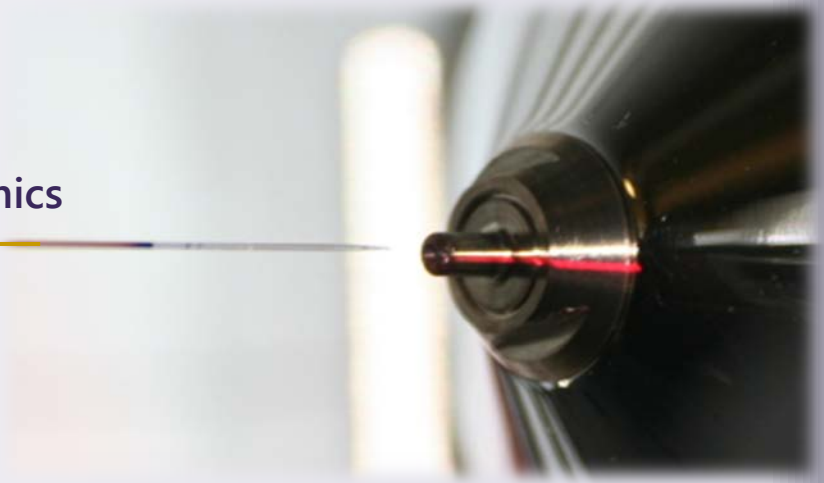
UWPR

Advancing Proteomics



UNIVERSITY *of* WASHINGTON
Proteomics Resource

Welcome to the Proteomics Workshop!

Course overview

Monday
10 am to noon: Proteomics Introduction (room C123B)
1 pm to 3 pm: Lab work in groups of 3-4 (B59)

Tuesday
10 am to noon: Data Analysis Introduction (room C123B)
1 pm to 3 pm: Lab work in groups of 3-4 (B59)

Wednesday
10 am to noon: Data analysis; please bring your personal notebook/laptop (room 110)
Pizza Lunch provided by ThermoFisher Scientific Thanks to Kevin Wheeler!!
1 pm to 3 pm: Instructors will be available to answer any questions
(room C123B and B59)

Introduction

- UWPR overview
- Proteomics overview
- Mass spec based proteomics
- Some examples

UWPR Overview

UWPR (University of Washington Proteomics Resource)

Governed by a committee of 11 UW PI's

Staff

- Priska von Haller: UWPR lab management
- Jimmy Eng: Bioinformatics management
- Tahmina 'Eva' Jahan: software developer
- Vagisha Sharma and Mike Riffle part time software developers

Resources

- Seven LC-MS systems
- Computing cluster
- Various software tools

Funding

- Costcenter
- State to support Proteomics Research at the UW

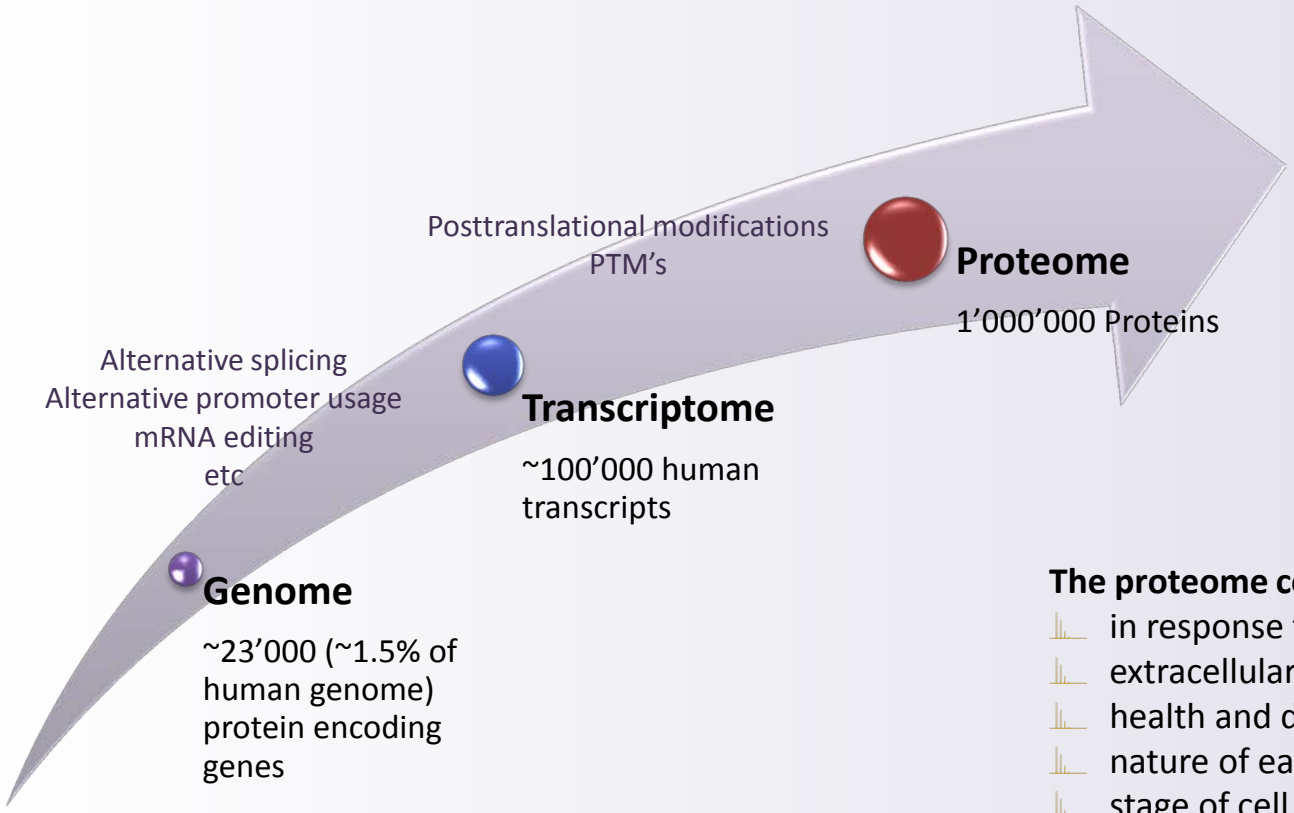
What is Proteomics?

"In the wonderland of complete sequences, there is much that genomics cannot do, and so the future belongs to **proteomics**:

The analysis of complete complements of proteins.
Proteomics includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and, ultimately, their function."

-Stan Fields in *Science*, 2001.

The Proteomics Challenge



The Challenge:

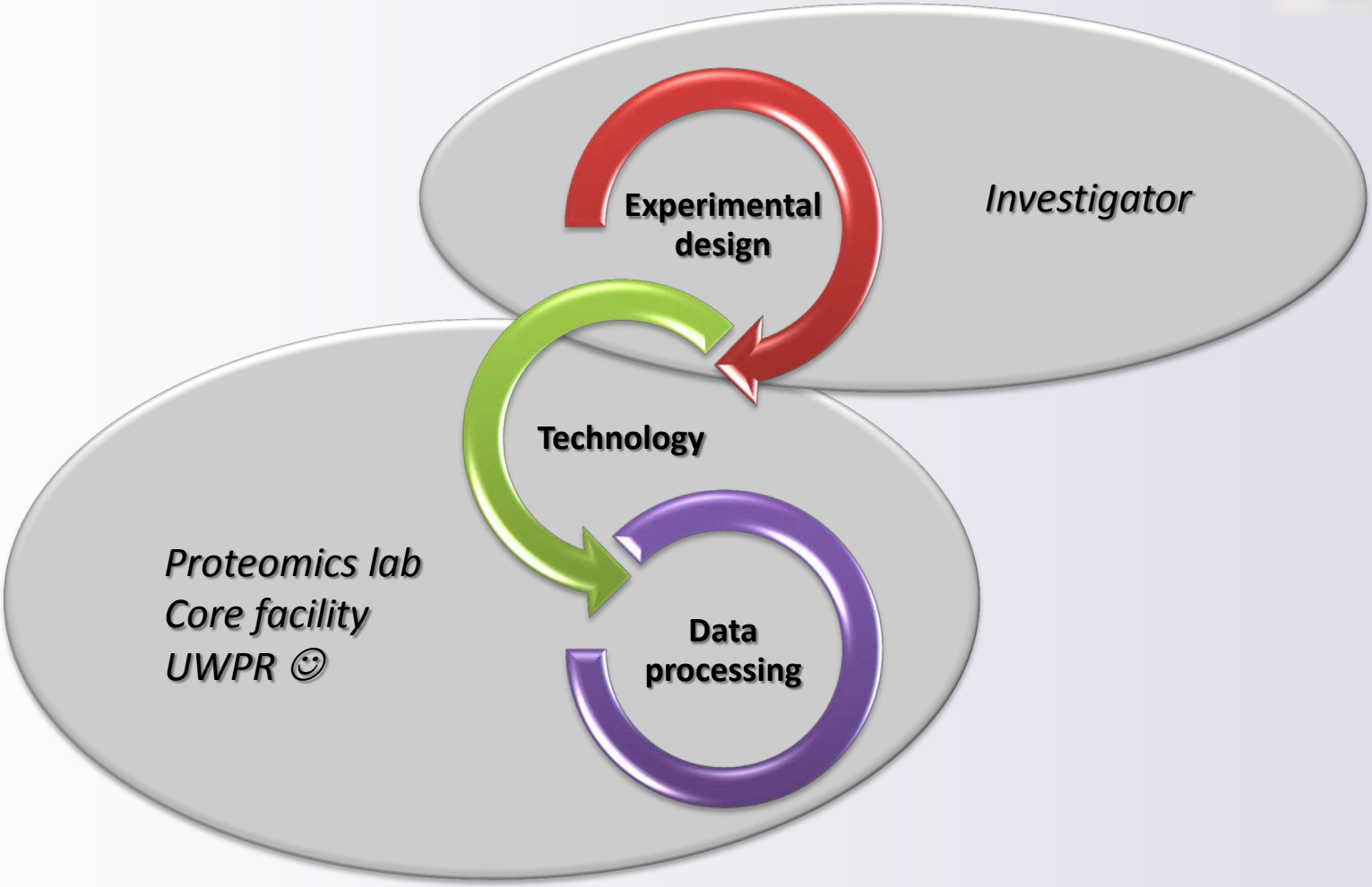
- Abundance (range >10^9)
- Modifications
- Structure
- Complexes
- Localization
- Turnover
- No Amplification

The proteome constantly changes

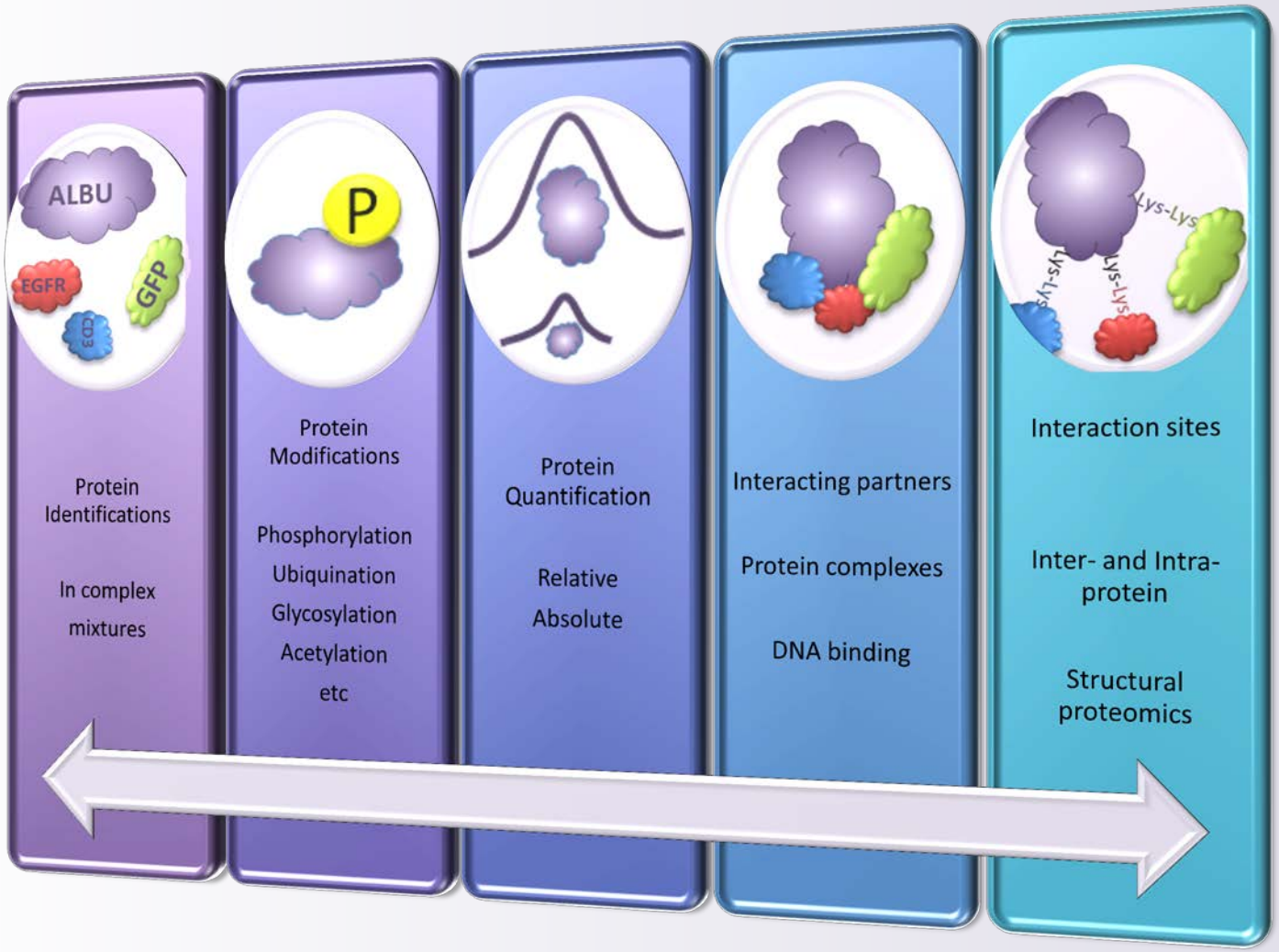
- in response to intra- and extracellular signals,
- health and disease,
- nature of each tissue,
- stage of cell development
- effects of drug treatment
- …….

As such, the proteome often is defined as
"the proteins present in one sample (tissue, organism, cell culture) at a certain point in time."

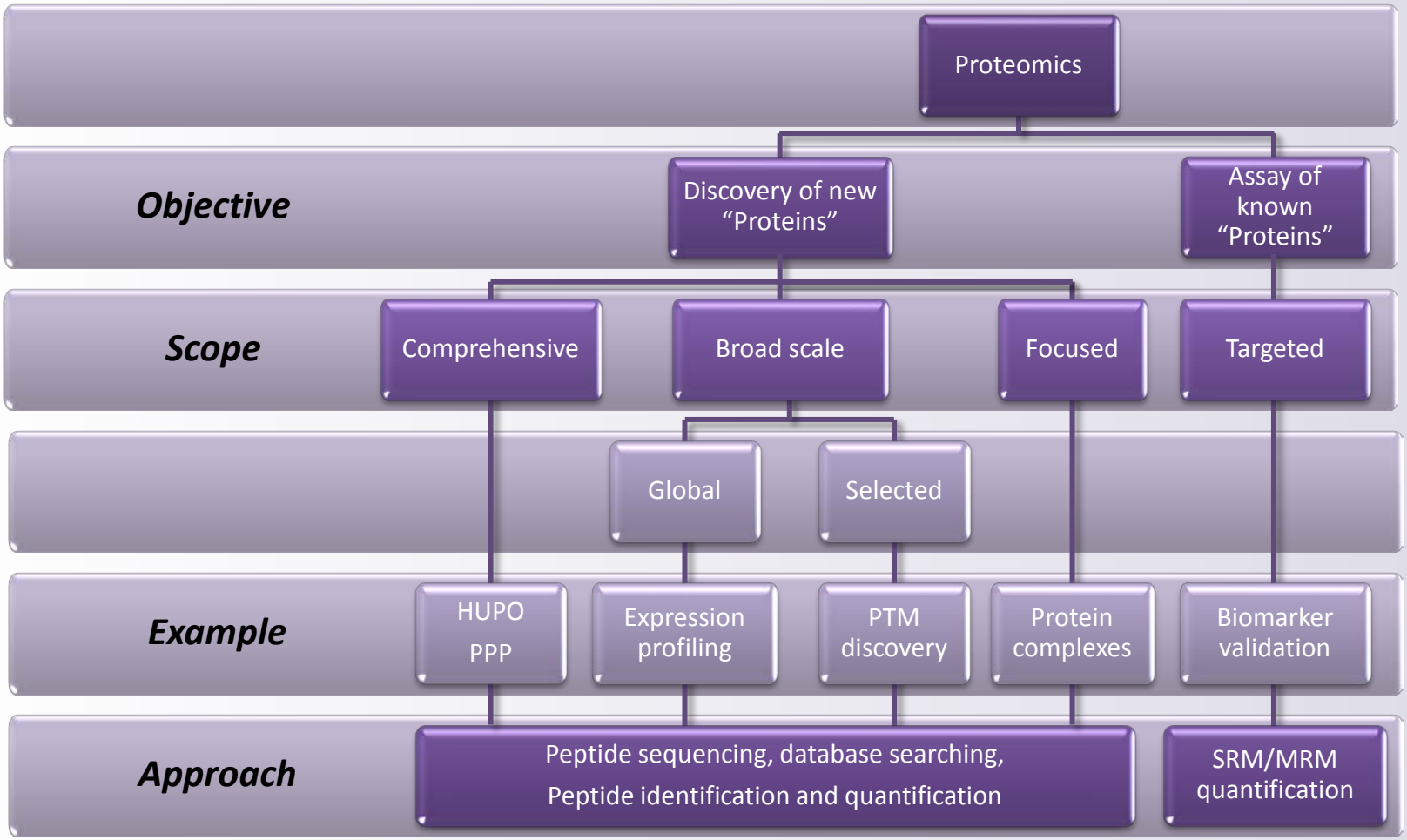
The Three Tiers of Proteomics



What kind of answers can we get?

