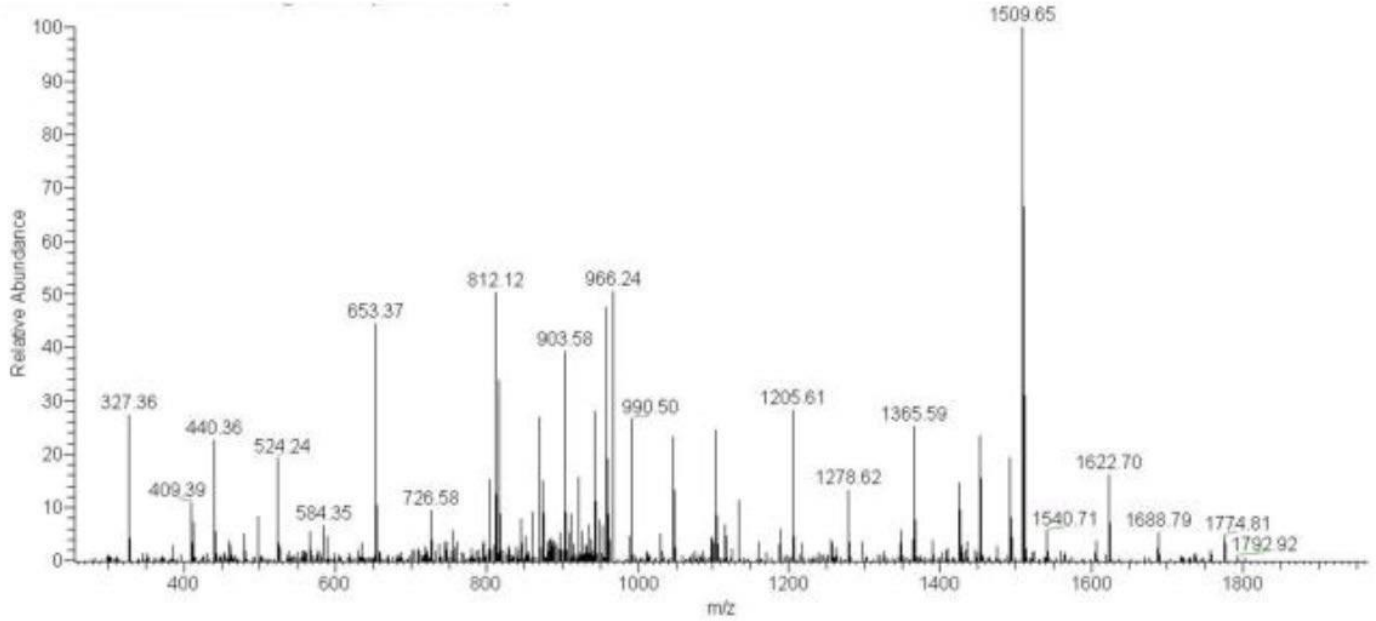
LECTURE 4: QUANTITATIVE MASS SPECTROMETRY-BASED PROTEOMICS

Quantitative Proteomics

- Quantitative proteomics aims at simultaneously quantitation of level differences between many proteins in different samples, not at measurement of their absolute concentrations.

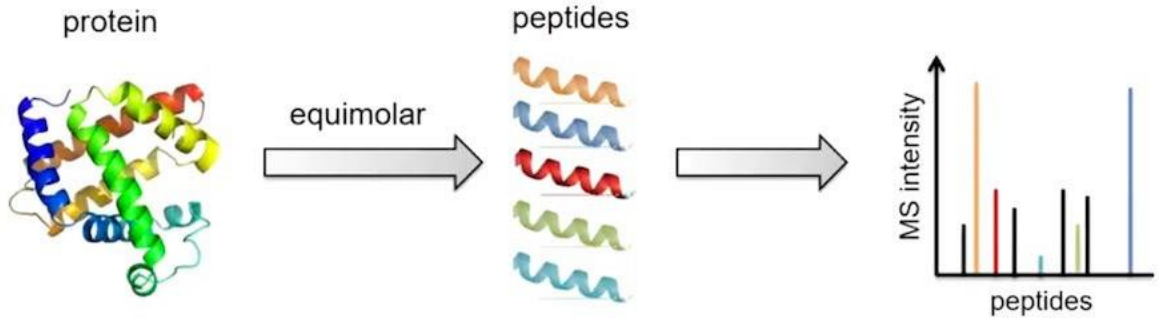
- Mass spectrum records a whole bunch of m/z

- BUT MS intensity does **NOT** tell us peptide abundance directly.



Why does MS^1 intensity not tell us peptide abundance directly?

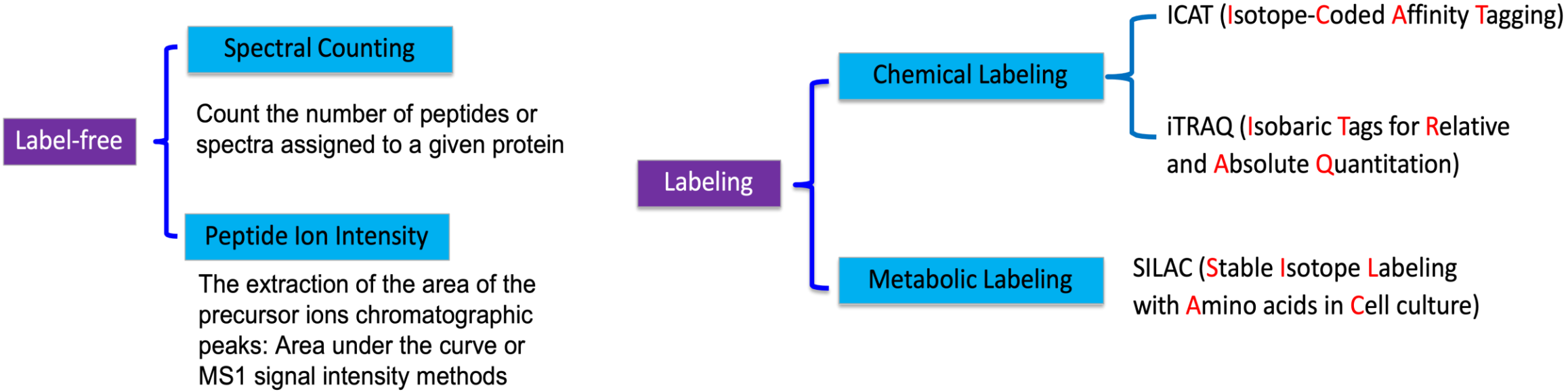
- There is a poor correlation between the amount of a peptide and the MS intensity in a single MS spectrum



- Many factors affect the MS intensity:
  - Peptide concentration
  - Day to day and long-term instrument reproducibility
  - **Digestion efficiency**
  - **Recovery during sample preparation**
  - **Ionization efficiency**
  - **Instantaneous matrix effects**

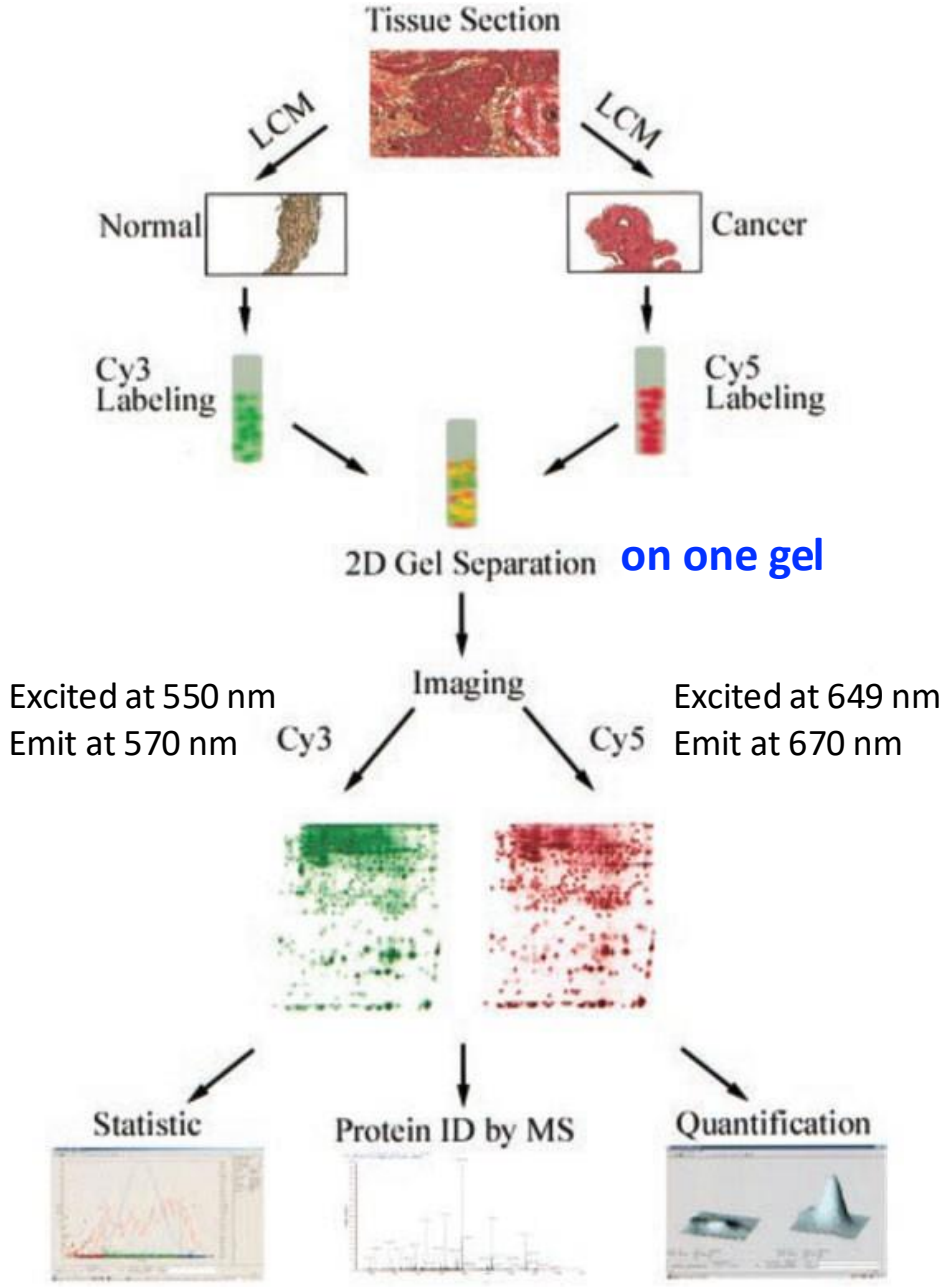
Quantitation Techniques in Proteomics

- Electrophoretic techniques
- Mass spectrometric techniques



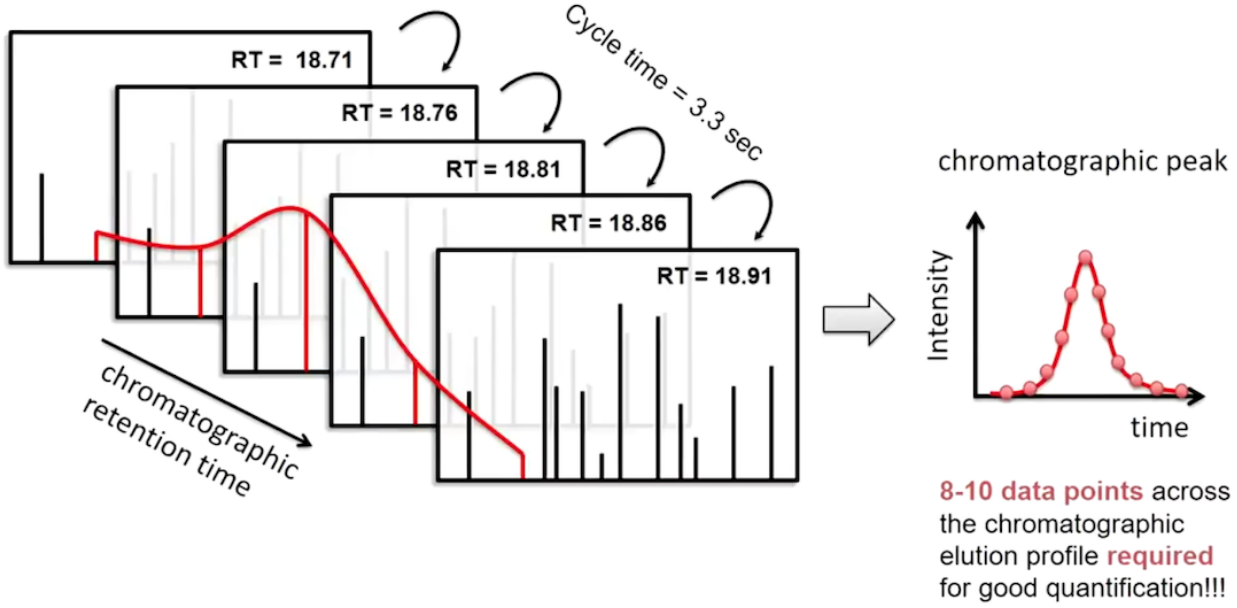
Electrophoretic Technique

- It is often difficult to obtain reproducible separations by standard 2D PAGE.
- Quantitative proteomics utilizes fluorescent dyes
  - Ease of use
  - High sensitivity (1 ng)
  - Low background (not staining the gel)
  - Compatibility with MS
  - Broad range of linearity
- Differential gel electrophoresis (DIGE) is used in quantitative proteomics.



