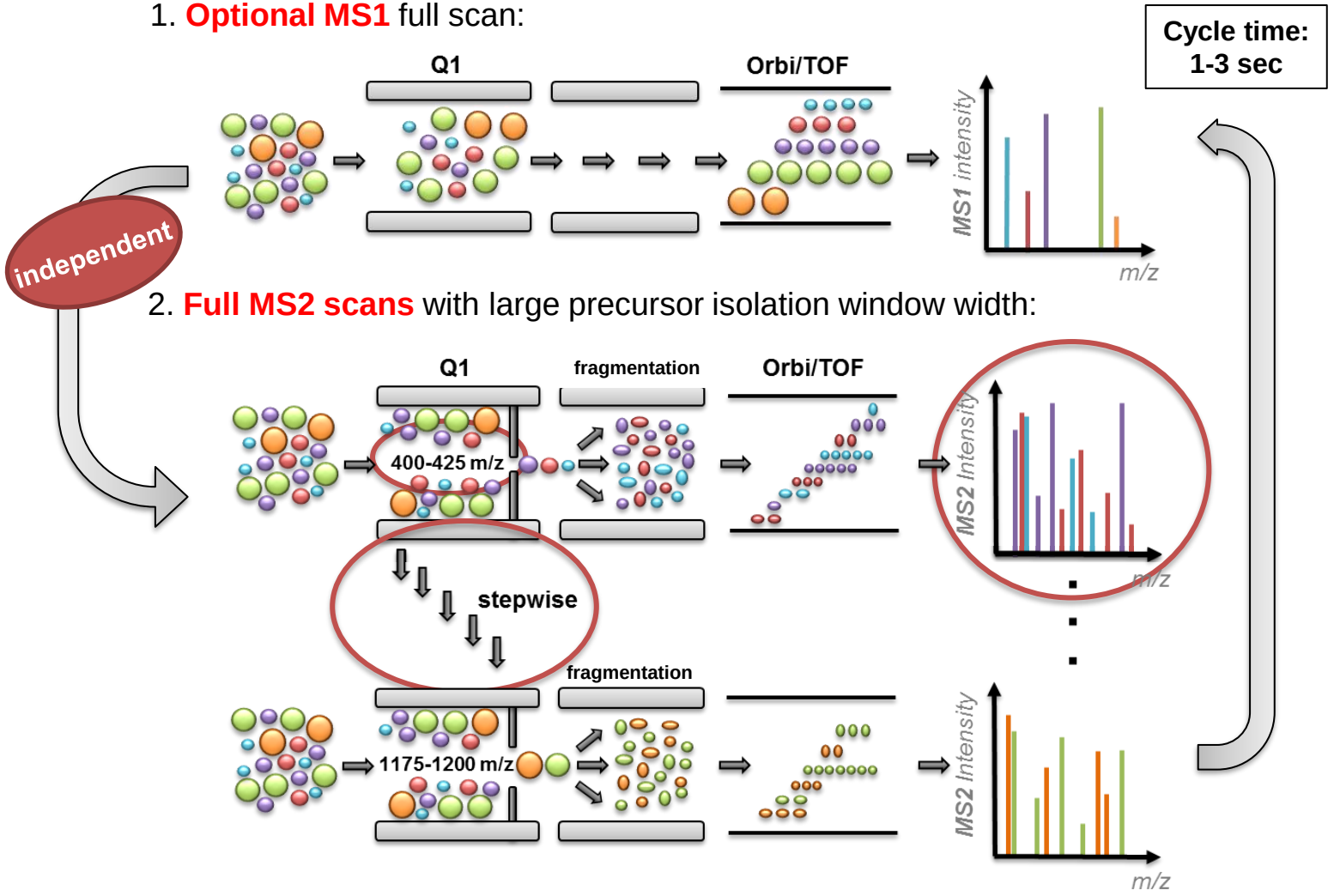


2. **Full MS2 scans** with large precursor isolation window width:

Slide adapted from Christina Ludwig

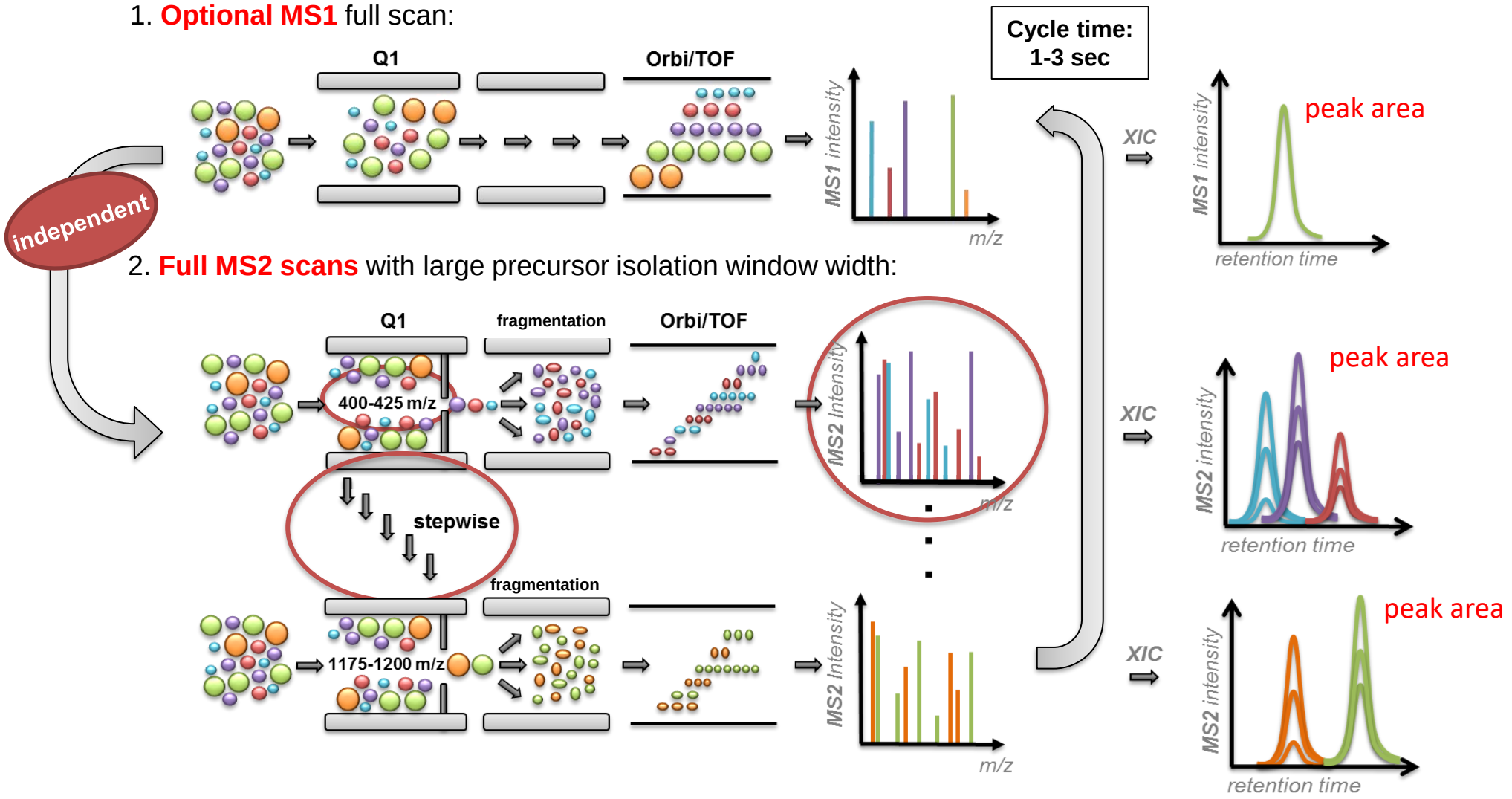
Principle of data independent acquisition - DIA