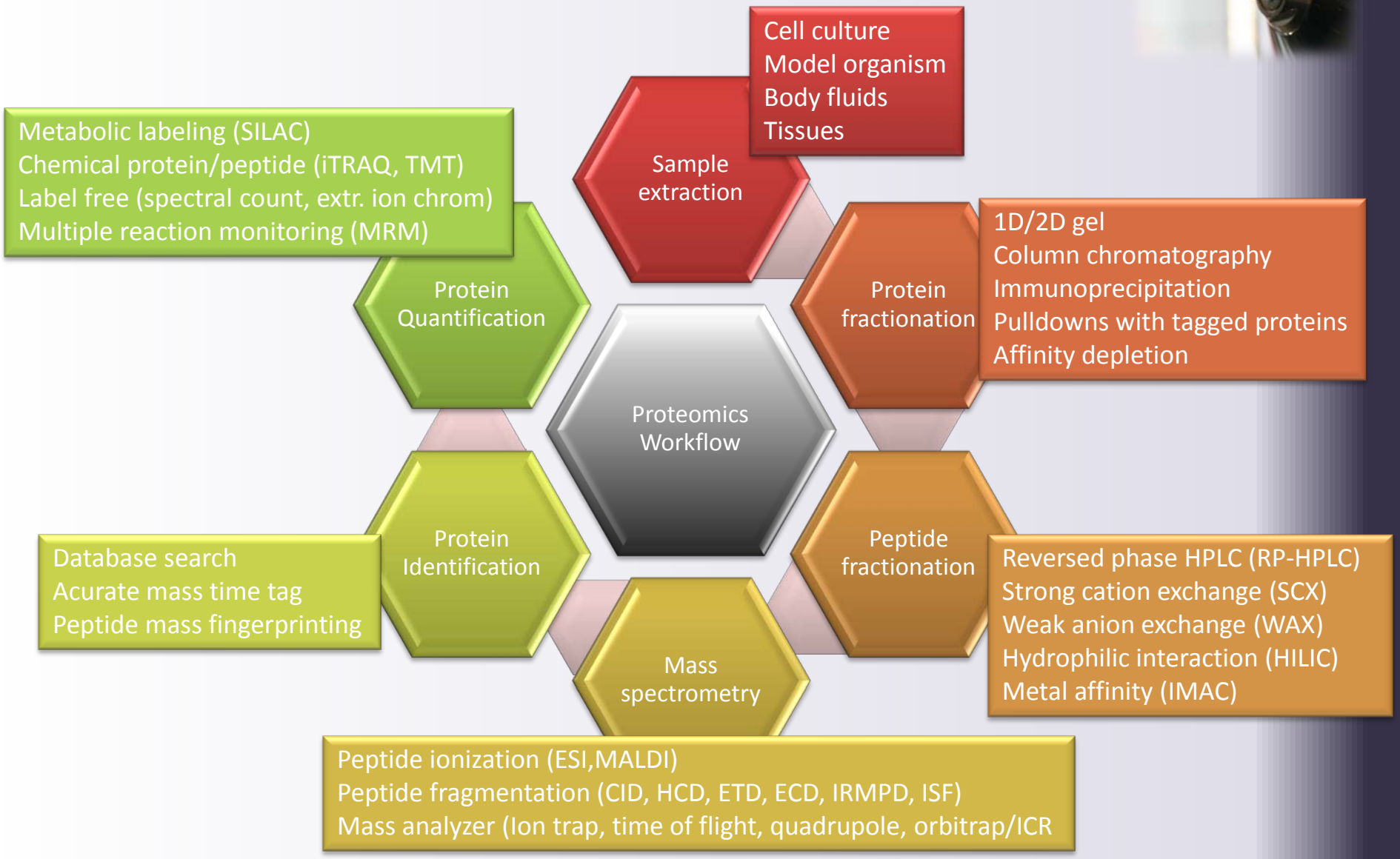


Conceptual proteomics experiments

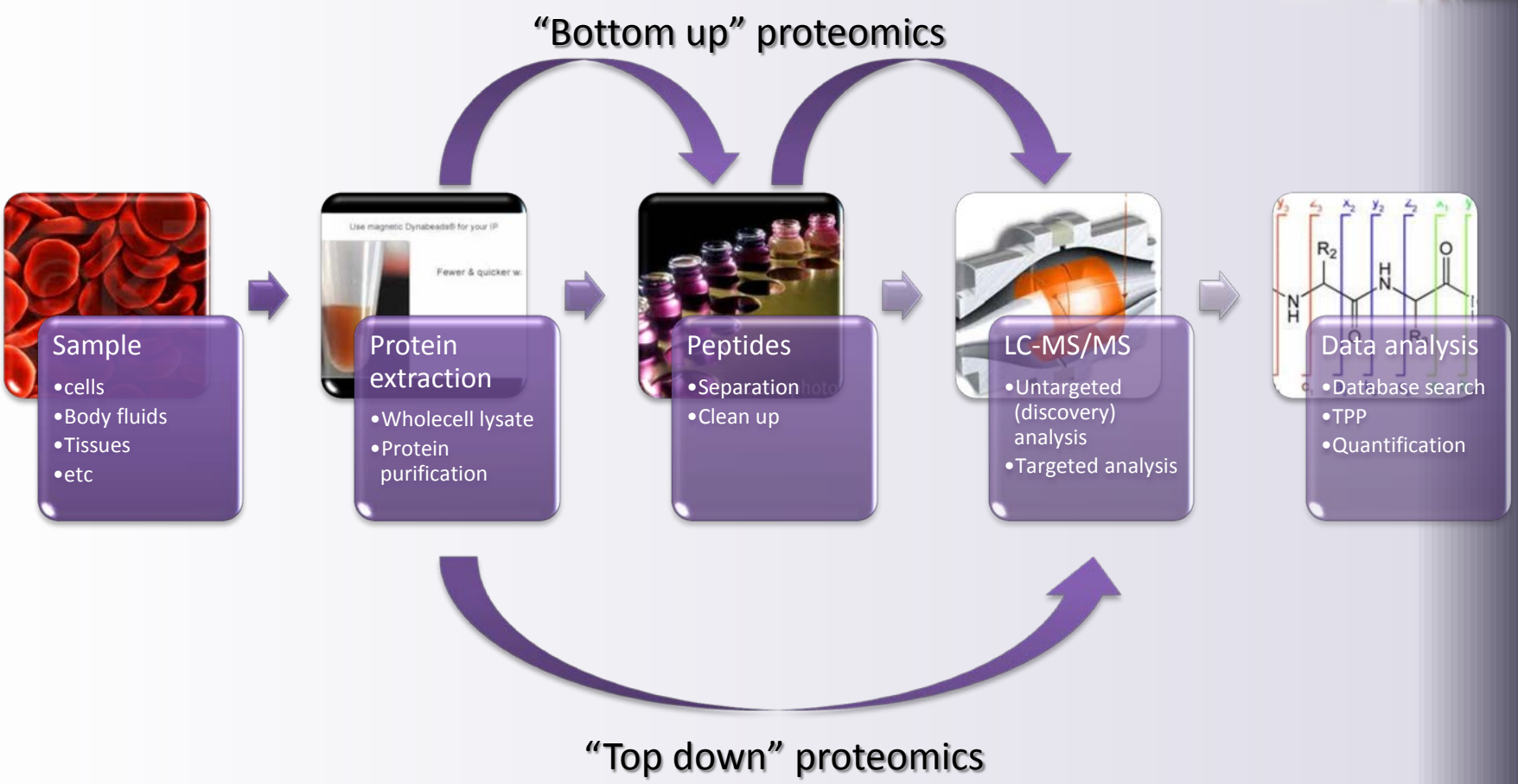


P. Mallick and B. Kuster, Nature biotechnology 28 (7), 695 (2010).

Proteomics Workflow

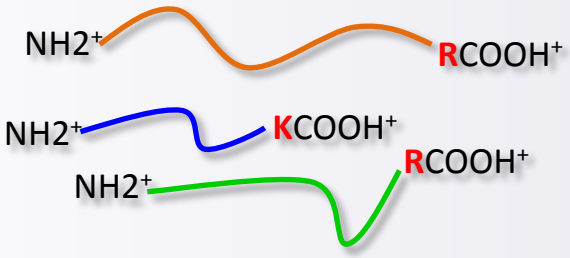


Bottom up Proteomics Workflow



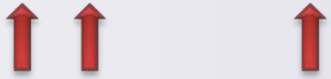
Proteins to Peptides

Enzymatic digest



Detectable size range
~ 8 – 25 amino acid or m/z <2000

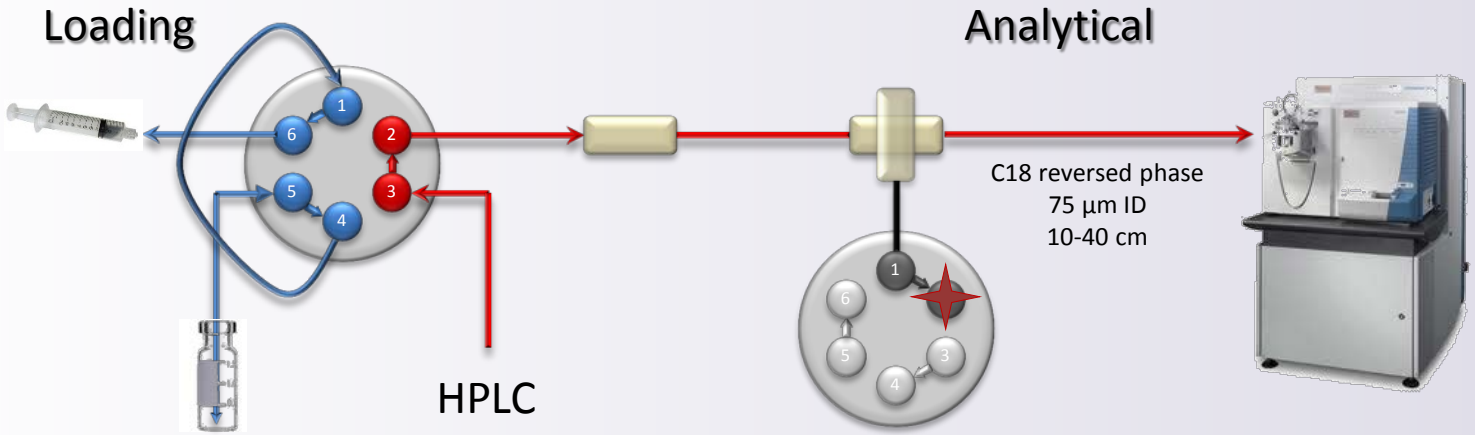
…..R.AR.ESAMPLER.SPEPTIDE…..



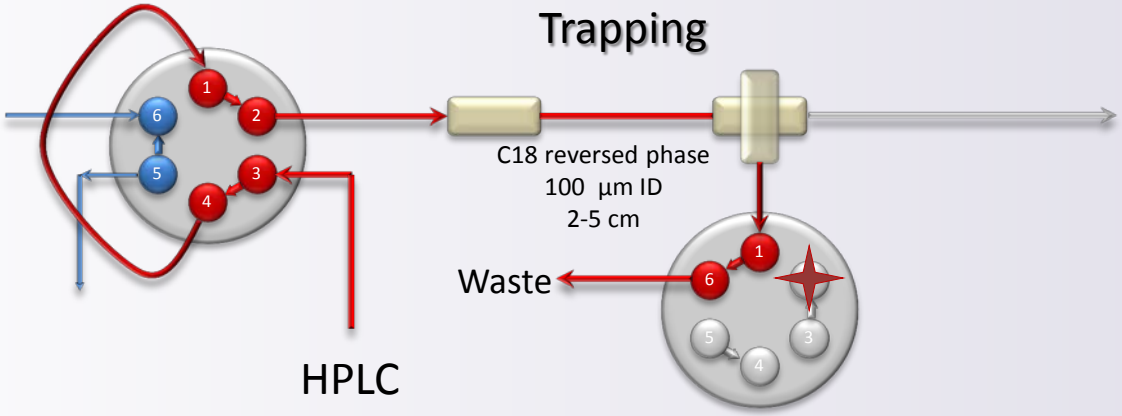
Trypsin cleaves C-terminal of Lys and Arg

Less common enzymes
ArgC
LysC
Pepsin
etc

The LC in LC-MS

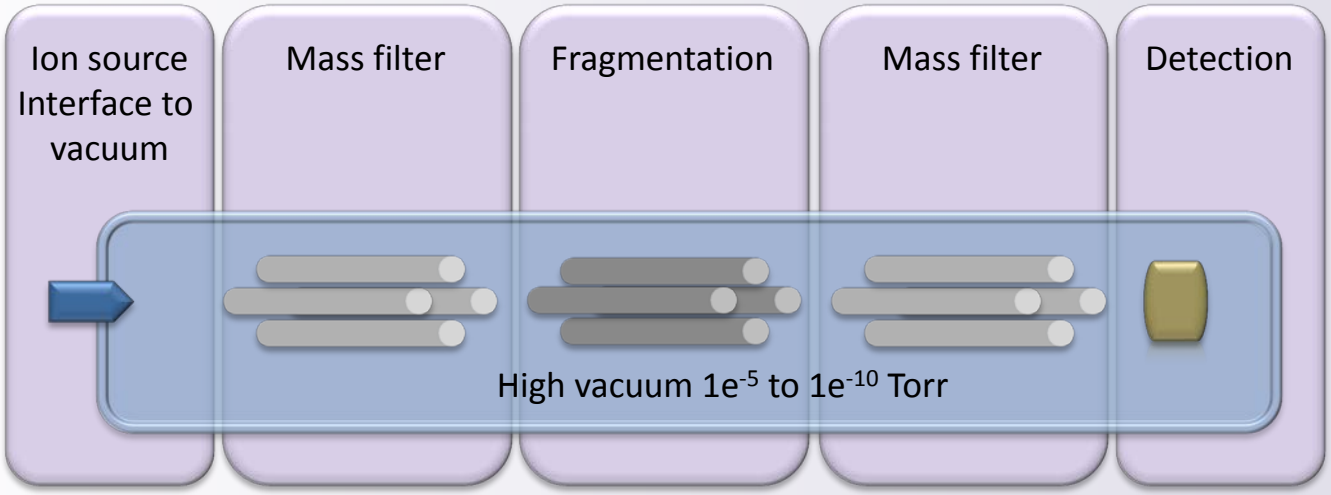


1-4 µg of peptide (1-5µl)
per LC-MSMS run

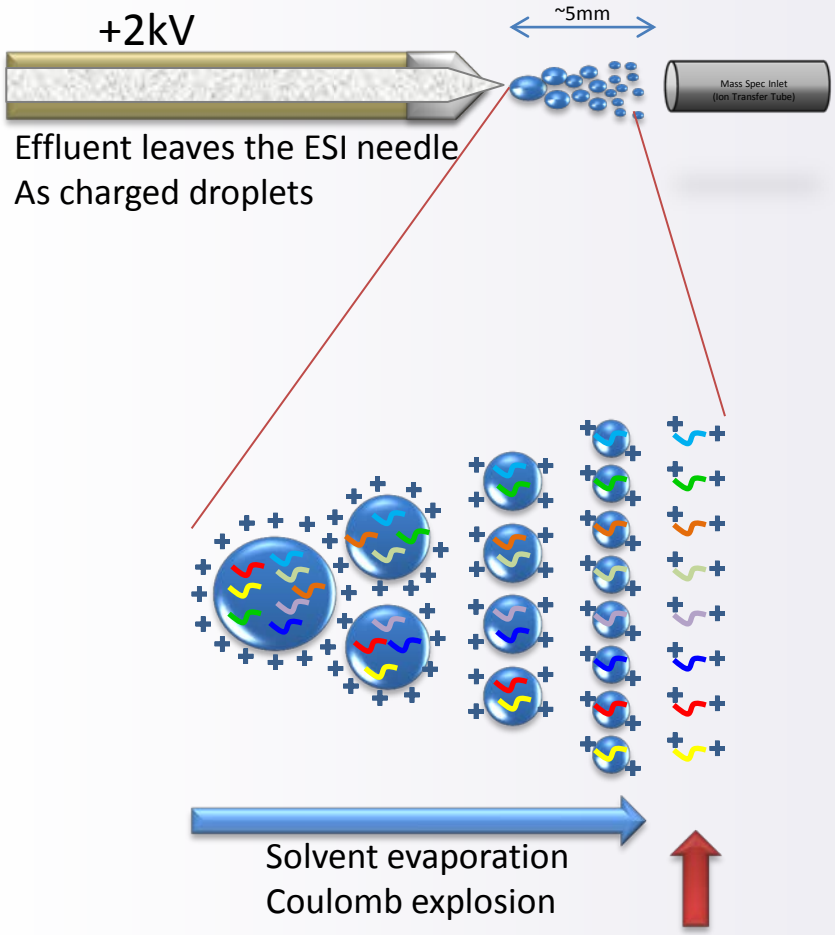


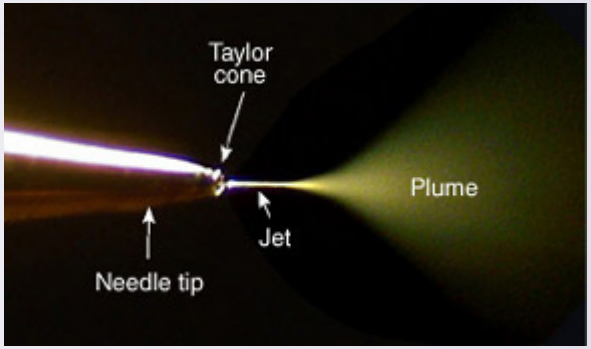
The MS in LC-MS

Basically a scale
measuring the mass of a charged molecule,
to be precise the mass to charge ratio or m/z