Quantitative LC-MS

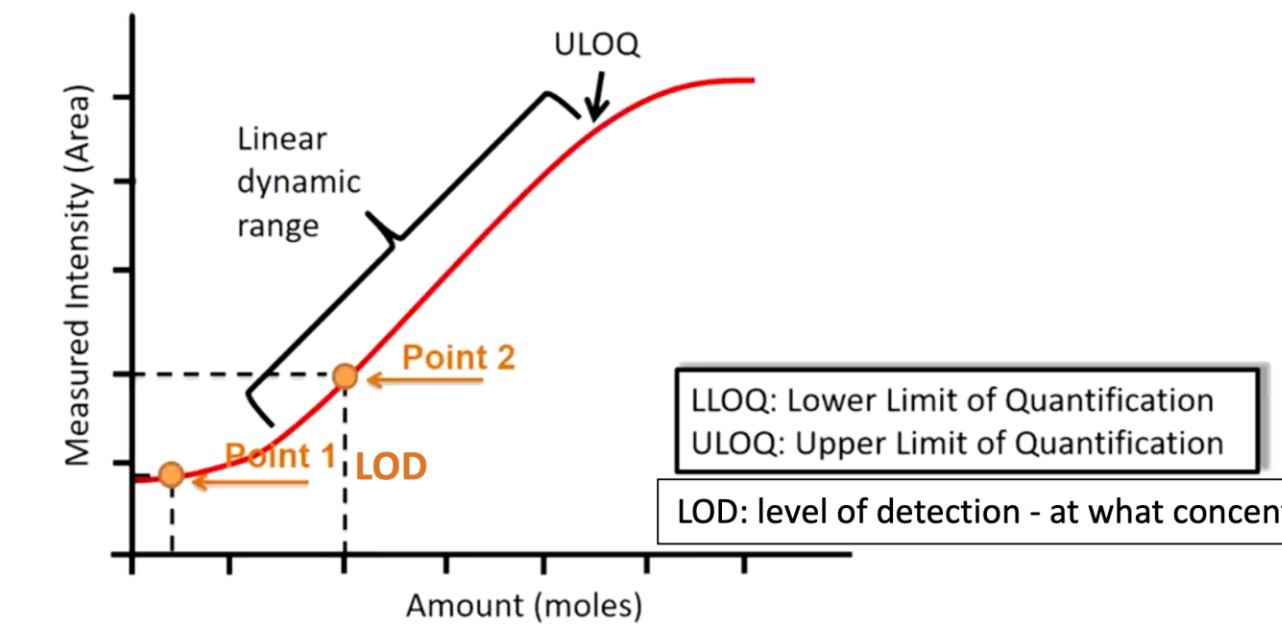
- Fixed volume of the sample is injected
- Analyte spreads out, elutes over a certain timespan from the column (peaks)
- Only a fraction of the analyte really enters the MS



Cycle Time: A Crucial Parameter In Chromatography-based Quantification

To achieve maximum benefit in an LC/MSn experiment, the series of events starting from the process of filling the MS analyzer through detecting the ions must be optimized for a chromatographic timescale. The time taken to complete this series of events is the analytical **cycle time**.

Mass Spectrometry-based Quantification

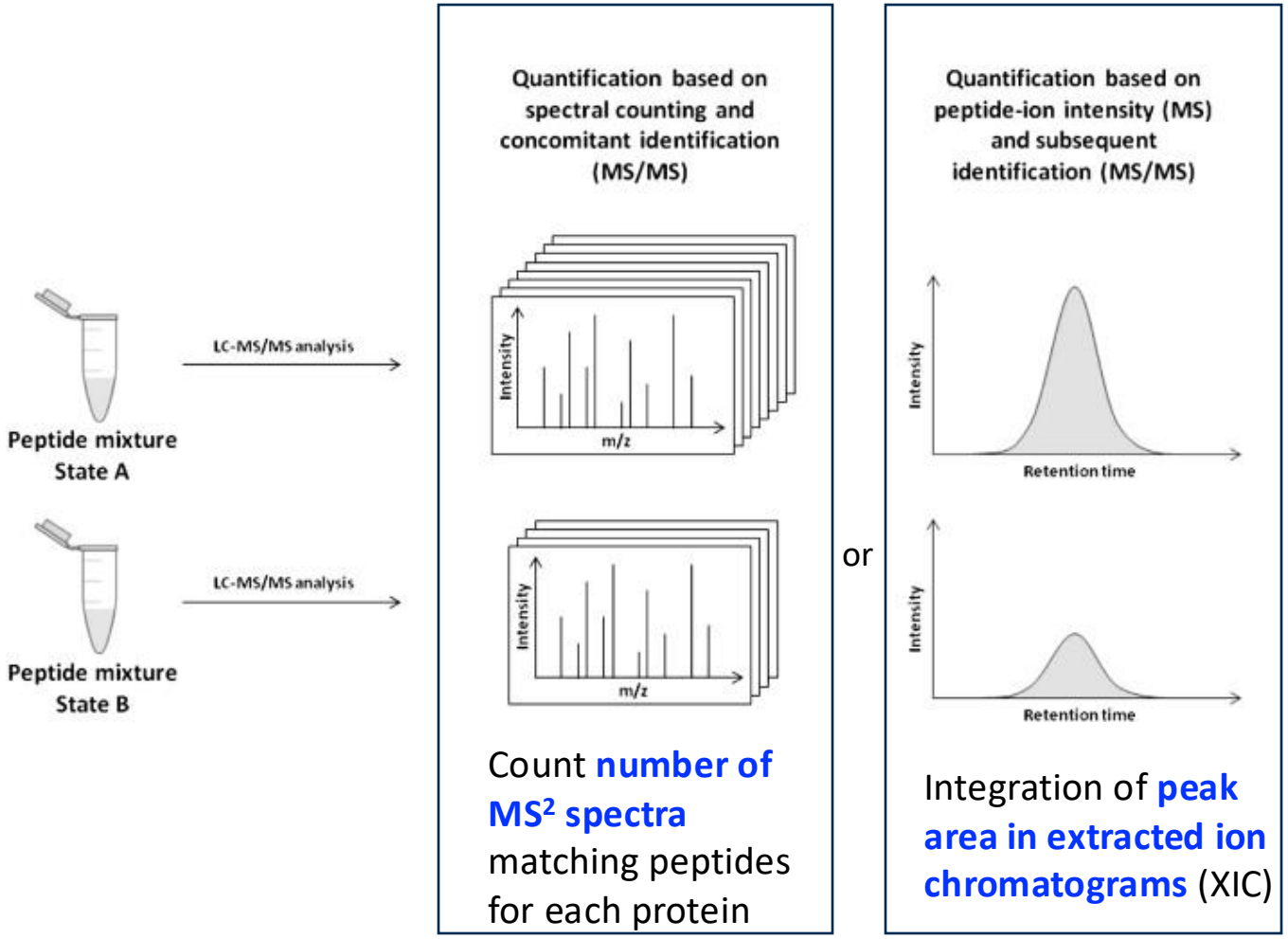


Chromatographic MS Signal Intensity Scales Linear With Peptide Abundance

- Accurate quantitative results can only be achieved when working within the linear dynamic range of every given peptide, respectively.
- The linear dynamic range and LLOQ and ULOQ are peptide and MS dependent

Label-free Methods: Spectral Counting or Peak Area

- Each sample is separately prepared and then subjected to individual LC-MS/MS runs.
- **Spectral Counting**: more abundant peptides are more likely to be observed and detected.
- **Peak Area**: signal intensities of ions after ESI correlate with ion concentrations.



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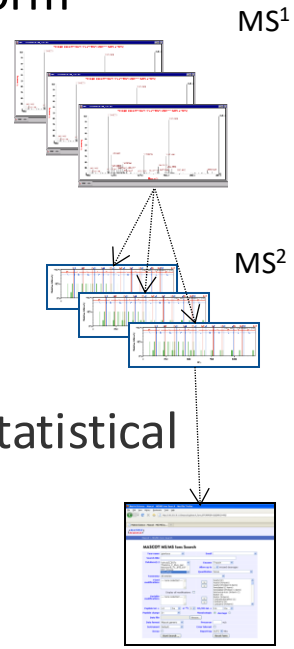
Spectral Counting

- **Method summary:**
  - MS^2 spectra queried against a sequence database to make peptide-spectrum matches (PSMs)
  - Peptide ions from more abundant proteins trigger many more MS^2 scans
  - Count PSMs matched to a given protein => approximate protein abundance
- **Requirements:**
  - Not much – system setup for LC-MS/MS (most tandem mass spectrometers can perform spectral counting)
- **Advantages:**
  - It's really easy to do!
- **Disadvantages:**
  - Unclear how accurate the protein abundance values are. Normalization and careful statistical evaluation are still needed.
    - Origin of MS2 spectra…
    - proteins with only a few observable peptides
    - the quantitative changes between experiments are small
  - Need lots of replicates to get statistical significance (how many…?)
  - Lots of machine time needed