1. **Optional MS1** full scan:



Slide adapted from Christina Ludwig

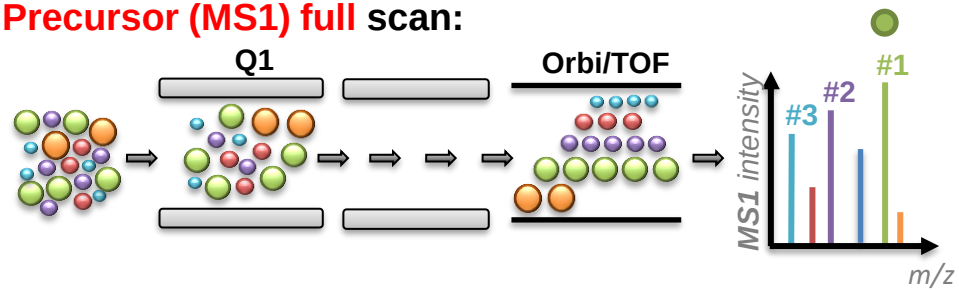
Principle of data independent acquisition - DIA



Slide adapted from Christina Ludwig

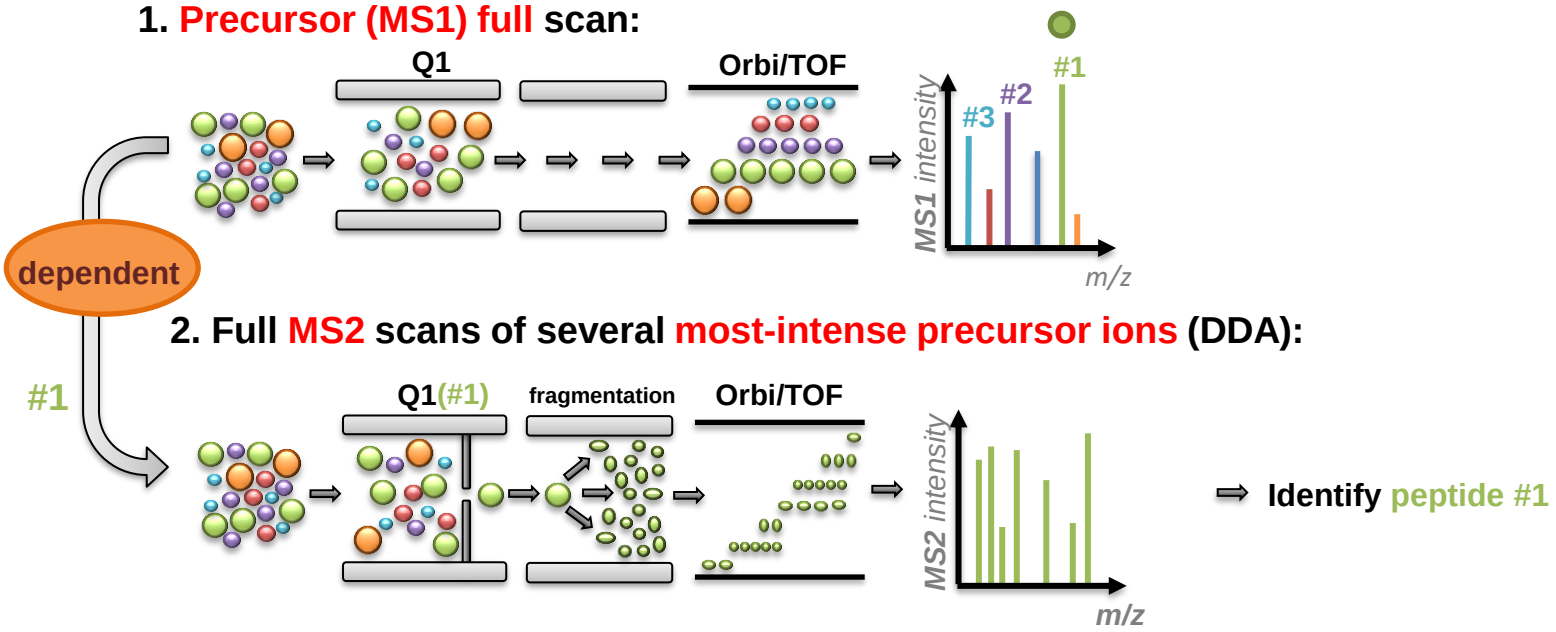
Principle of data dependent acquisition - DDA

