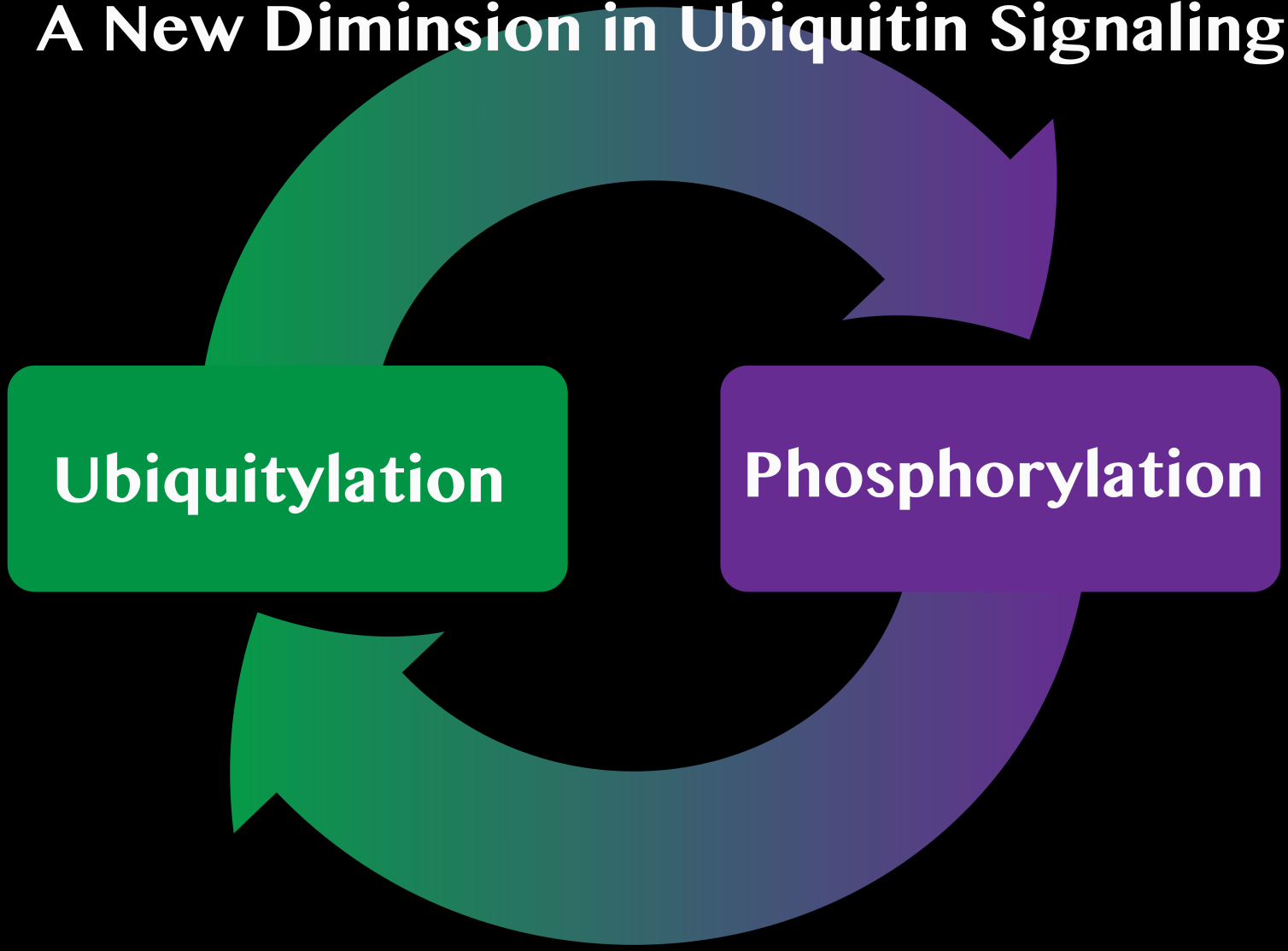
Assignments for Next Monday:

Nathan (Lab work Day 1, 9/28)

Ryan (Journal Club on [Wauer et al, 2015], 9/28)

Phosphorylation of Ubiquitin: A New Diminsion in Ubiquitin Signaling

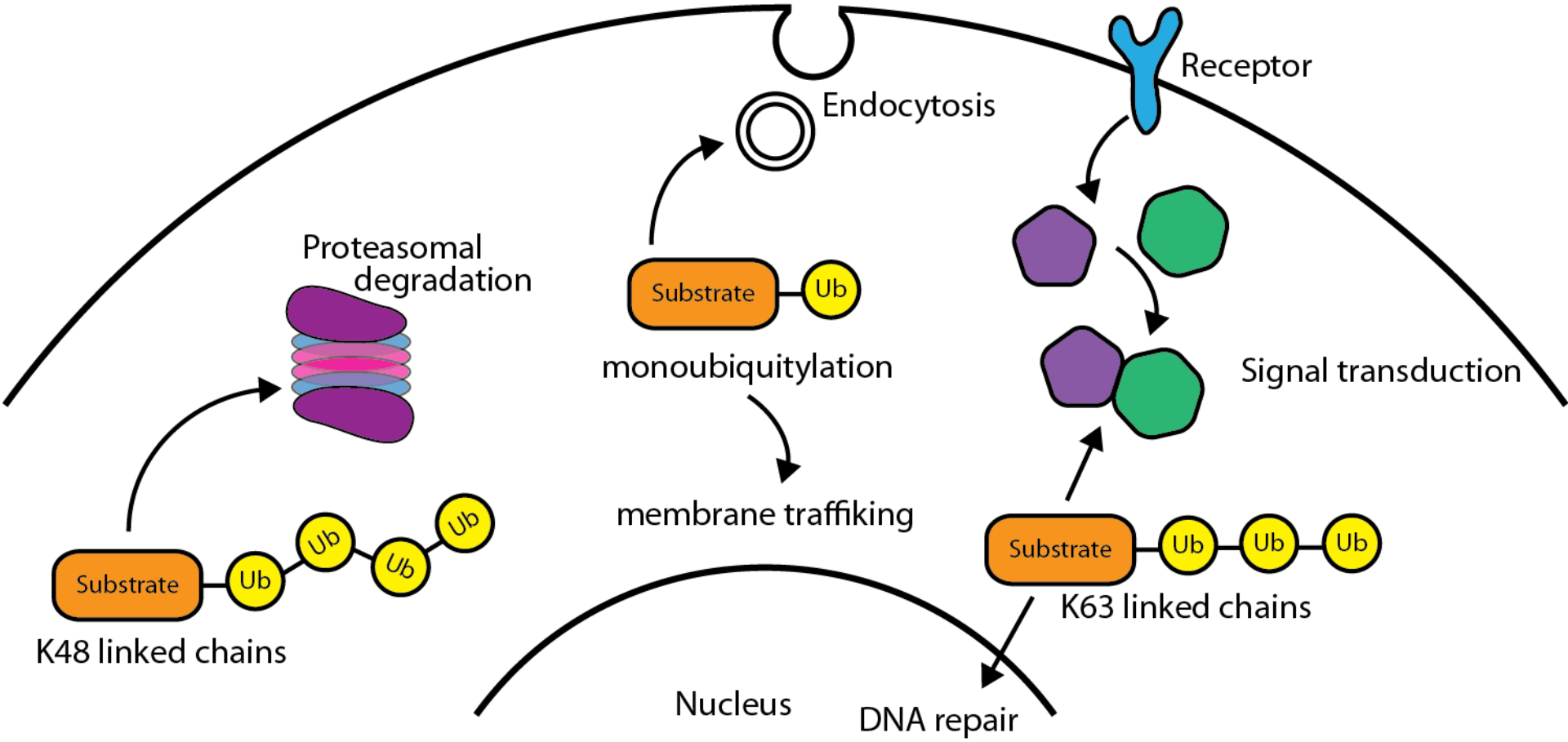


Danielle Swaney
Assitant Adjunct Professor, QB3

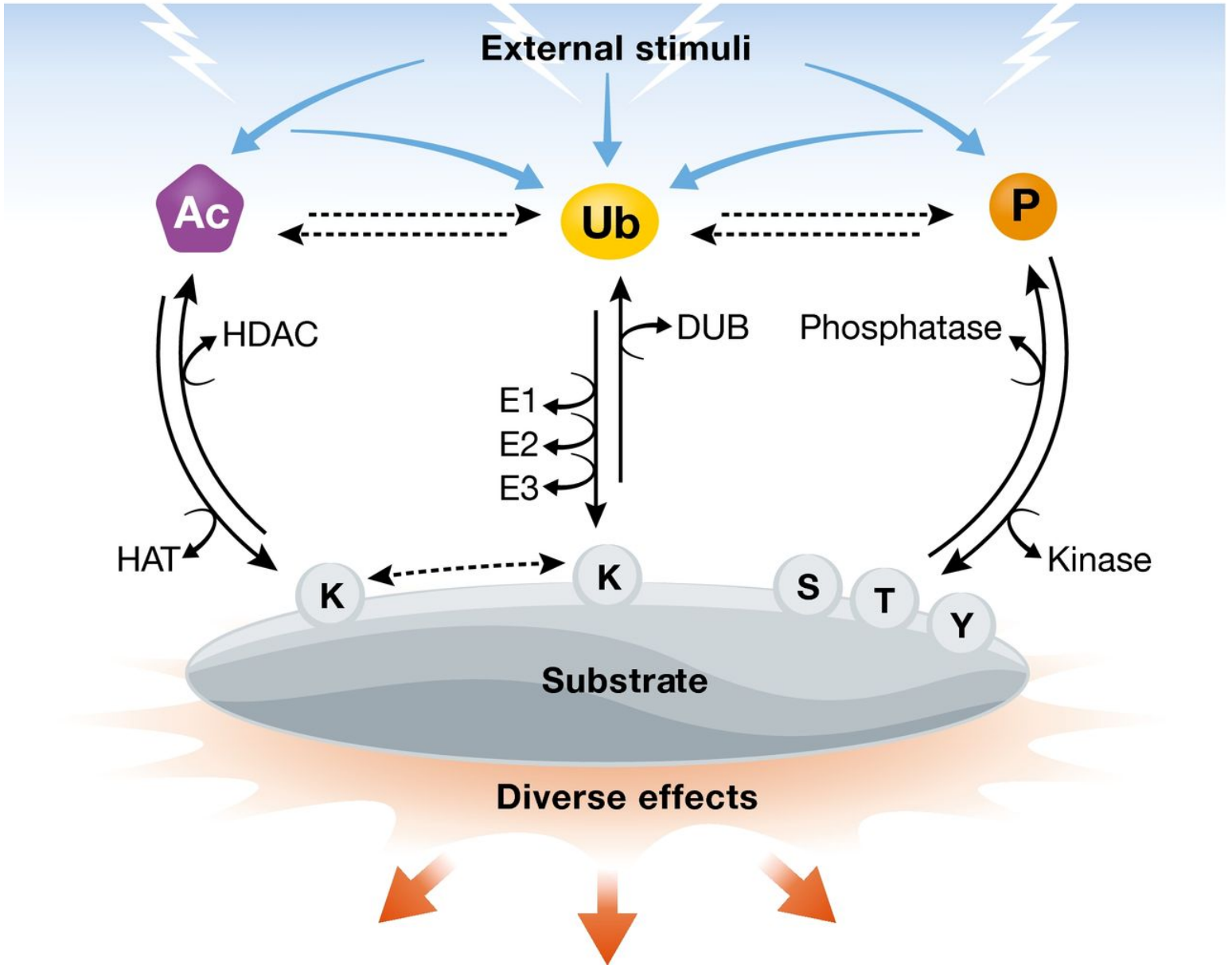
Outline

- Rationale for mass spectrometry project
- Approaches to study kinase-substrate interactions
- Introduction to mass spectrometry

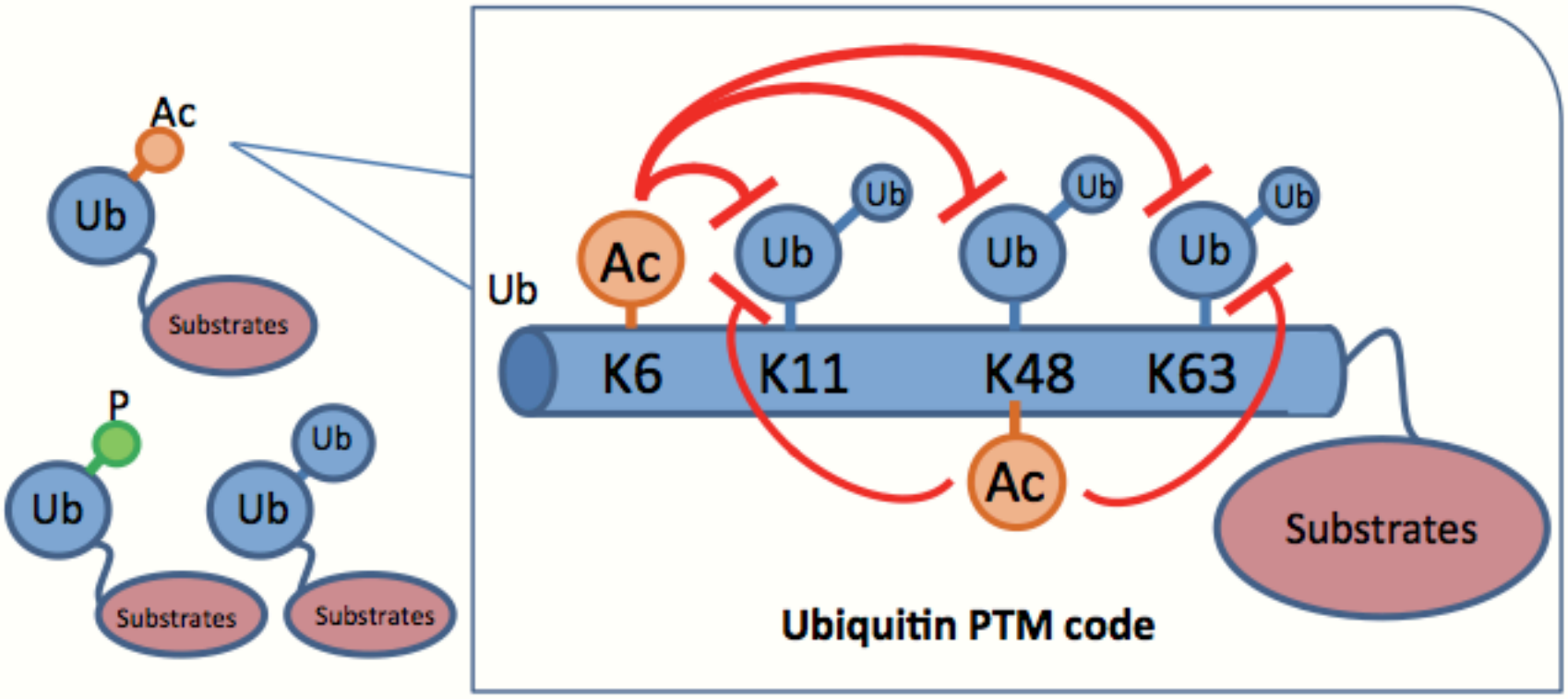
Ubiquitin is a protein post-translational modification with a wide variety of roles



Ubiquitin itself can be modified by other post-translational modifications

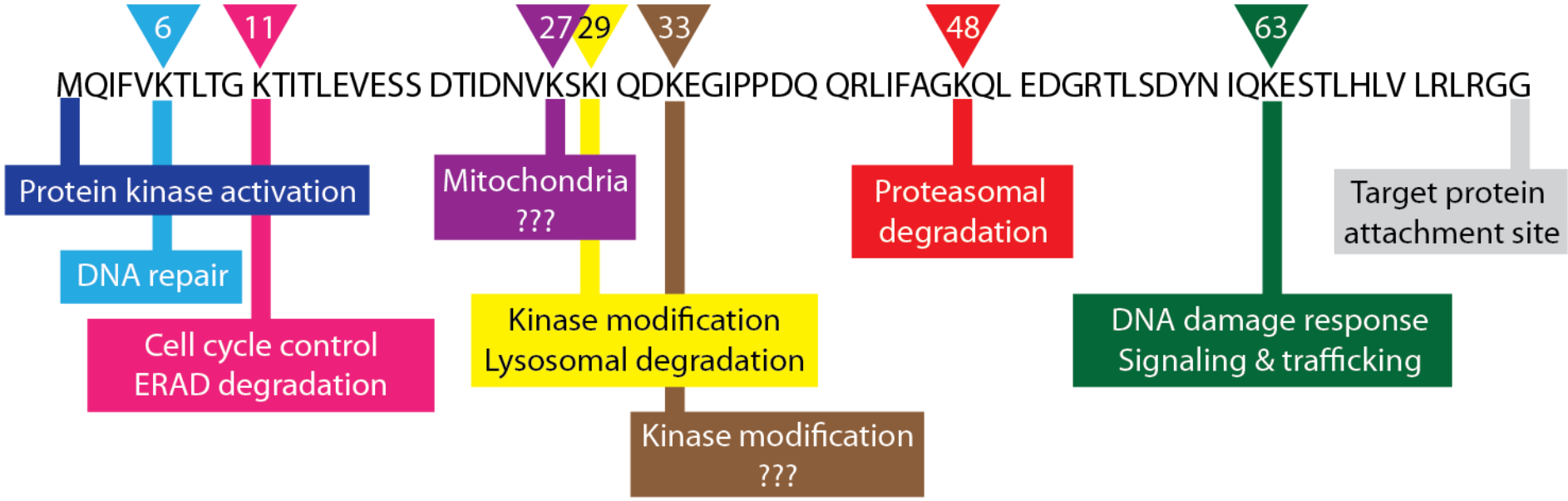


Lysine acetylation of ubiquitin – blocks ubiquitin chain elongation

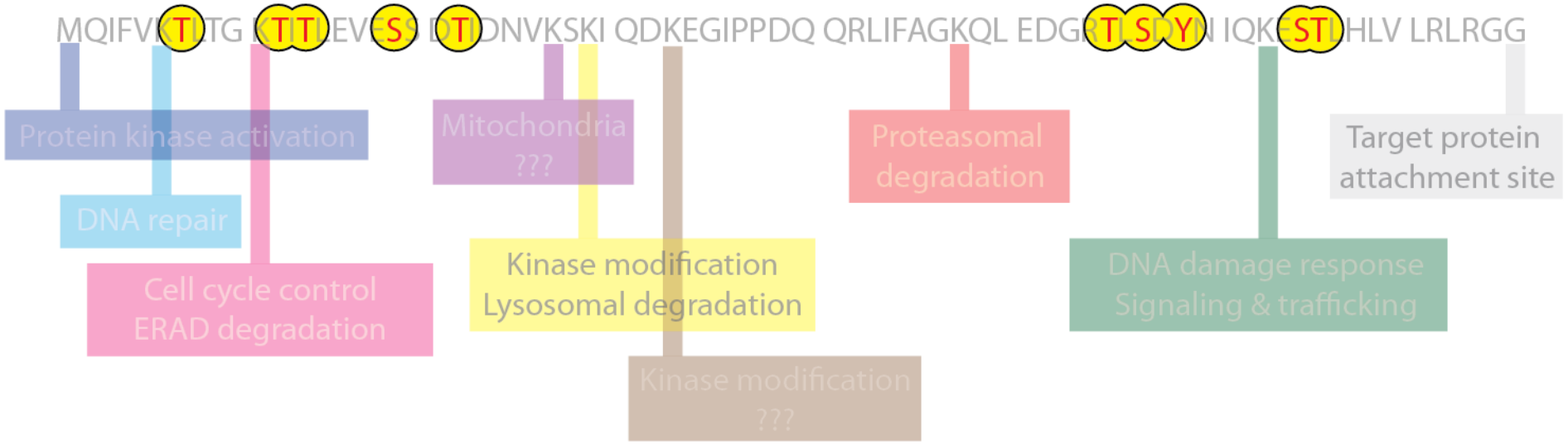


Ohtake, F. *et al.* Ubiquitin acetylation inhibits polyubiquitin chain elongation. *EMBO reports* (2014). doi:10.15252/embr.201439152