Electrospray Ionization (ESI)





www.newobjective.com

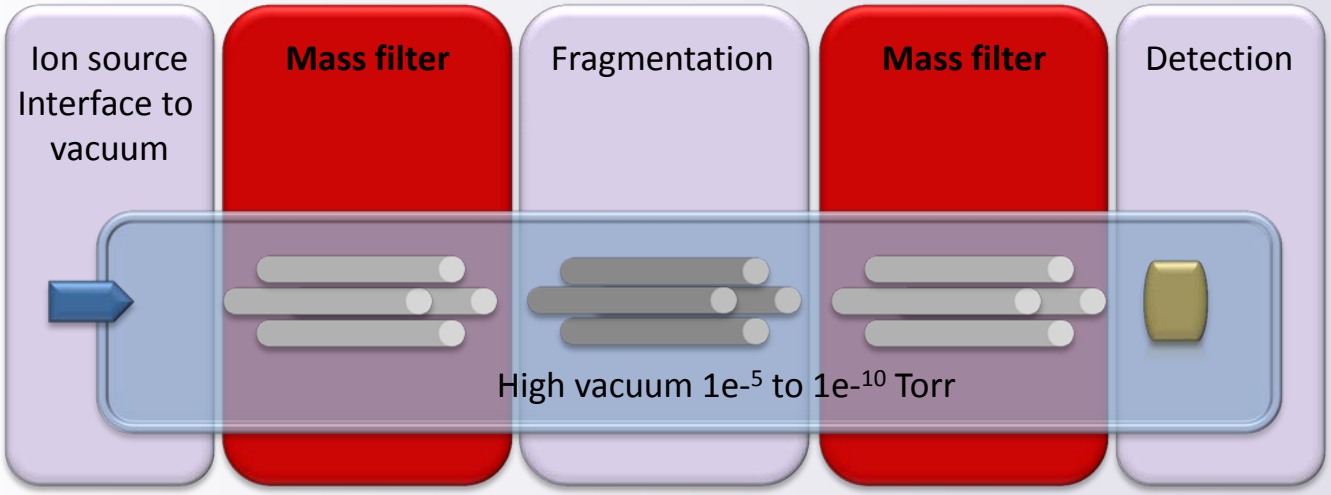
Mass Spec Inlet
(Ion Transfer Tube)

Other ionization techniques include:

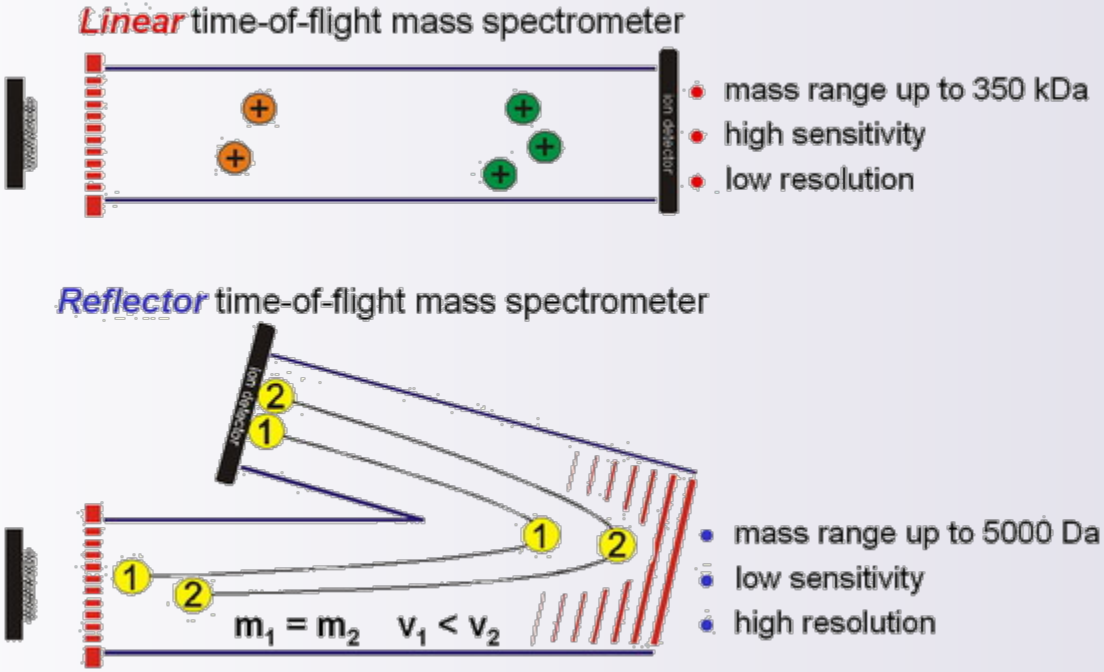
- MALDI (matrix assisted laser desorption ionization)
- SAWN (surface acoustic wave nebulization)
- CI (chemical ionization)
- DESI (desorption ESI)

Mass Filters and Analyzers

Basically a scale
measuring the mass of a charged molecule,
to be precise the mass to charge ratio or m/z



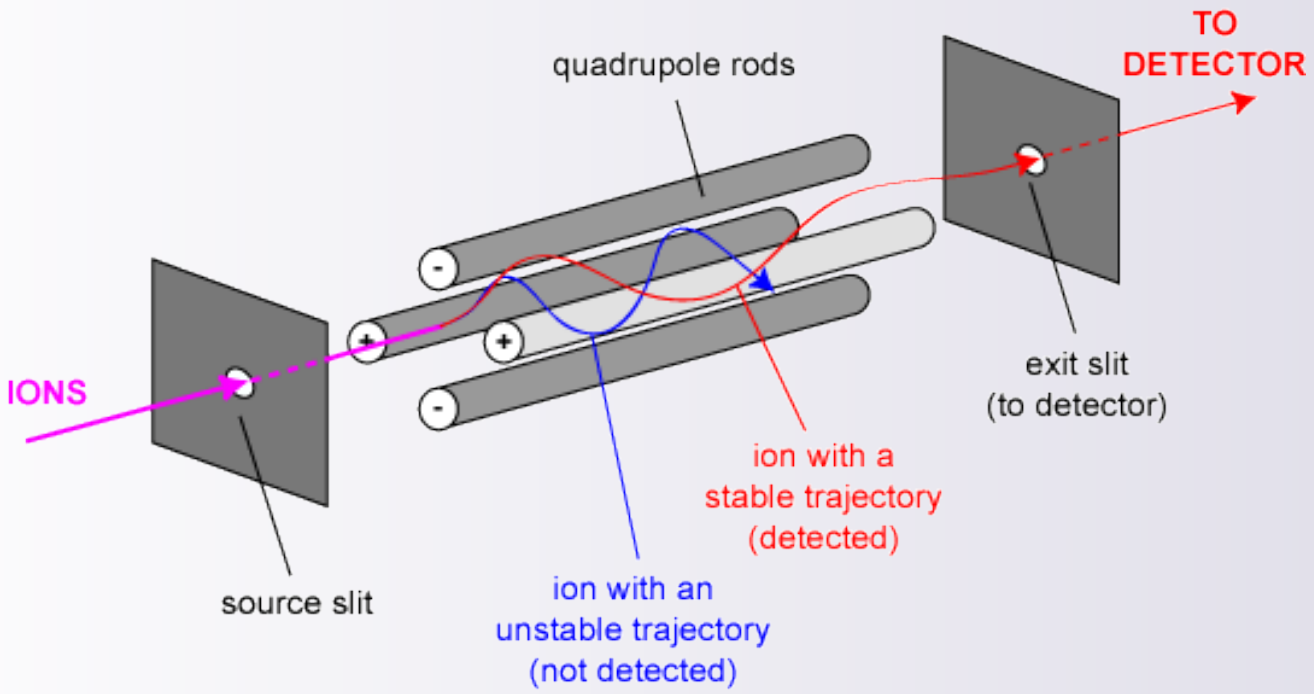
TOF (time of flight)



Mass range: 5000 Da
Resolution: Up to ~10000
Accuracy: 0.1-0.01%

http://www.anagnostec.eu/maldi-tof-ms/technology.html

Quadrupole

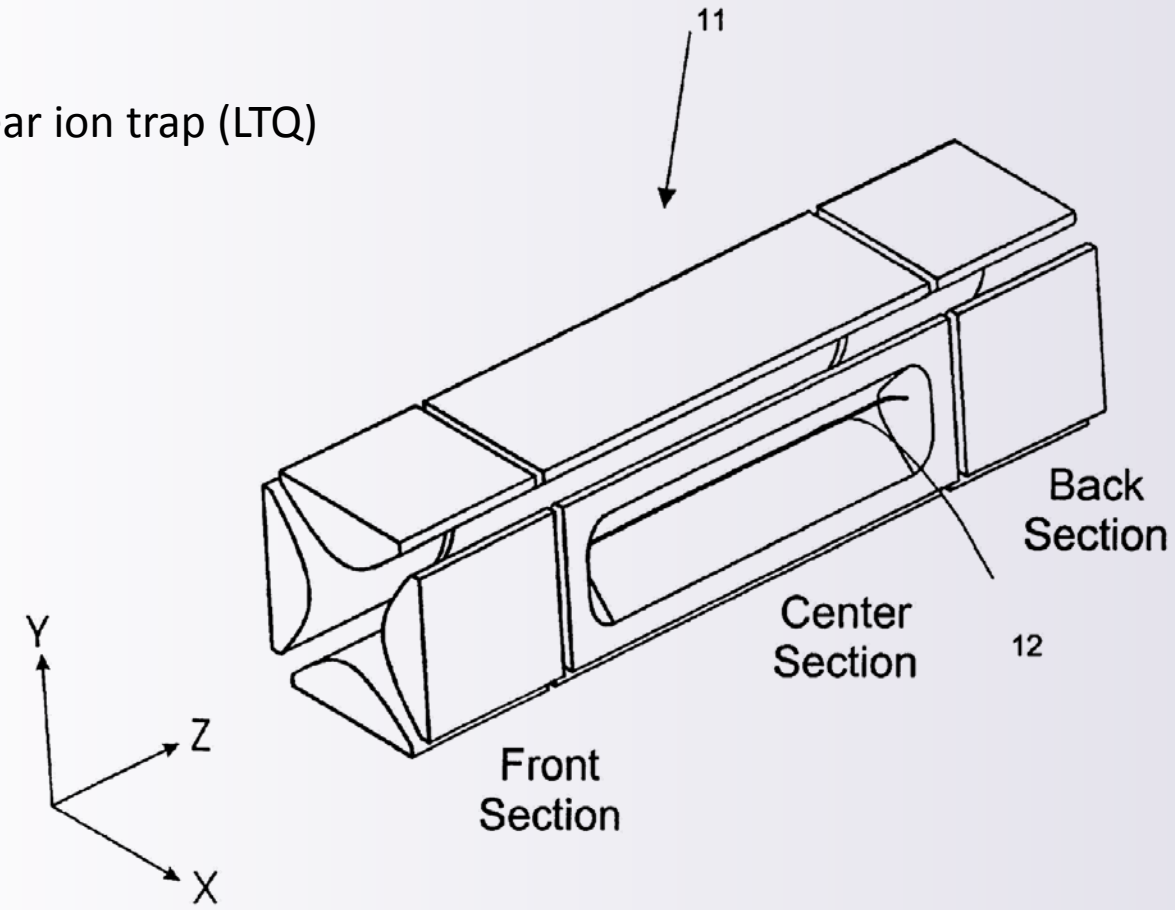


Mass range: <3000 m/z
Resolution: Up to ~2000
Accuracy: 0.1%

http://www.chm.bris.ac.uk/ms/theory/quad-massspec.html

Ion trap

Linear ion trap (LTQ)

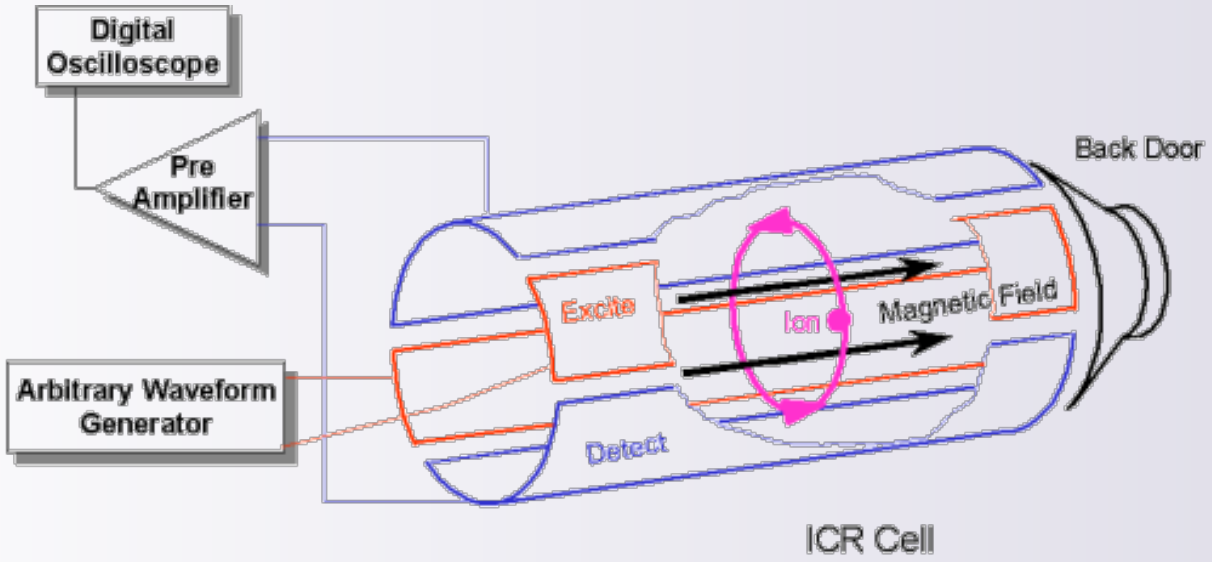


Mass range: typical <2000, extended <4000 m/z
Resolution: Up to ~10000
Accuracy: 0.1-0.01%

http://www.freepatentsonline.com/6797950.html

FT-ICR

FT-ICR (Fourier Transform Ion Cyclotron Resonance)