1. Precursor (MS1) full scan:



Slide adapted from Christina Ludwig

Principle of data dependent acquisition - DDA



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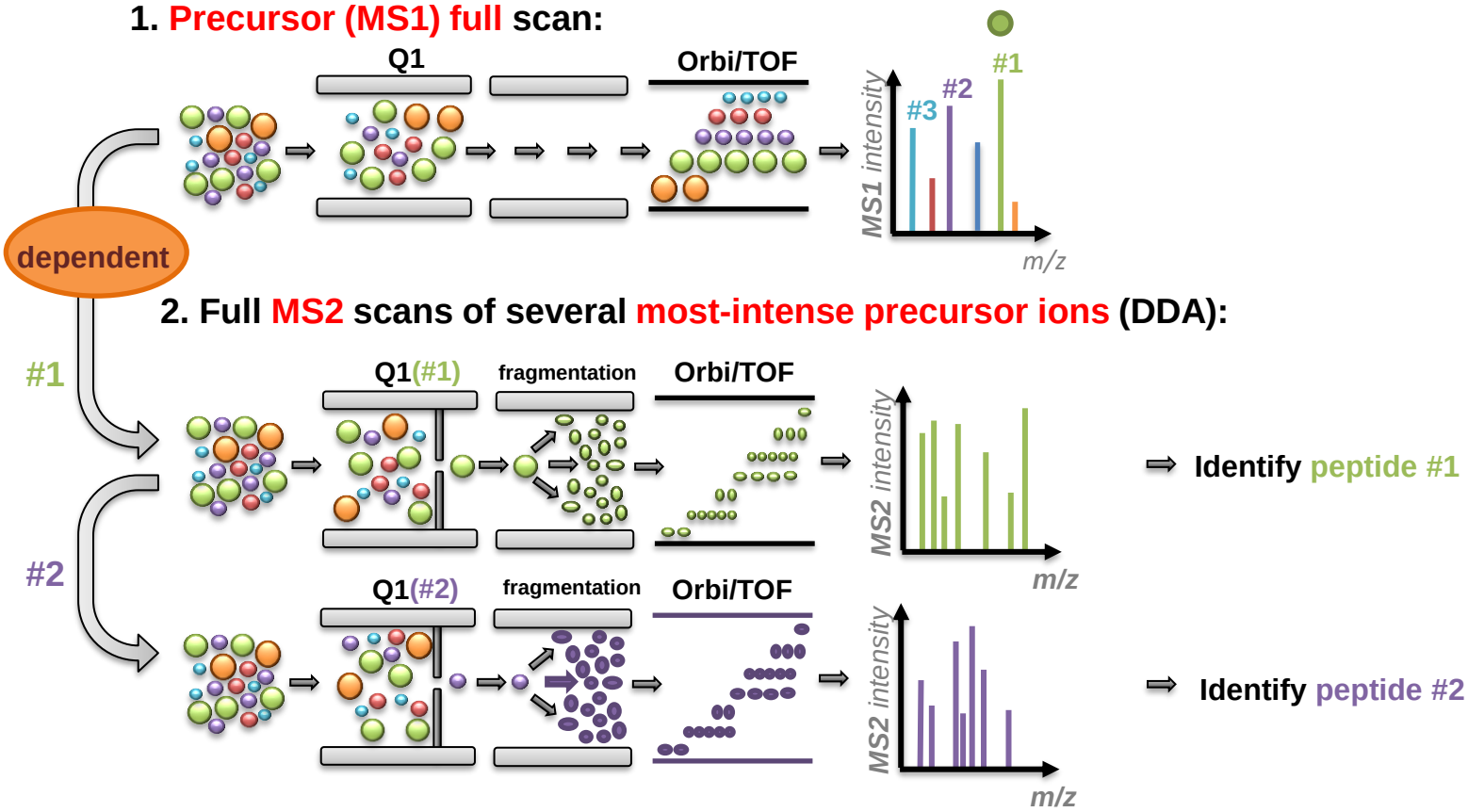

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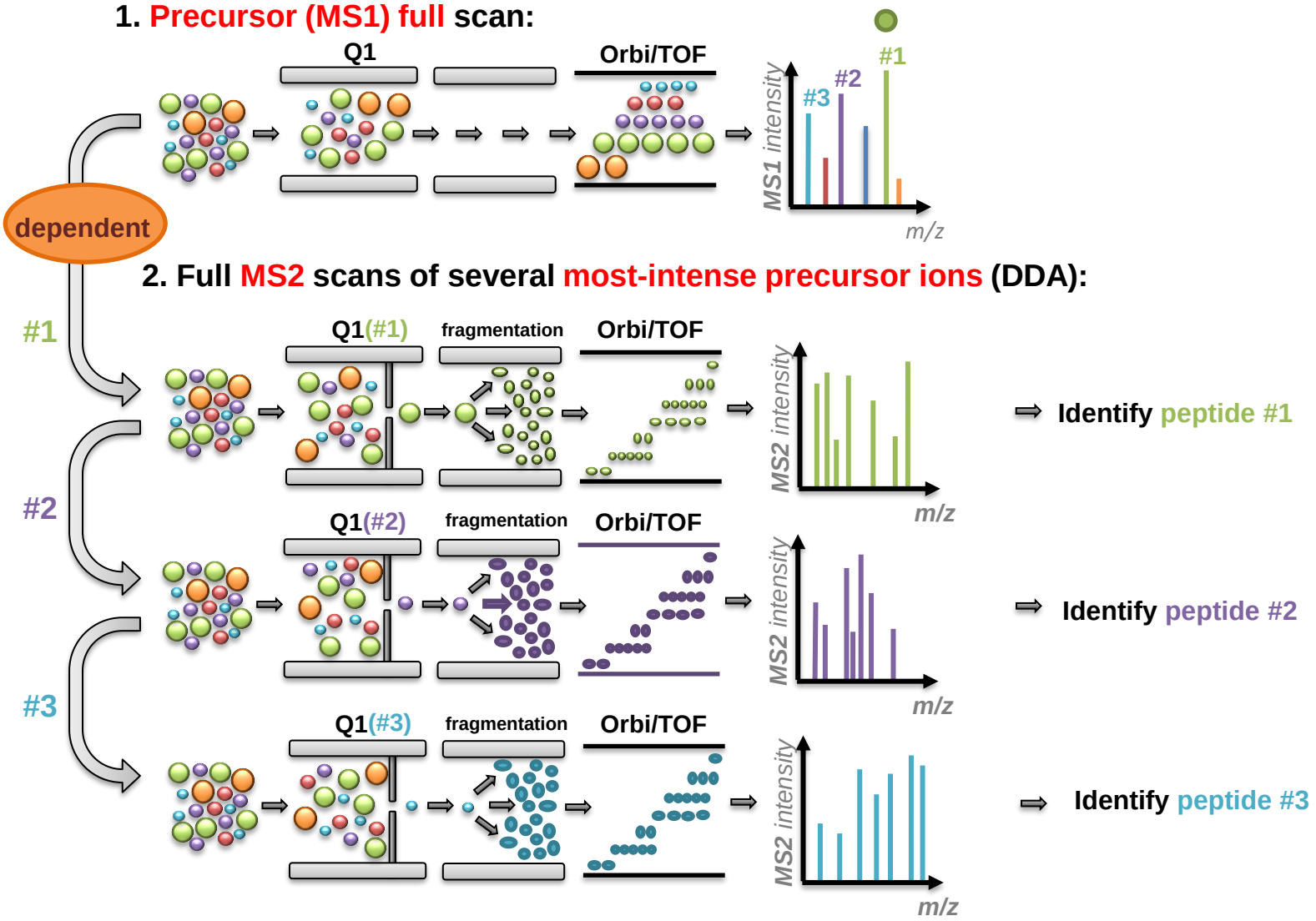