The Origin of Spectra Count

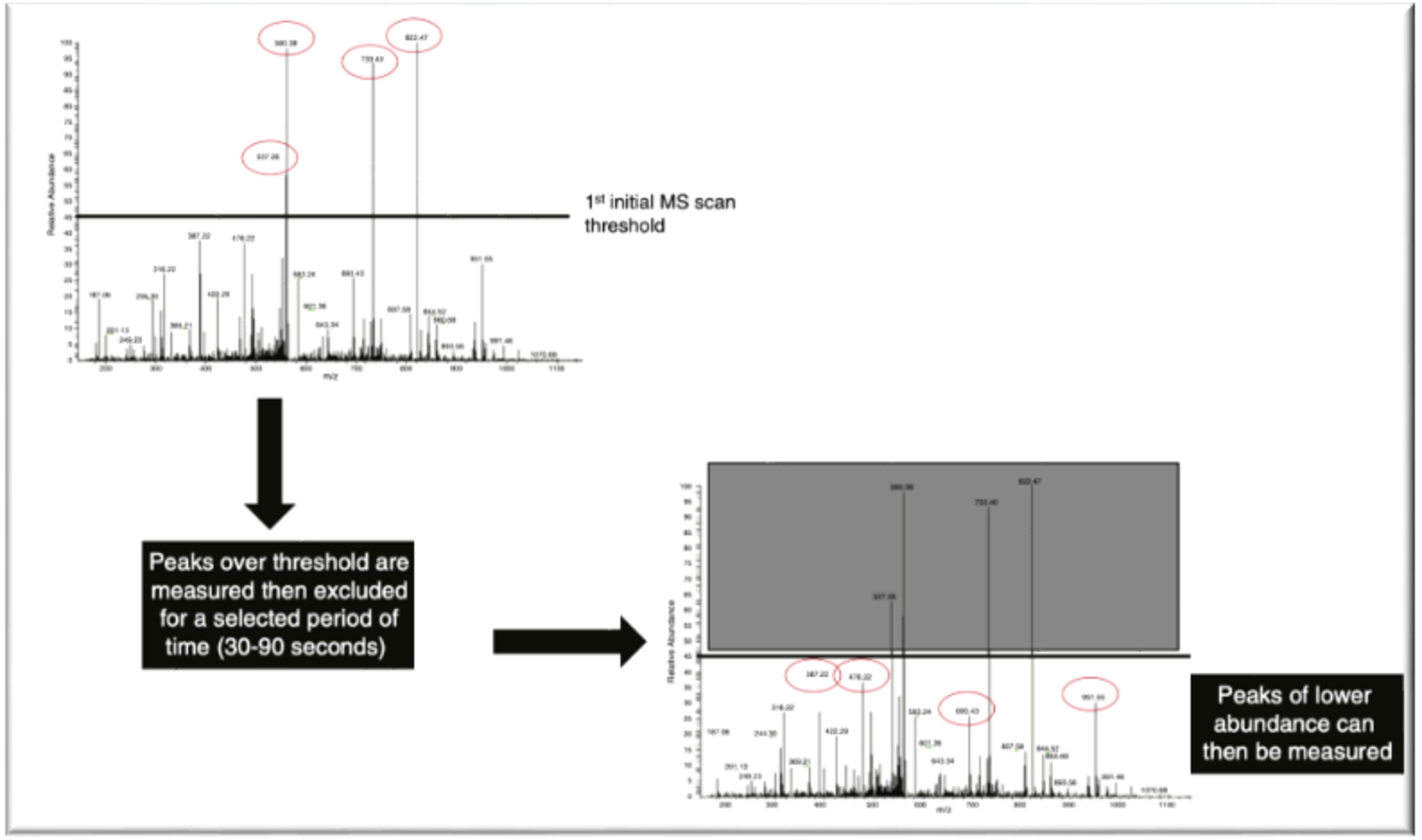
- Fully digested tryptic peptides can be positively charged at both *N*- and *C*-terminus upon electrospray ionization. Some tryptic peptides, containing histidine or extra arginine/lysine due to missed cleavage, can be 3^+ charged.
- The MS^2 spectra that matched to a protein is a combination of:
  - spectra from different partial tryptic peptides and full tryptic peptides,
  - spectra from the same peptide with different charges,
  - spectra of the same peptide with variable modifications,
  - repeated spectra from the same peptide due to expired dynamic exclusion.

Note: At some situations, spectra that are potentially matched to partial tryptic peptides or peptides with PTMs are not included during data analysis by spectral interpretation software.

Dynamic exclusion is a software technique, allowing the mass spectrometer to more efficiently identify peptides in a sample.

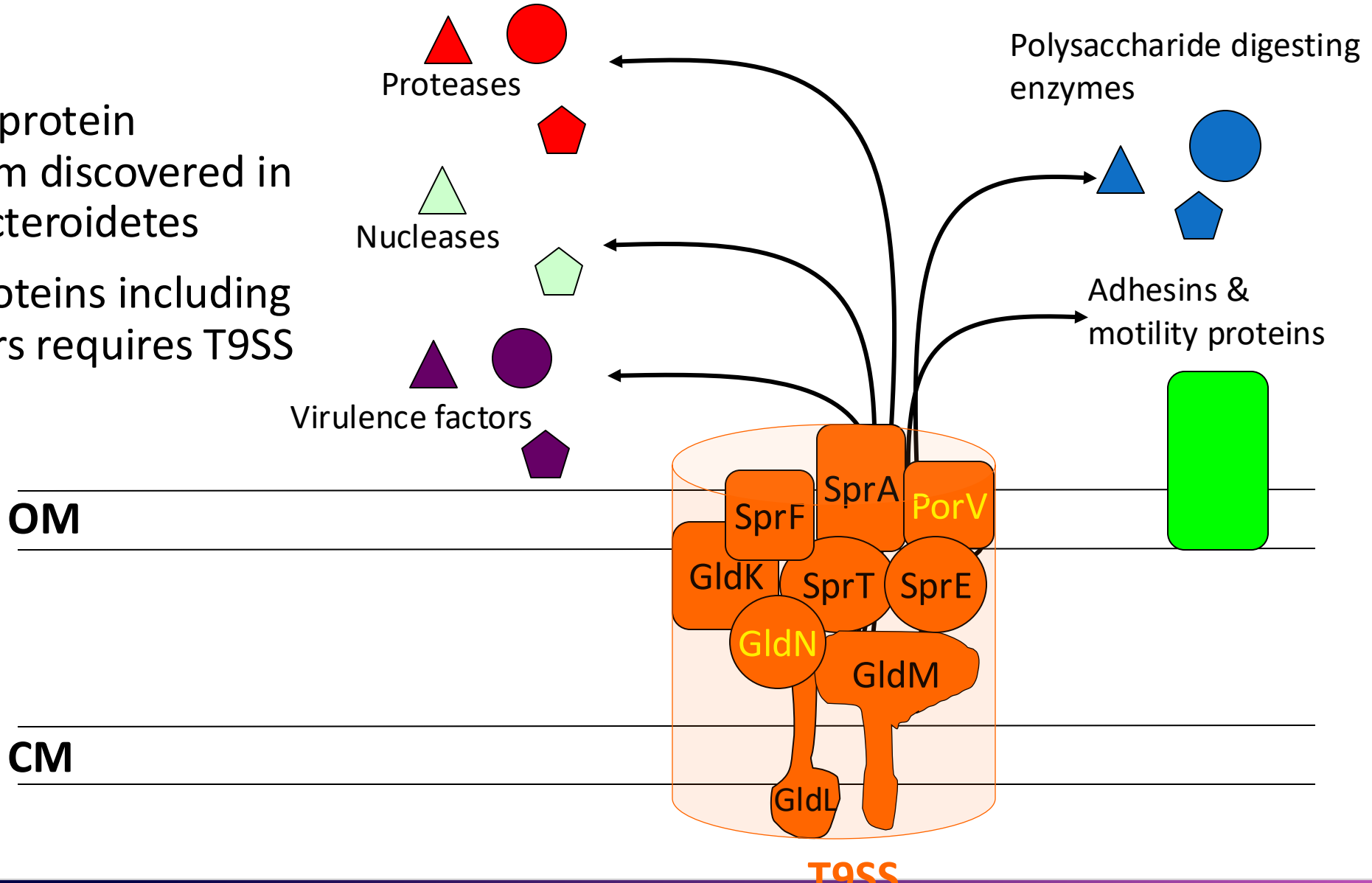
The first scan measures the ions with the highest intensity (most abundant). These masses are added to a temporary ‘exclusion’ list for a period

Once the high intensity peaks have been sequenced and excluded the MS can measure peaks under the threshold, thereby detecting less abundant peptides.



Case Study: Type IX Secretion System (T9SS)

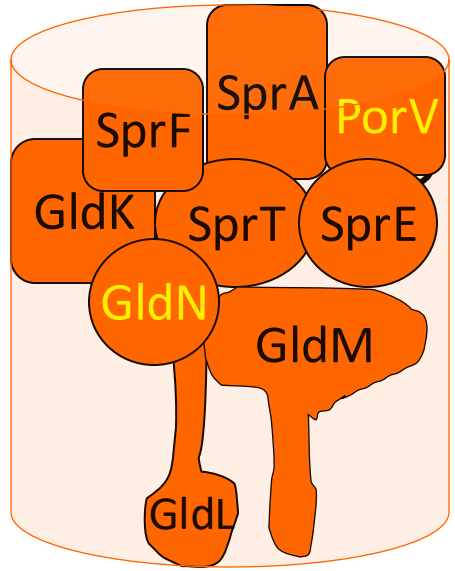
- T9SS is a novel protein secretion system discovered in the phylum Bacteroidetes
- Secretion of proteins including virulence factors requires T9SS



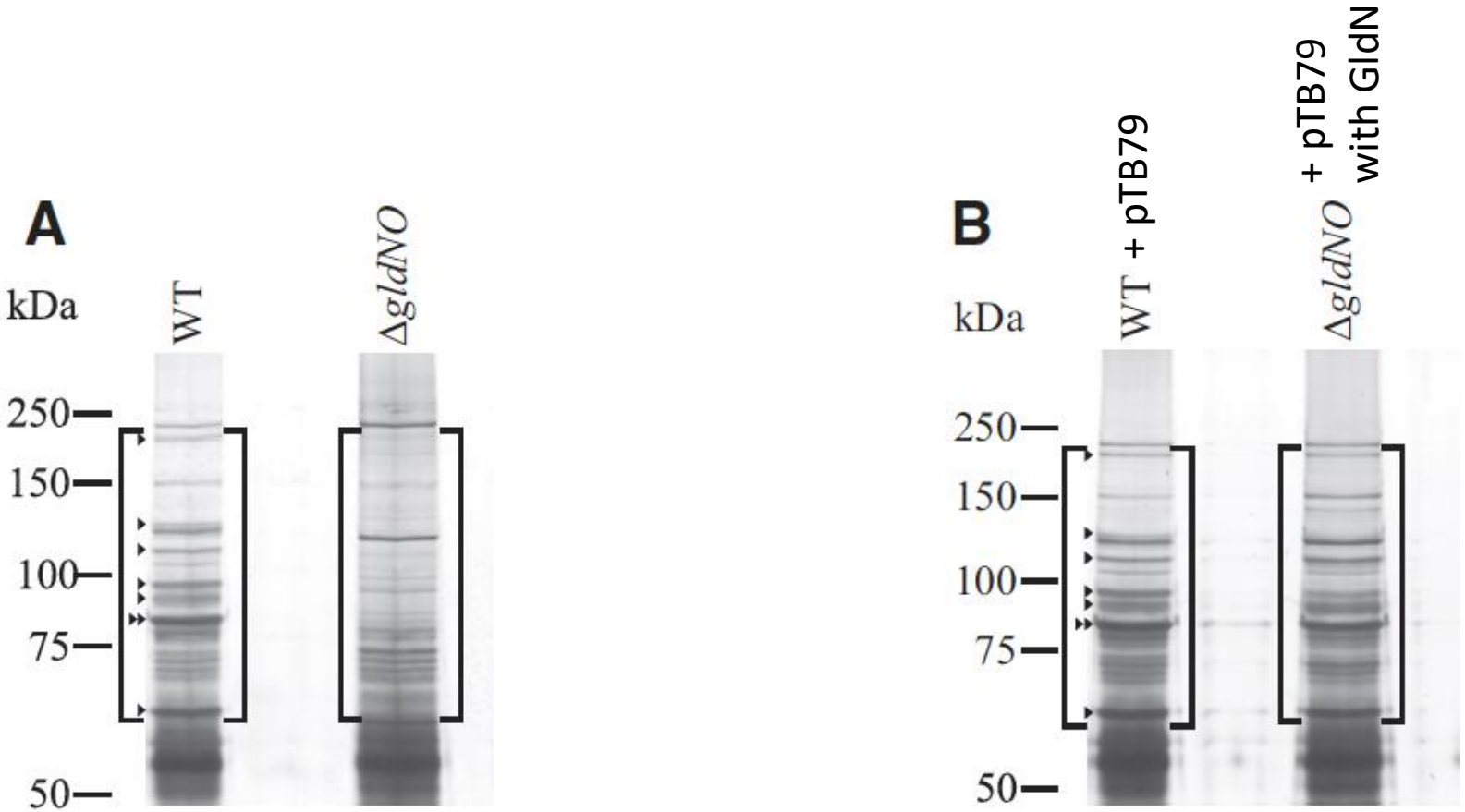
Purpose and Strategy

- **Purpose**: to identify which proteins are secreted by the bacterial type IX secretion system (T9SS) and their abundance