Functions of ubiquitin



Nearly every S/T/Y on ubiquitin (the most conserved protein) is phosphorylated and <u>conserved</u> from human to yeast



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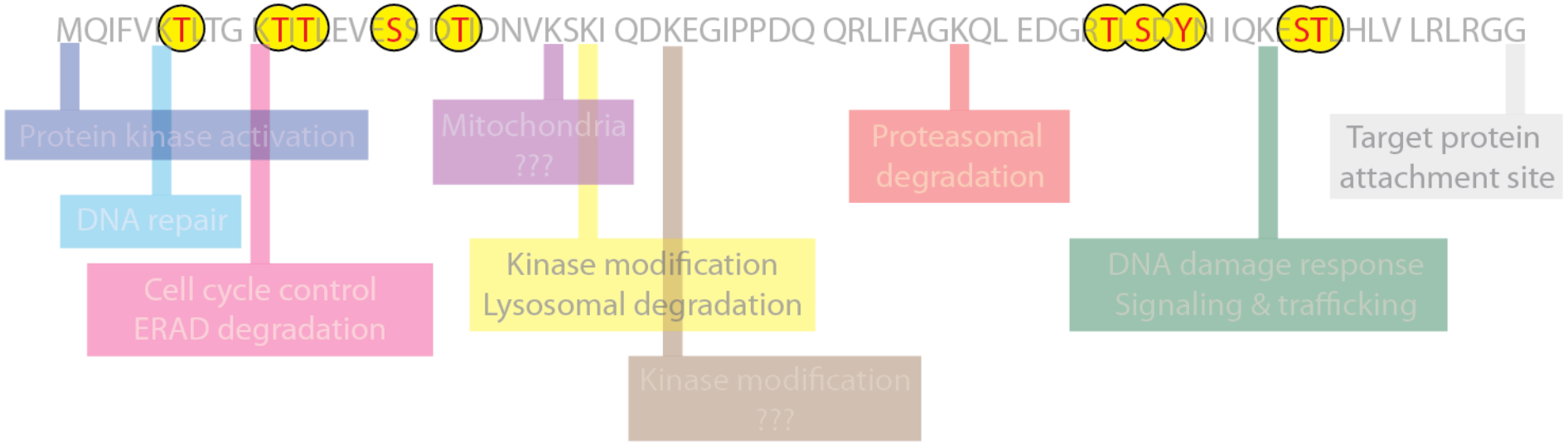
What kinases phosphorylation ubiquitin?



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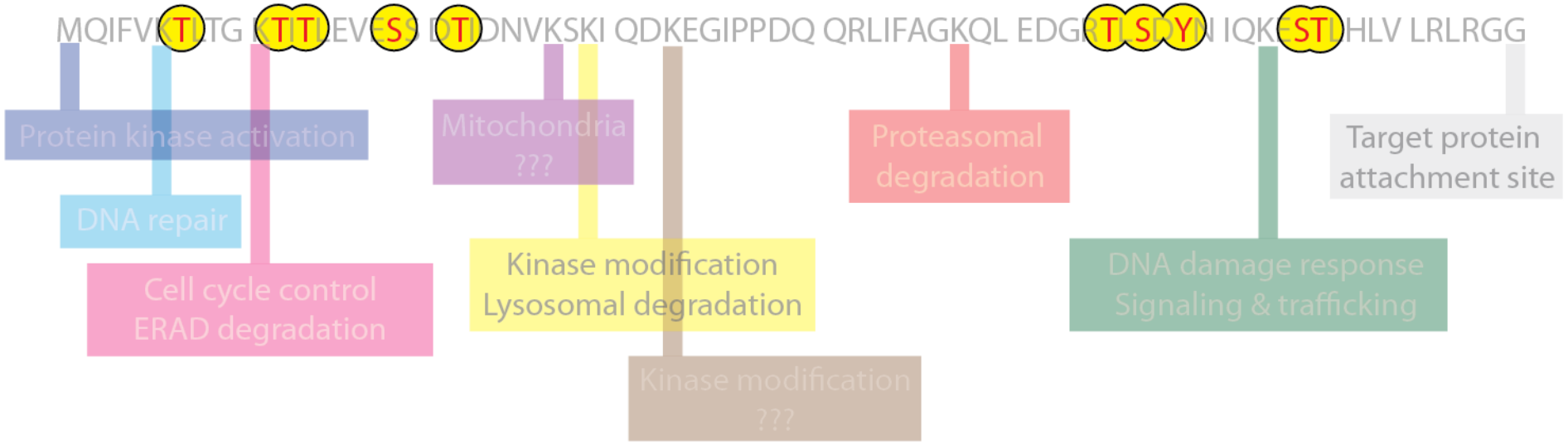

How does phosphorylation regulate ubiquitin function?

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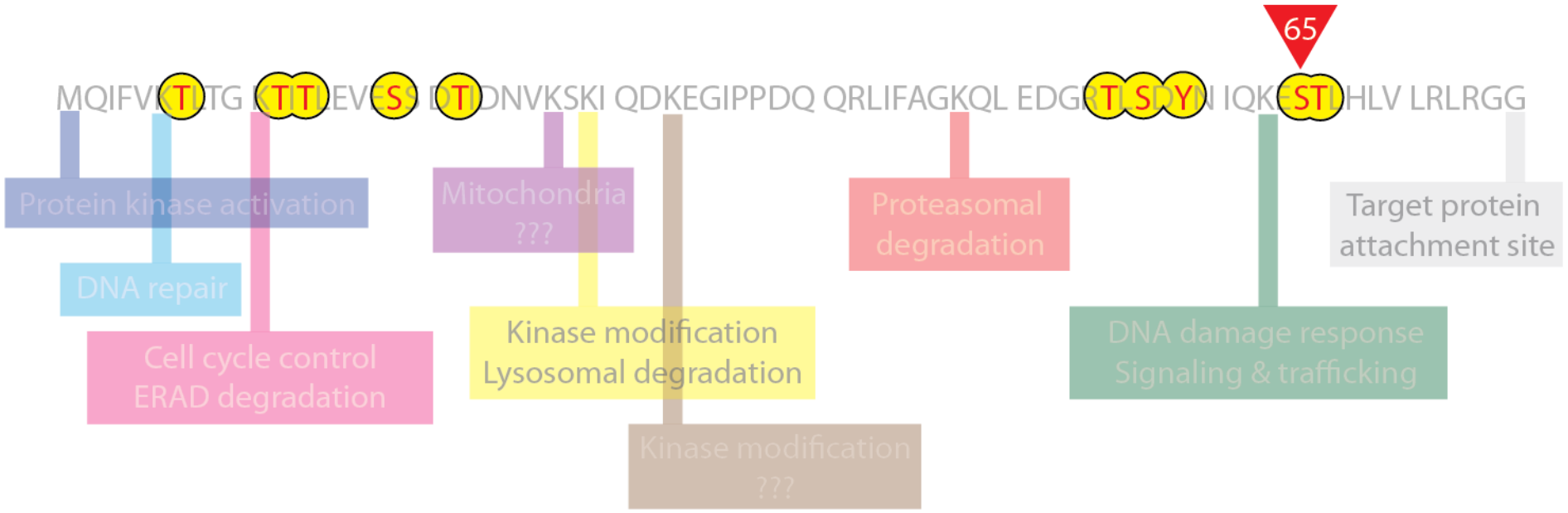
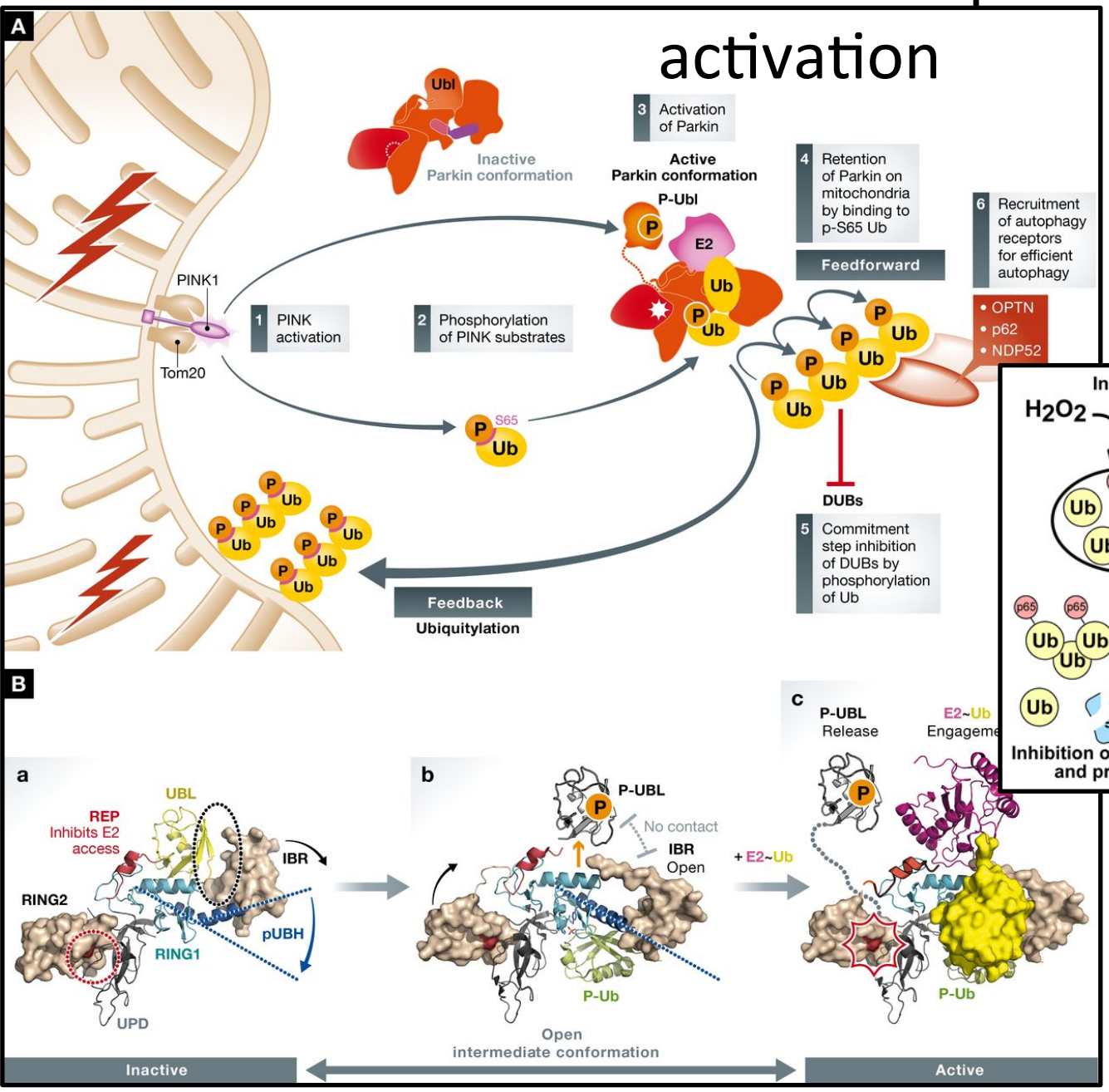
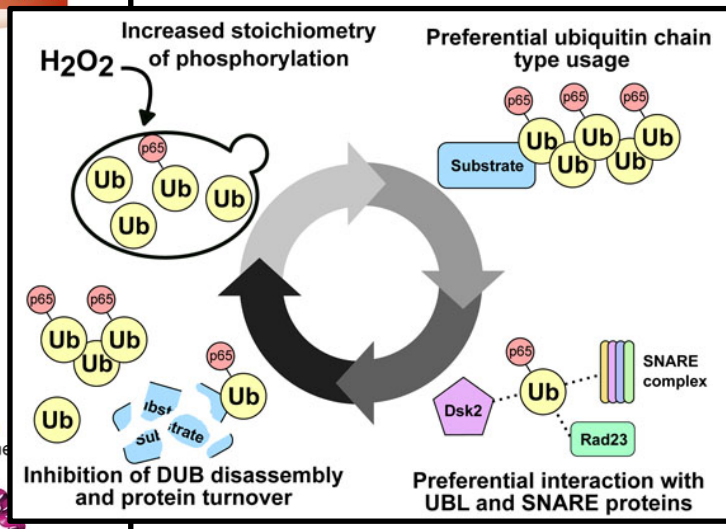


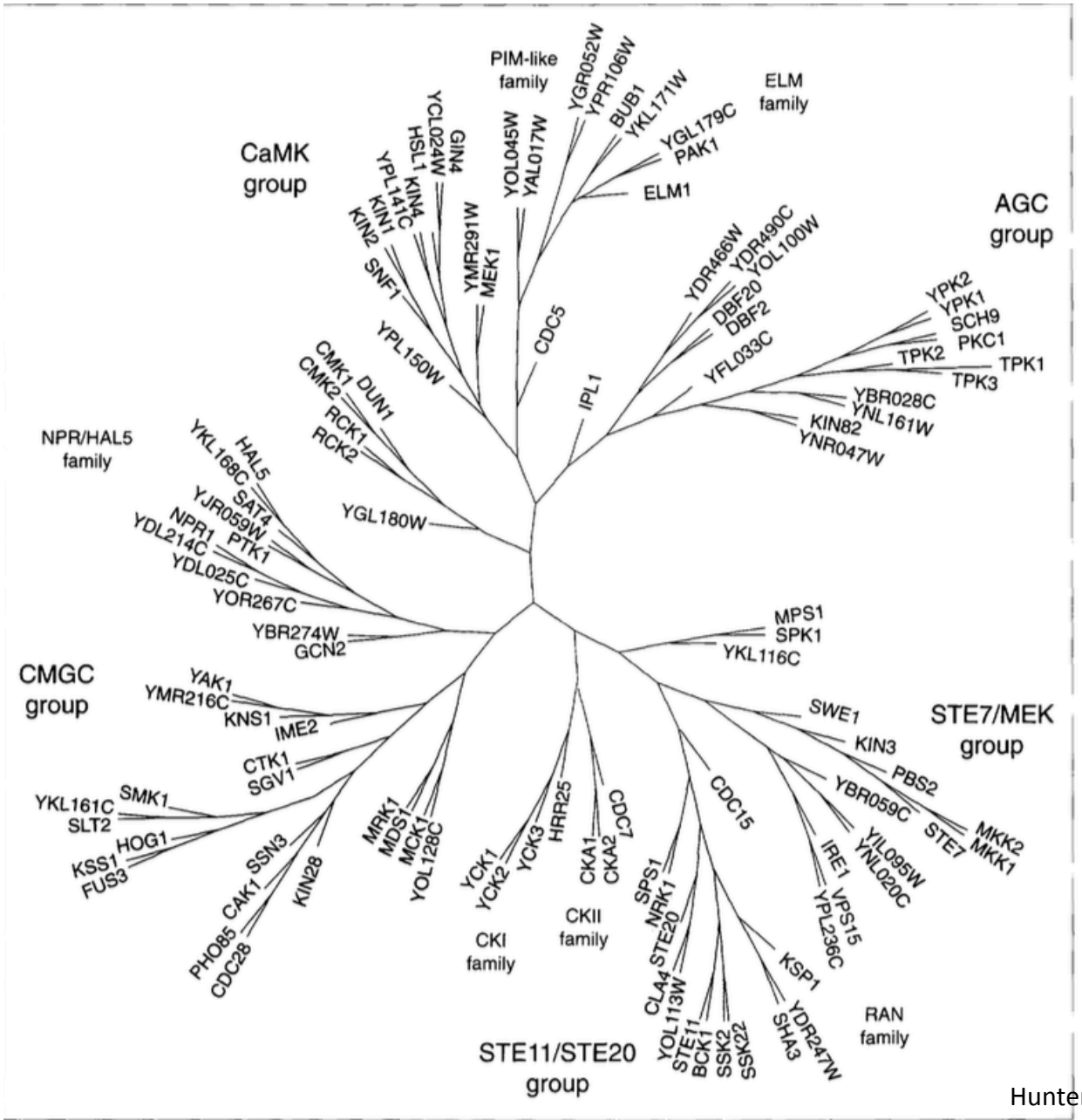
Human: Pink1 kinase → Ub S65p → Parkin E3 ligase activation



Ub S65p also exists in yeast, despite no Pink1 ortholog. What is the kinase?



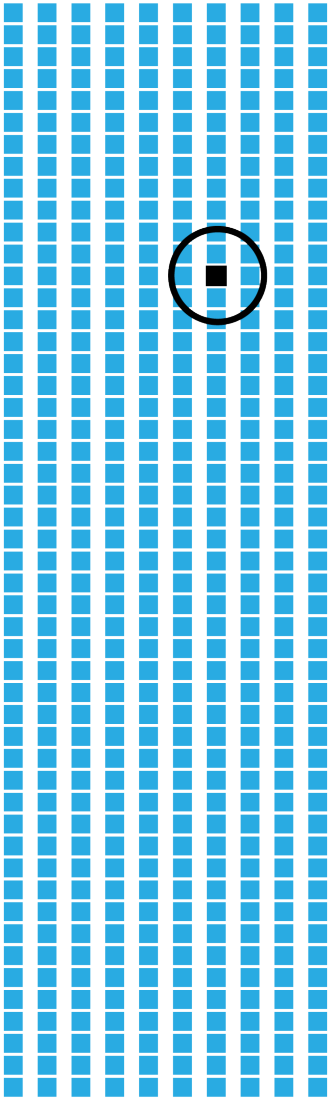
Approaches to study kinase-substrate interactions



Hunter & Plowman, TIBS, 1997.

Connecting enzymes and substrates is challenging

> 500 Protein Kinases

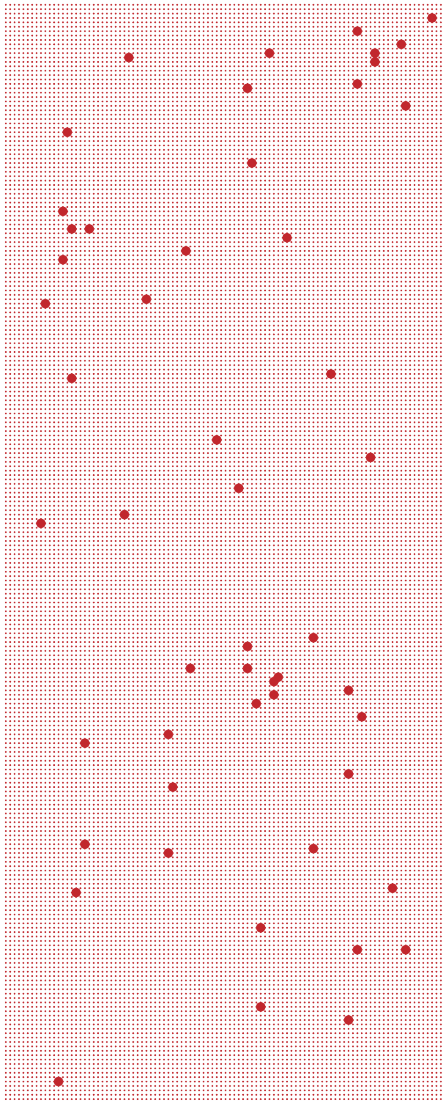


How can we connect these?