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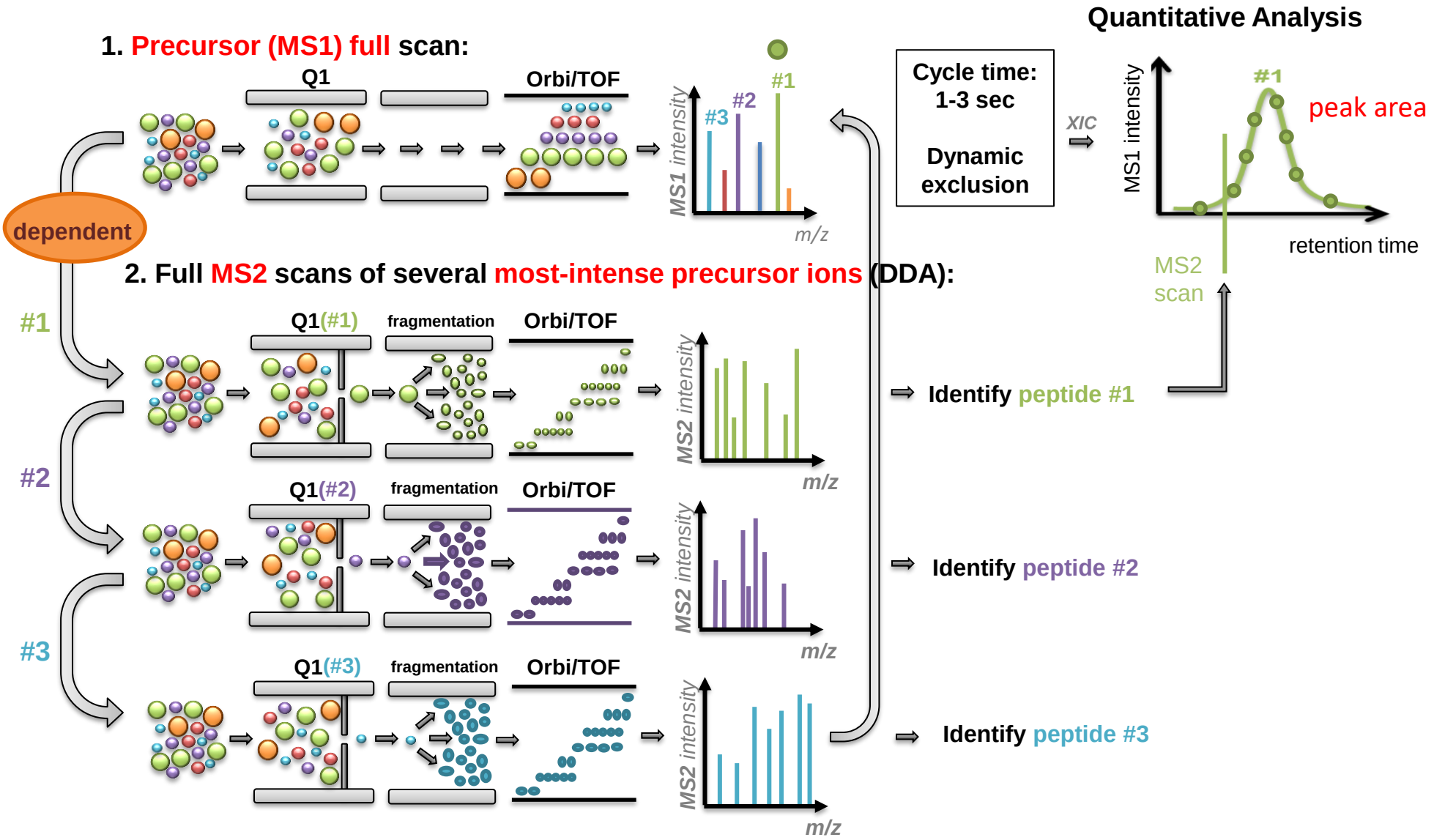


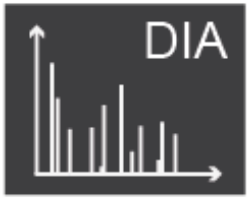
Slide adapted from Christina Ludwig

Experimental question

When should I use which acquisition method?

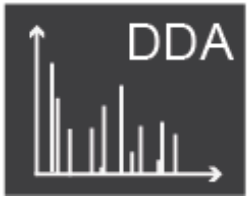
- Precise, and reproducible quantification
- No technical missing values

- Prior knowledge
- Small set of defined targets



- Global analysis
- Precise and reproducible quantification

- (Prior knowledge / spectral library)
- Highly complex MS2 spectra
- FDR control can lead to false positive identifications



- Global analysis
- No prior knowledge needed

- Bias towards more intense peptides/proteins
- Technical missing values

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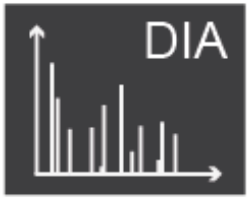

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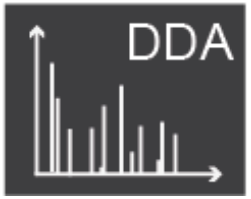


- Global analysis
- No prior knowledge needed

- Bias towards more intense peptides/proteins
- Technical missing values

Tutorial Aims: **1) Compare identifications for a limited set of peptides**
2) Compare quantitative accuracy