- **Strategy**: Create T9SS mutants (Δ*gldNO* and Δ*porV*) and complementation → Cell culturing → SDS-PAGE of secreted extracellular proteins → Enzymatic in-gel digestion → LC-MS/MS



Soluble extracellular proteins of wild-type and mutant cells



Kharade, S. S., & McBride, M. J. (2015). *197*(1), 147–158. https://doi.org/10.1128/JB.02085-14

TABLE 5 Candidate proteins secreted by the T9SS identified by LC-MS/MS analysis of cell-free culture fluid

Locus tag/protein name	Mol mass[b] (kDa)	Predicted localization[c]	CTD[d]	Predicted protein function[e]	Spectrum count for:		
					Wild type	Δ*gldNO* strain	Δ*gldNO* strain with pTB79
Fjoh_0074	123.1	OM, E	TIGR04183	Nuclease/phosphatase	42	3	108
Fjoh_0601	208.2	OM			115	0	84
Fjoh_0602	279.3	OM			68	0	38
Fjoh_0604	144.2	E			47	0	39
Fjoh_0606	409.5	OM			163	0	172
Fjoh_0808/RemA	154.0	E	TIGR04183	Motility adhesin	38	0	47
Fjoh_0886	99.1	E	TIGR04183	Peptidase	12	0	19
Fjoh_1022	51.1	E	TIGR04183	Licheninase	6	0	6
Fjoh_1123	121.9	E, OM	TIGR04131		34	0	10
Fjoh_1188	152.7	E, OM	TIGR04183		49	0	104
Fjoh_1189	181.4	E	TIGR04183	Lectin	74	0	112
Fjoh_1208	112.5	E	TIGR04183	α-Amylase	45	0	66
Fjoh_1231	97.8	E	TIGR04183	Pectate lyase	9	0	13
Fjoh_1269	94.3	E, OM	TIGR04183		27	4	43
Fjoh_1408[f]	106.0	E	TIGR04183	α-Amylase	2	0	4
Fjoh_1645[f]	258.1	E	TIGR04131		2	0	6
Fjoh_2150	39.0	E, OM	TIGR04183		6	0	6
Fjoh_2273	93.3	E	TIGR04131		4	0	5
Fjoh_2389[f]	57.7	E, OM	TIGR04183	Peptidase	2	0	7
Fjoh_2667	129.7	OM			28	0	7
Fjoh_2687	155.8	E			26	1	26
Fjoh_3108	30.9	OM, E, P			7	0	10
Fjoh_3246	299.4	OM, E	TIGR04183		12	0	77
Fjoh_3324	105.3	E	TIGR04183	Carbohydrate binding	16	1	40
Fjoh_3729	195.1	OM			46	0	32
Fjoh_3777	128.1	OM, E	TIGR04183	Deacylase	10	0	25
Fjoh_3952	330.6	E	TIGR04131		22	0	11
Fjoh_4174	102.5	E	TIGR04183	Carbohydrate binding	40	5	40
Fjoh_4176	95.4	E	TIGR04183	Carbohydrate binding	48	3	65
Fjoh_4177	144.9	E	TIGR04183	Glycoside hydrolase	22	0	35
Fjoh_4750	158.1	E	TIGR04131		13	0	3
Fjoh_4819	112.5	C, OM, P		Glycoside hydrolase	34	0	5
Fjoh_4934	84.8	E	TIGR04131		11	1	7

Kharade, S. S., & McBride, M. J. (2015). *197*(1), 147–158. https://doi.org/10.1128/JB.02085-14

Peak Area/Peptide Ion Intensity Based Protein Quantification

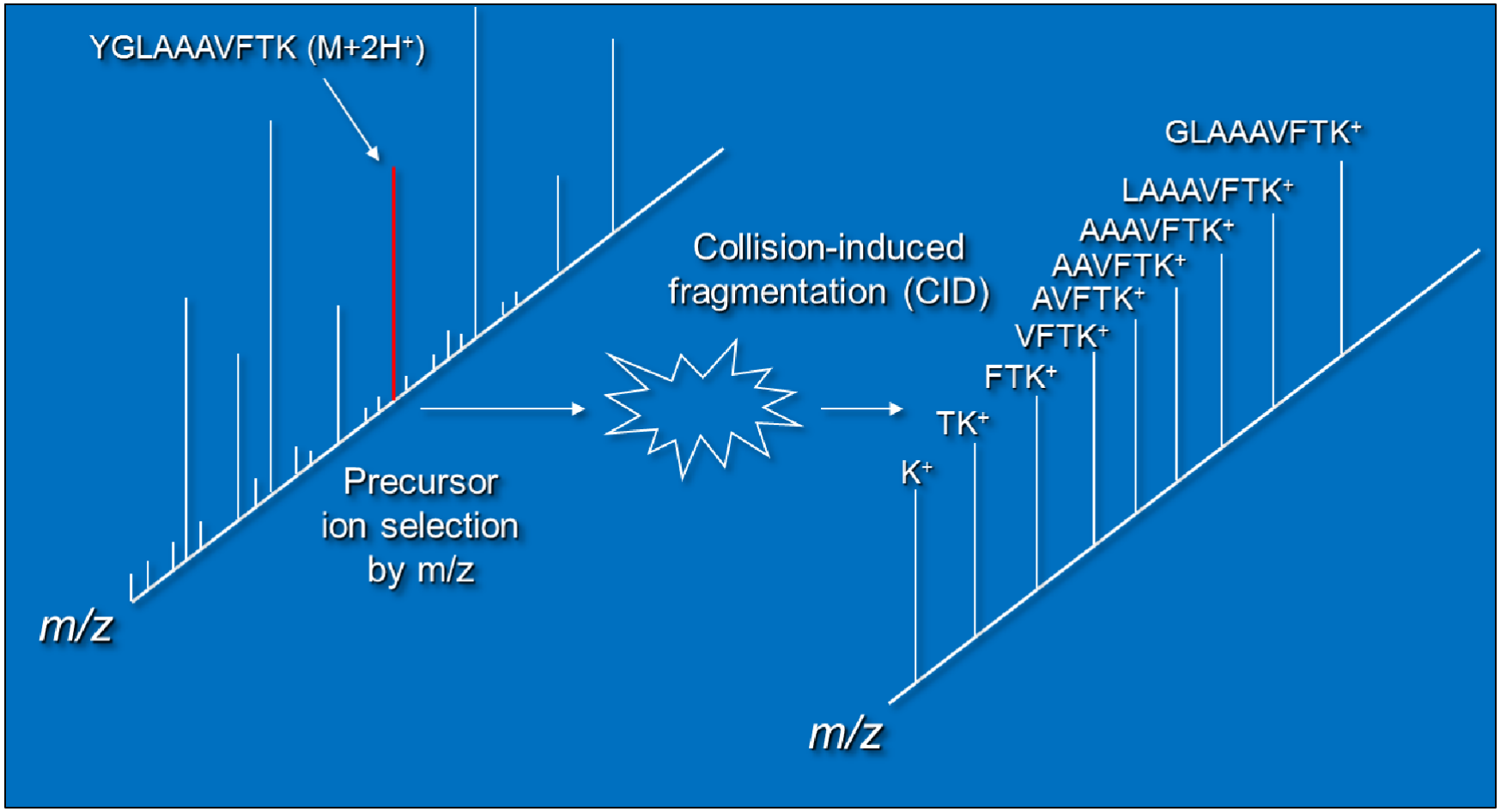
- MS signal intensity for the peptide at a certain time is proportional to the concentration eluting off the column
- The area under the chromatographic peak is proportional to the total amount of analyte eluting and thus to the amount in the sample. Hence, we want to integrate over time.

- **Method summary:**
  - Compare **MS^1 peptide ion abundance** across runs;
  - Specialist software must align different parallel LC-MS runs
  - Calculate ratios from aligned MS1 data

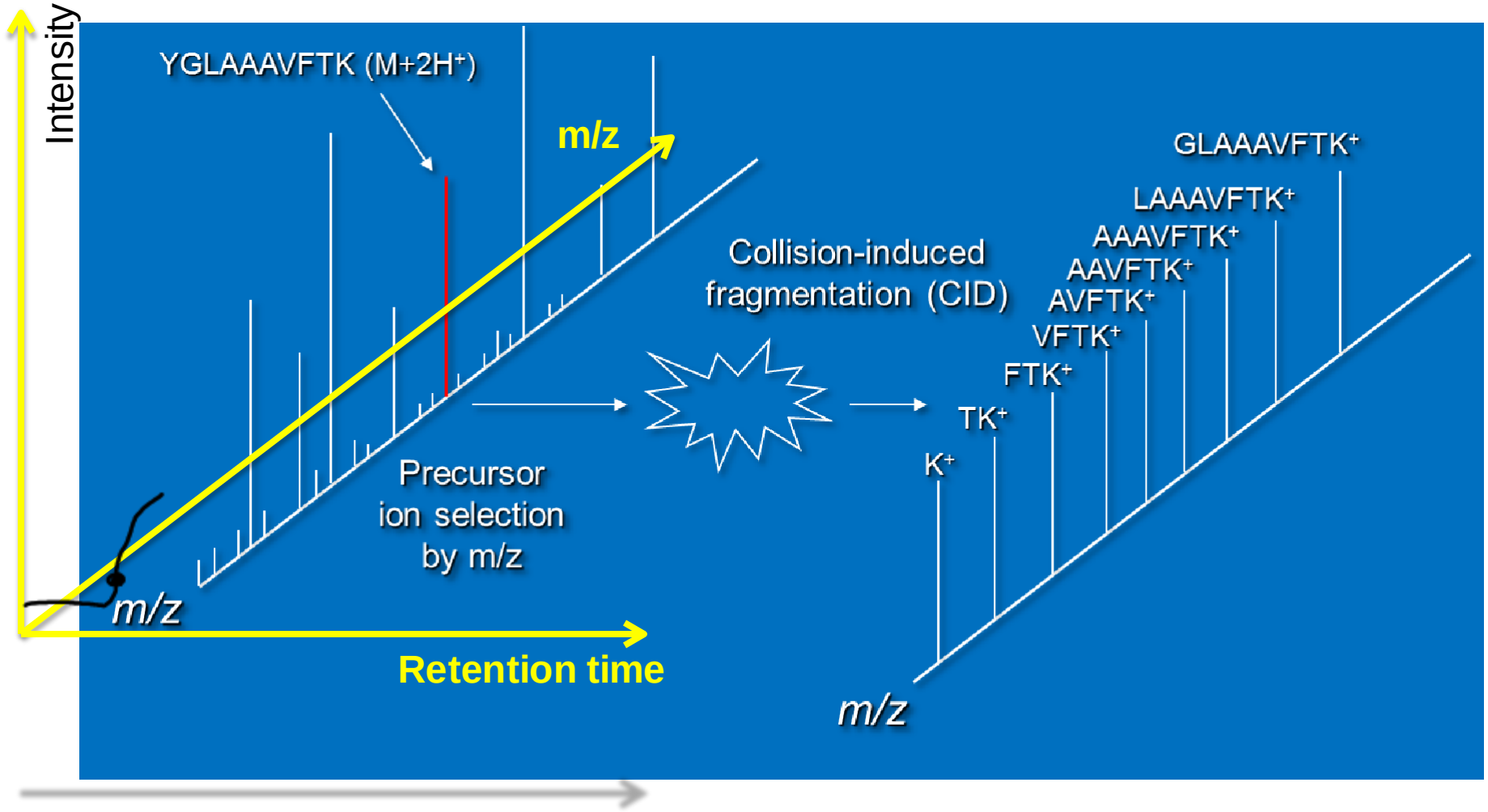
Peak Area/ Peptide Ion Intensity Based Protein Quantification