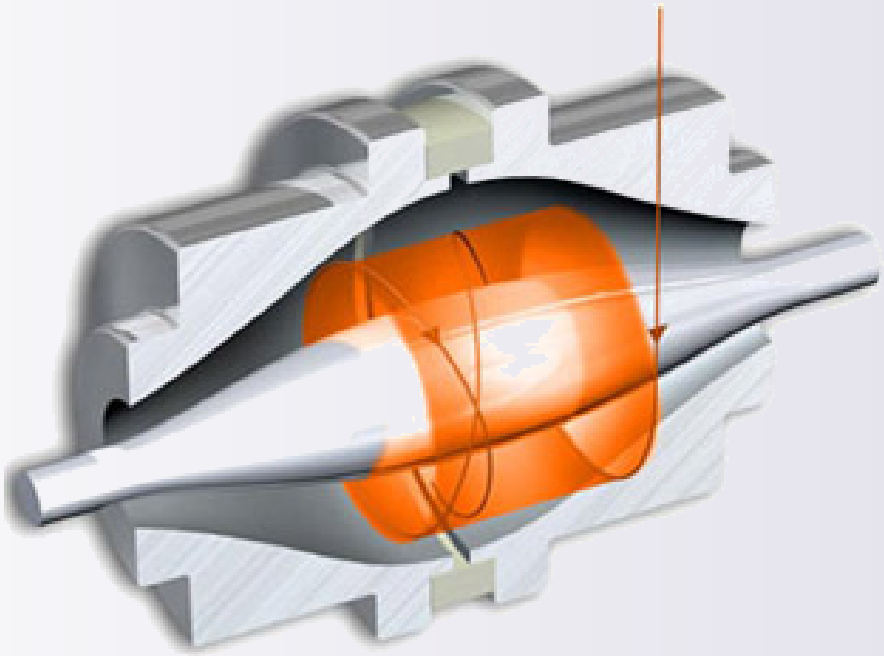


Mass range: typical <2000, extended <4000 m/z
Resolution: Up to ~1'000'000
Accuracy: <5ppm

http://www.photon.t.u-tokyo.ac.jp/~maruyama/fticr/ft-icr.html

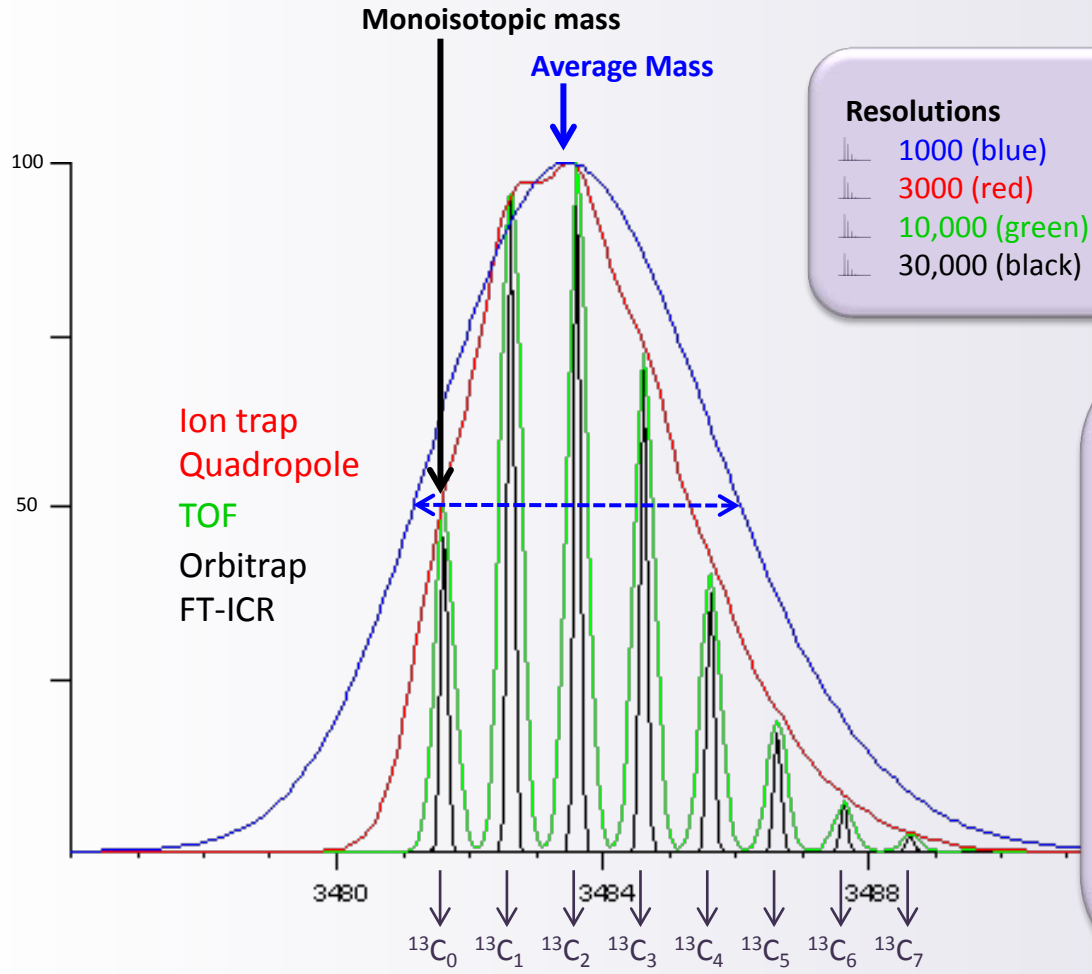
Orbitrap

Orbitrap the new FT mass analyzer



Mass range: typical <2000, extended <6000 m/z
Resolution: Up to ~240’000
Accuracy: <2ppm

Resolution and Mass Accuracy



Resolutions

- 1000 (blue)
- 3000 (red)
- 10,000 (green)
- 30,000 (black)

Mass / peak width at 50% = Resolution
3484 / 5 = 697
3484 / 3.5 = 995
3483.5 / 0.5 = 6'967
3483.5 / 0.15 = 23'223

Mass accuracy

True mass 1000.0000
Measured mass 1000.0020
Δmass = 0.0020

Error = (0.002 / 1000) x 10^6 = 2 ppm

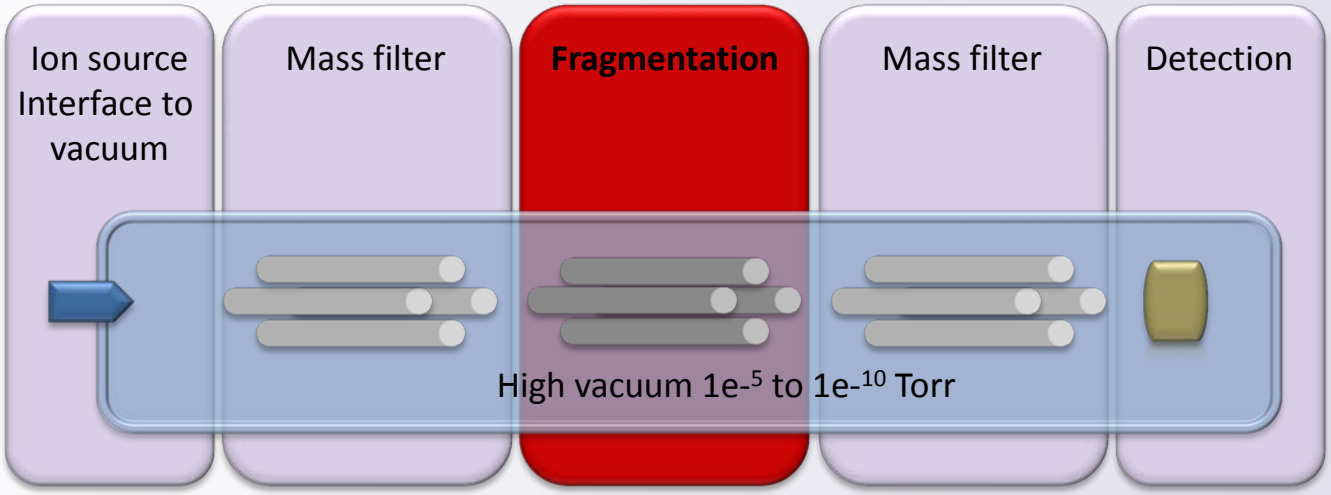
At m/z 1000.0000

3 → 3 ppm
2 → 20 ppm
1 → 100 ppm

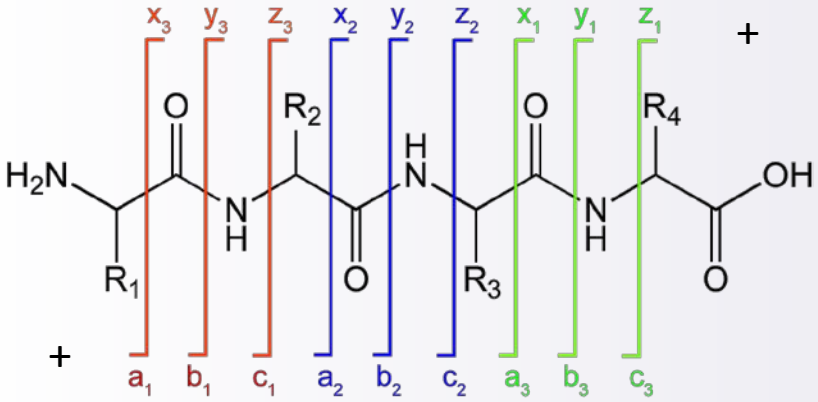
http://mascot.proteomix.org/help/mass_accuracy_help.html

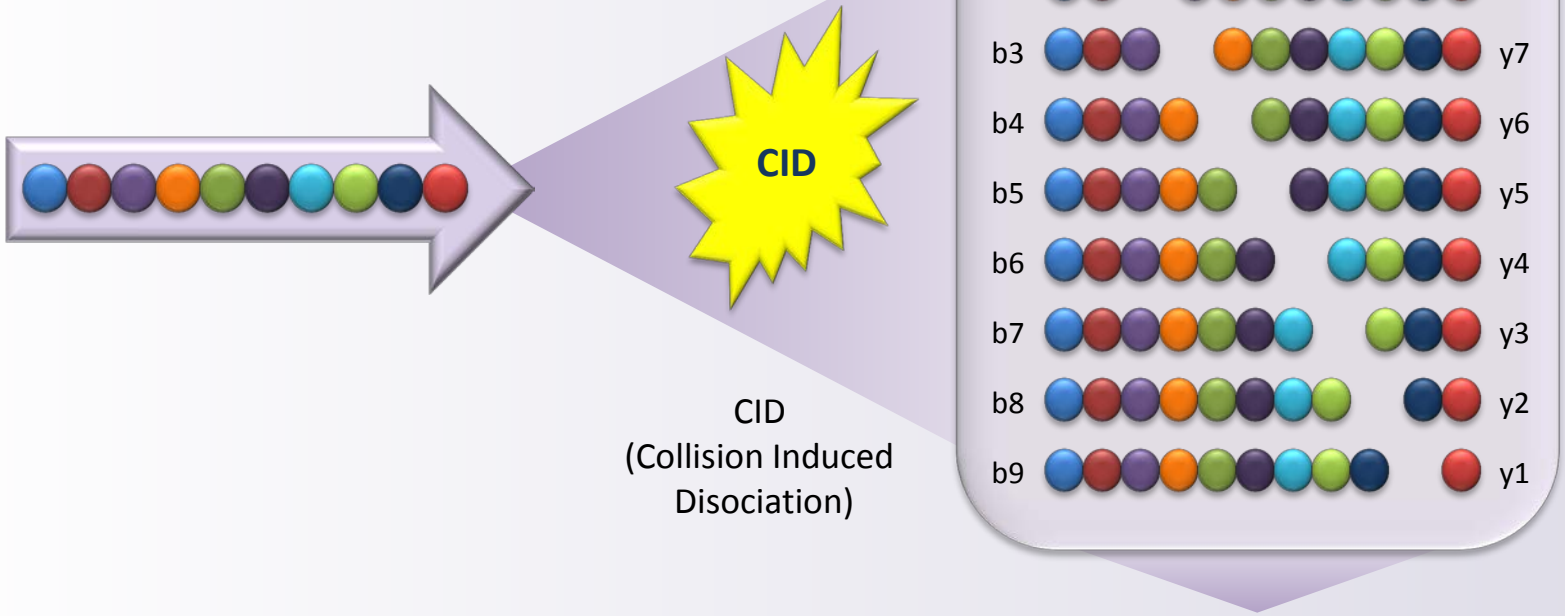
Fragmentation

Basically a scale
measuring the mass of a charged molecule,
to be precise the mass to charge ratio or m/z



Peptide Fragmentation

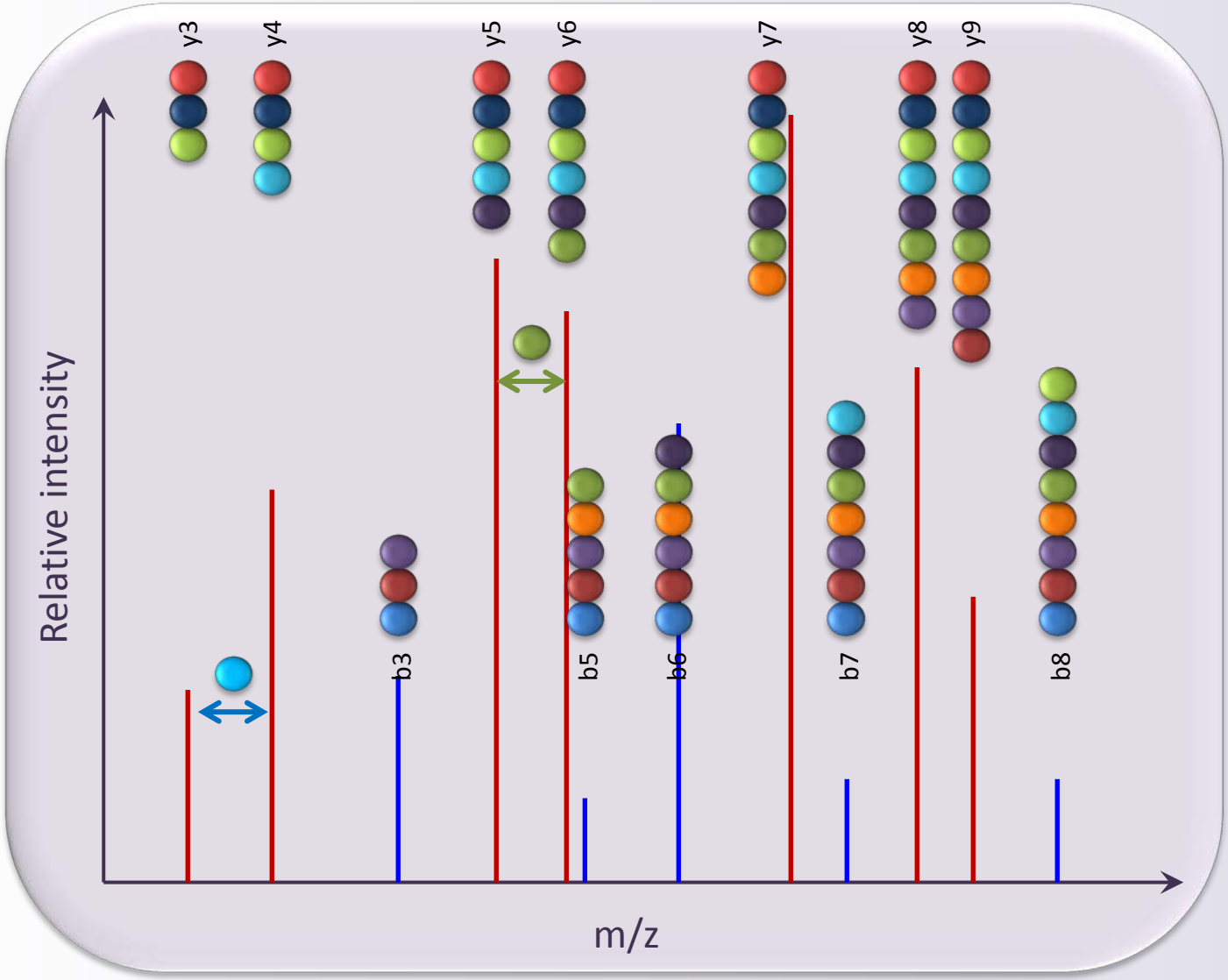




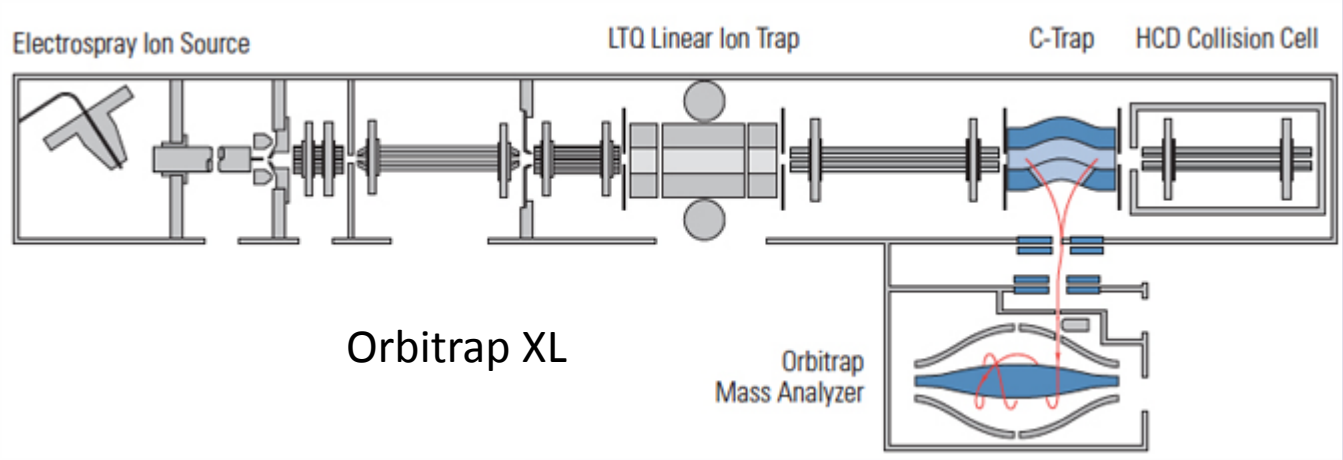
CID
(Collision Induced Disociation)

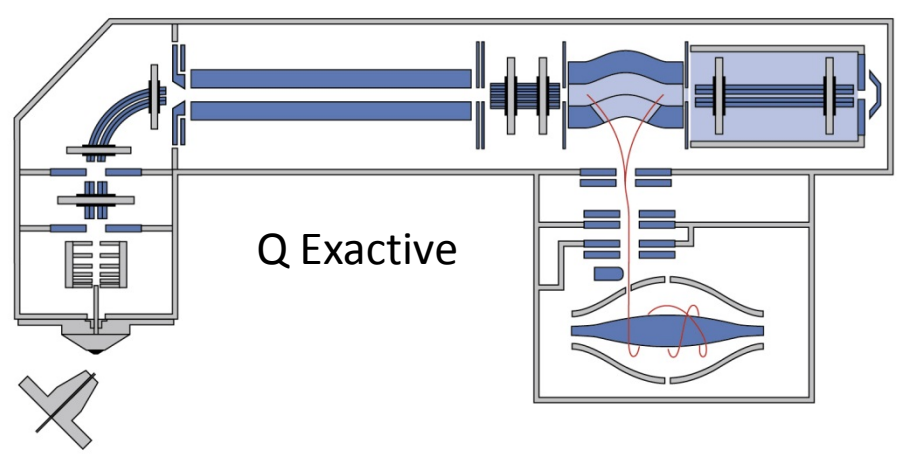
Peptide Identification

CID spectra



Schematic of a mass spec





http://planetorbitrap.com/

How does a mass spec work?