Overview

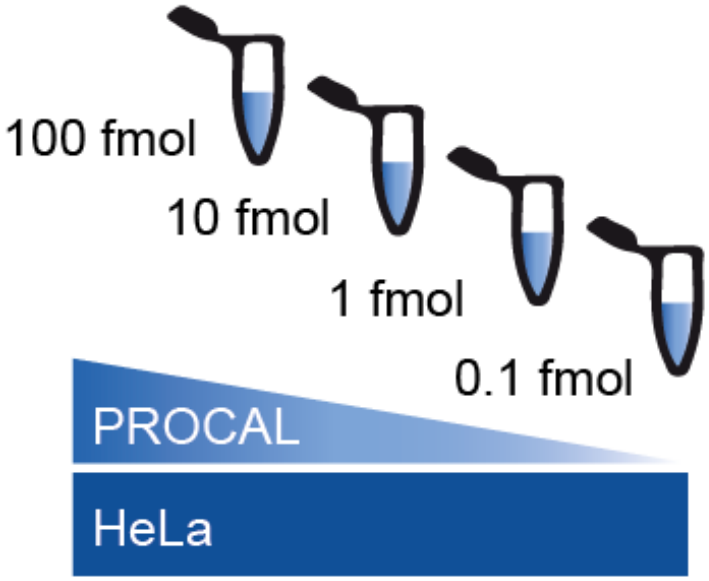
A) Datatypes – DDA, DIA, PRM

B) **Description of tutorial dataset**

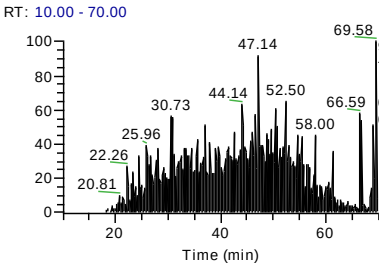
C) Skyline: “Targeted data evaluation”

Tutorial Dataset

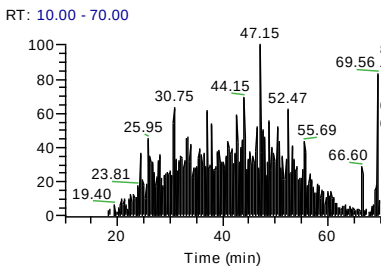
PROCAL peptide dilution in HeLa background



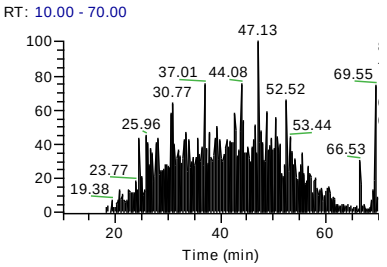
100 ng HeLa + 0.1 fmol PROCAL



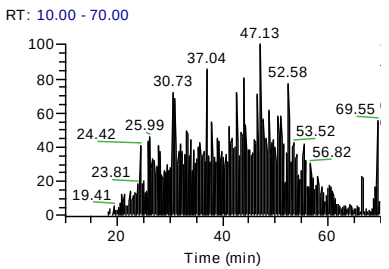
100 ng HeLa + 1 fmol PROCAL

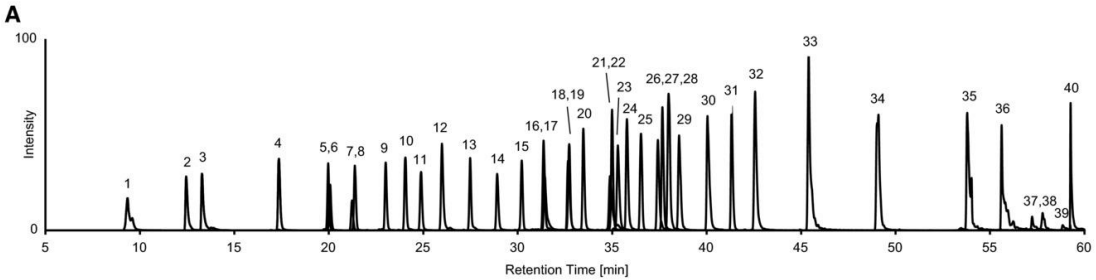


100 ng HeLa + 10 fmol PROCAL



100 ng HeLa + 100 fmol PROCAL

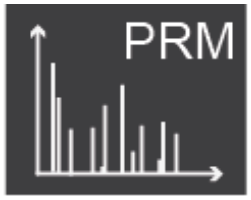


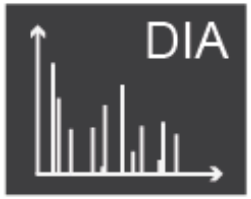


Zolg., et al. (2017) *PROCAL: A Set of 40 Peptide Standards for Retention Time Indexing, Column Performance Monitoring, and Collision Energy Calibration.* ***Proteomics***

Tutorial Dataset

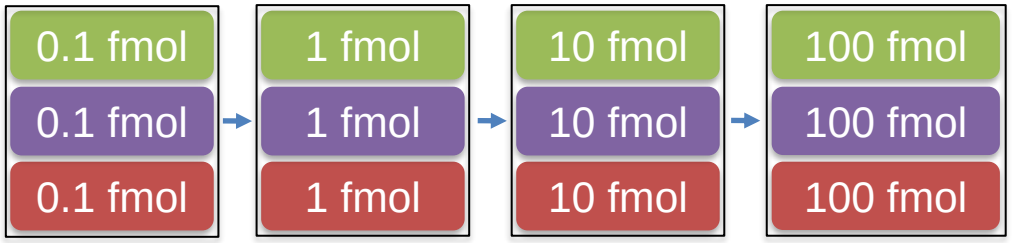
Acquisition Methods on Q-Orbitrap system (Eclipse)





40 PROCAL peptides
2+

Parameter	PRM	DIA	DDA
MS1			
Orbitrap Resolution	60,000	120,000	60,000
Scan Range (m/z)	360-1300	360-1300	360-1300
Max IT (ms)	50	50	50
Norm.AGC Target	100%	100%	100%
MS2			
Isolation Window	1.3	-	1.3
NCE (%)	30	30	30
Orbitrap Resolution	30,000	30,000	15,000
Scan Range (m/z)	140-2000	200-1800	-
Max IT (ms)	120	54	22
Norm.AGC Target	400%	1000%	200%
Cycle time	-	-	2
Dynamic exclusion	-	-	30s
Windows	-	40 variable	



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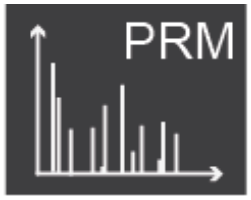

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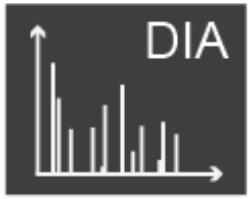
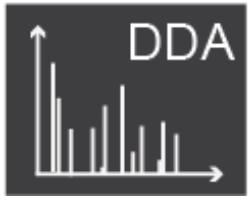


DIA-NN database search
(DIA-NN library prediction)