Two lines of evidence are typically required:

1. *in vivo*
   - Overexpression

> 25,000 Phosphosites



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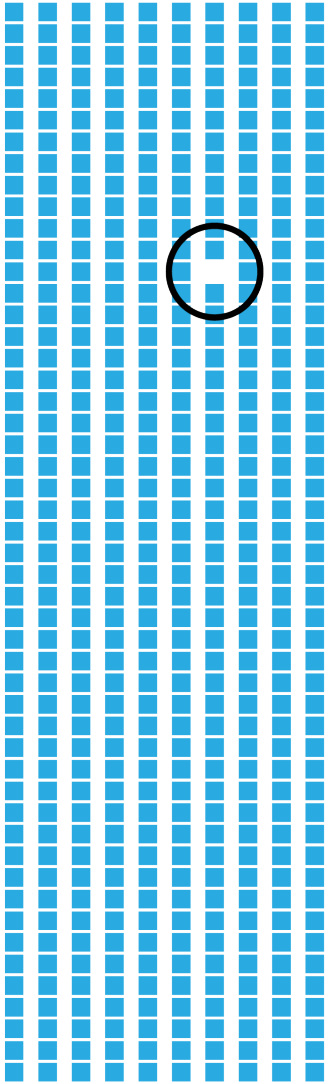
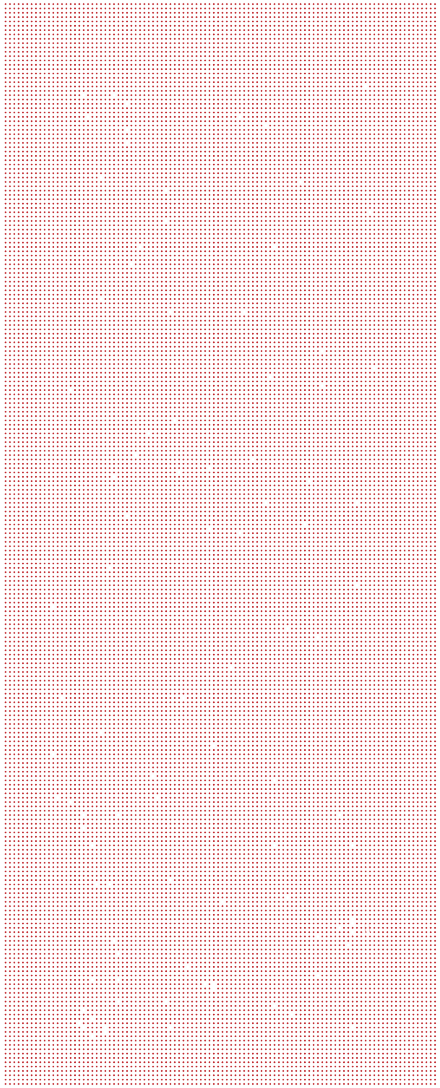


How can we connect these?

Two lines of evidence are typically required:

1. *in vivo*
   - Overexpression
   - Knockdown

> 25,000 Phosphosites



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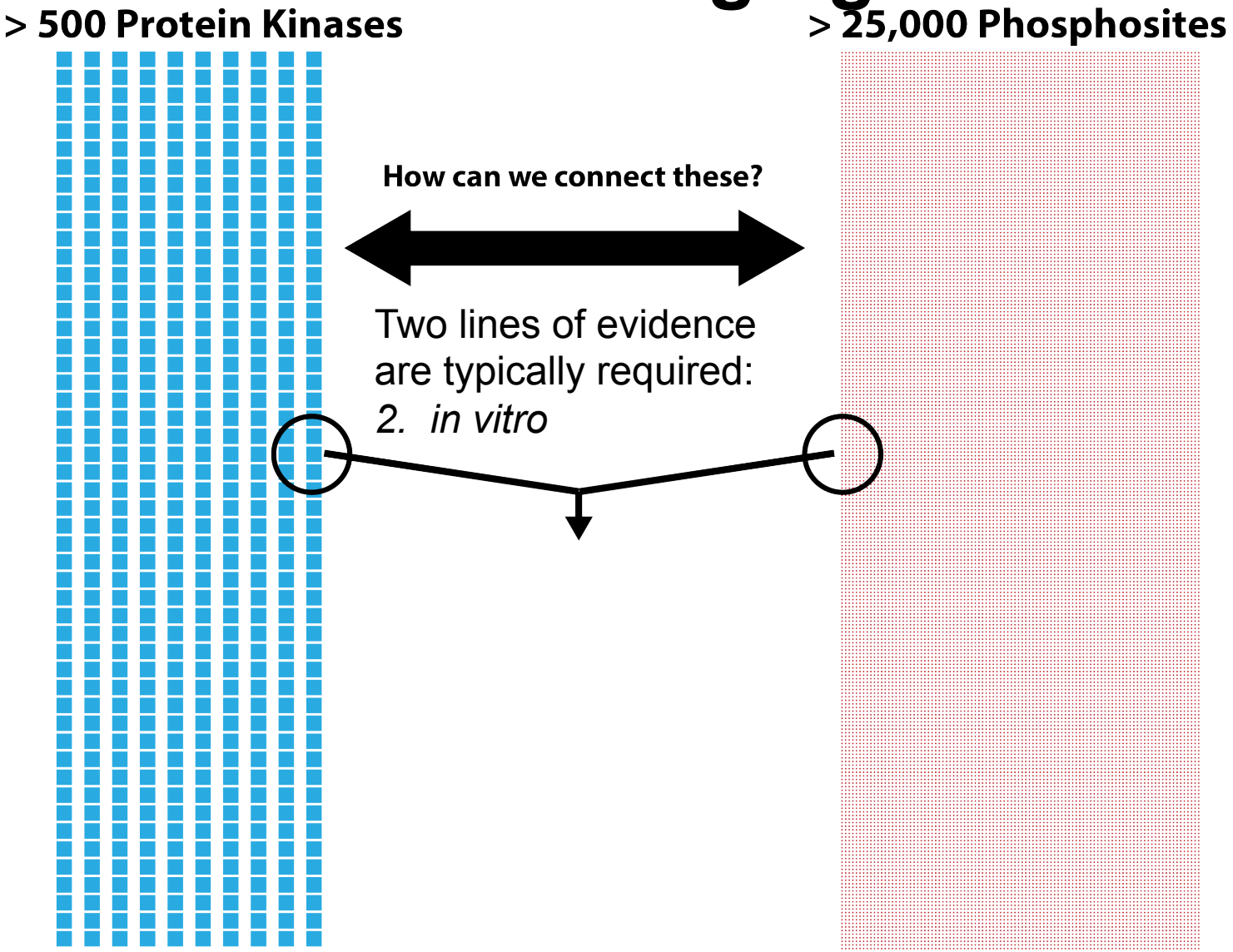
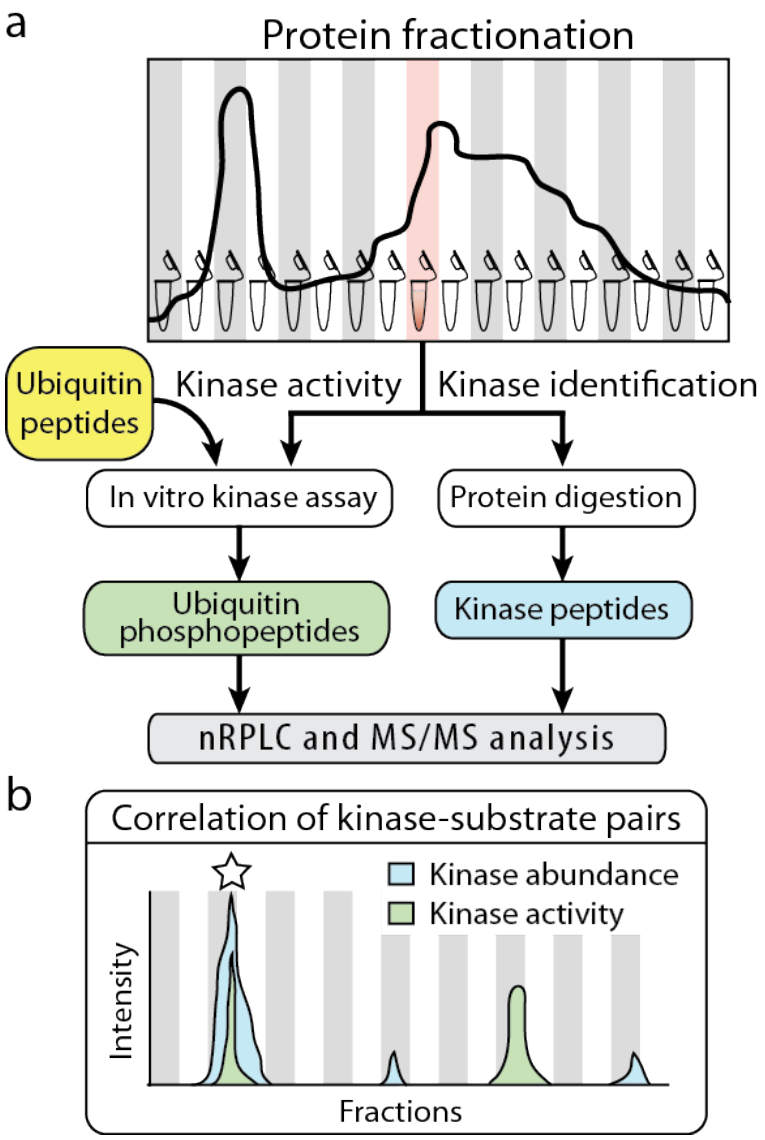


Kinase activity profiling approach



Kubota, K. *et al.* Sensitive multiplexed analysis of kinase activities and activity-based kinase identification. *Nat Biotechnol* **27,** 933–940 (2009)

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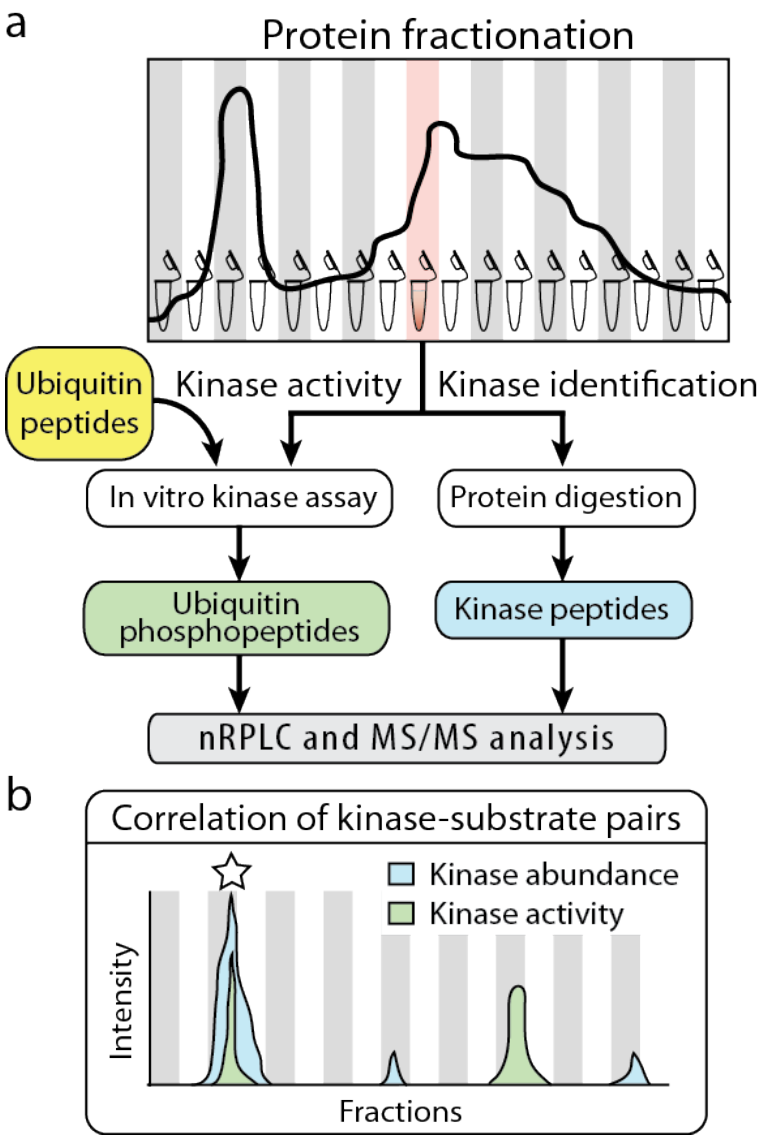
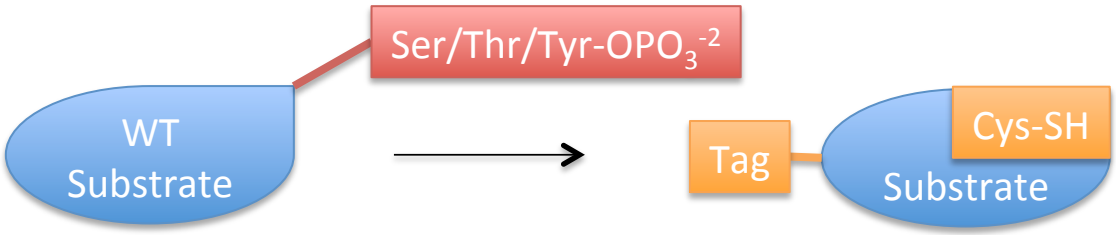


PROS: Un-biased
CONS: labor intensive, high-false positive

Kubota, K. *et al.* Sensitive multiplexed analysis of kinase activities and activity-based kinase identification. *Nat Biotechnol* **27,** 933–940 (2009)

Chemical Biology approach (Shokat method)



Riel-Mehan, M. M. & Shokat, K. M. A Crosslinker Based on a Tethered Electrophile for Mapping Kinase-Substrate Networks. *Chemistry & Biology* **21,** 585–590 (2014).

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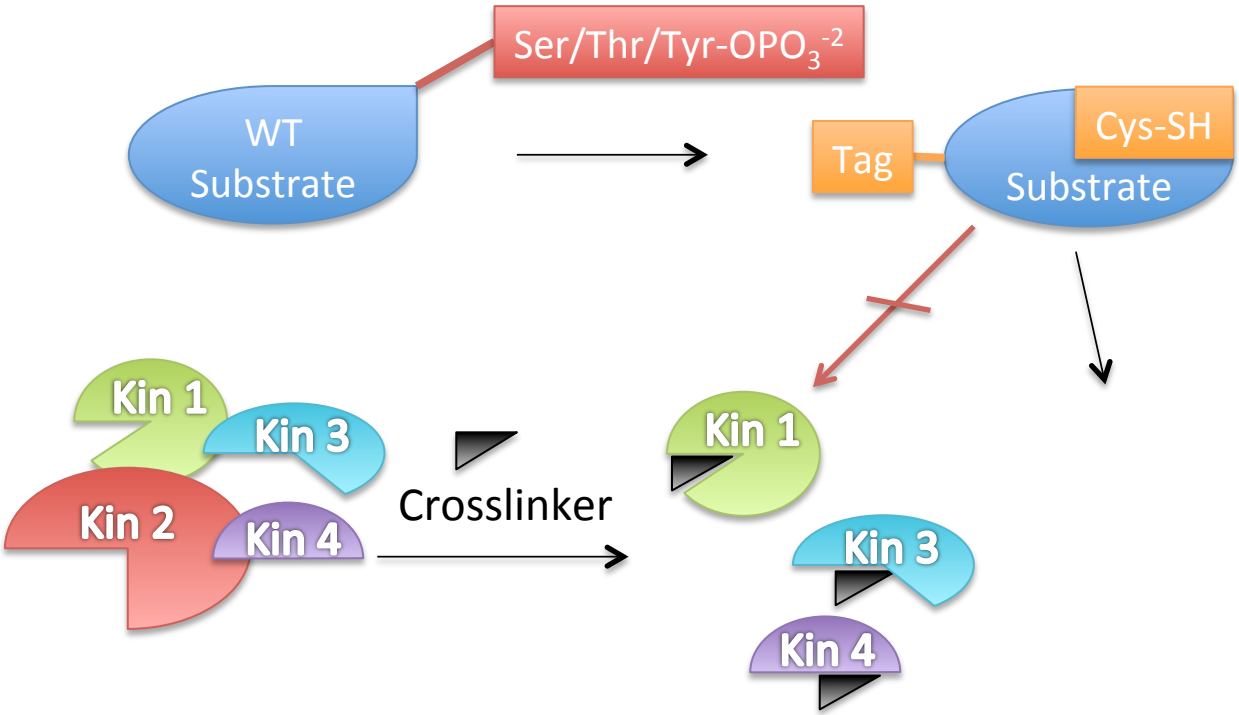

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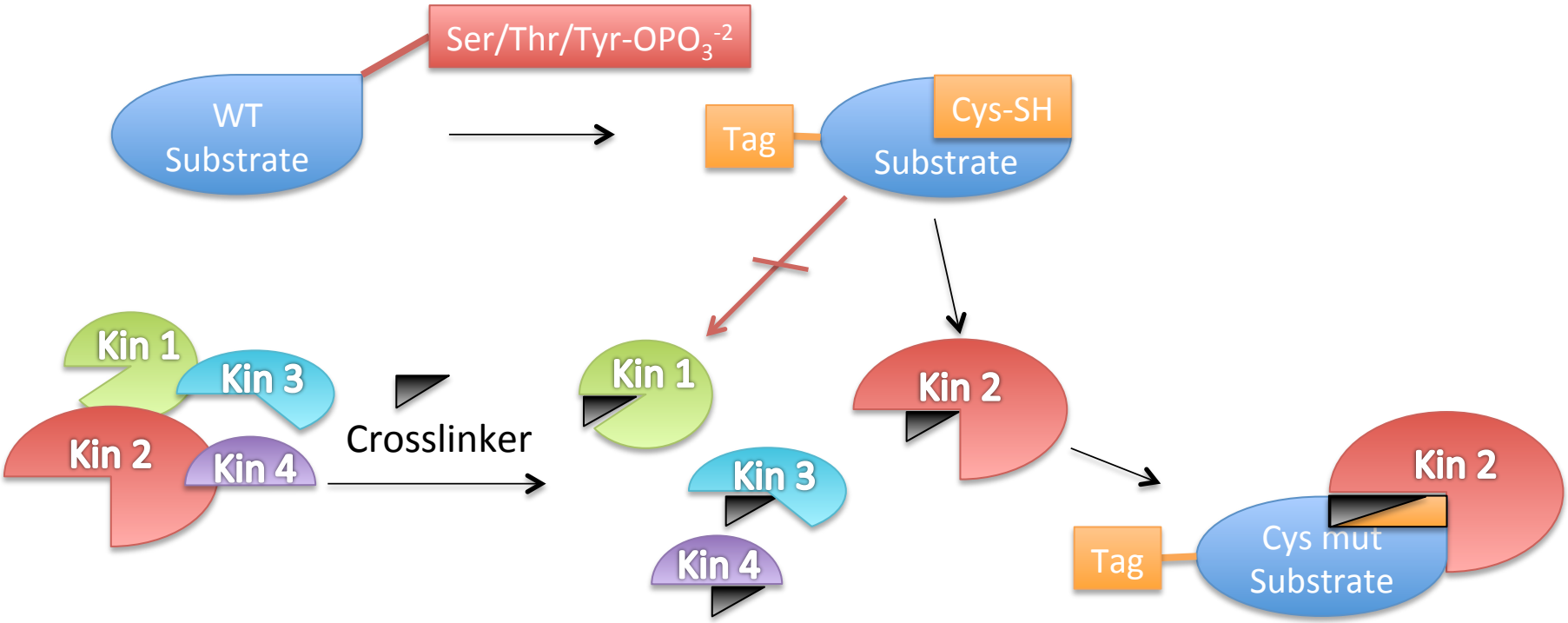