How about a short movie ….

Time for a break!!

Sample prep

Depends on the sample origin and the experiment
Highly variable and constantly changing

Divide and conquer
Extract and separate proteins
Digest proteins
Separate and clean up peptides

Clean up:
No (low) detergent & low/no salt

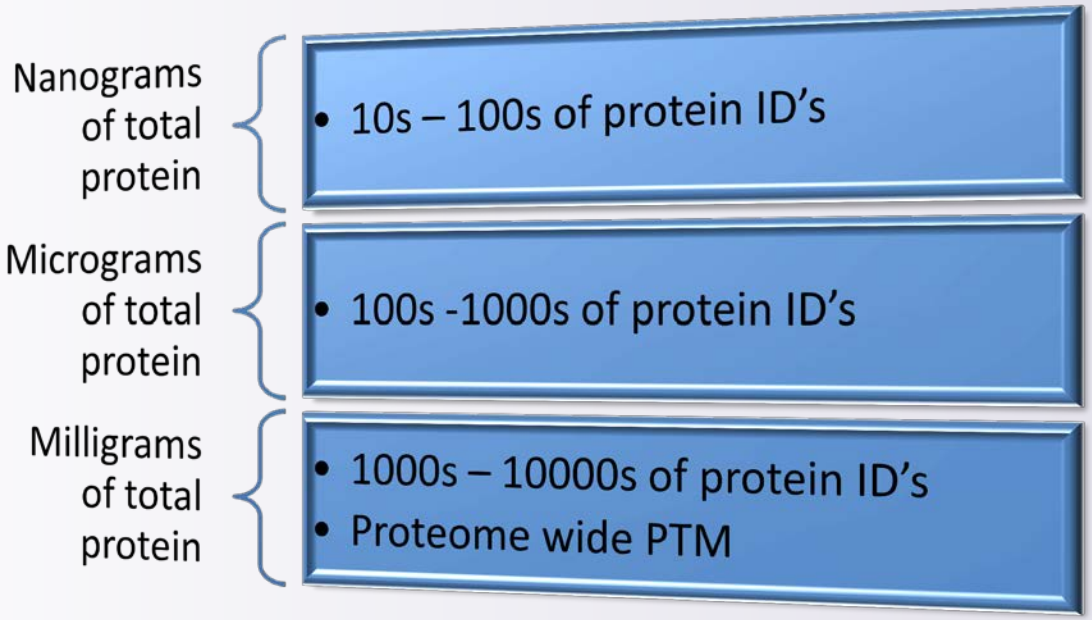
high amounts of salts and detergents must be removed before MS analyses.

e.g. protein precipitation, ultrafiltration, solid phase extraction, SDS-PAGE, etc

There are as many protocols as there are proteomics researchers

Shotgun proteomics

How much protein do I need?

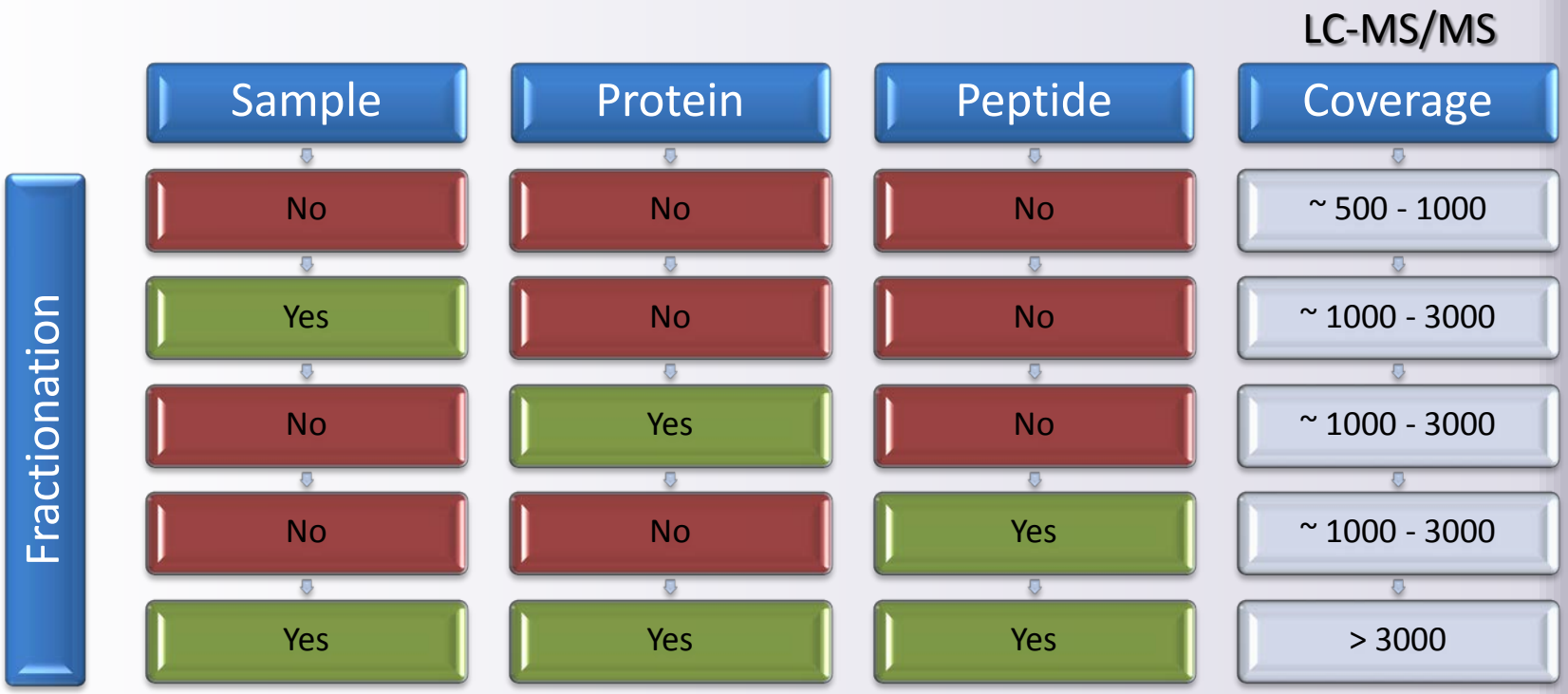


Results strongly depend on sample preparation, complexity and dynamic range.
Typically, one-tenths as many proteins are identified from serum than from cell lines or tissues.

Shotgun proteomics

Coverage:

- Basic protein ID's
- PTM ID's
- Label free quantification



Divide and conquer

Shotgun Proteomics Exp 1

1e7 cells in 0.5ml PBS
- add 0.5 ml of 10mM Tris pH 7.5, 10M urea, 0.1% Rapigest
- Sonicate for 5min

Lysate
- Centrifuge for 10 min

Cleared lysate
- reduce (10 mM TCEP; 20 min; 37°C)
- alkylate (10 mM iodoacetamide; 20 min; dark; RT)

Red/alk proteins
- Dilute to urea <1.5M with 100mM ammonium bicarbonate
- Add Trypsin (1:100 w:w), 6hrs, 37°C

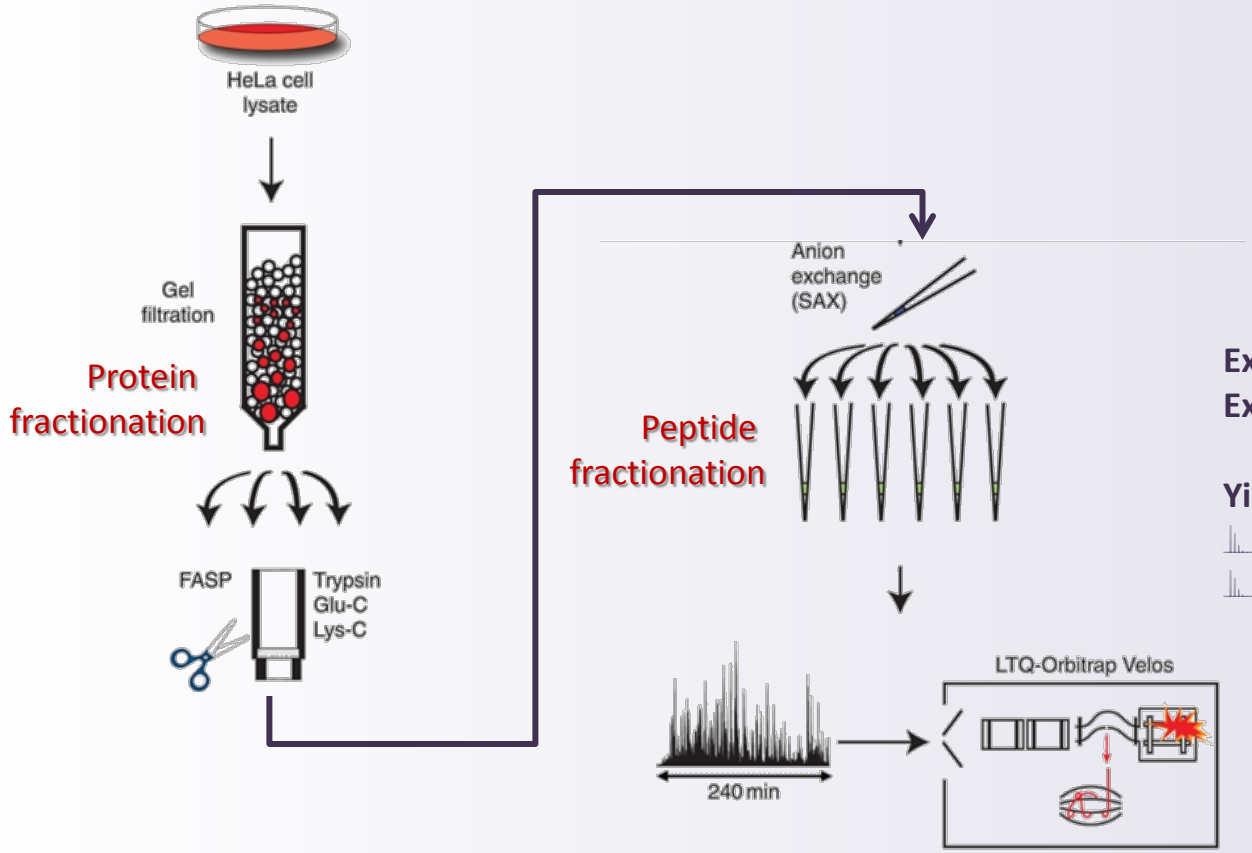
Peptides
- Clean up C18 reversed phase spin columns
- Fractionate and cleanup

Peptide fractionation

Peptide fractionations: 127 LCMS runs
~ 175000 peptides
> 10,000 Proteins

M. Beck, A. Schmidt, J. Malmstroem et al., *Molecular systems biology* **7**, 549 (2011).

Shotgun Proteomics Exp 2



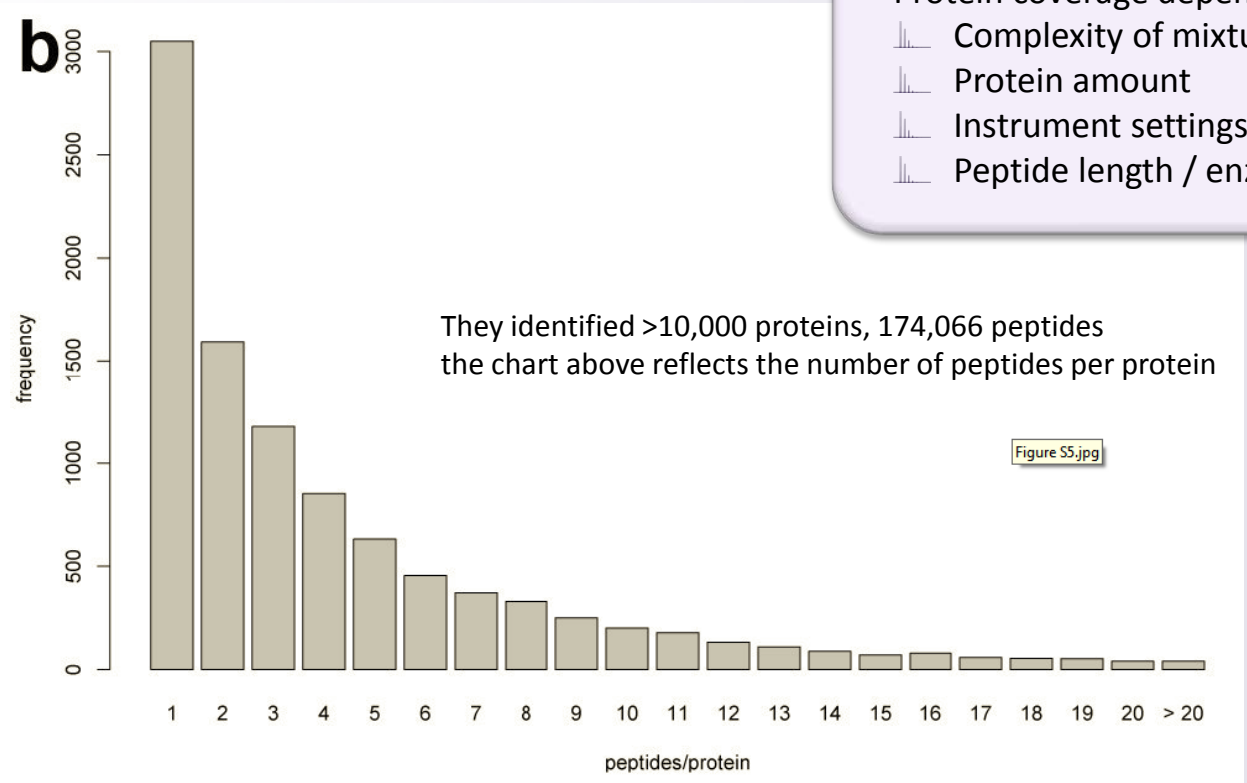
Exp 1: 126 fractions 21 days
Exp 2: 72 fractions 12 days

Yield:
- **Peptides 187,006 and 163,784**
- **Protein 10596 and 10255**

N. Nagaraj, J. R. Wisniewski, T. Geiger et al., *Molecular systems biology* **7**, 548 (2011).

Protein coverage?