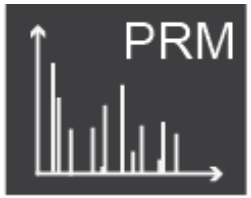
MaxQuant database search

Parameter	Settings
Spectral library prediction	PROCAL.fasta; human reference UP000005640 Swiss Prot fasta ; contaminants.fasta Trypsin/P; 1 Missed cleavages 0 Maximum number of variable modifications
DIA search	
Spectral library	PROCAL predicted library (.speclib)
Peptide length range	7-30
Precursor m/z range	360-1300
Fragment ion m/z range	200-1800
MBR	**TRUE → new .speclib library file**
Protein inference	Genes
Neural network classifier	Single-pass mode
Quantification strategy	Robust LC (high precision)
Cross-run normalization	RT-dependent
Library generation	Smart profiling
Speed and RAM usage	Optimal results

Parameter	Settings
Type	Standard
Enzyme	Trypsin/P
Missed clevages	1
Modifications	Fixed: Carbamidomethyl (C) Variable: Oxidation (M);Acetyl (Protein N-term)
Label-free quantification	None
Sequences	PROCAL.fasta; human reference UP000005640 Swiss Prot fasta
Match between runs	FALSE
PSM FDR	1%
Protein FDR	1%
Min peptide length	7

Tutorial Dataset

Acquisition Methods on Q-Orbitrap system (Eclipse)



40 PROCAL peptides
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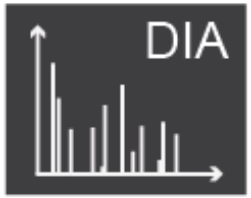

DIA-NN database search
(DIA-NN library prediction)

MaxQuant database search

raw data
.fasta

report.tsv
.speclib (MBR)

mqpar
msms.txt

Targeted data analysis

For other database search options check out:
https://skyline.ms/wiki/home/software/Skyline/page.view?name=building_spectral_libraries

Overview

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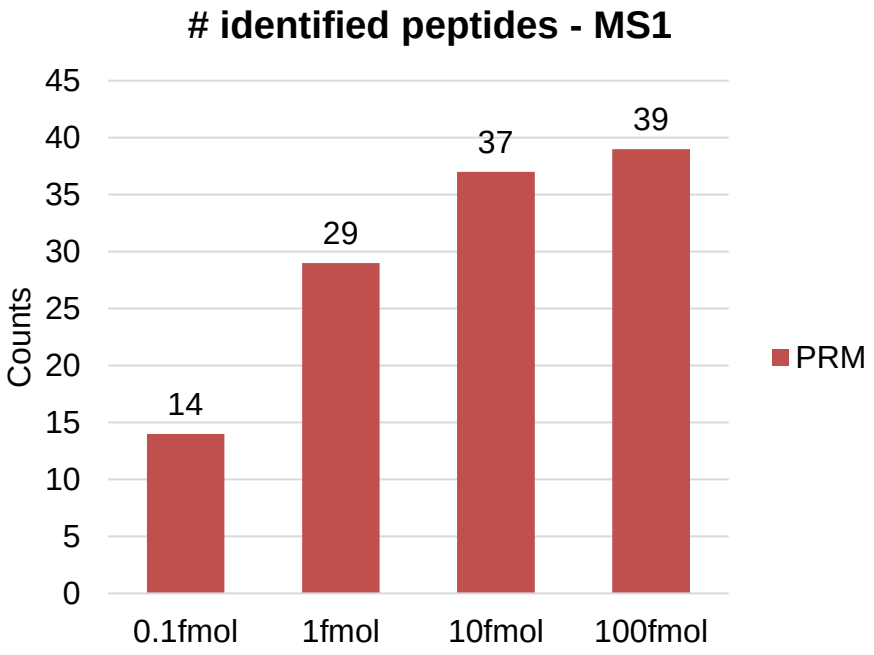
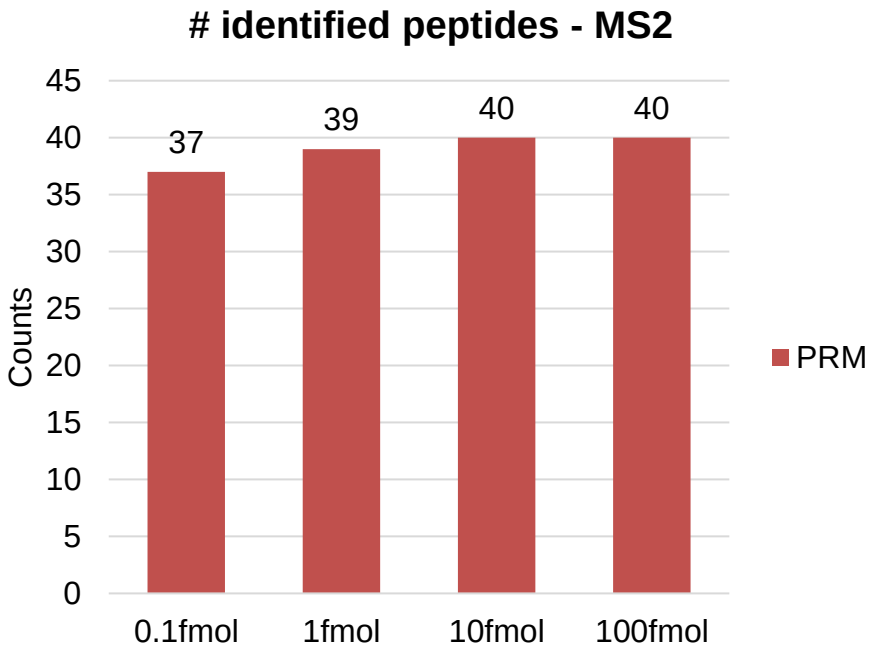
Skyline -tutorial