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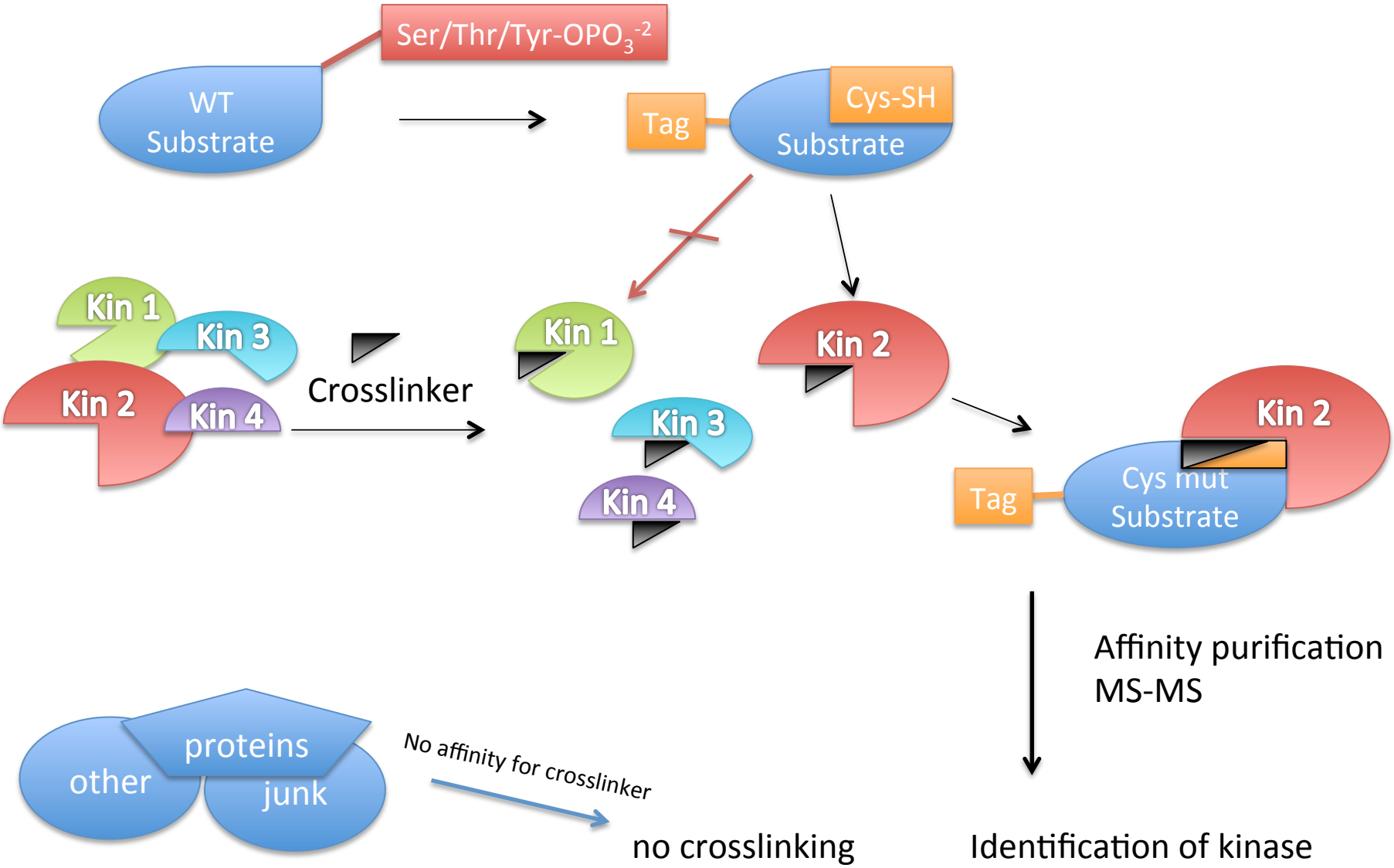

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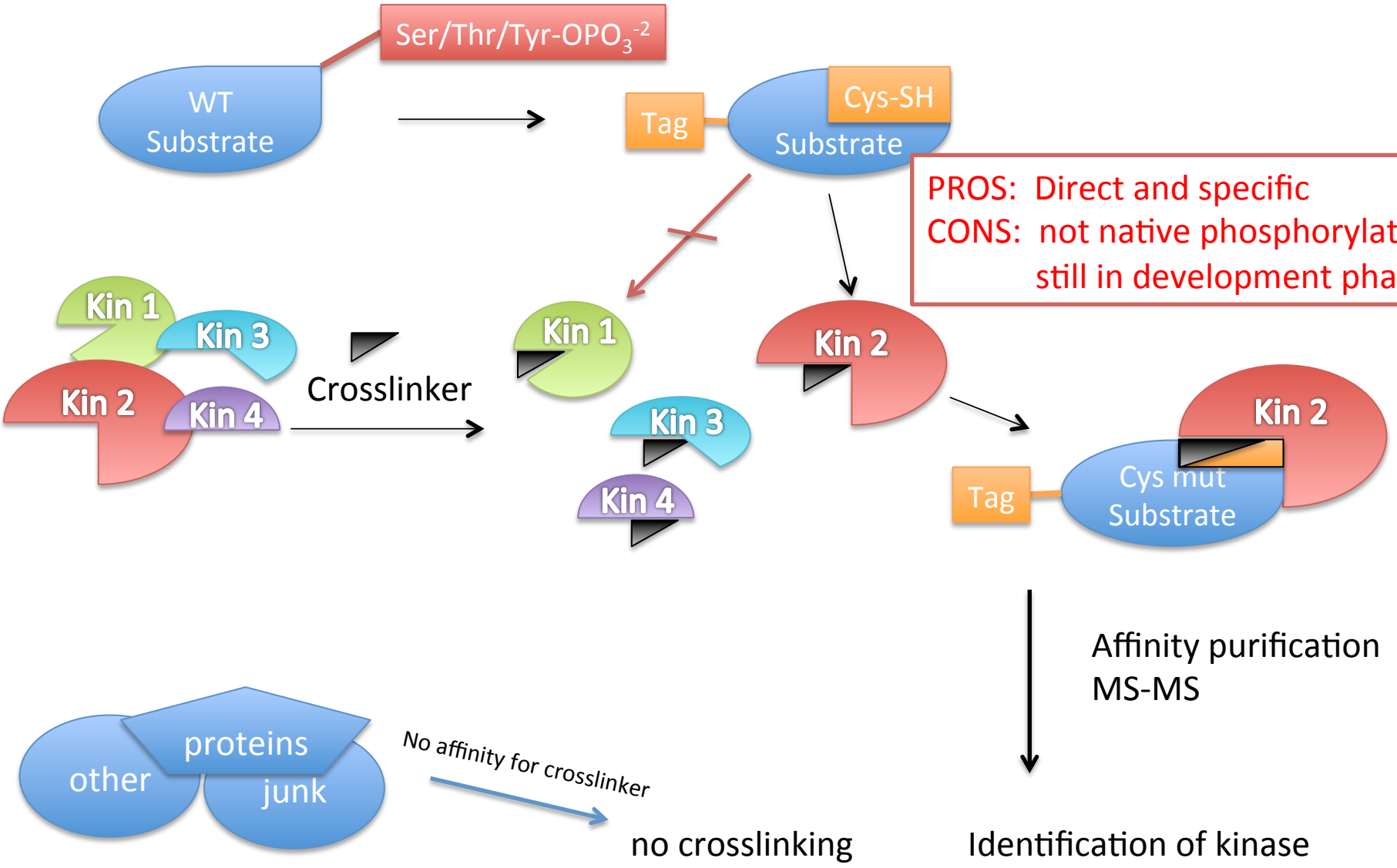
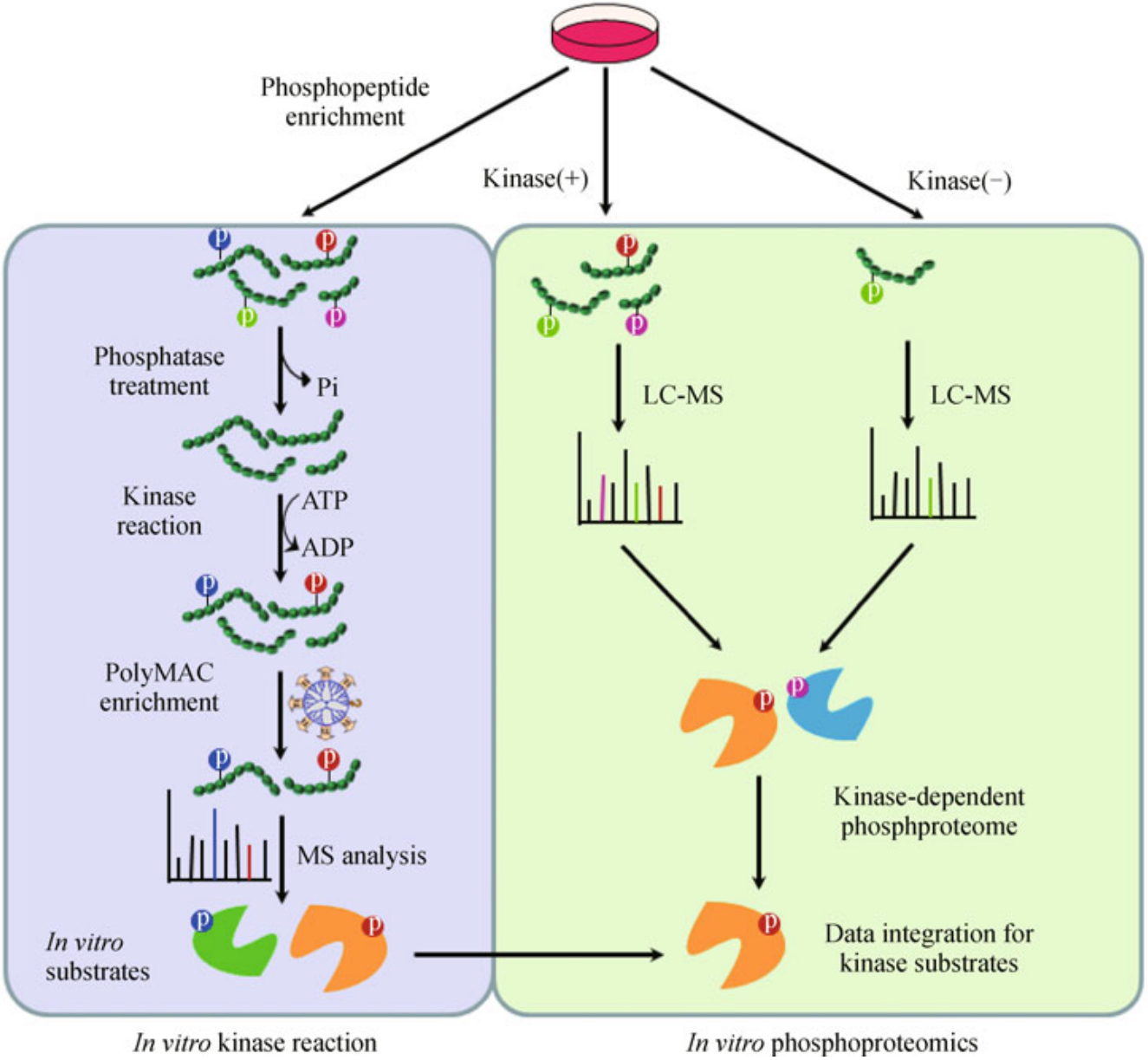


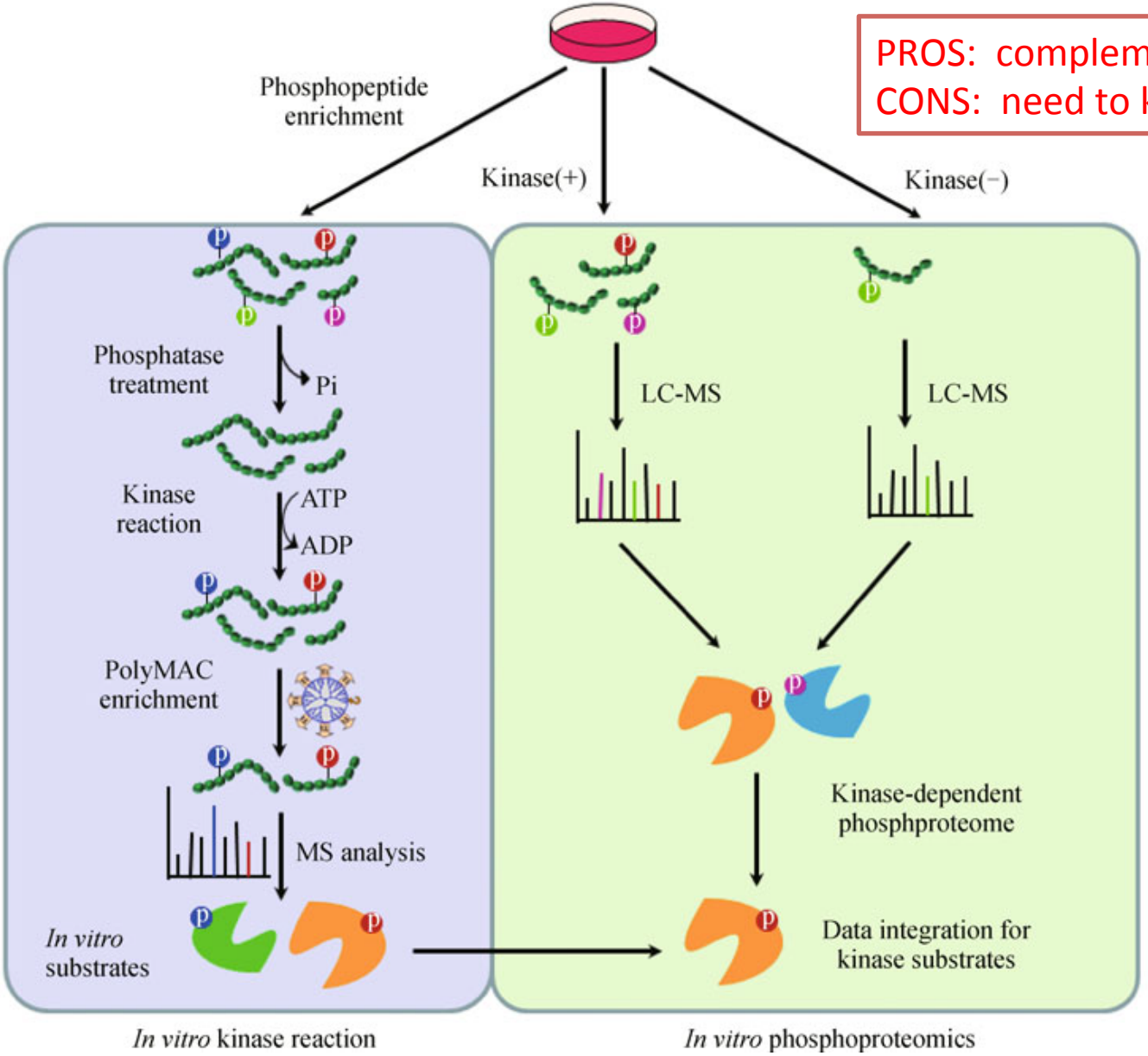
Kinase directed approaches



Xue, L. & Tao, W. A. Current technologies to identify protein kinase substrates in high throughput. *Front. Biol.* **8,** 216–227 (2013).

Kinase directed approaches

PROS: complementary in vivo and in vitro
CONS: need to know kinase already



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Selection of kinases for this course: protein array approach

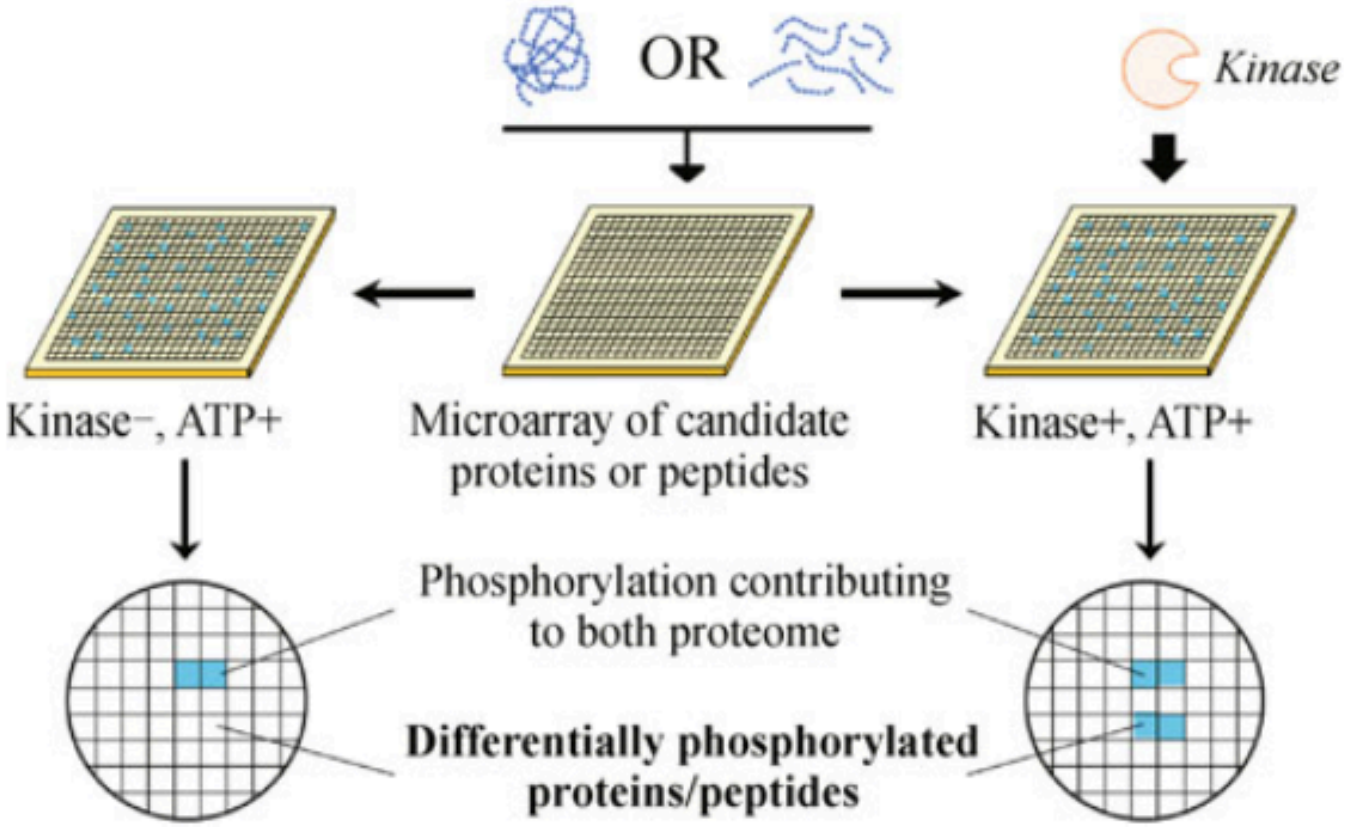

Figure 2 Kinase assay based on protein array or peptide array. Protein/peptide collections are spotted on the microarray, followed by the incubation with a purified active kinase under the reaction condition. Phosphorylation is detected by various methods.