- **Advantages:**
  - In theory, should be more accurate than spectral counting – uses real intensity data
  - No complicated labelling protocols
- **Disadvantages:**
  - Data processing is fairly CPU intensive
  - Only works well if experimental system has high technical and biological reproducibility
- **Requirements:**
  - Very good LC delivery system (must be reproducible)
  - High resolution mass spec (Orbitrap etc...)
  - Good PC for running software

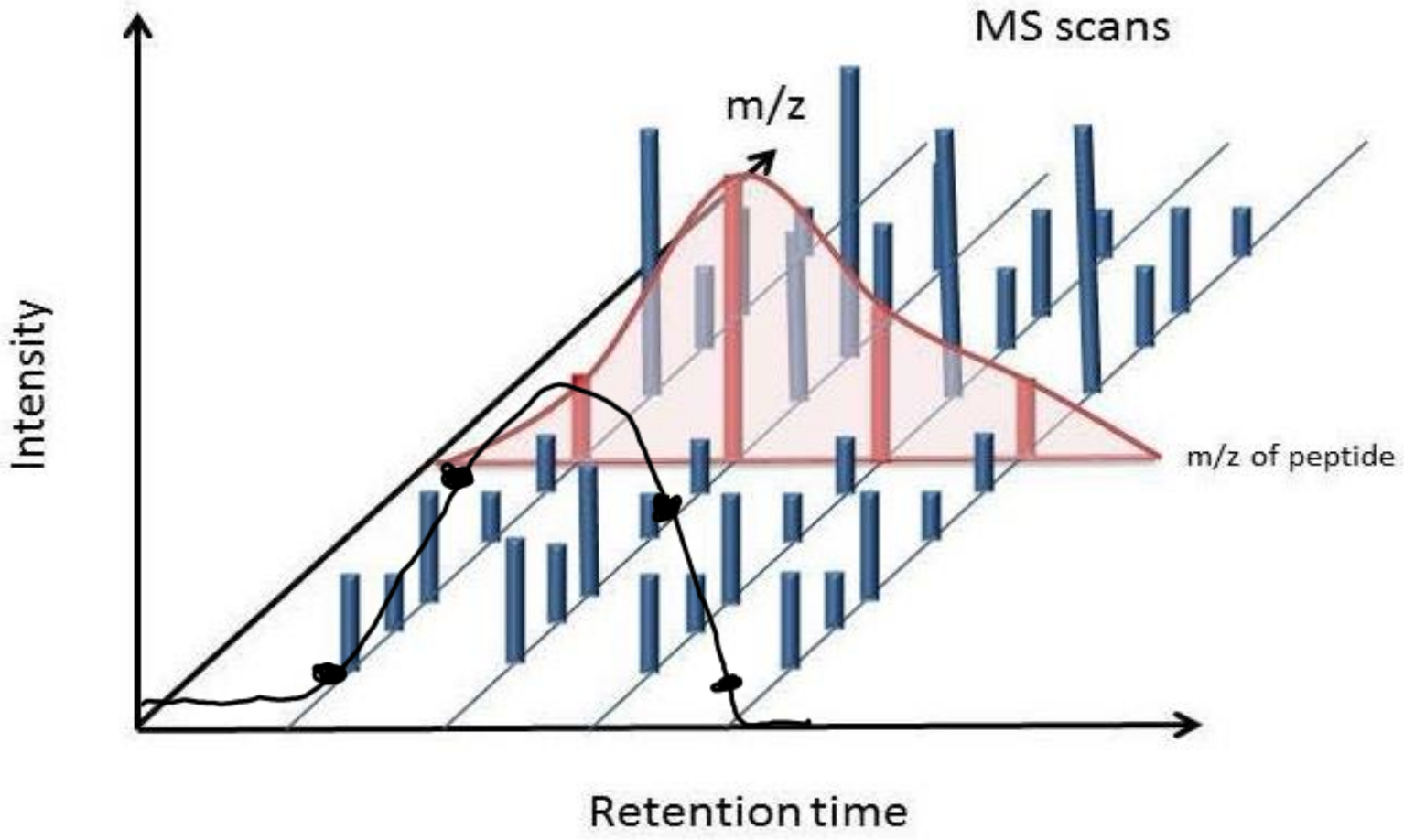
Acquired Data



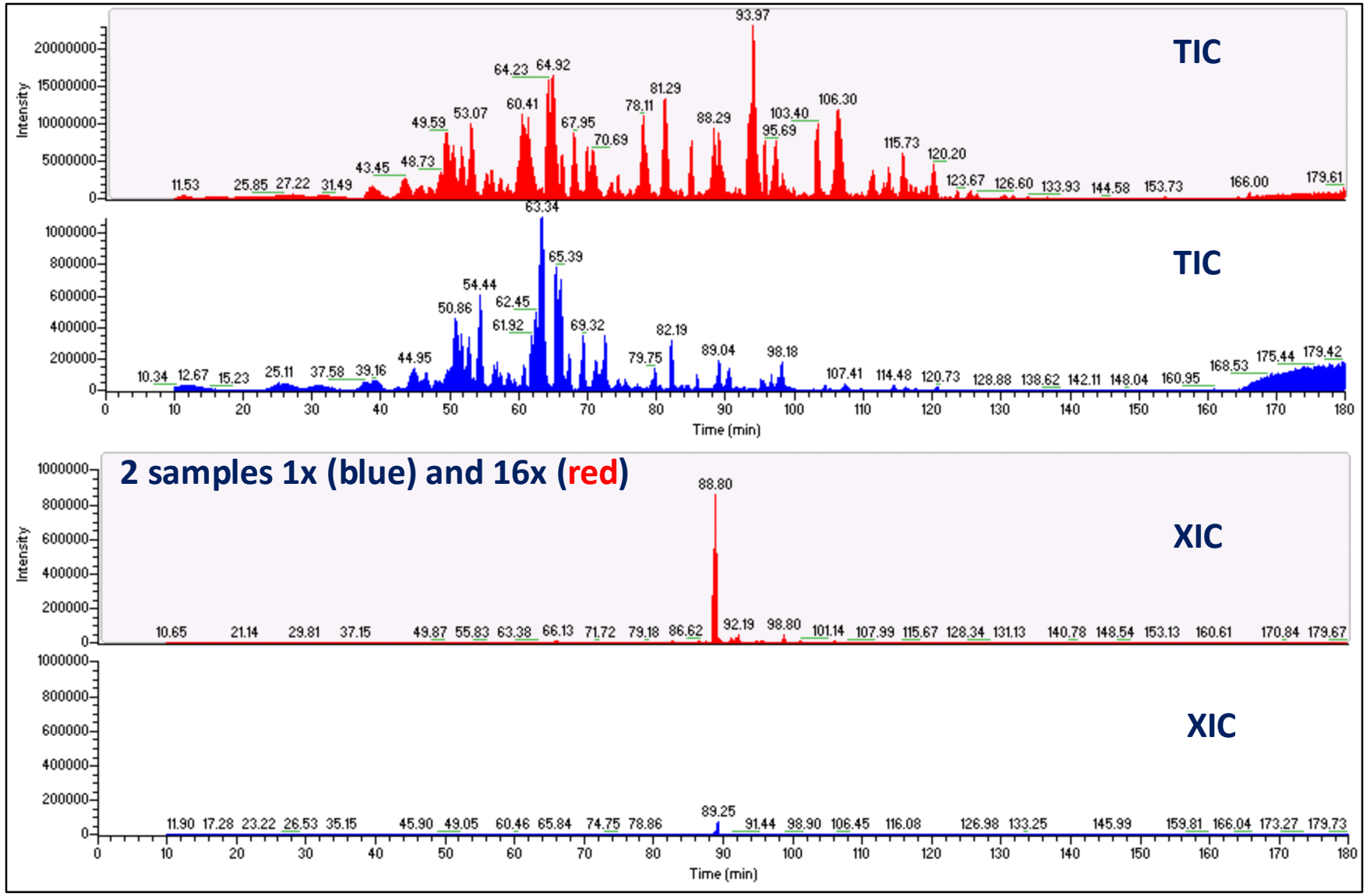
Acquired Data



Acquired Data ?



Peptide Ion Intensity



The total ion current (TIC) chromatogram represents the summed intensity across the entire range of masses being detected at every point in the analysis

In an **extracted-ion chromatogram (XIC** or EIC), also called a reconstructed-ion chromatogram (RIC), one or more m/z values representing one or more analytes of interest are extracted from the entire data set for a chromatographic run.

LC-MS Chromatographic Alignment

- To extract the peptide peak area, two basic parameters, m/z and retention time, must be determined.
- Typically, the m/z value is measured reproducibly in low resolution mass spectrometers such as the LTQ linear ion trap MS and extremely reproducibly in high resolution mass spectrometers such as LTQ-Orbitrap.
- Retention time of peptides can shift between experiments
- LC-MS maps can contain millions of peaks
- In label-free quantification, maps thus need to be aligned in order to find the corresponding peaks

What should be considered?

- **Fact 1:** A single chromatographic condition, e.g., one specific column with a specific mobile phase and gradient, will not be optimal for each of the thousands of peptides in a single injection of a complex sample.
  → Multiple runs.
- **Fact 2:** A peptide shared by different proteins → the intensity of this peptide should be divided by sharing frequency.
- **Fact 3:** Systematic bias → normalization and filtration of unquantifiable peptides

Label-free Methods: spectral counting or peak area

Pros:

- Simple workflow

  - No complicated (or expensive) labelling or tagging protocol

- Whole proteome analysis
- Comparison of multiple states (relative quantification)

Cons:

- Still can be expensive
  - Need plenty of replicates to get statistical power (machine time!)
- Reproducible sample prep, chromatography and MS performance is critical for this approach
- Not straightforward to validate results from big data sets
- Low abundance proteins hard to measure accurately

Applications of Label-free Quantitative methods

- Identifying expression profiles in different biological processes
- Diagnosing certain diseases and cancer biomarkers
- Monitoring changes in certain biological process proteomes
- Studying protein interaction networks

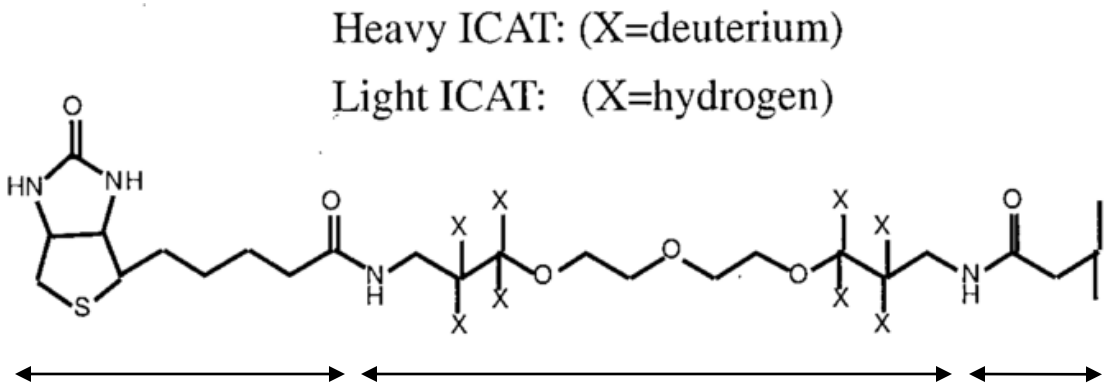
Stable-isotope Labeled Methods

- Provide a useful means of determining the relative expression level of individual proteins between samples with high precision (coefficients of variation less than 10%).