Skyline – summary peak identifications

Filter
MS1 idotp > 0.9
MS2 dotp > 0.7





Skyline – summary peak identifications

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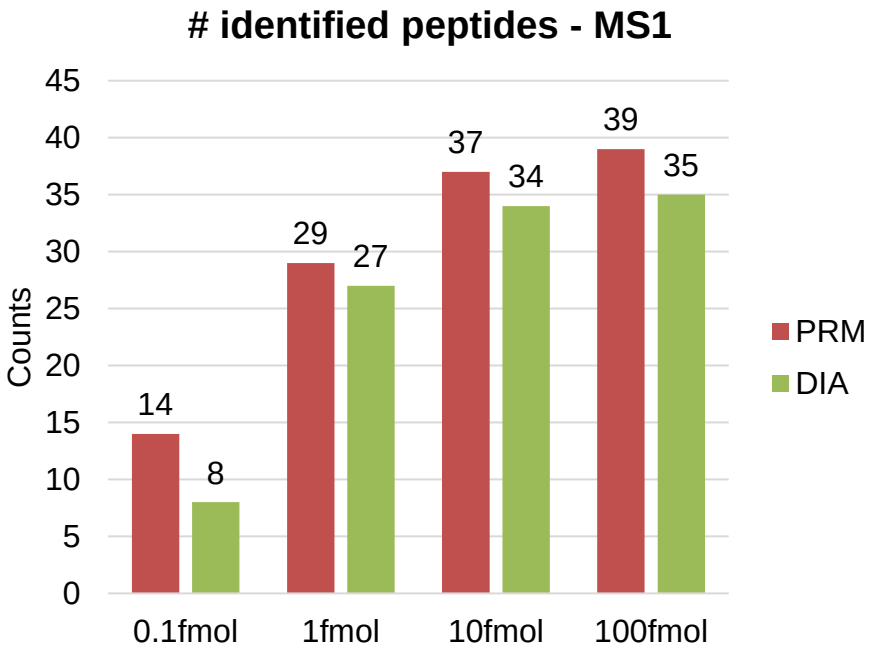

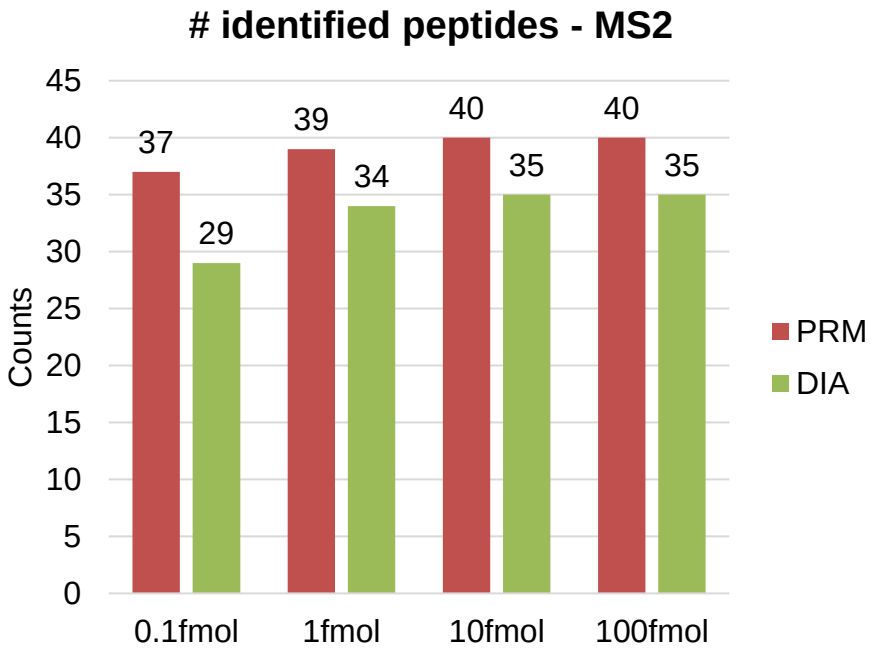

Skyline – summary peak identifications

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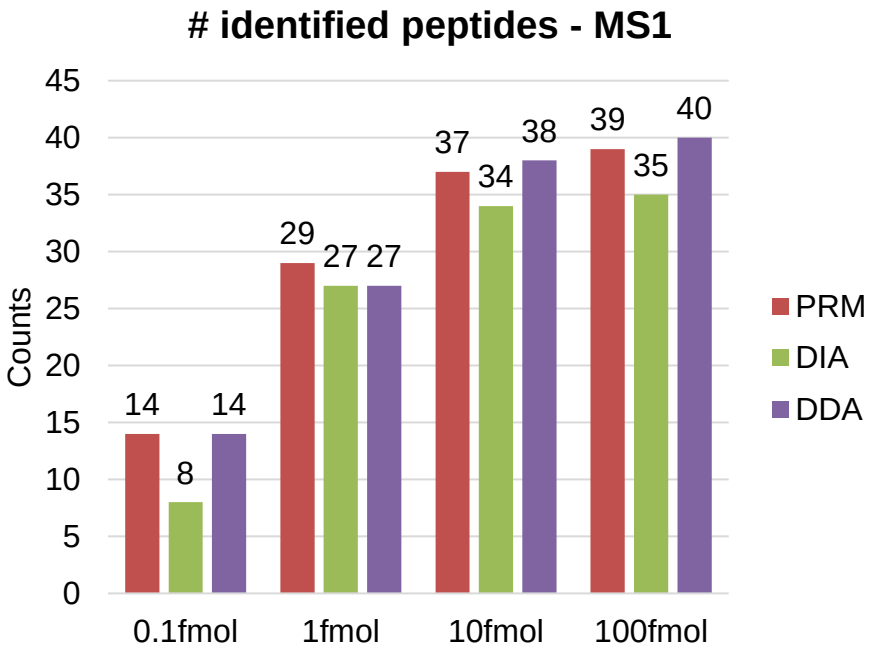