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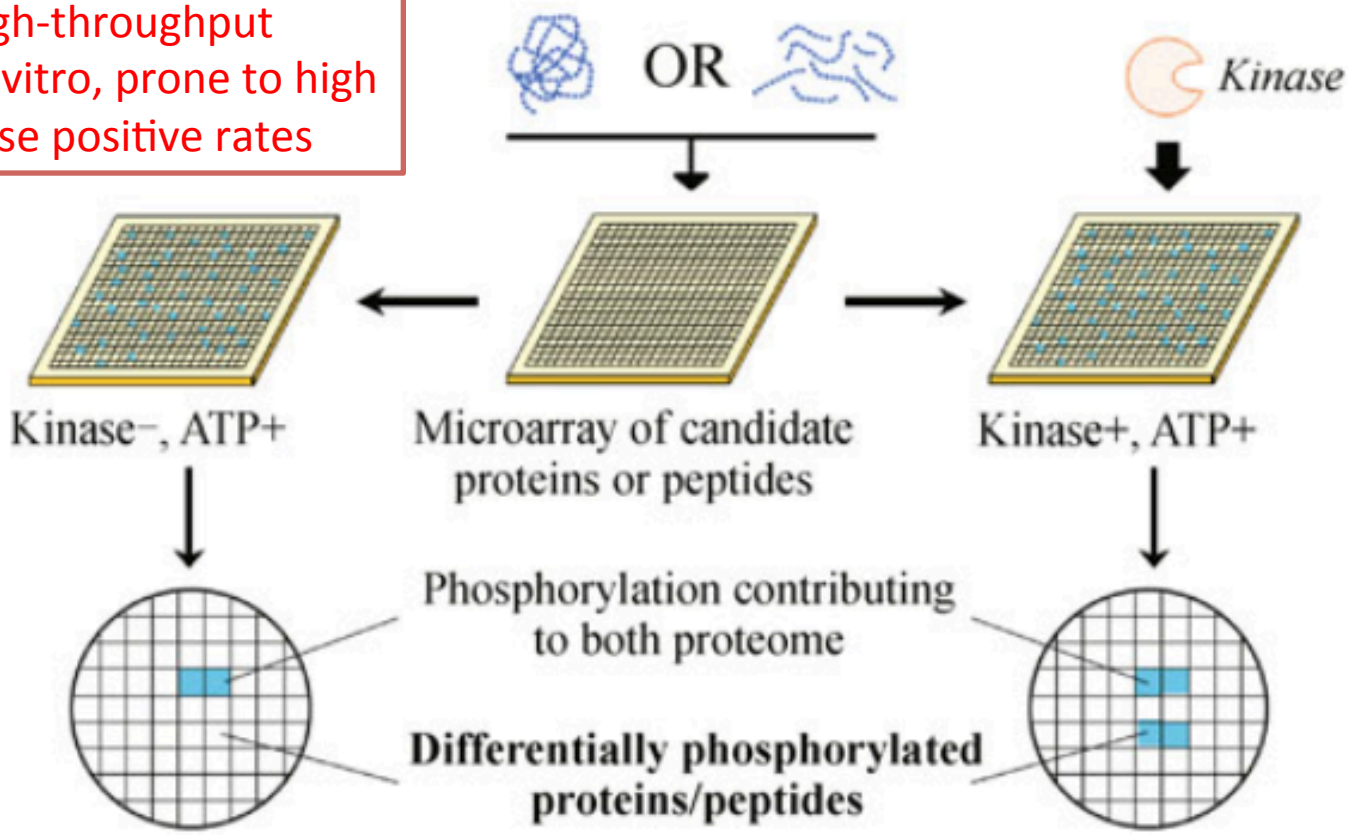
Newman, R. H. *et al.* Construction of human activity-based phosphorylation networks. *Molecular Systems Biology* **9,** 655–655 (2013).

Selection of kinases for this course: protein array approach

PROS: High-throughput
CONS: In vitro, prone to high false positive rates



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In short, well-established methods to map kinase-substrate relationship require one of the following:

(1) Prior knowledge of kinase
→ substrate hunting

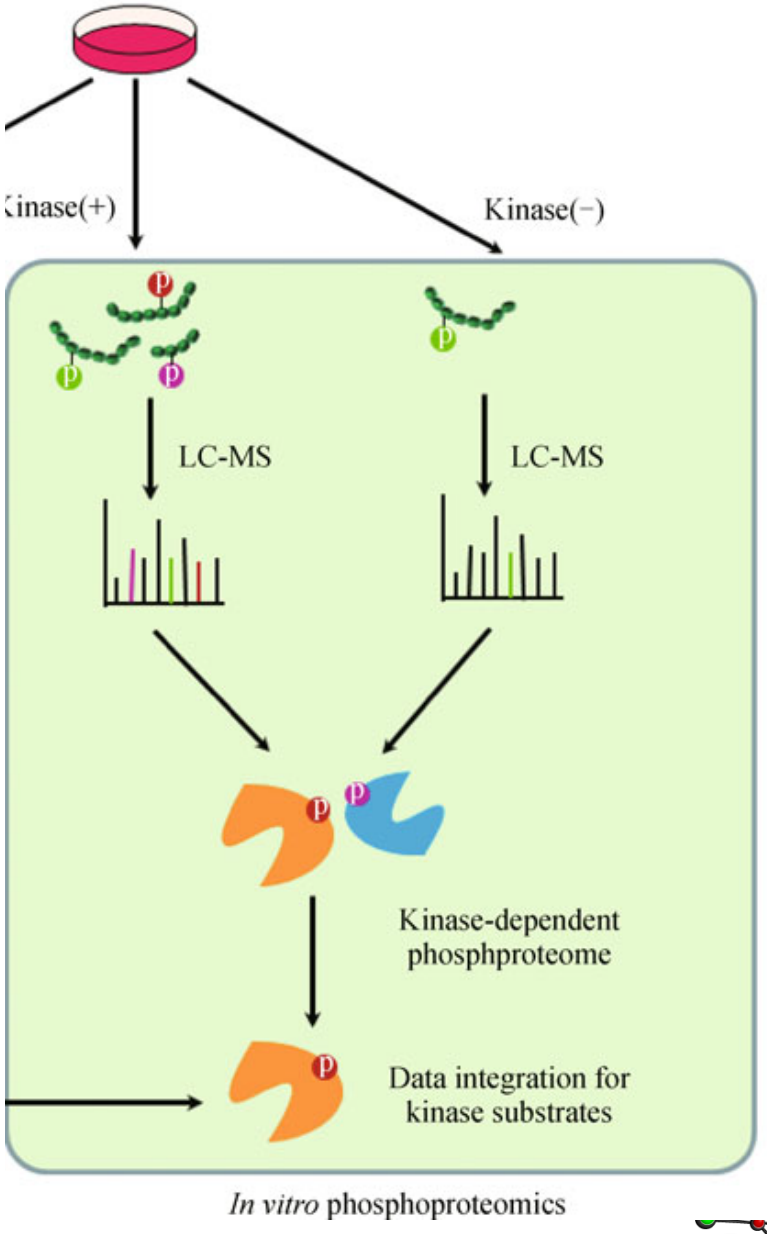
(2) A labor intensive brute force approach
→ kinase hunting

(3) Serendipitous luck

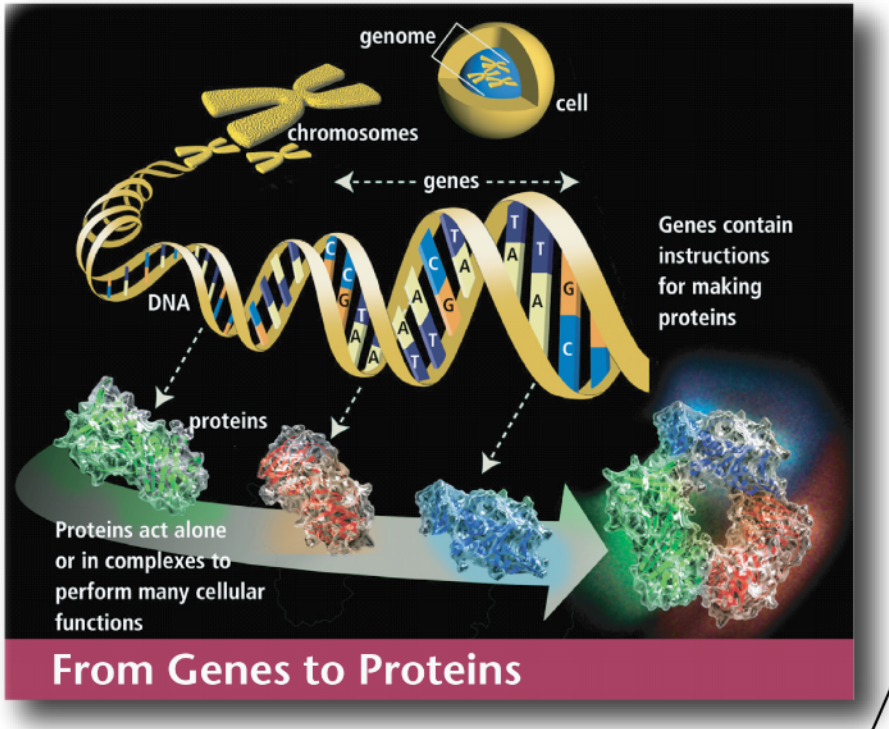
*****note other approaches do exist: phage display, yeast 2-hybrid, genetic interaction. But they all suffer from one of the primary CONS listed for methods described here.**

Selection of kinases for this course: protein array results directing a kinase-directed approach

Paper	Human Kinase	Yeast kinase?
Human kinase array	NEK2	KIN3
Human kinase array and YEAST kinase array	WEE1	SWE1
Yeast kinase array		CMK1
Yeast kinase array		TPK1
Yeast kinase array		ALK1
Yeast kinase array (Youle autophagy paper connected Pink1, phosphoubiquitin, and autophagy signaling)		ATG1



From genes to proteins



www.doegenomes.org

- The human genome codes ~ 25,000 proteins
  after modification > 500,000
- the human body consists of 10^{14} cells
- each cell makes ~ 15,000 different proteins

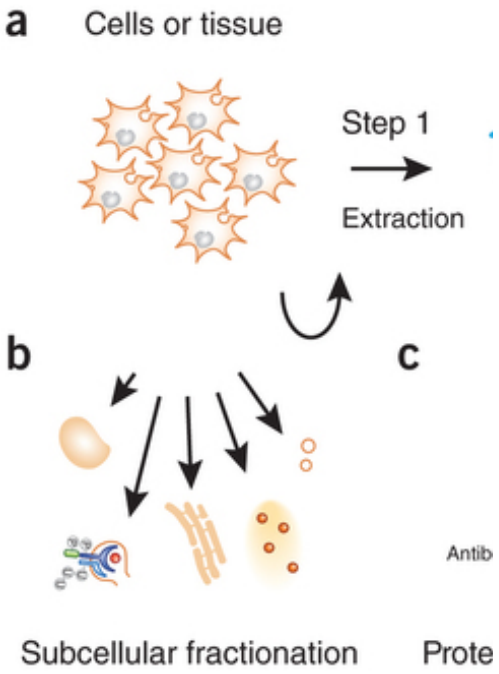
- *what are they?*
- *where are they?*
- *how many copies are present?*
- *what is their function?*
- *when are they made?*
- *what proteins do they interact with?*
- *how are they modified?*

Mass spectrometry based proteomics

Velos Orbitrap

Orbitrap Fusion

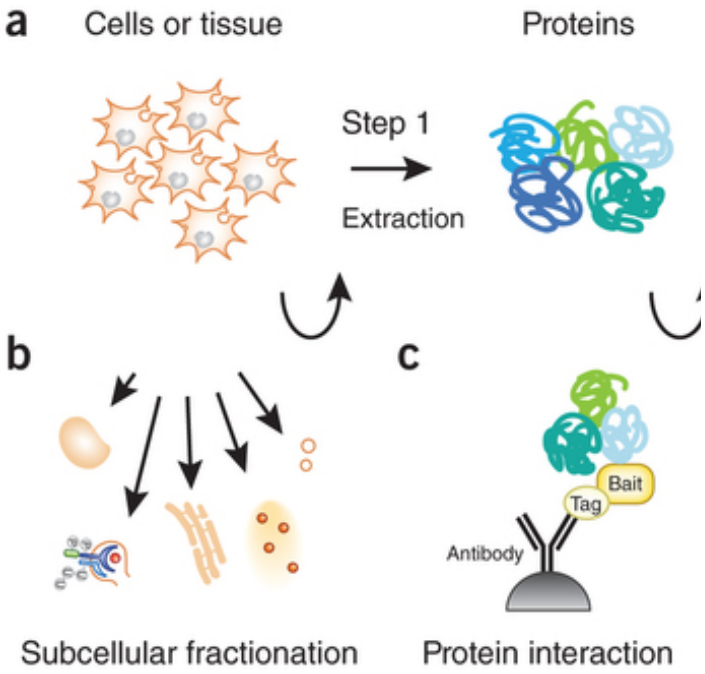
Proteomics Workflow



Considerations:

- Qualitative: what proteins are there?
- Quantitative: What differences in proteins or PTMs between conditions?
  - Different cell types
  - Kinase KO
  - Chemical perturbation
  - Etc.

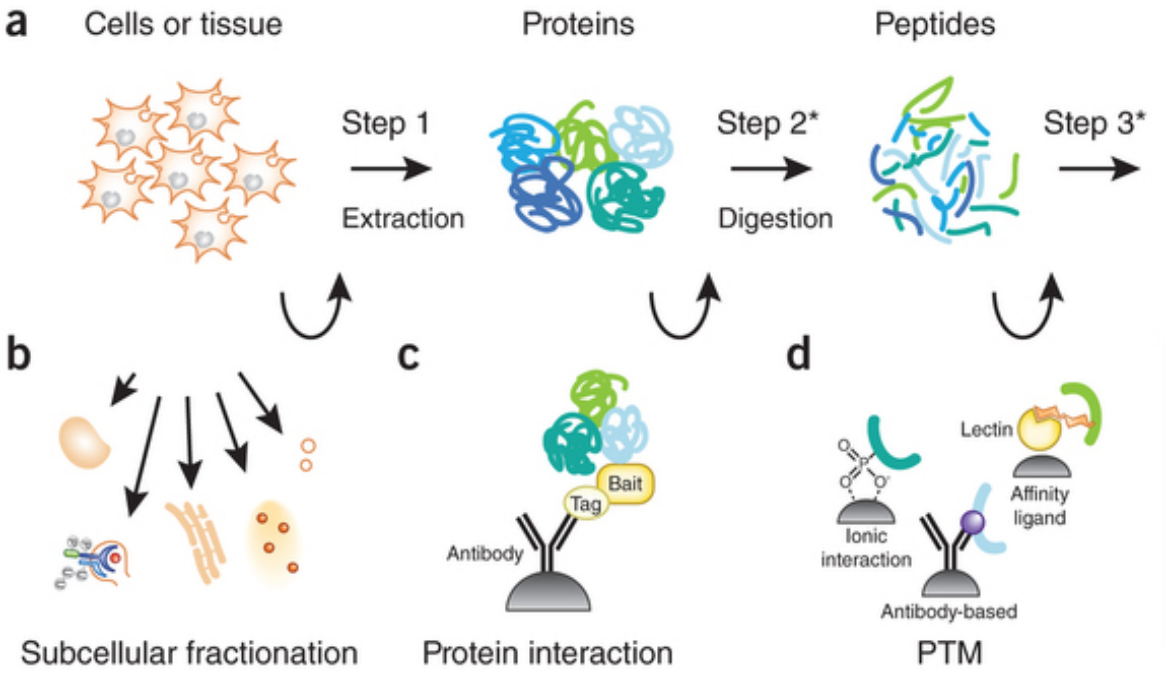
Proteomics Workflow



Considerations:

- Native or denaturing?
- Protein purification
- PTM stability

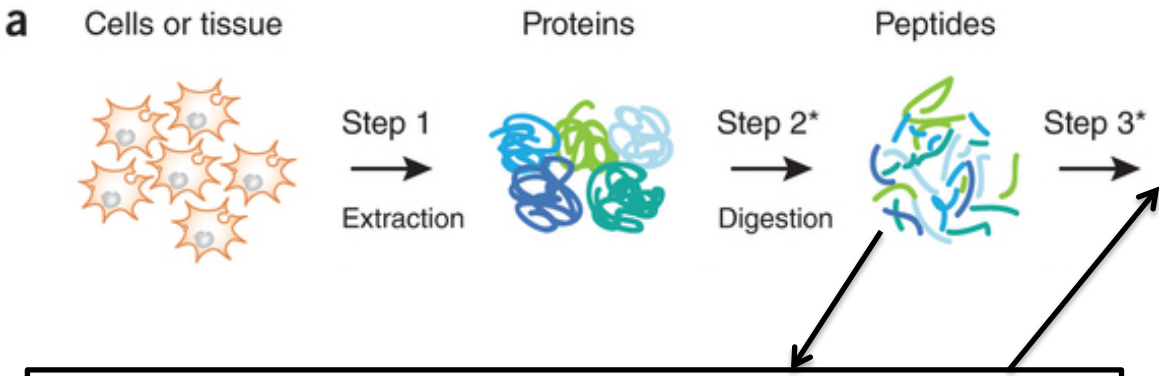
Proteomics Workflow



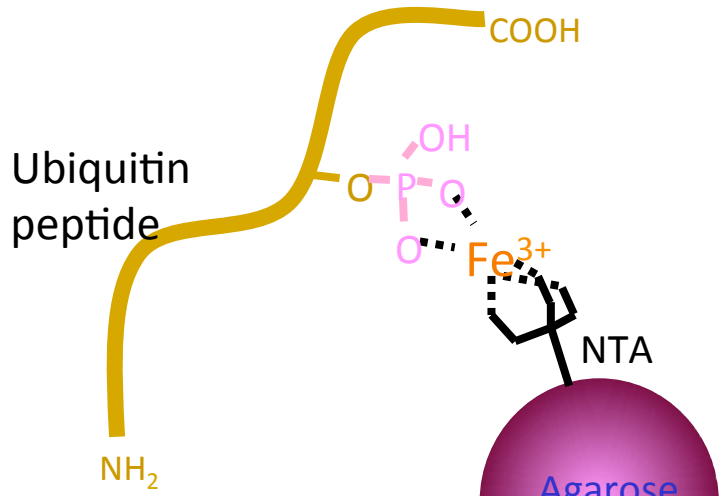
Considerations:

- PTM purification
- Fractionation to reduce complexity

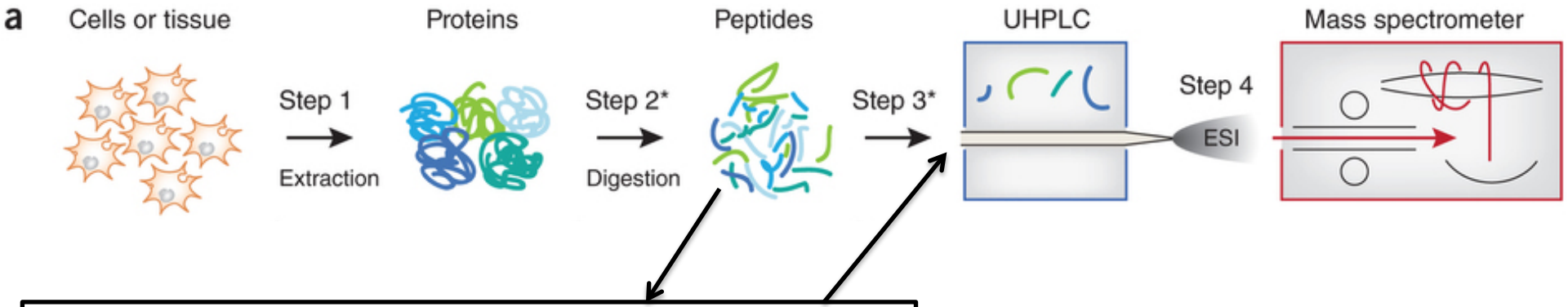
Proteomics Workflow



Phosphopeptide enrichment
Immobilized Metal Affinity Chromatography (IMAC)



Proteomics Workflow



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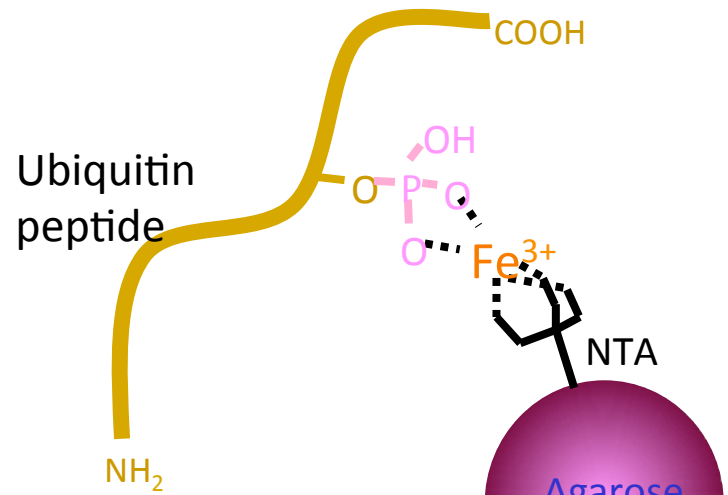

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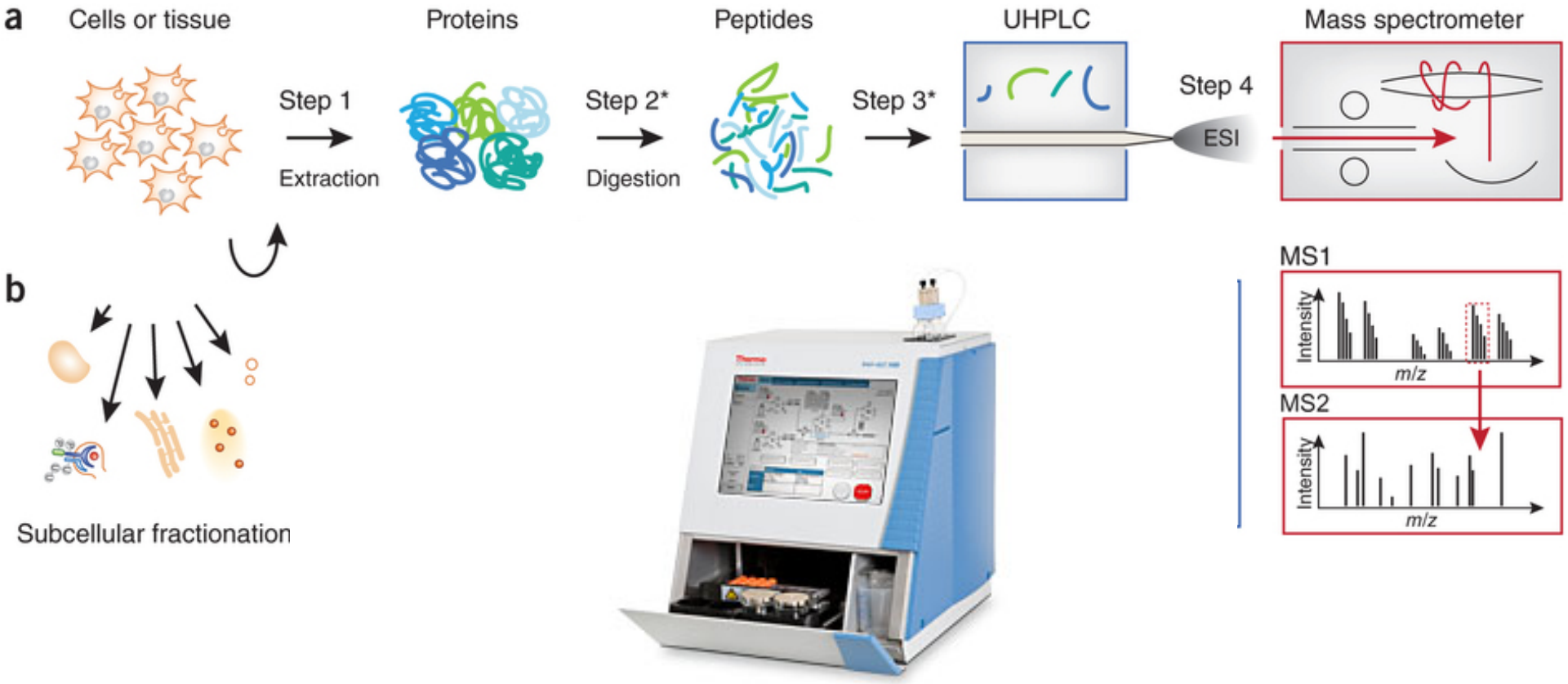
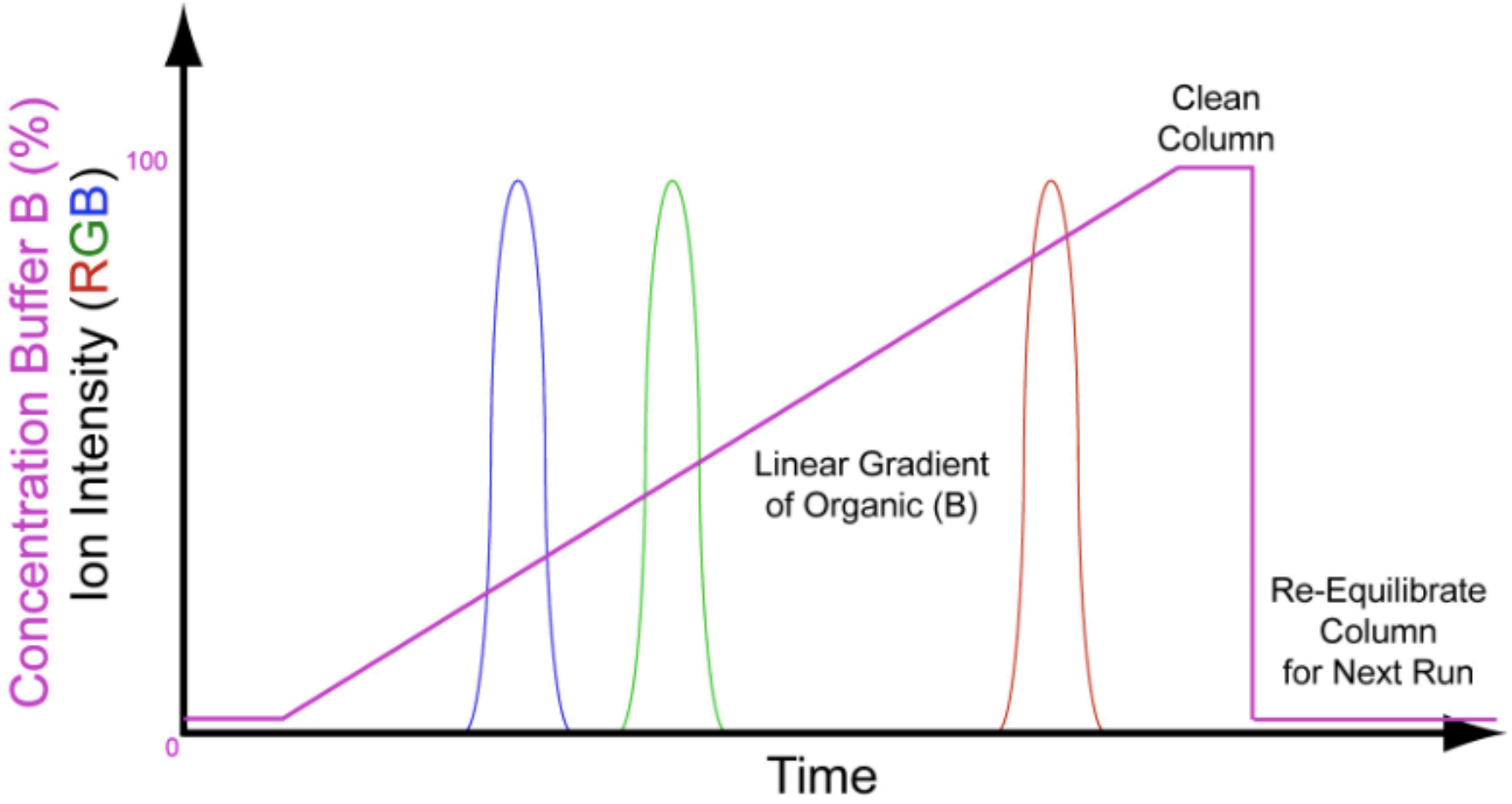
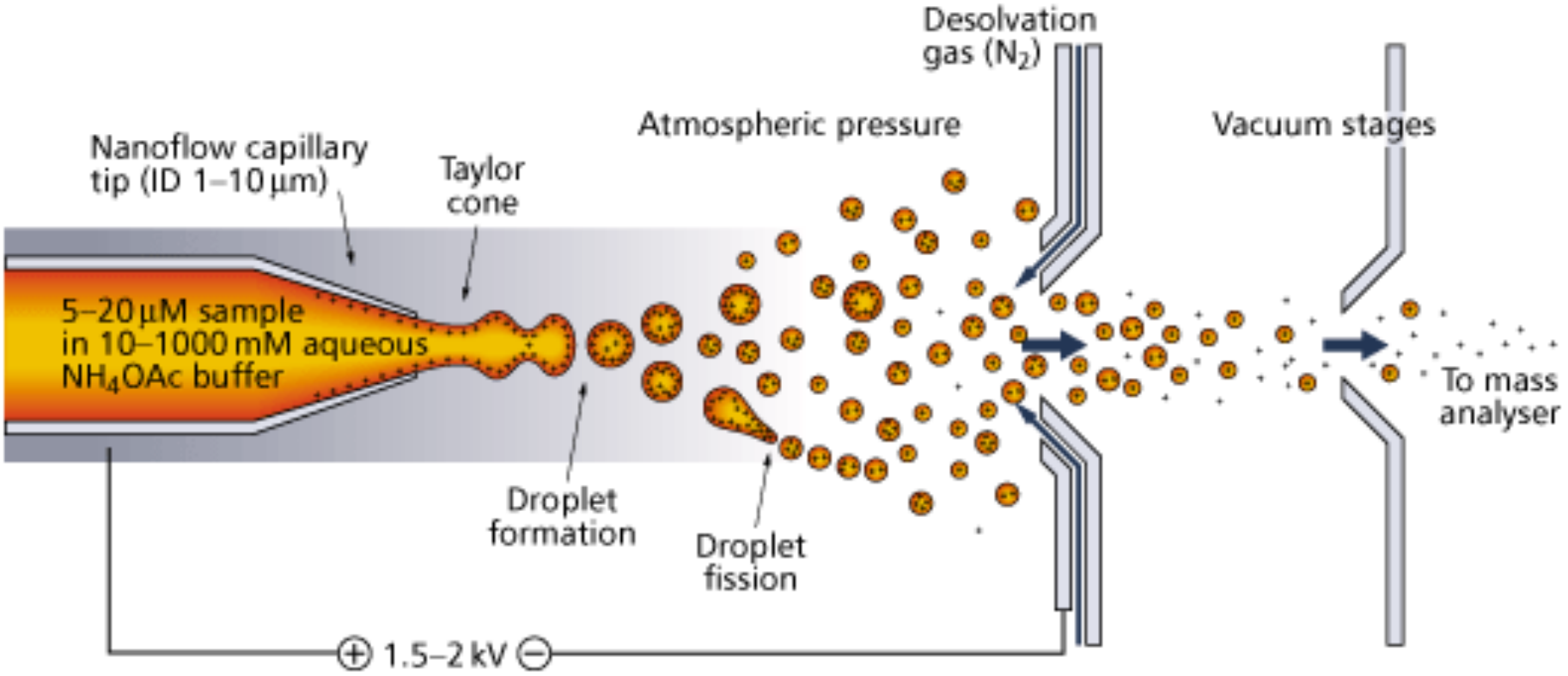


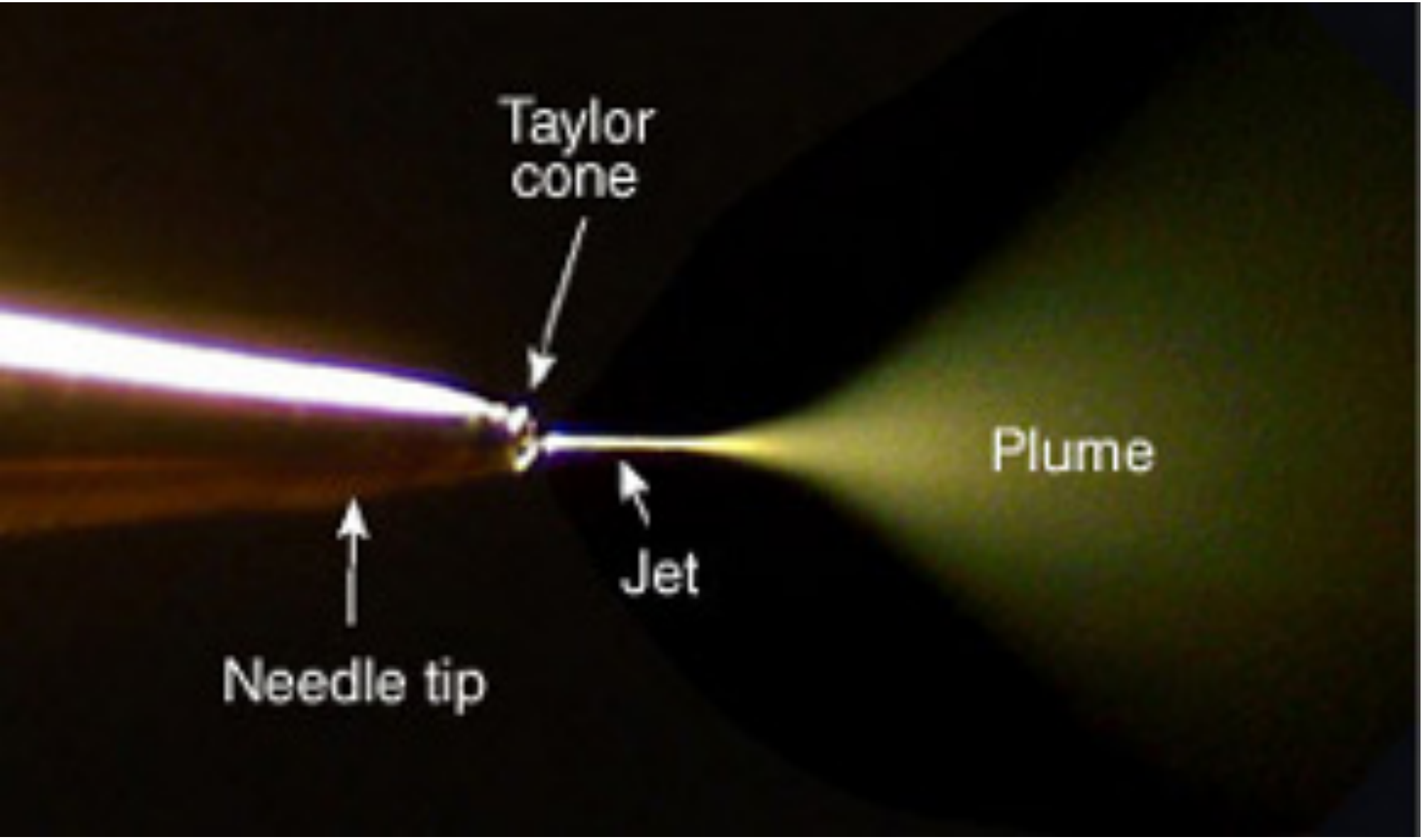
Reversed-Phase HPLC

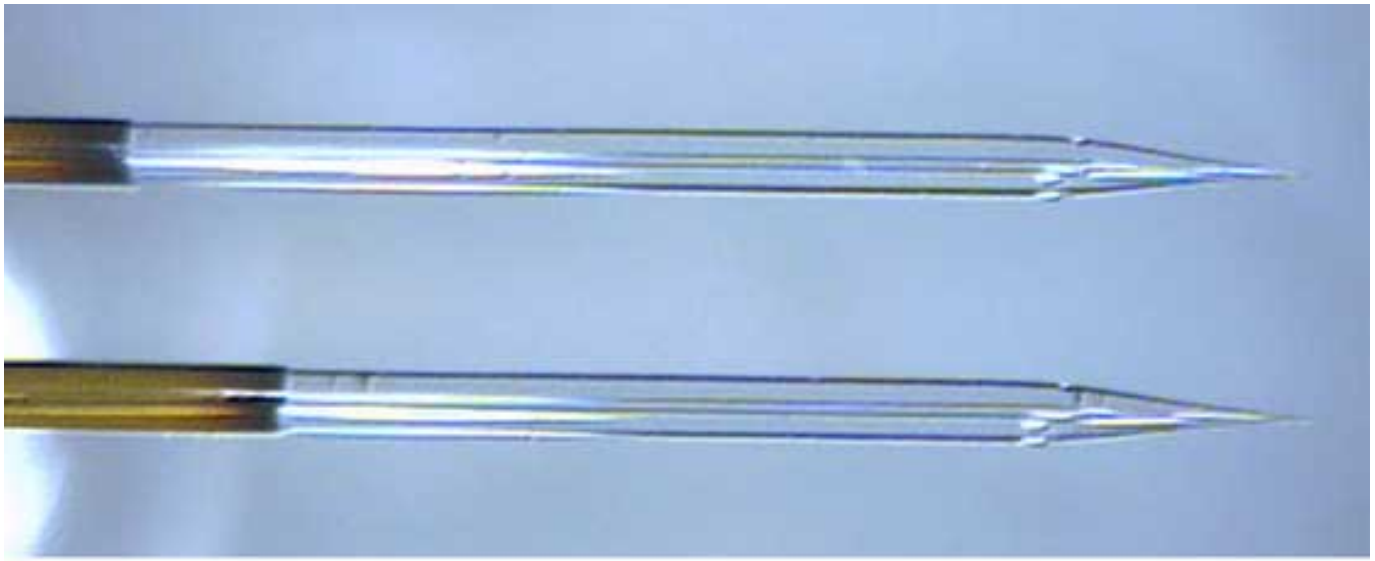
Load peptides: 100% water, 0.1% formic acid
Elute with increasing gradient of acetonitrile, 0.1% formic acid