- Because two or more samples tagged with different numbers of stable isotopes can be mixed before any processing steps, sample-to-sample recovery differences are eliminated.

- Also allow post-translational modifications, splice variations and mutations (often unnoticed in immunoassays) to be detected and identified, increasing the clinical relevance of the assay and avoiding the issues of non-specific binding and cross-reactivity observed in immunoassays.

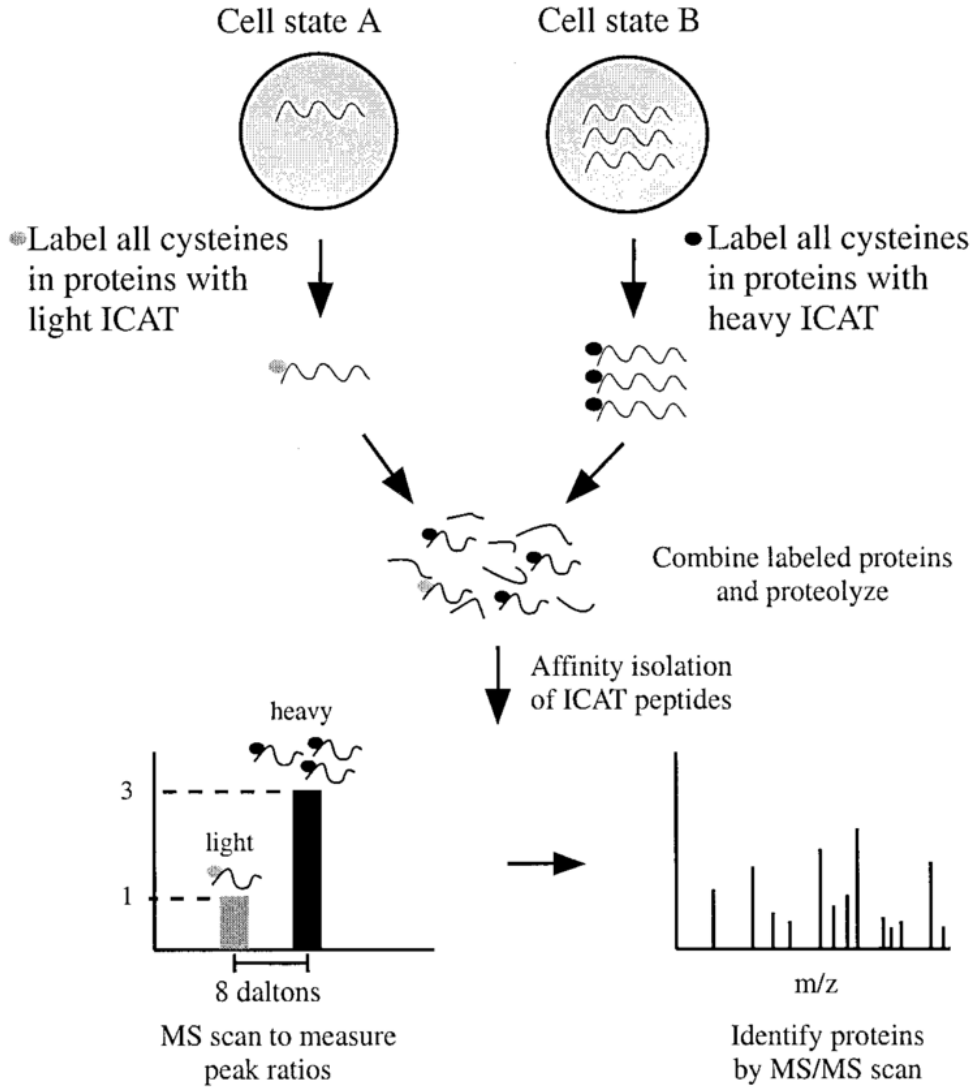
Labeling: ICAT (Isotope-Coded Affinity Tagging)



Biotin moiety, which permits isolation of the labeled molecules by affinity chromatography (interact with avidin immobilized)

Linker containing light (X= H) or heavy (X=D) isotopes

Iodoacetamide moiety, which binds to cysteine thiol group



Quantitate relative protein levels by measuring peak ratio

Identify peptide by sequence information

ICAT (Isotope-Coded Affinity Tagging)

Advantages

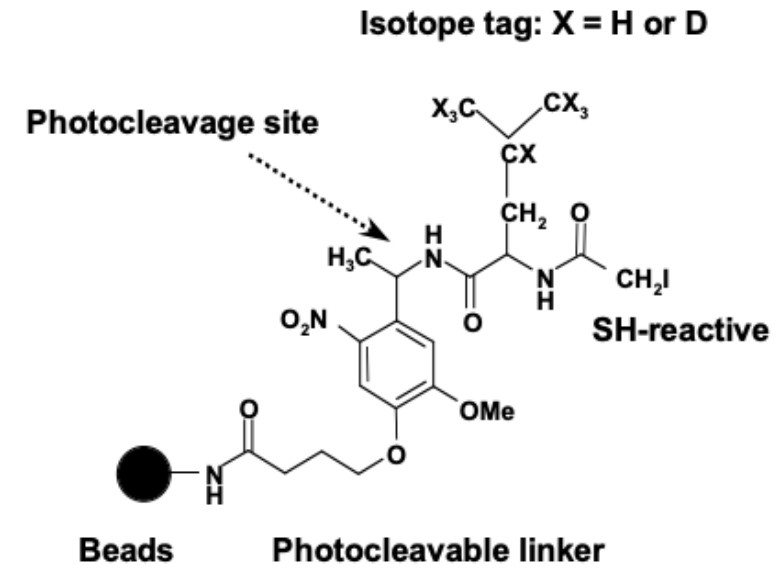
- High specificity
- High sensitivity
- Applicability to samples of different origin (cell, tissues, fluids)
- Effective labeling in the presence of guanidine, SDS, or urea

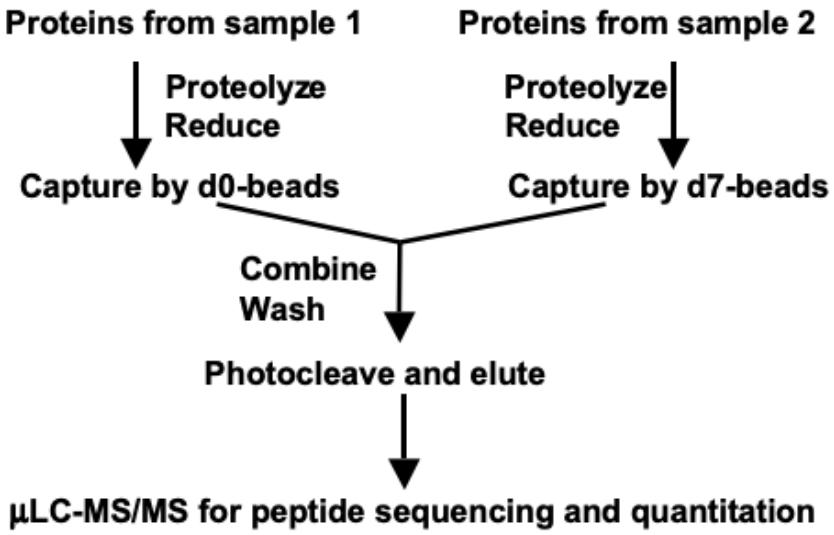
Disadvantages

- It doesn’t allow for quantification of proteins that do not contain Cys residues.

Solid-phase ICAT

- Faster and easier to conduct, because it doesn't require isolation of labeled peptides by chromatographic methods.
- Better, but it's expensive and light sensitive.

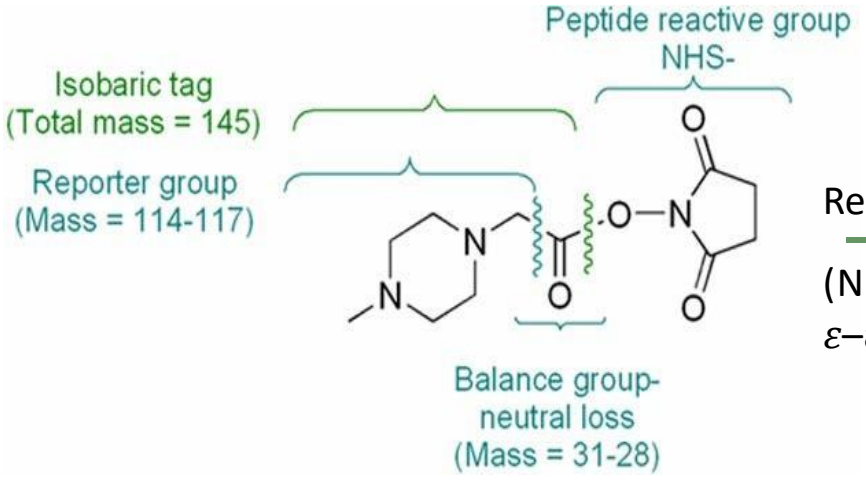




Zhou *et al.*, Nature Biotech. 2002, 19, 512

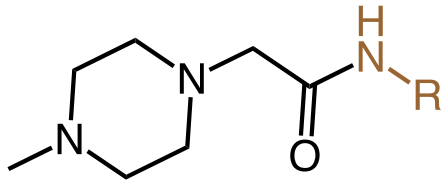
Labeling: iTRAQ (Isobaric Tags for Relative and Absolute Quantitation)

- iTRAQ permits simultaneous analysis of 2-8 samples.
  - TMT– 6-plex
  - iTRAQ-8 – 8-plex
- It is based on labeling of peptides with isobaric tags but produce different fragment ions during fragmentation.

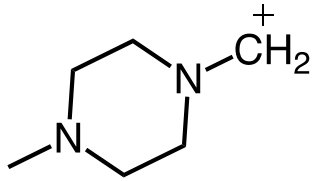


React with NH_2 groups

(N termini and
ε–amino groups of Lys)