Protein coverage depends on
- Complexity of mixture
- Protein amount
- Instrument settings
- Peptide length / enzyme used



They identified >10,000 proteins, 174,066 peptides
the chart above reflects the number of peptides per protein

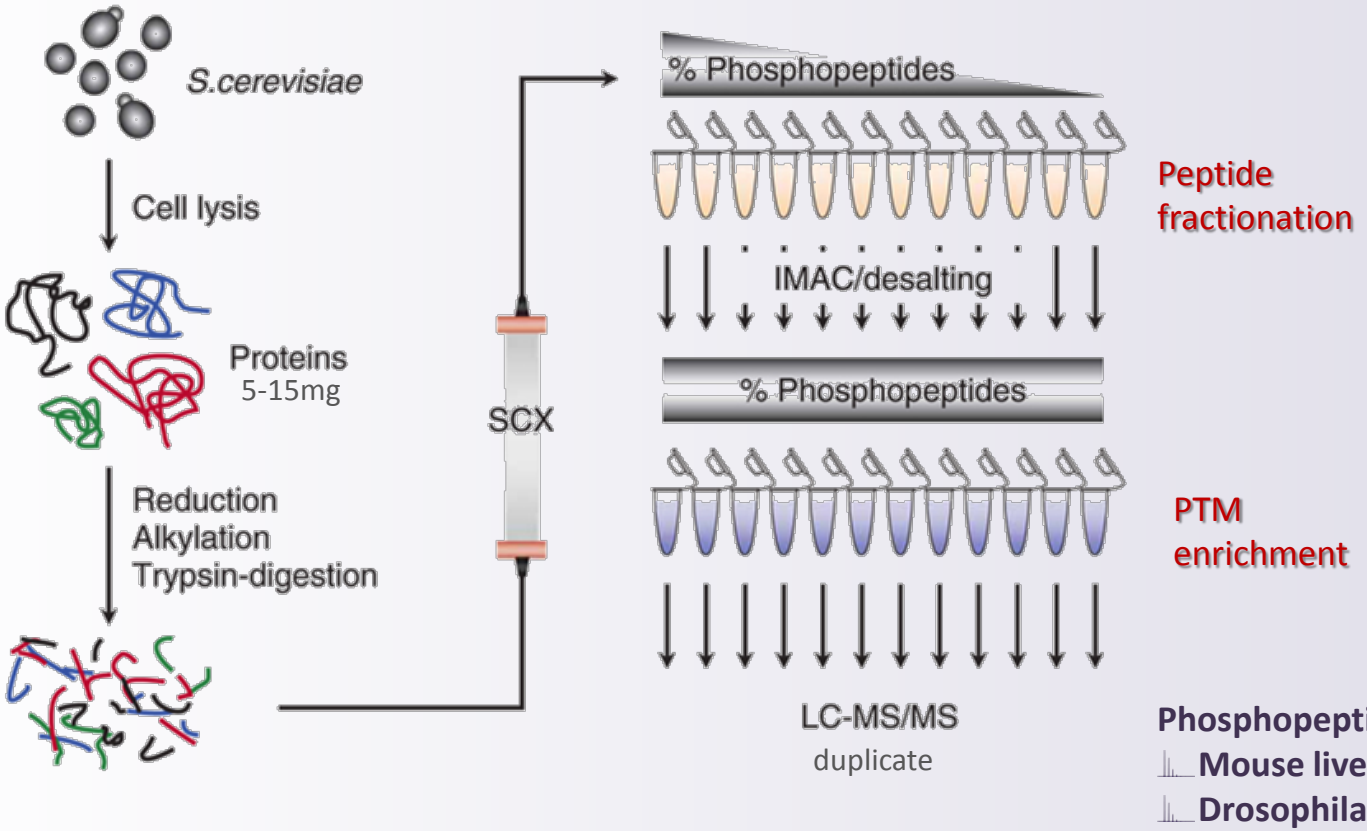
Figure S5.jpg

M. Beck, A. Schmidt, J. Malmstroem et al., *Molecular systems biology* **7**, 549 (2011).

In complex mixtures many low abundance proteins will be identified by only a single unique peptide

Shotgun PTM analysis

Example: phosphorylation



Peptide fractionation

PTM enrichment

Phosphopeptide yield:

- **Mouse liver 5500**
- **Drosophila embryos 13000**

Judit Villen is a PI in UW Dept. Genome Sciences:

Villen, J. and S. P. Gygi (2008) "The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry." *Nat Protoc* **3**:1630-8.

Quantitative proteomics

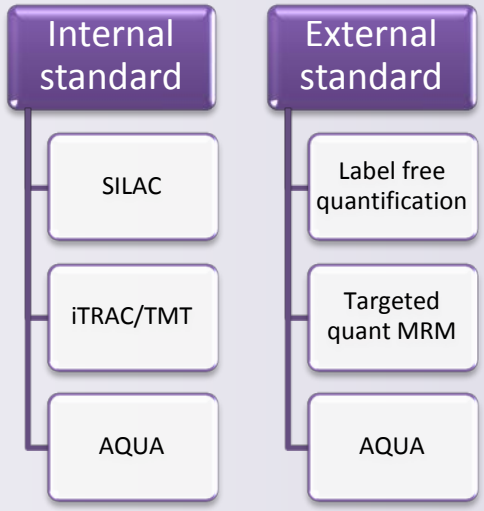
Bad News…. mass spec is not a quantitative tool

- Peptide ionization efficiency is sequence specific
- Co-eluting peptides affect each others ionization

To quantify we need an internal or external standard with identical physiochemical properties

Internal standards = stable isotopic labeling

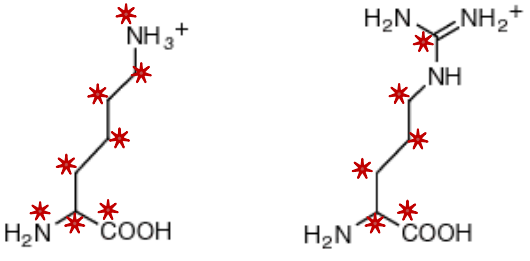
External: Measuring the same peptide in two consecutive runs



SILAC

SILAC Stable isotope labeling by amino acids in cell culture

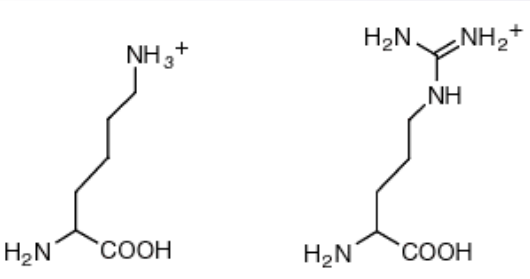
Metabolic labeling

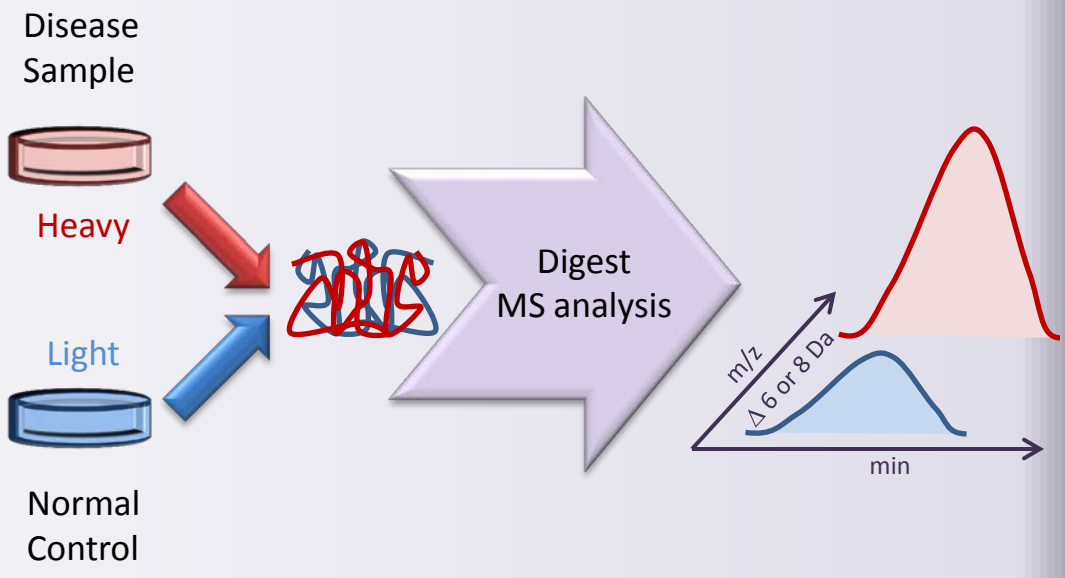


Lys-$^{13}C_6$ $^{15}N_2$ Arg-$^{13}C_6$

Δ 8 Da Δ 6 Da

Lys-$^{12}C_6$ $^{14}N_2$ Arg-$^{12}C_6$





Good for small changes (10-50%)

NOTE:

1+ peptide Δ m/z = 6 or 8

2+ peptide Δ m/z = 3 or 4

3+ peptide Δ m/z = 2 or 2.6

Publications from a UW lab discribing SILAC:

S. E. Ong, *Analytical and bioanalytical chemistry* (2012) Review

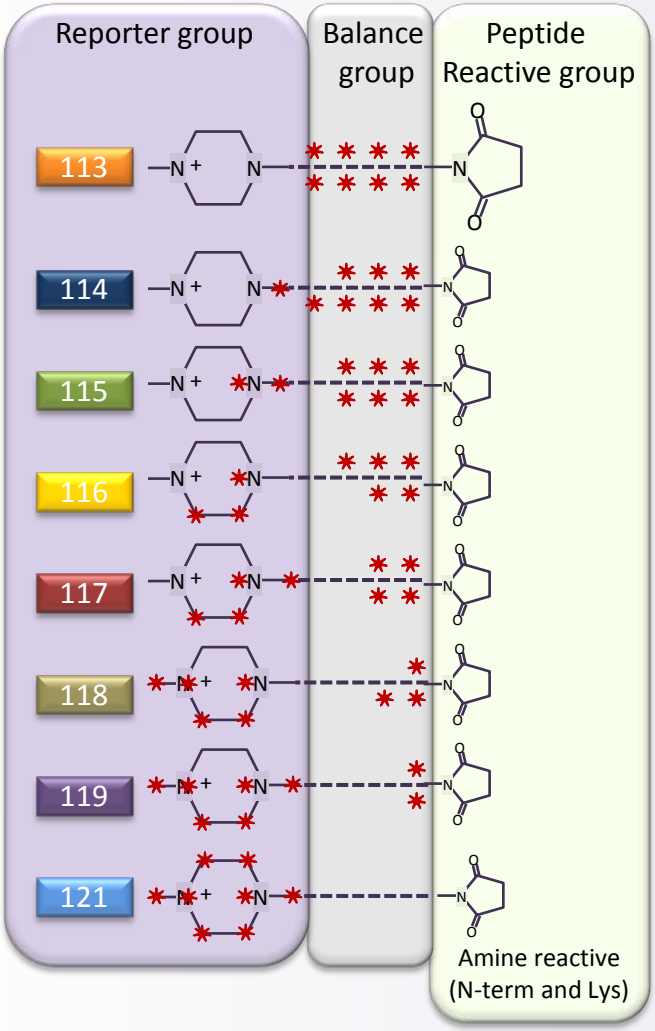
J. D. Chavez, M. R. Hoopmann, C. R. Weisbrod et al., *PloS one* **6** (5), e19892 (2011).

M. R. Hoopmann, J. D. Chavez, and J. E. Bruce, *Analytical chemistry* **83** (22), 8403 (2011).

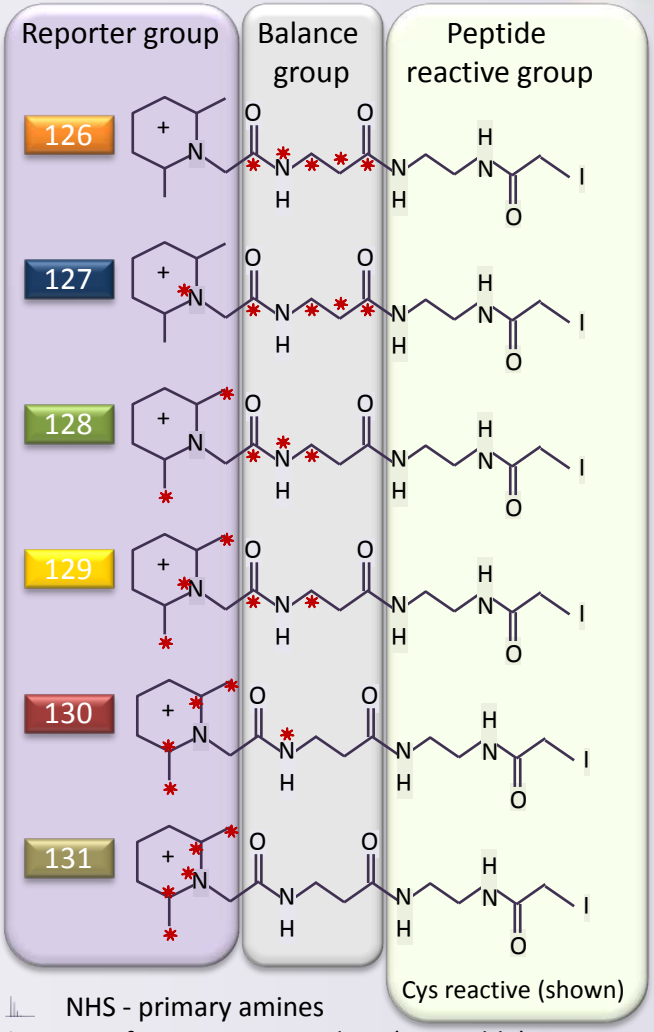
www.silac.org

Isotopic labeling iTRAQ and TMT

iTRAQ (isobaric tags for relative and absolute quantification)



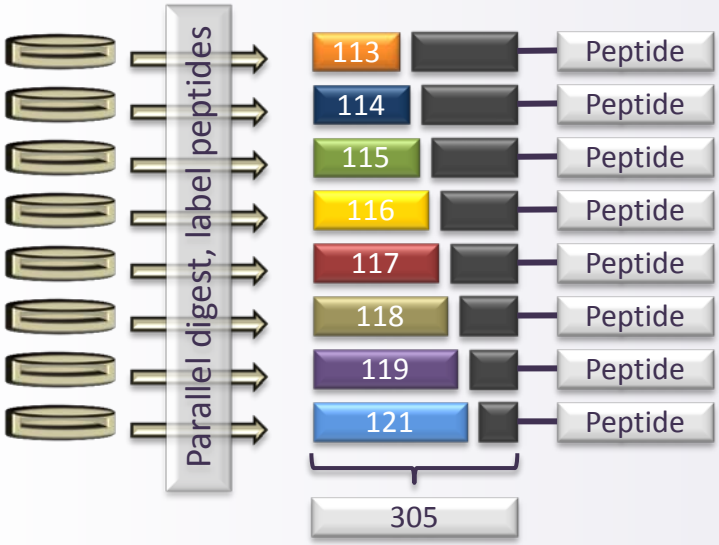
TMT (tandem mass tag)



- NHS - primary amines
- cys - free cysteine residues (reversible)
- iodo - free cysteine residues (irreversible)*
- hydrazine - aldehydes & ketones*

Isotopic labeling

iTRAQ, TMT

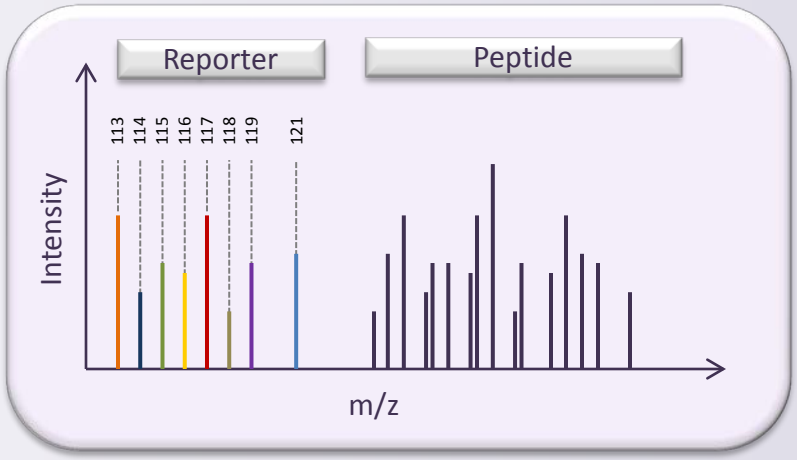


Pros:

- Applicable to all sample types
- Relatively easy
- Multiplexing (up to 8)

Cons:

- Cost
- Separate sample processing



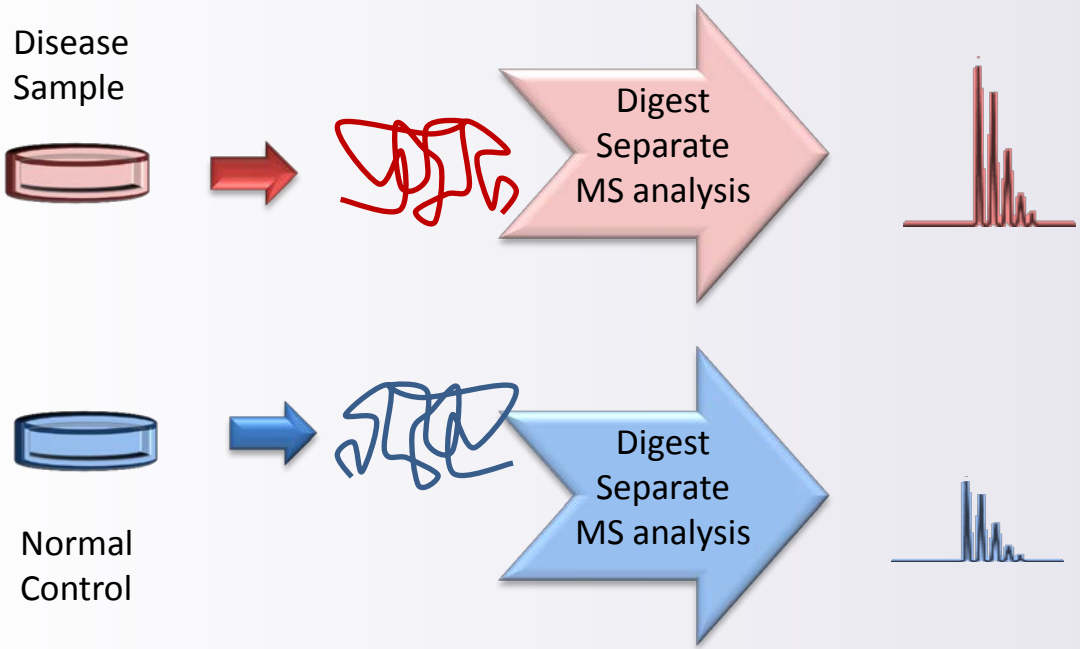
LC-MS/MS analysis

Good for moderate changes (50-200%)

Publications from a UW lab discribing iTRAQ:
A. T. Navare, P. Sova, D. E. Purdy et al., *Virology* **429** (1), 37 (2012)

Label free quantification

Relative quantification



- Extract ion chromatograms
- Spectral counting

Pros:

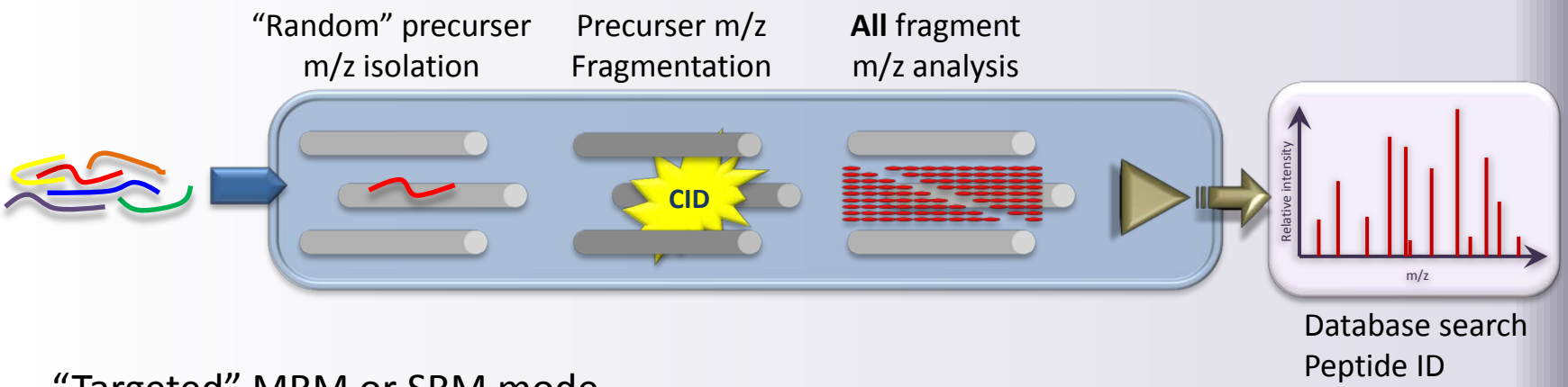
- Applicable to all sample types
- Cheap
- Multiplexing (“infinite”)

Cons:

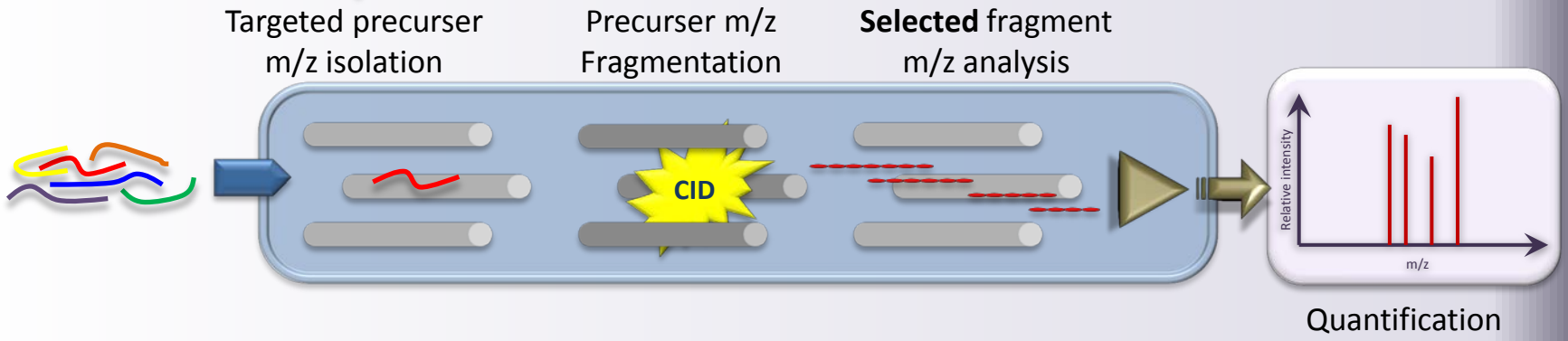
- Susceptible to technical variation

Targeted quantification

"Discovery" MS/MS mode

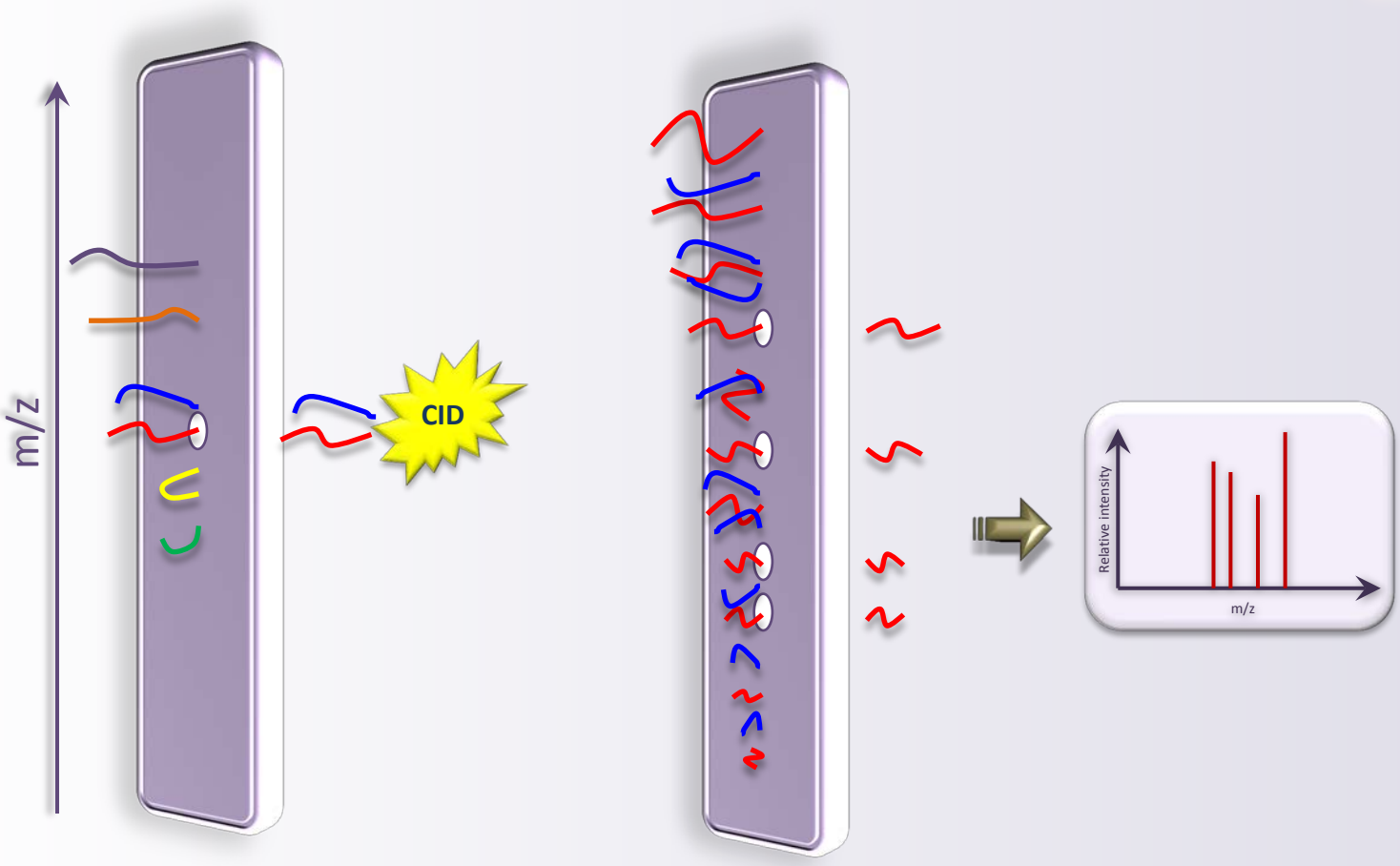


"Targeted" MRM or SRM mode



MRM multiple reaction monitoring
SRM selected reaction monitoring

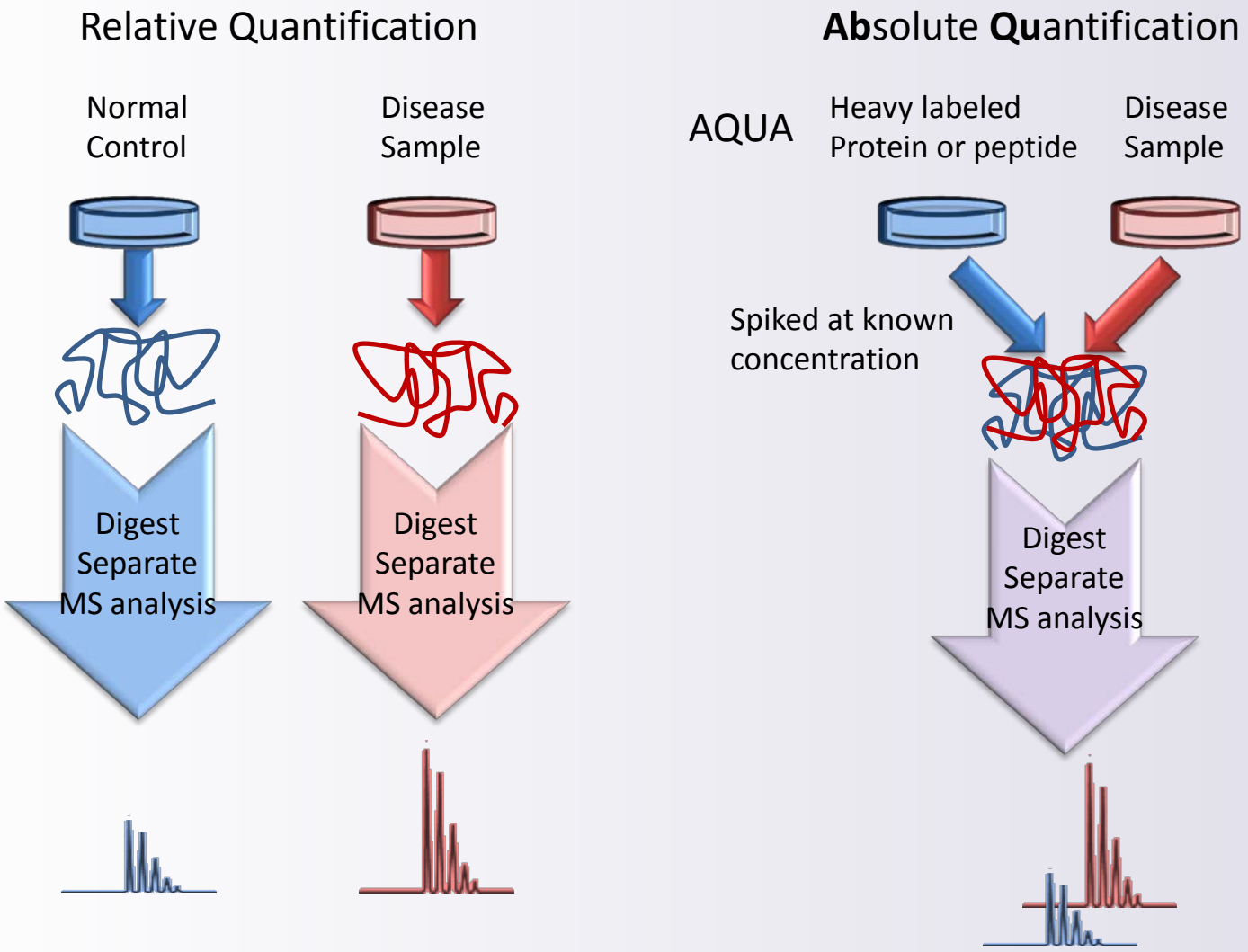
Targeted Quantification/Identification



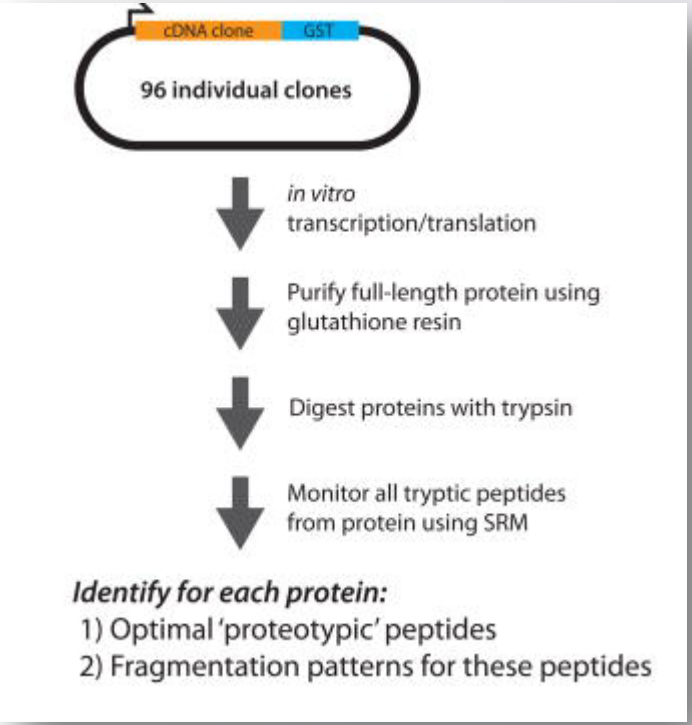
Known peptide
Precursor m/z

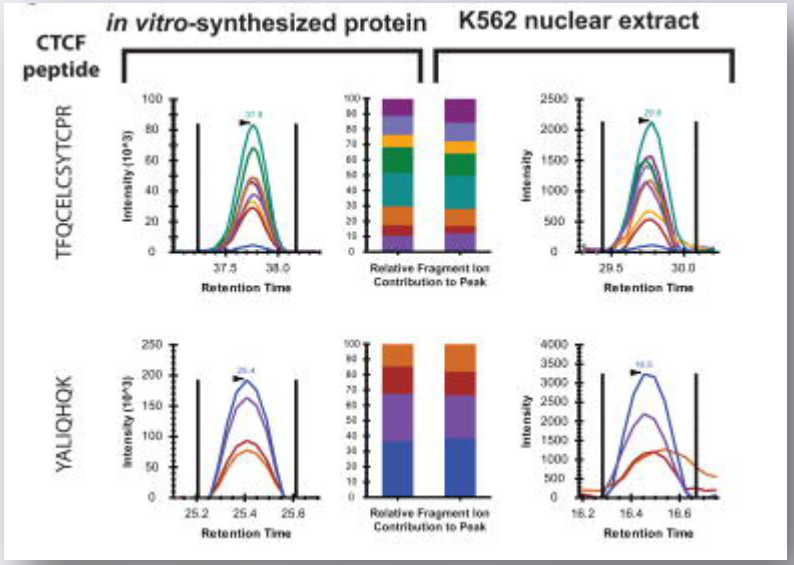
Known fragment m/z

Targeted quantification



Targeted Identification





Or in silico prediction:

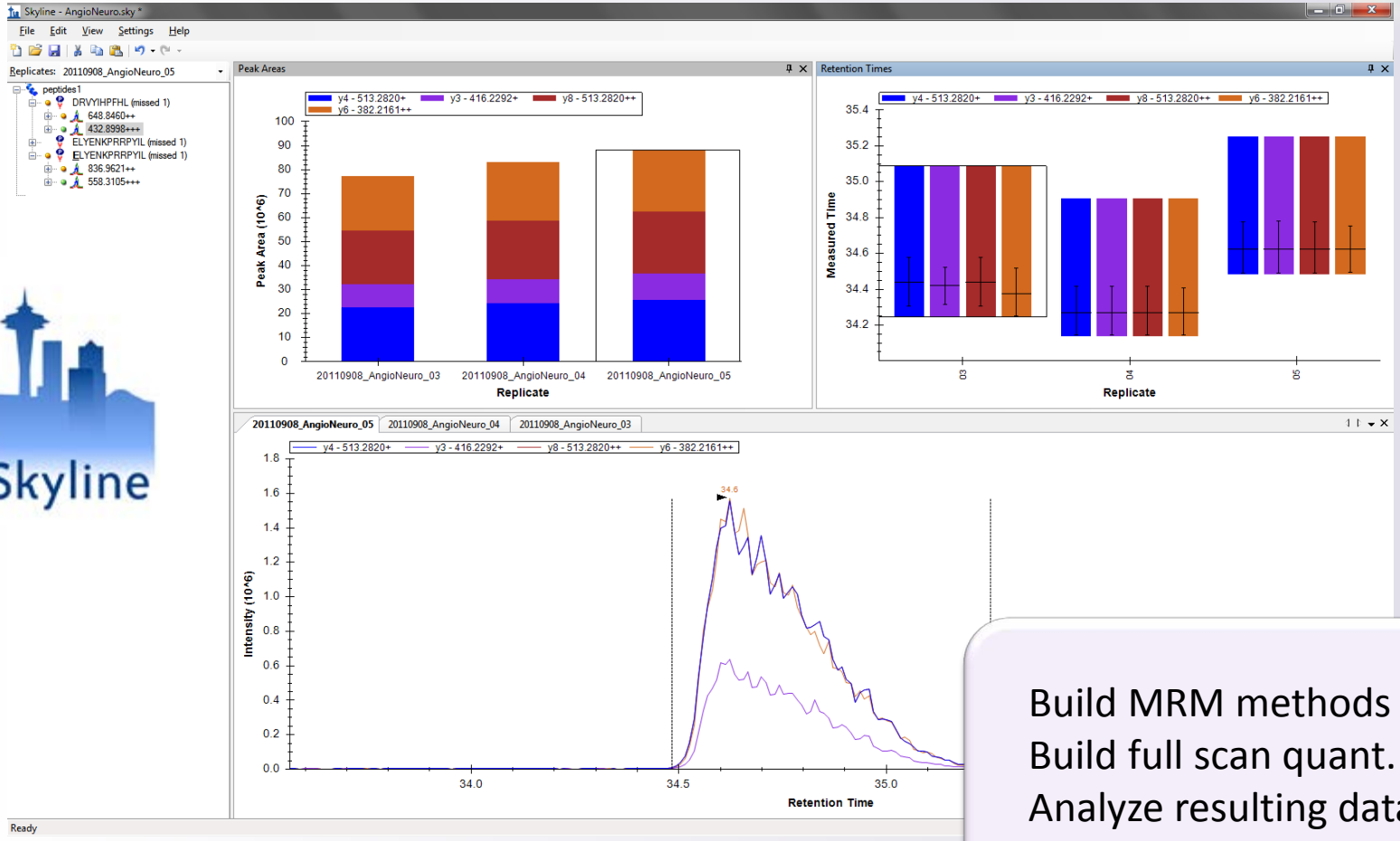
- Peptide precursor m/z
- Fragment m/z

Identification and quantification of

- Low abundance proteins
- Predicted proteins

Rapid empirical discovery of optimal peptides for targeted proteomics. Stergachis AB, MacLean B, Lee K, Stamatoyannopoulos JA, **MacCoss** MJ. Nat Methods. 2011 Nov 6;8(12):1041-3.

Skyline



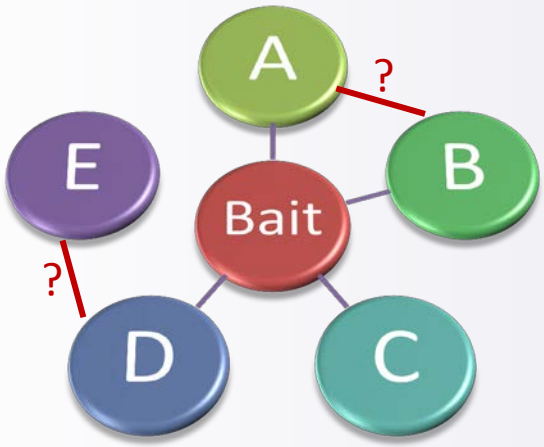
Skyline

Build MRM methods
Build full scan quant. Methods
Analyze resulting data

https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view

Protein-protein interactions

Affinity purification coupled with mass spectrometry



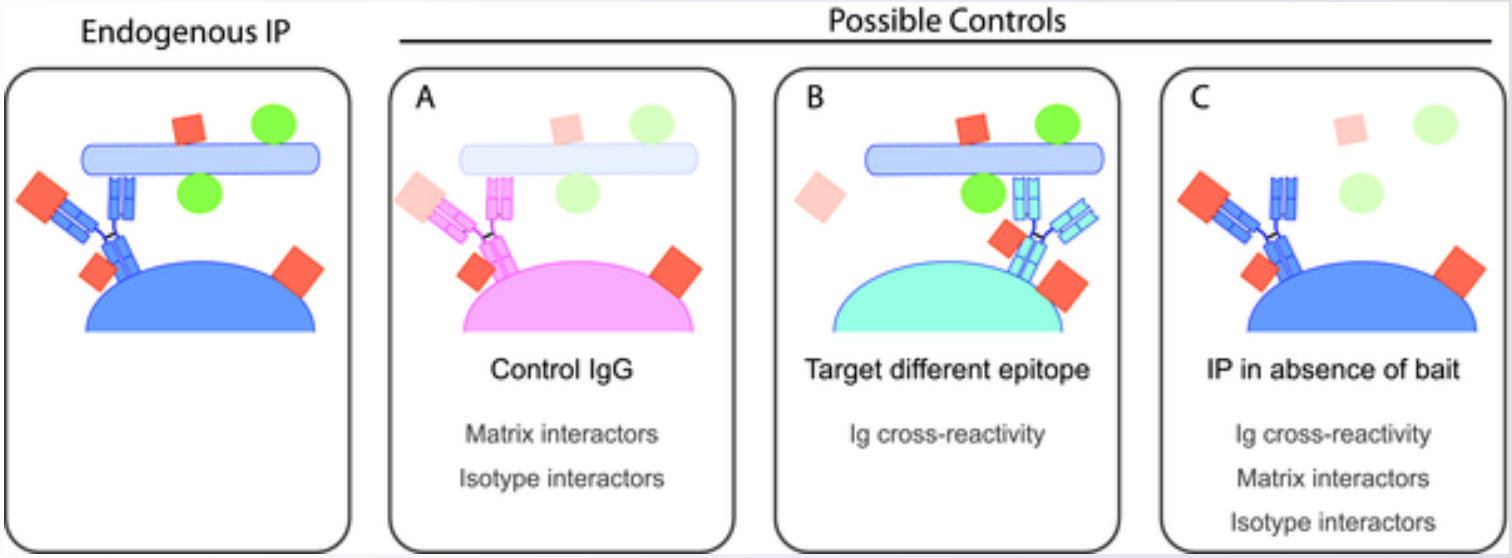
Pull down bait with specific antibody (IP)
Or
Express tagged bait and pull down with
Tag specific reagents

Complex organization is difficult to assess
Bait identifies A, B, C, D, E
Pull down A identifies bait, B and X?
Pull down B…..
Pull down C……..
Pull down D……..
etc

Beyond hairballs: The use of quantitative mass spectrometry data to understand protein-protein interactions.
A.C. Gingras and B Raught, FEBS letters2012 Apr 10. [Epub ahead of print]

Protein-protein interactions

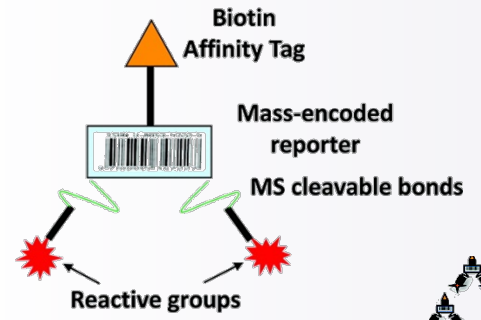
Typical controls for IP experiments:



Analyze sample and control and compare peptide/protein list
Or
Combine with isotopic labeling (SILAC), mix and analyze together

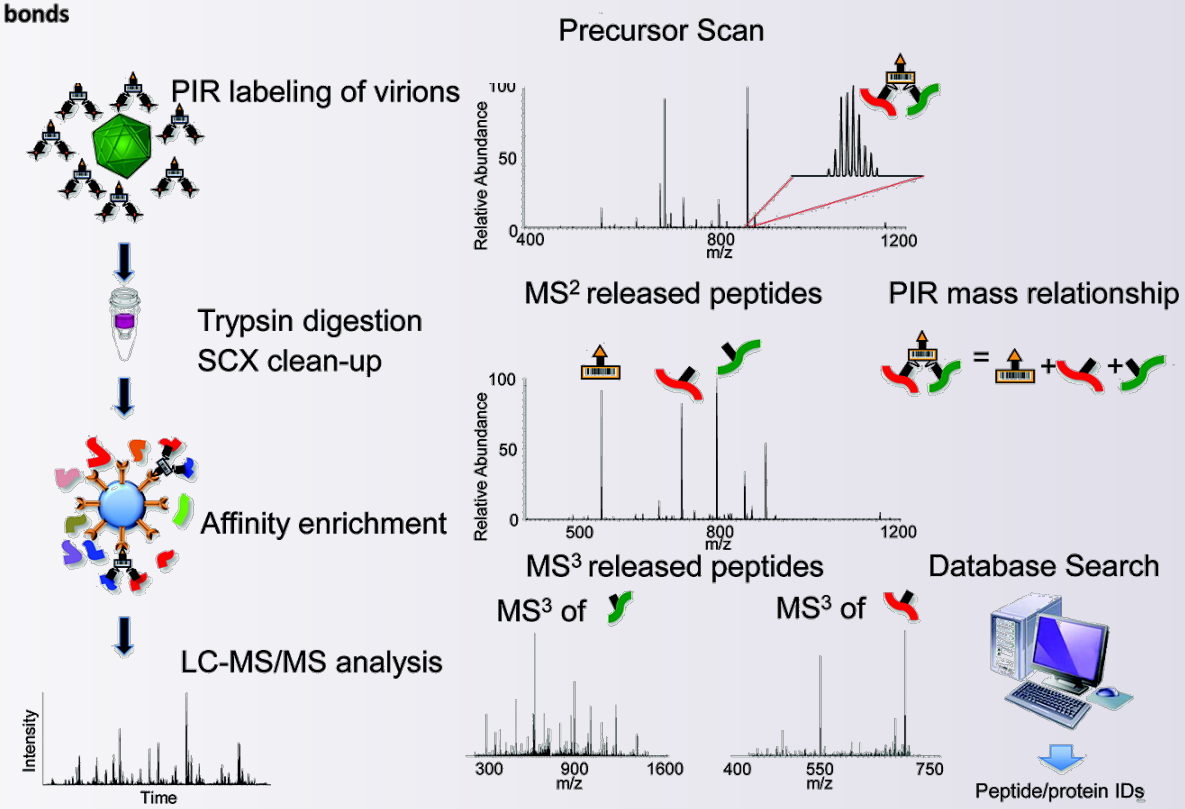
W. H. Dunham, M. Mullin, and A. C. Gingras, Proteomics 12 (10), 1576 (2012).

Protein-protein interactions



Schematic of the PIR molecule

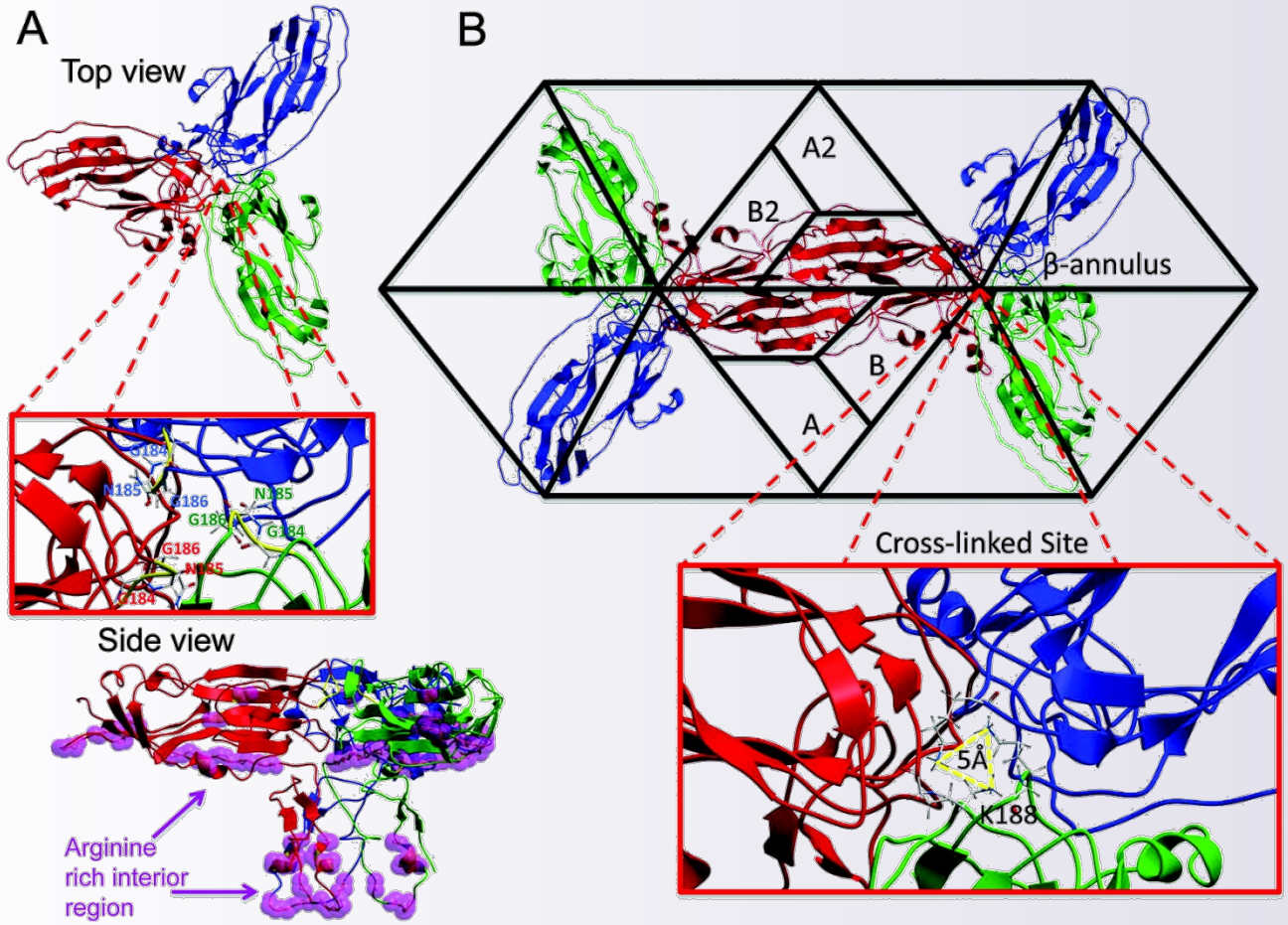
Cross linking strategy



Juan D. Chavez; Michelle Cilia; Chad R. Weisbrod; Ho-Jong Ju; Jimmy K. Eng; Stewart M. Gray; James E. Bruce; *J. Proteome Res.* **2012,** 11, 2968-2981.
For a review on cross linking see J. E. Bruce, Proteomics 12 (10), 1565 (2012).

Protein-protein interactions

Interaction partners and structural information



Juan D. Chavez; Michelle Cilia; Chad R. Weisbrod; Ho-Jong Ju; Jimmy K. Eng; Stewart M. Gray; James E. Bruce; *J. Proteome Res.* **2012,** 11, 2968-2981.
For a review on cross linking see J. E. Bruce, Proteomics 12 (10), 1565 (2012).

What's next?

Your Project goes here!!!

☺

Lets have a quick look at the
UWPR Informatics Platform

What's next?

We'll meet in the lobby at 1pm
To go have fun in the lab….