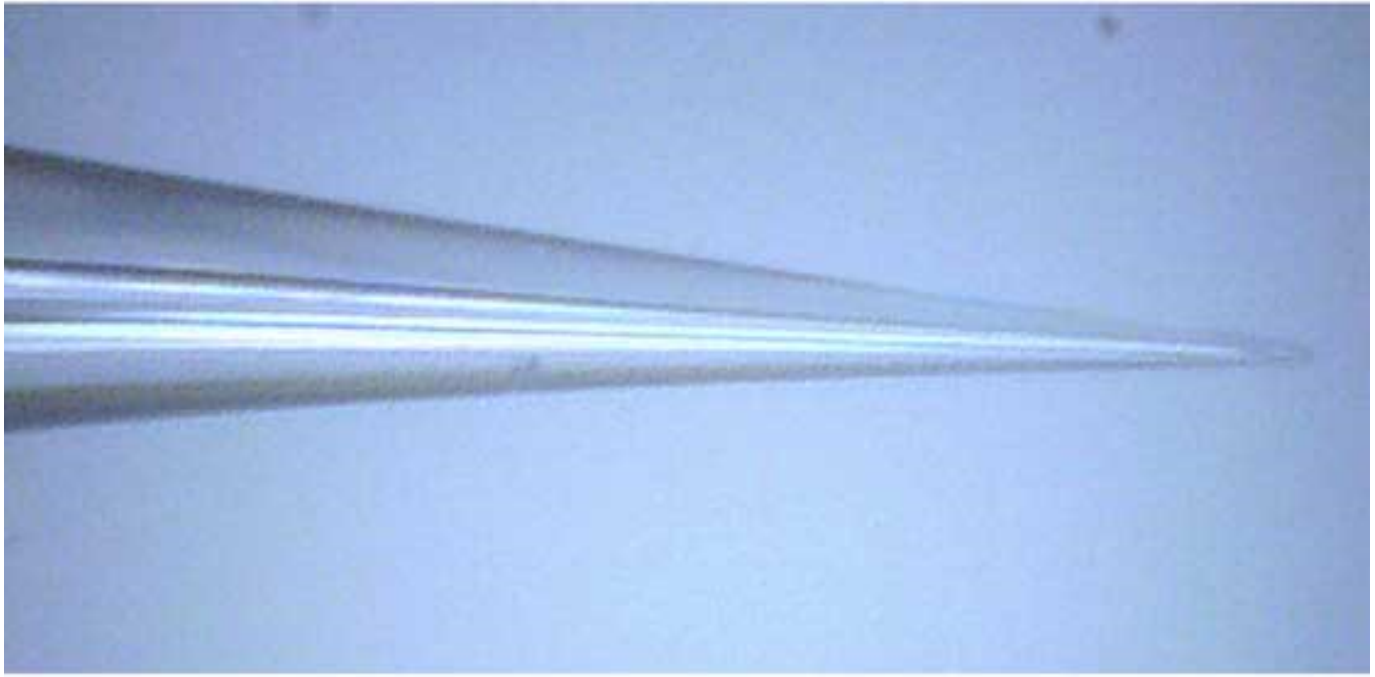
Getting your sample into mass spec – electrospray ionization




Taylor
cone
Plume
Jet
Needle tip

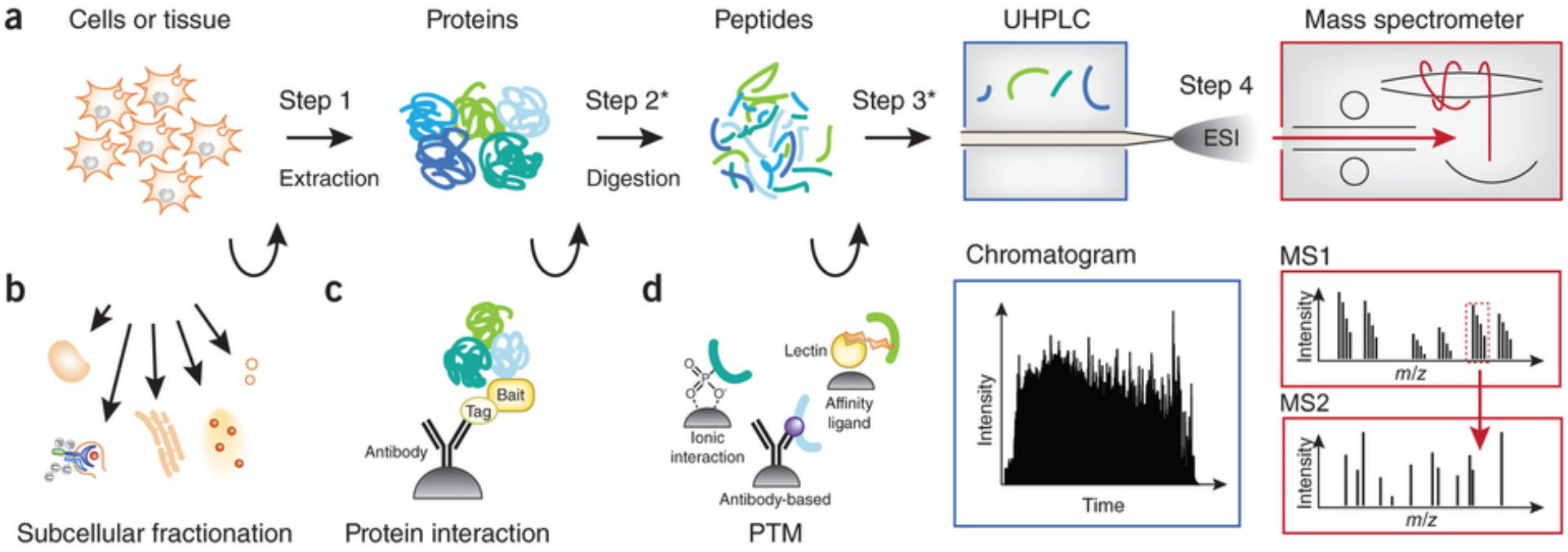


Electrospray Tip - 20x



Electrospray Tip - 400x

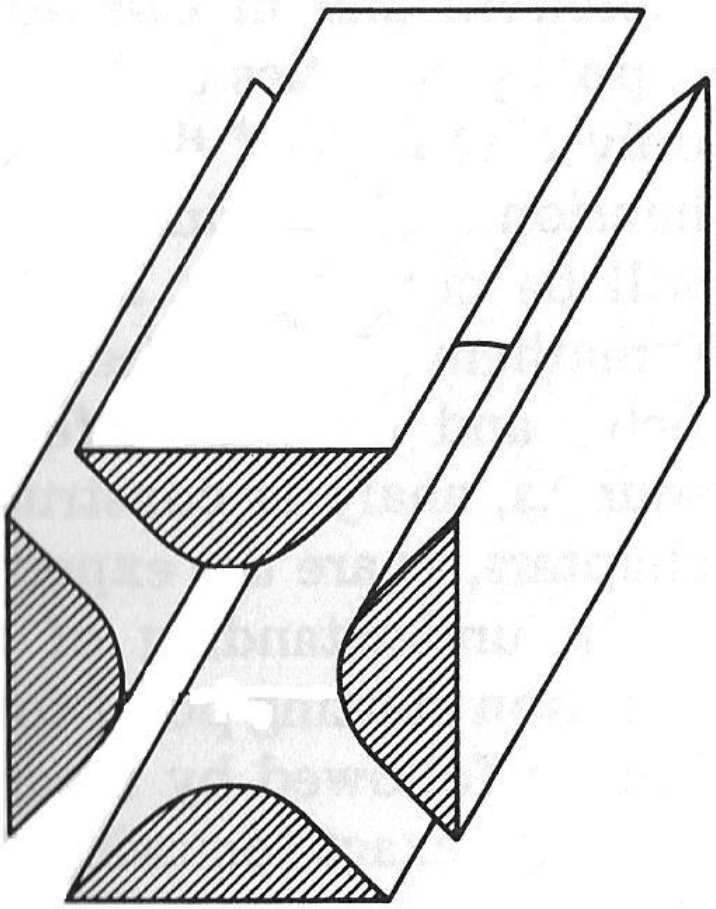
Proteomics Workflow

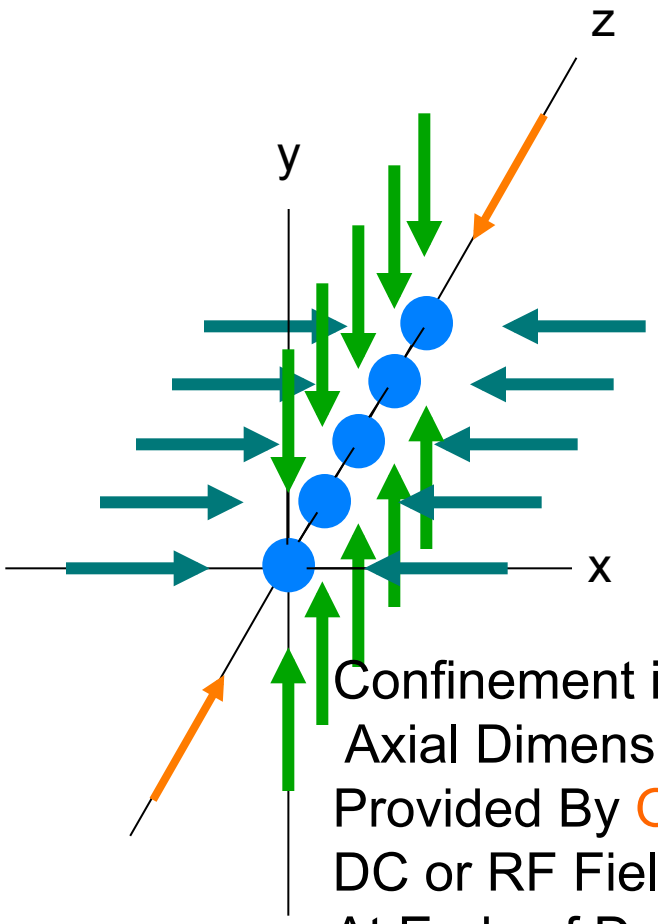


Considerations:

- Chemical nature of peptides of interest (PTM or un-modified)
- Complexity and dynamic range of mixture

RADIO FREQUENCY TWO DIMENSIONAL QUADUPOLE LINEAR ION TRAP

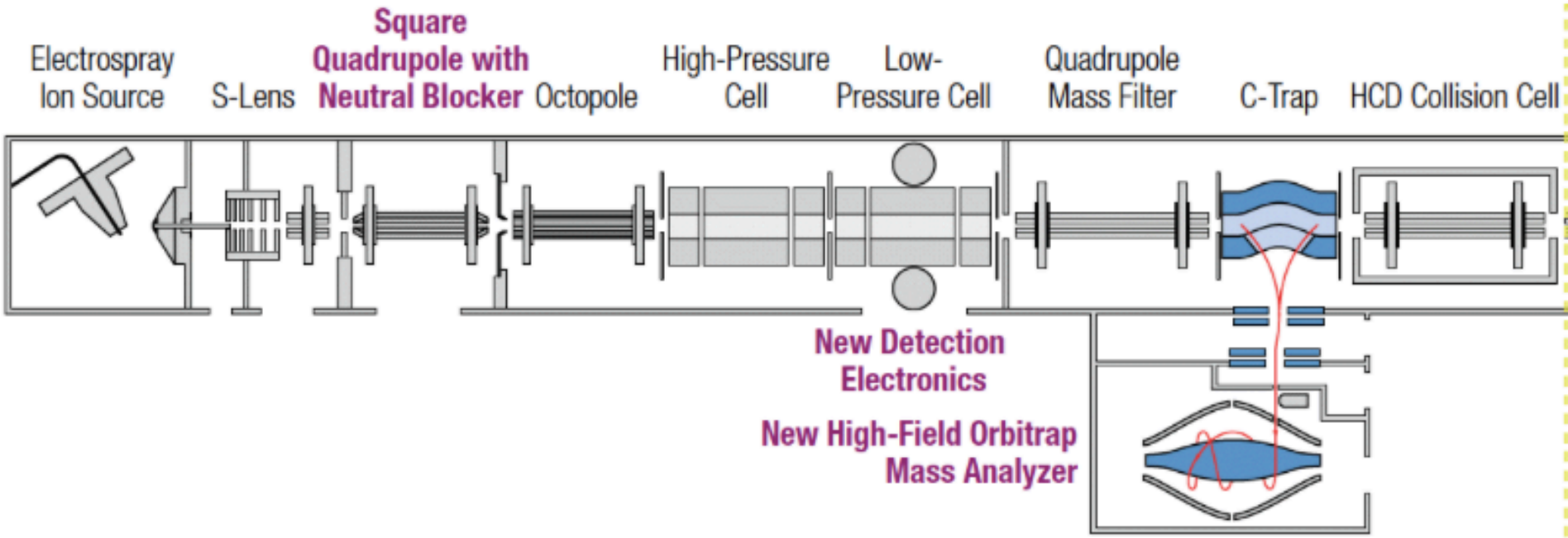


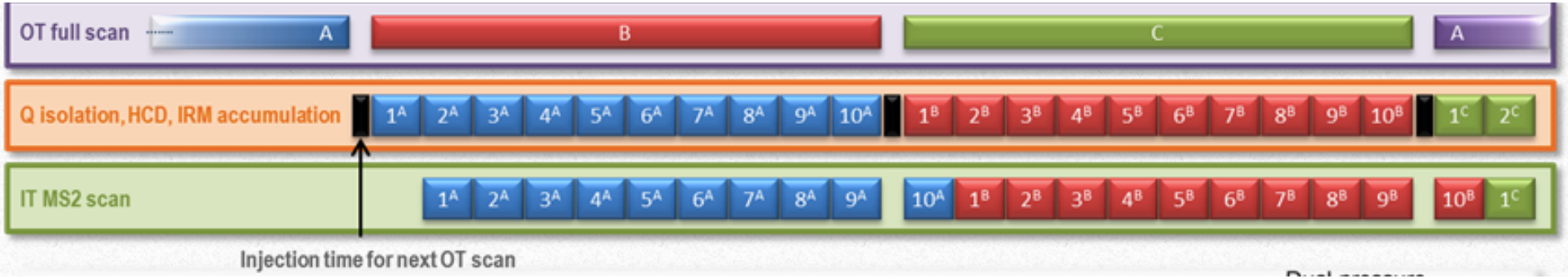


Confinement in Axial Dimension Provided By OTHER DC or RF Fields At Ends of Device

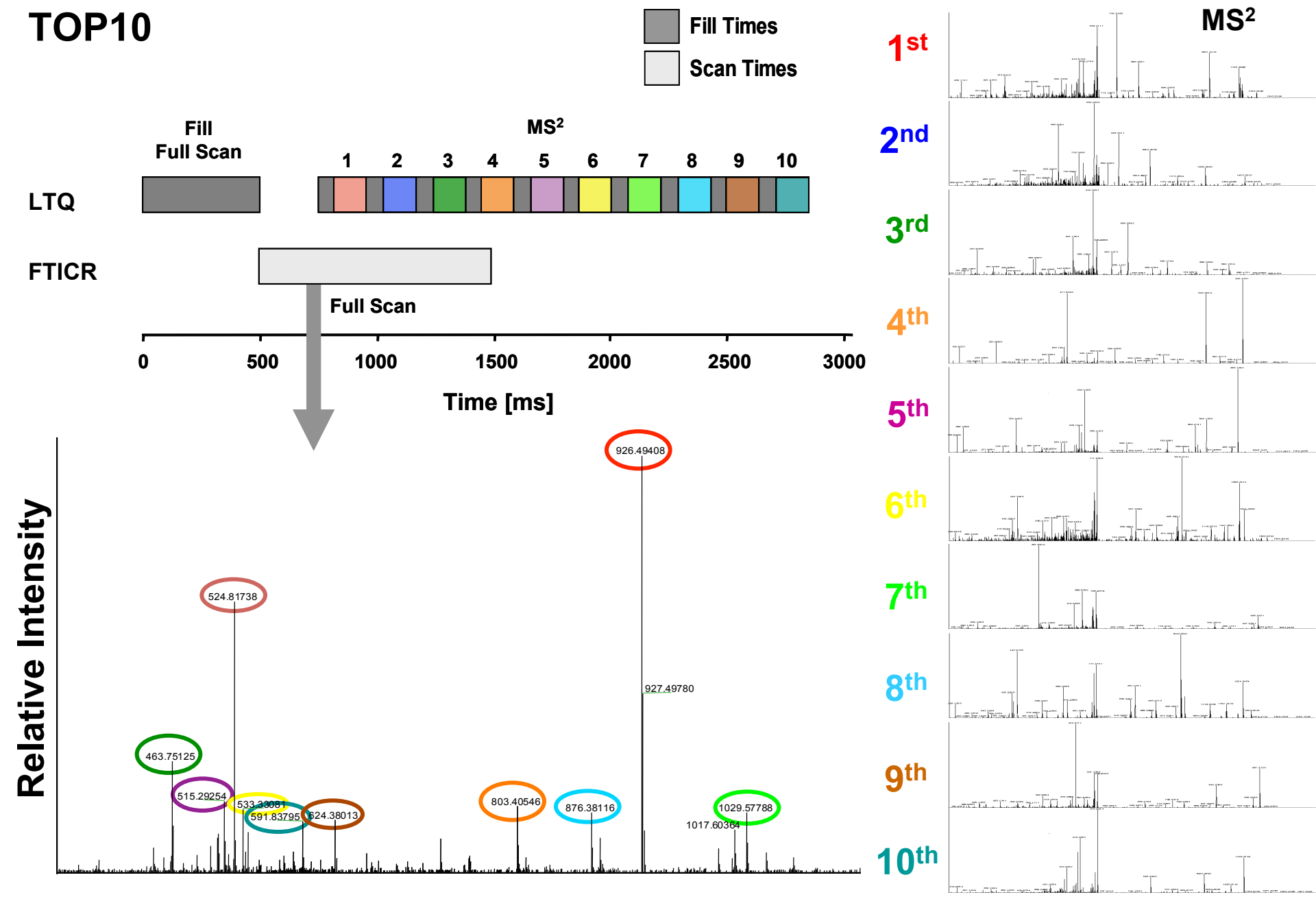
Figure From
Quadrupole Mass Spectrometry and Its Applications
P.H. Dawson Ed., Reprinted AIP Press 1995