MS/MS
fragmentation



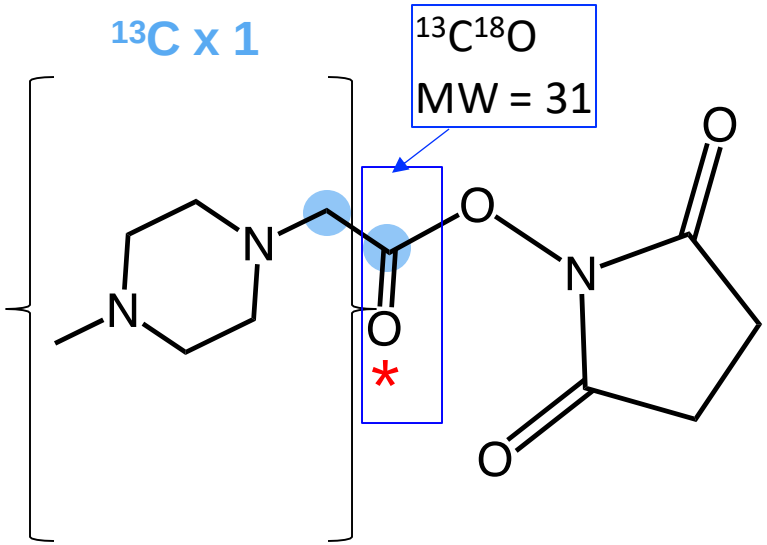
Reporter ion

+

Rest of molecule

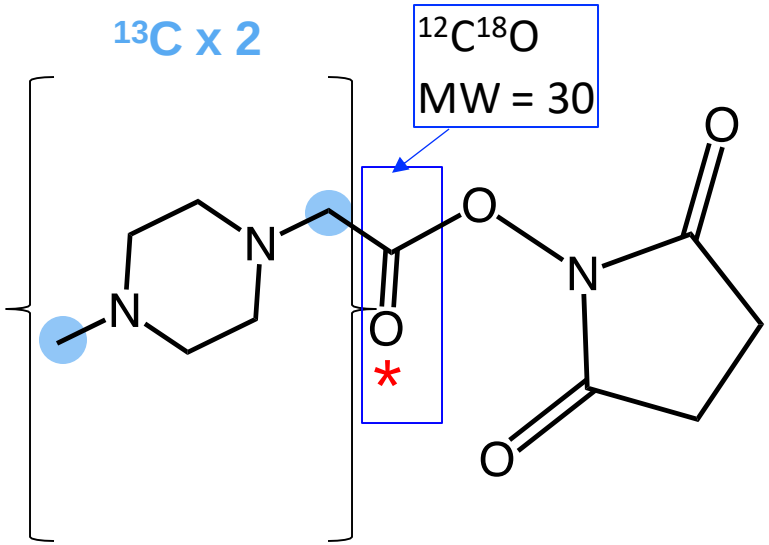
iTRAQ Reagents

^{13}C x 1



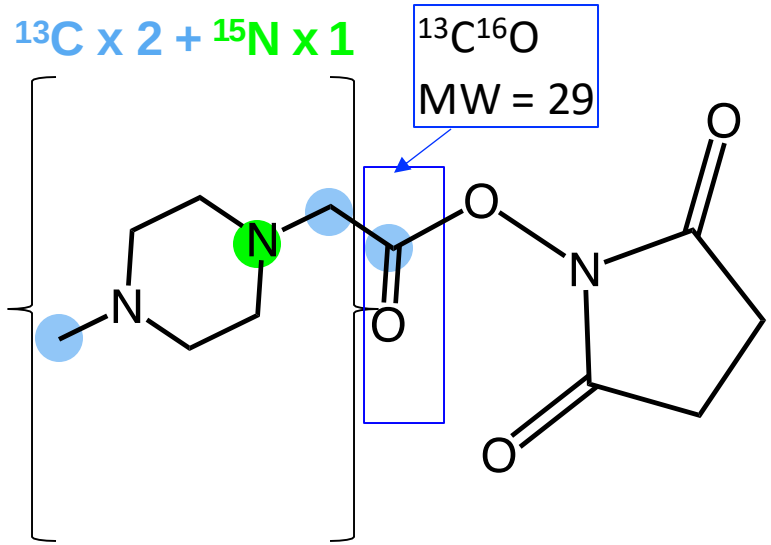
Produces an ion of MW = 114
after fragmentation

^{13}C x 2



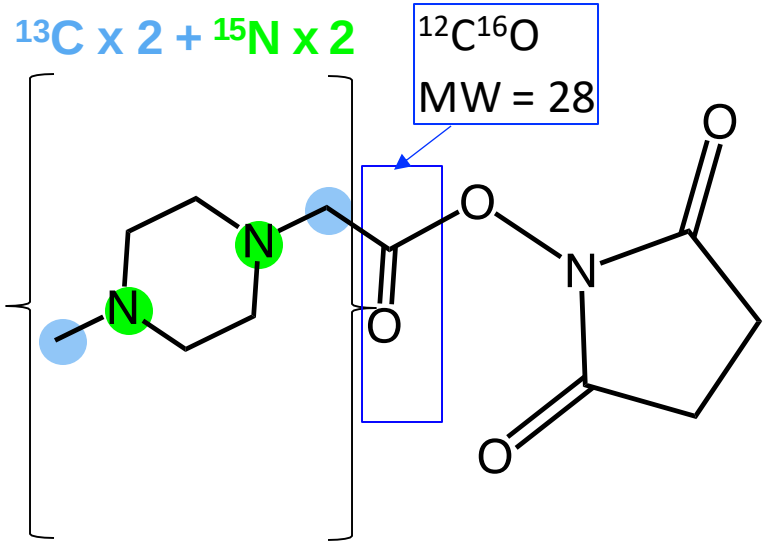
Produces an ion of MW = 115
after fragmentation

^{13}C x 2 + ^{15}N x 1



Produces an ion of MW = 116
after fragmentation

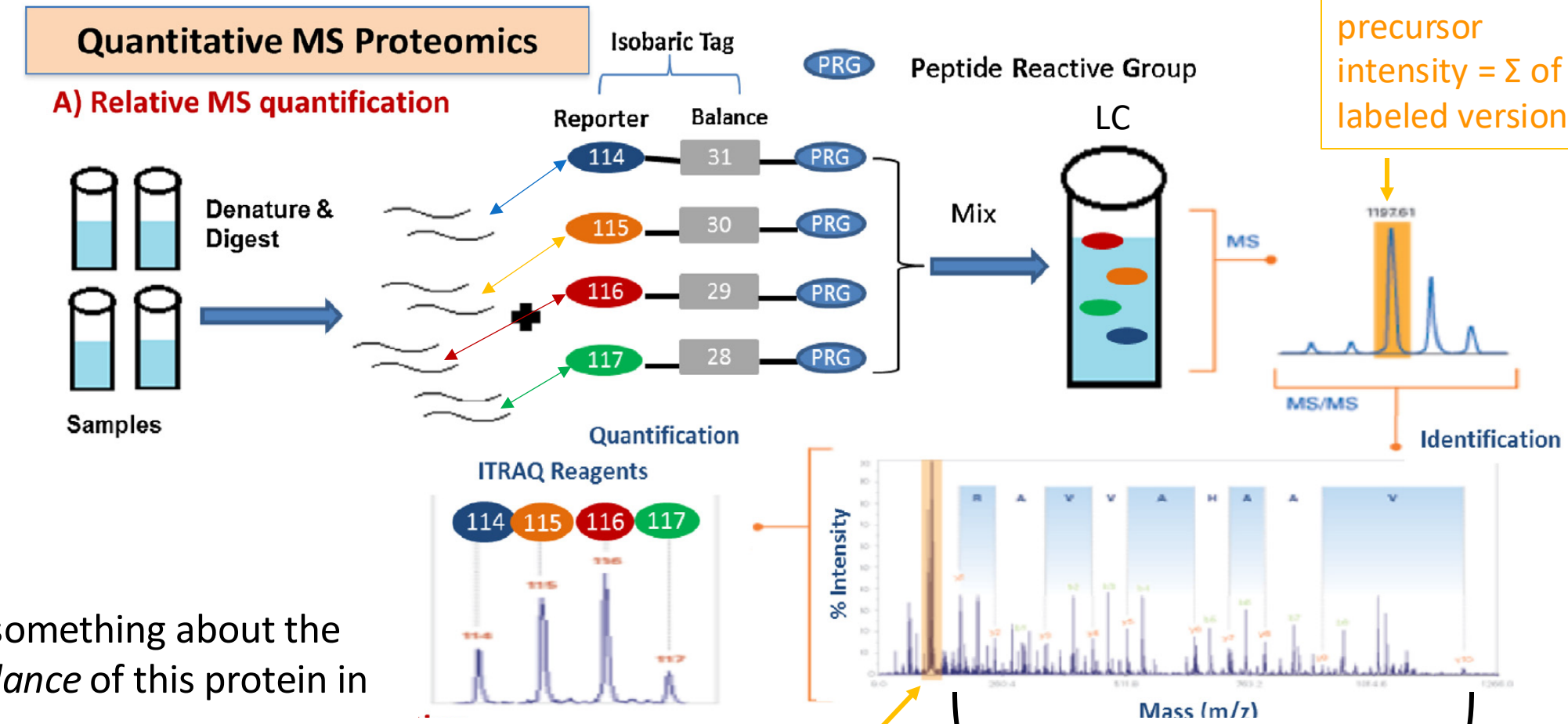
^{13}C x 2 + ^{15}N x 2



Produces an ion of MW = 117
after fragmentation

General Workflow with iTRAQ Method

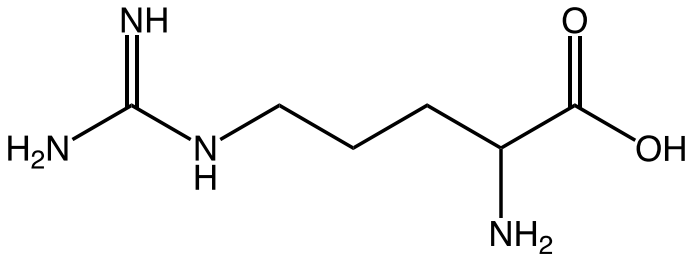
same peptide from 4 different samples: Observed precursor intensity = Σ of all labeled versions



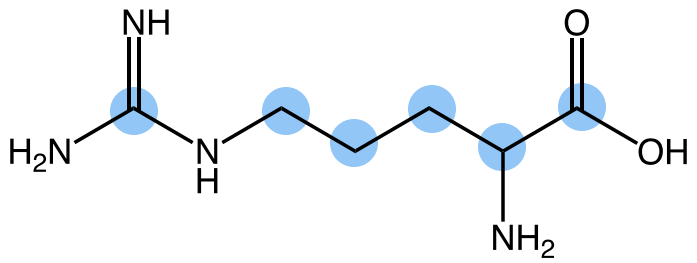
- **Ratio** tells us something about the *relative abundance* of this protein in the 4 samples

Labeling: SILAC (Stable Isotope Labeling with Amino Acids in Cell Culture)

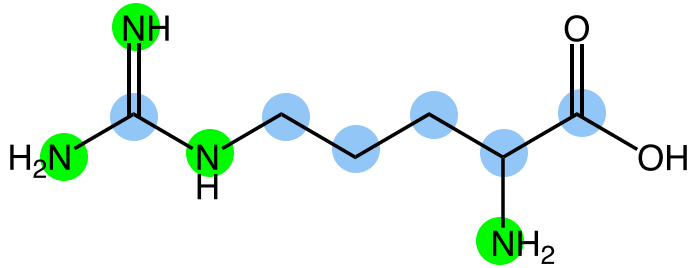
^{13}C ^{15}N



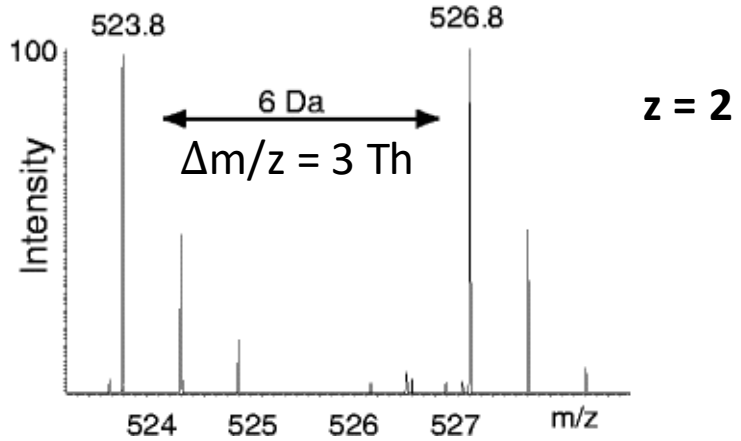
Arg_{+0} (Light)



Arg_{+6} (Medium)