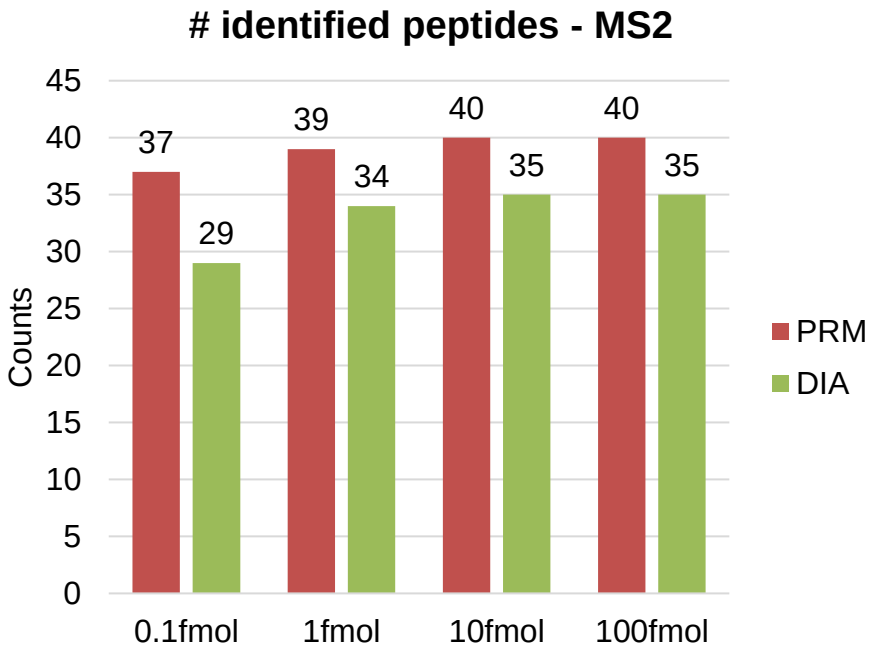

Skyline – summary Quan

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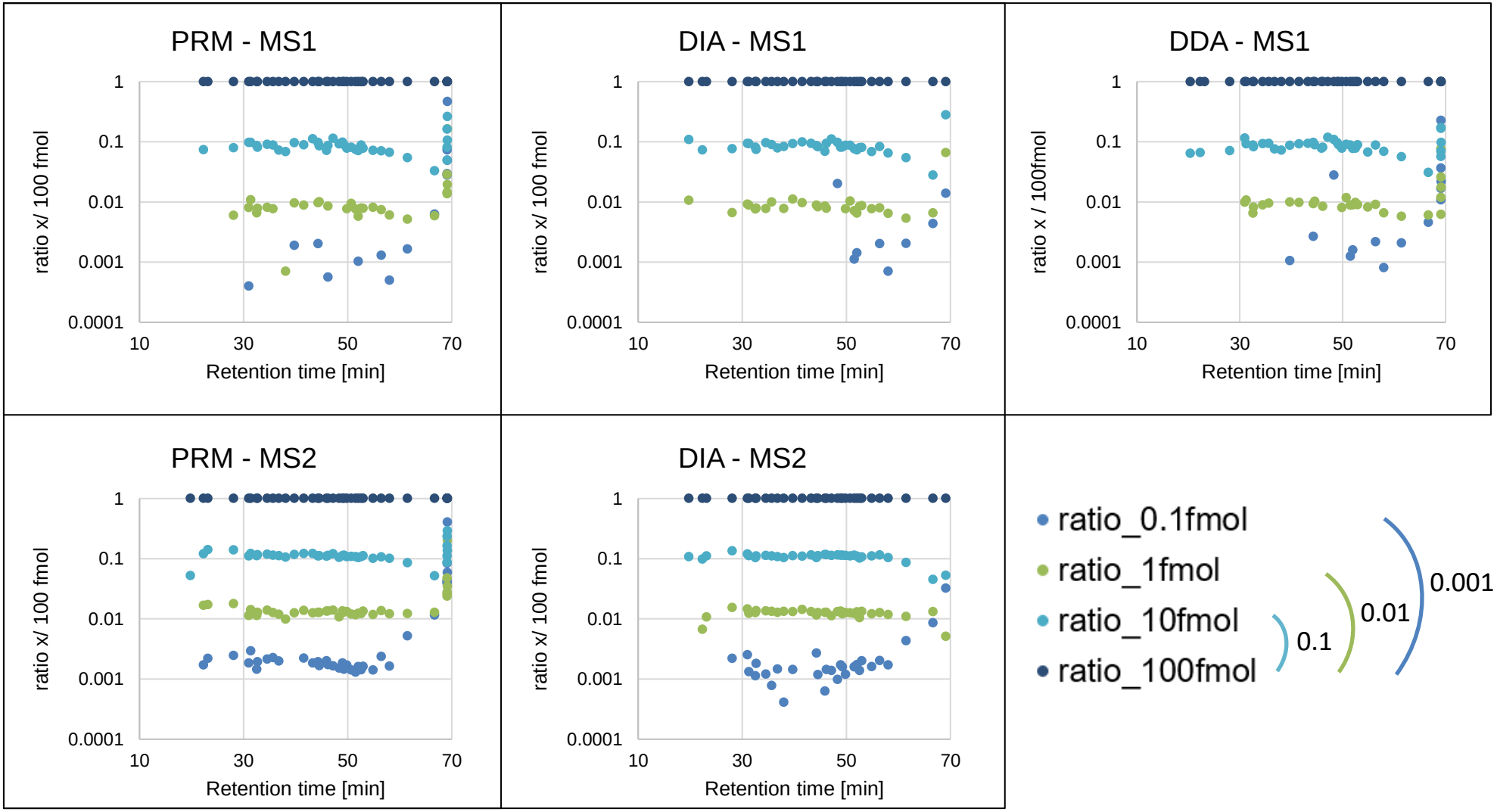
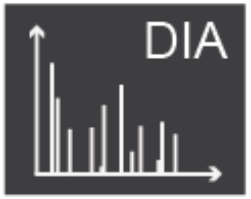
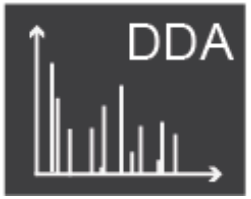


Comparison

When should I use which acquisition method?





Peak identifications MS1*	Peak identifications MS2*	Quantitative accuracy MS1	Quantitative accuracy MS2
++	++	+	++
+	+	+	+
++	-	+	-

*based on DIA-NN or MaxQuant search output

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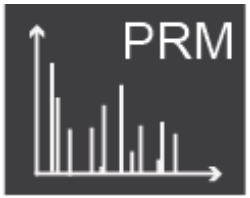
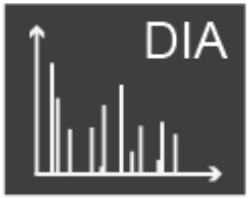




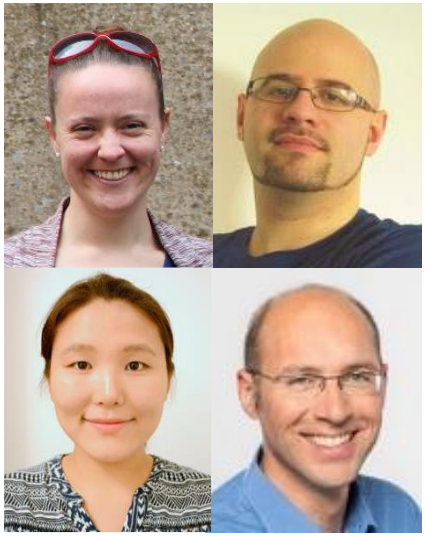
Peak identifications MS1*	Peak identifications MS2*	Quantitative accuracy MS1	Quantitative accuracy MS2
++	++	+	++
+	+	+	+
++	-	+	-

*based on DIA-NN or MaxQuant search output

… that depends on your sample type, experimental setup and research question!

Acknowledgement

BayBioMS@MRI Team
TUM University Hospital



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DFG Deutsche Forschungsgemeinschaft
German Research Foundation

Death Decisions
TRR 353

Klinikum rechts der Isar
Technische Universität München

The End

Questions?