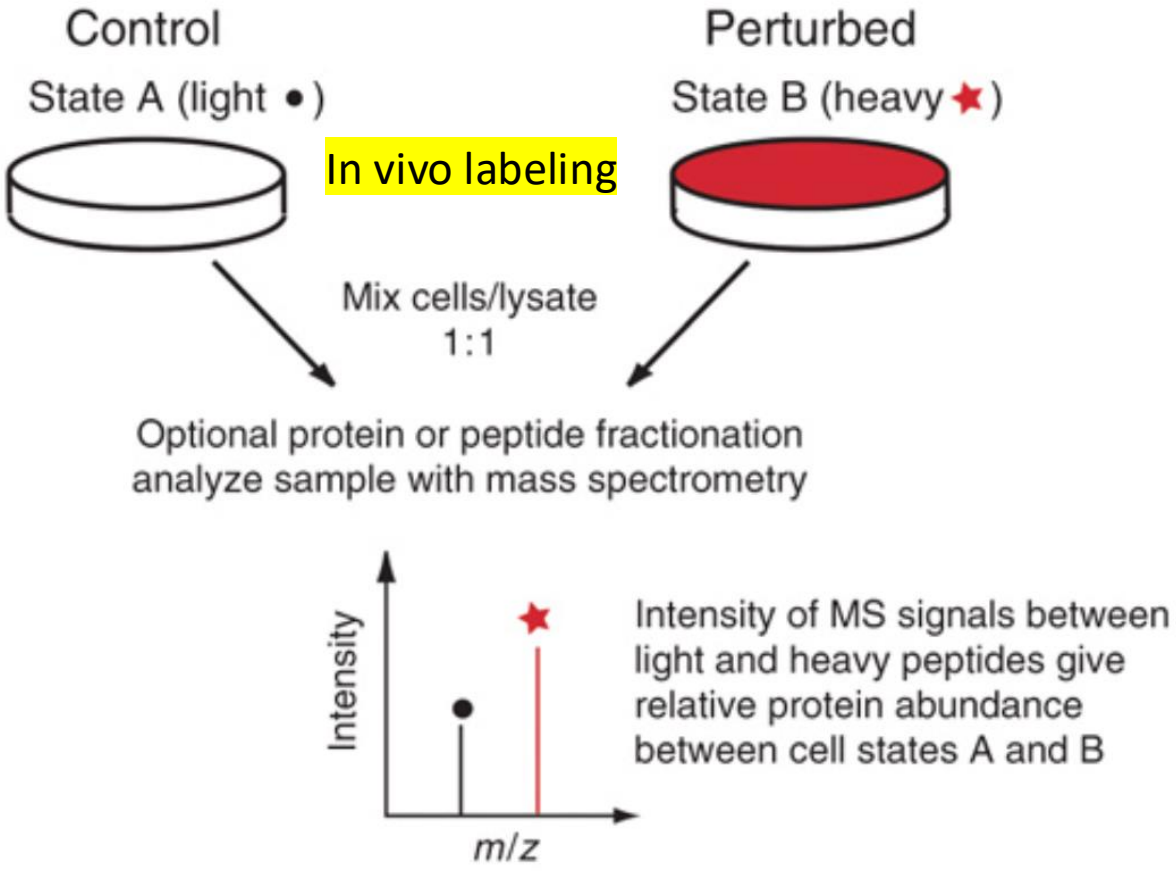


Arg_{+10} (Heavy)



z = 2

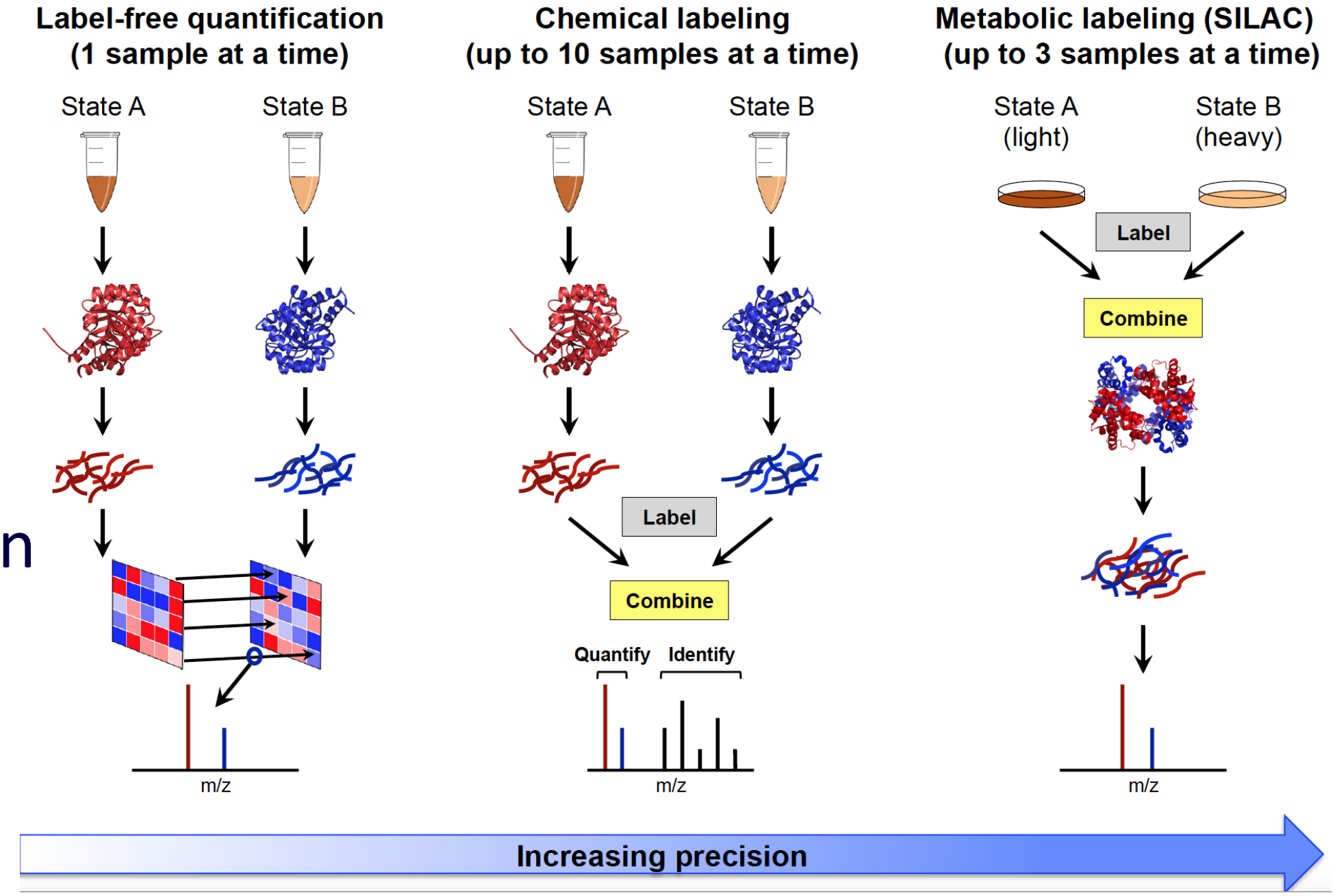
SILAC (Stable Isotope Labeling with Amino Acids in Cell Culture)



Drawbacks:

- Limited plex level (3 max)
- The method does not allow for the analysis of proteins directly from tissue.
- The stable-isotope enriched media are costly and may themselves affect cellular growth and protein production.
- The increase in nominal mass because of stable-isotope incorporation is not known until the sequence is determined.

Summary of MS-based Quantification



Label versus Label-free

Label (cons and pros)

- Stable isotopes are expensive and not suitable for clinical samples

- But… less experimental variation if samples are mixed
- Improved quantitative precision and accuracy
- Improved confidence in peak identification

Label-free (cons and pros)

- Need lots of replicates to get statistical power
- So lots of time on MS instrument – therefore also can be expensive
- Fairly new technique therefore not enough high-quality published studies showing best practice

- No labelling needed
- No limit on the number of samples
- Applicable to any kind of samples

Challenges in LC-MS Platform

- Highly reproducible LC-MS analysis (retention time shift, fluctuations in MS signal intensity, peptide identification in separated MS/MS)
- Complex samples (overlapping signals, misaligned peptides)
- Large sample size (column degradation)

Definition of Absolute vs Relative Protein Quantification

Relative quantification

- Relative comparison of the same protein between samples
- ≥2 samples
- Output: protein ratio

Absolute quantification

- Comparison of the same protein between samples and different proteins within the same sample
- ≥1 sample
- Output: protein concentration (copies/cell, fmol/µg extract, ng/mL body fluid)

The multitude of quantitative MS-application

- Which type of quantitative mass spectrometric approach is most suited for my project?

  - What type of MS platform do I have access to?
  - How precise and accurate do my quantitative results have to be?
  - What type of sample am I working with?
  - How large is my project (number of samples)
  - Budget (costs and time)
  - Do I need relative or absolute quantitative data?

Application: Protein Biomarker Development

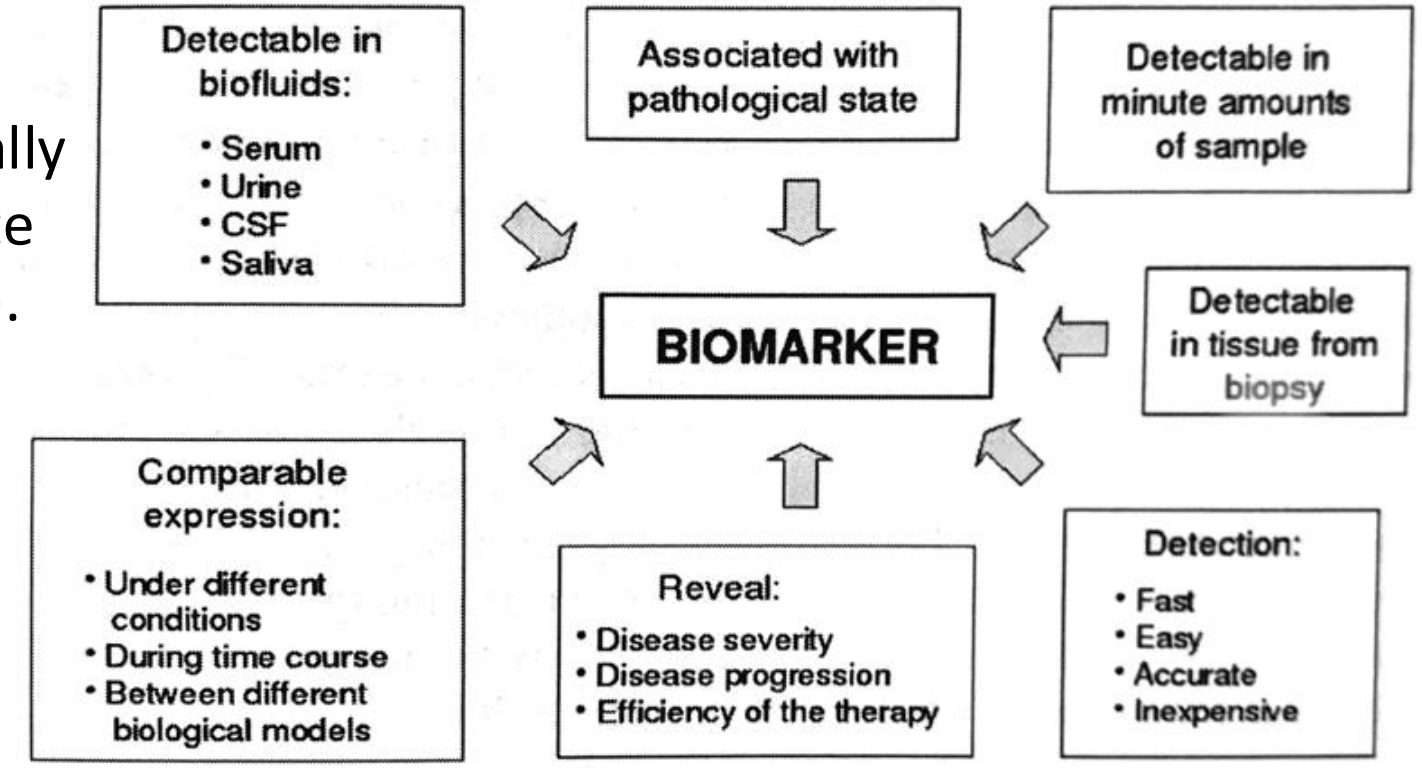
- Biomarker: molecular signature representing a state of a living organ/cell
  - Its expression is associated specially with a particular pathological state (e.g., level affected, modification).

-- Diagnostics

-- Prognostics

-- Target identification

-- Monitoring drug efficacy



nature protocols

REVIEW ARTICLE

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Check for updates

Tutorial: best practices and considerations for mass-spectrometry-based protein biomarker discovery and validation

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