Mass spec schematic and duty cycle

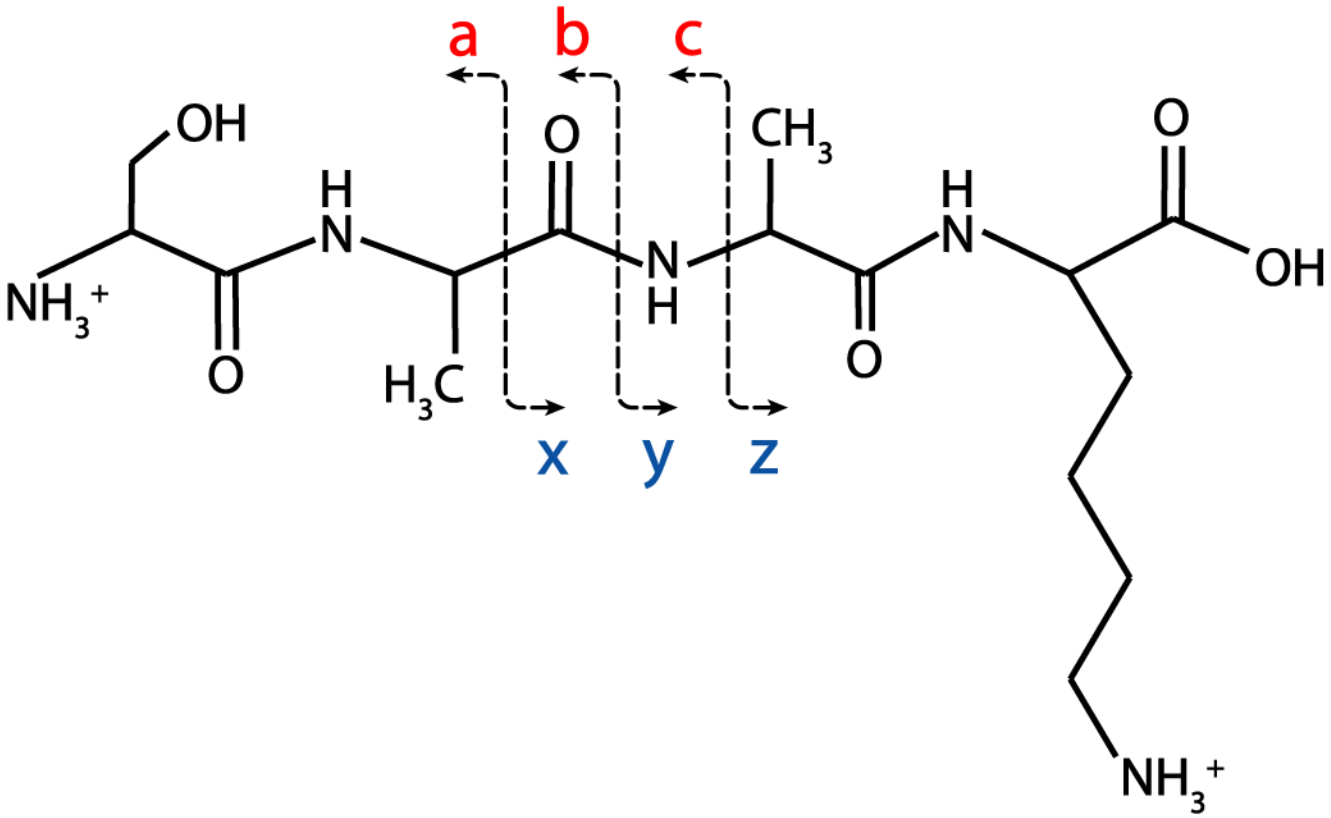


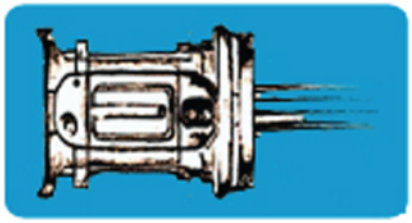


"shotgun sequencing"



Fragmentation nomenclature

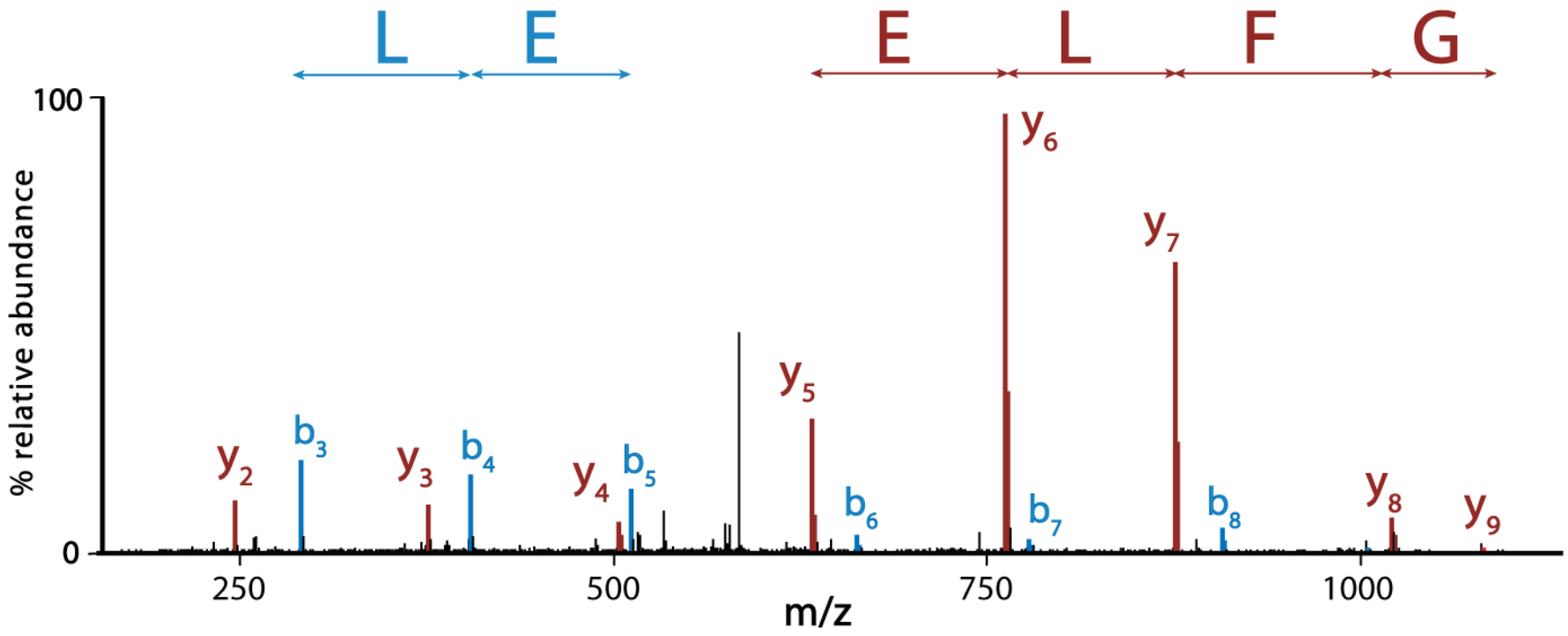




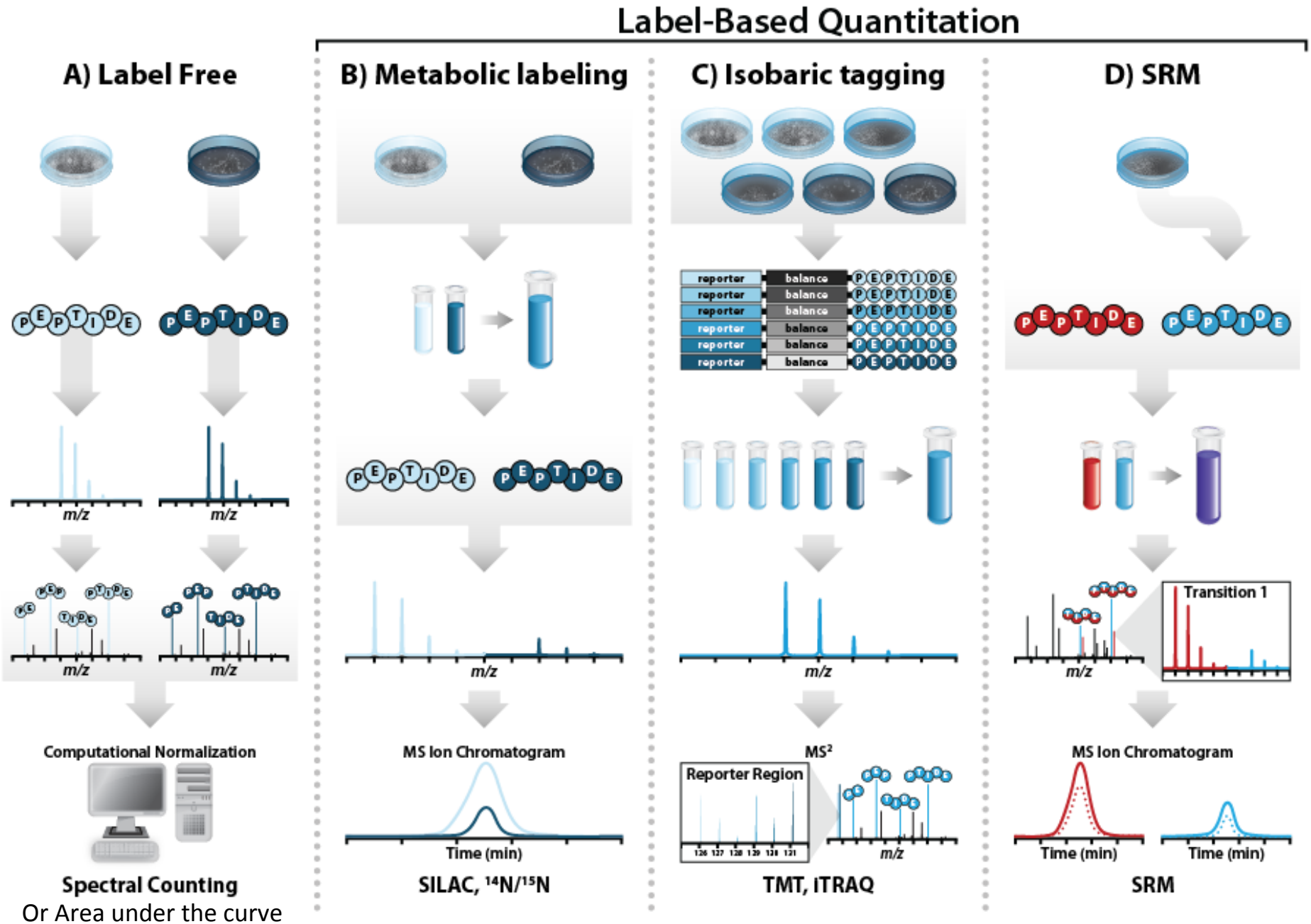
Peptide Sequencing (MS/MS)

collision-activated dissociation (CAD)

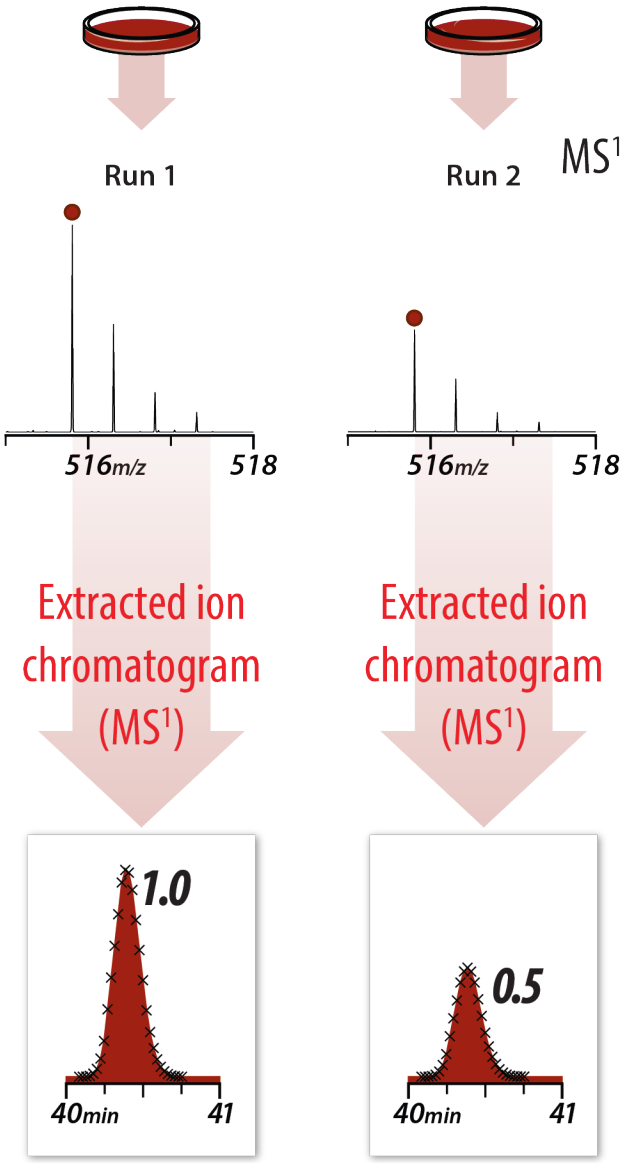
b^+	88	145	292	405	534	663	778	907	1020	1166
	S	G	F	L	E	E	D	E	L	K
y^+	1166	1080	1022	875	762	633	504	389	260	147



Mass spec operation animation:


Label-Based Quantitation
A) Label Free
B) Metabolic labeling
C) Isobaric tagging
D) SRM
PEPTIDE
m/z
reporter
balance
Computational Normalization
MS Ion Chromatogram
Time (min)
MS²
Reporter Region
Transition 1
Spectral Counting
Or Area under the curve
SILAC, ¹⁴N/¹⁵N
TMT, iTRAQ
SRM

Label free quantitation AUC



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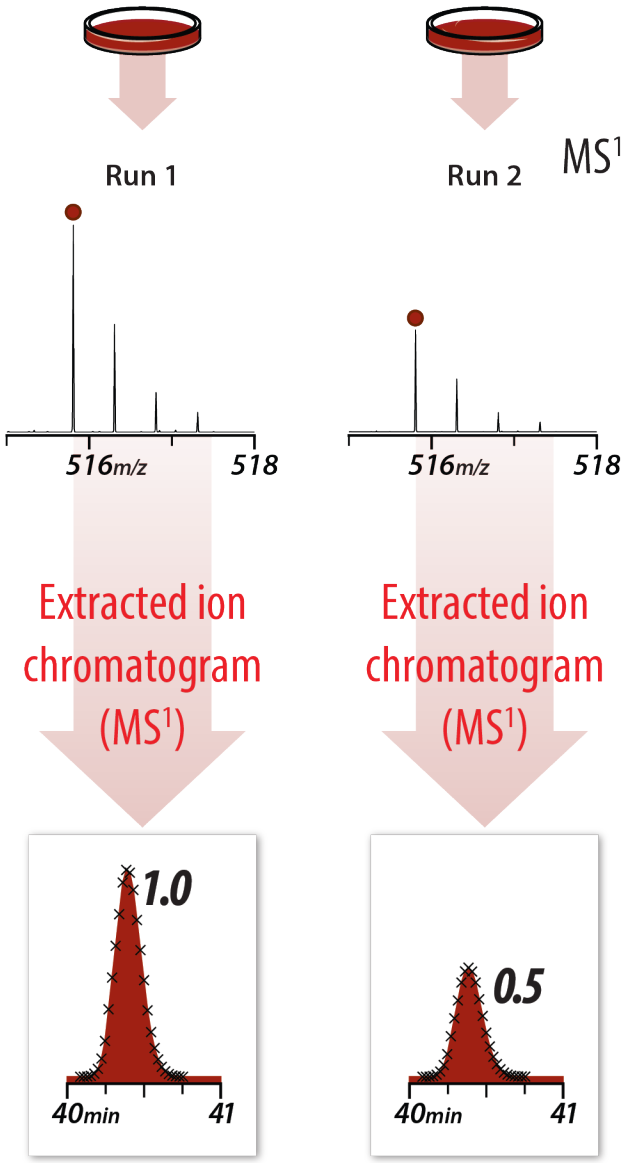
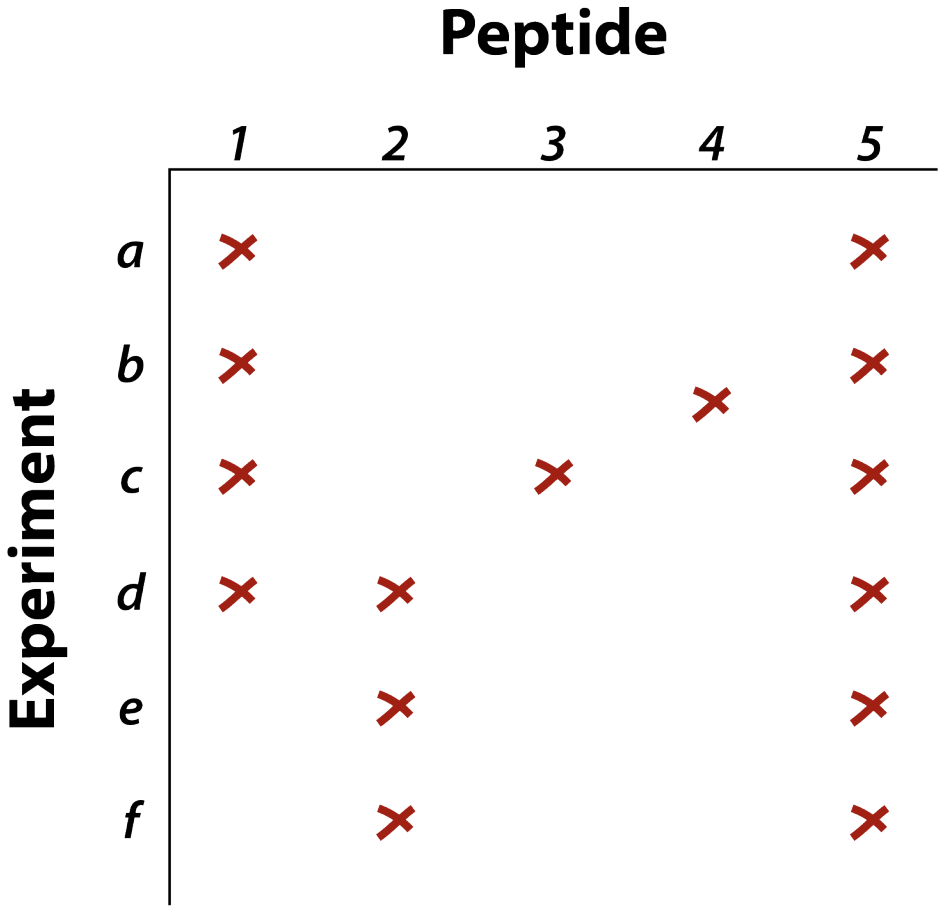
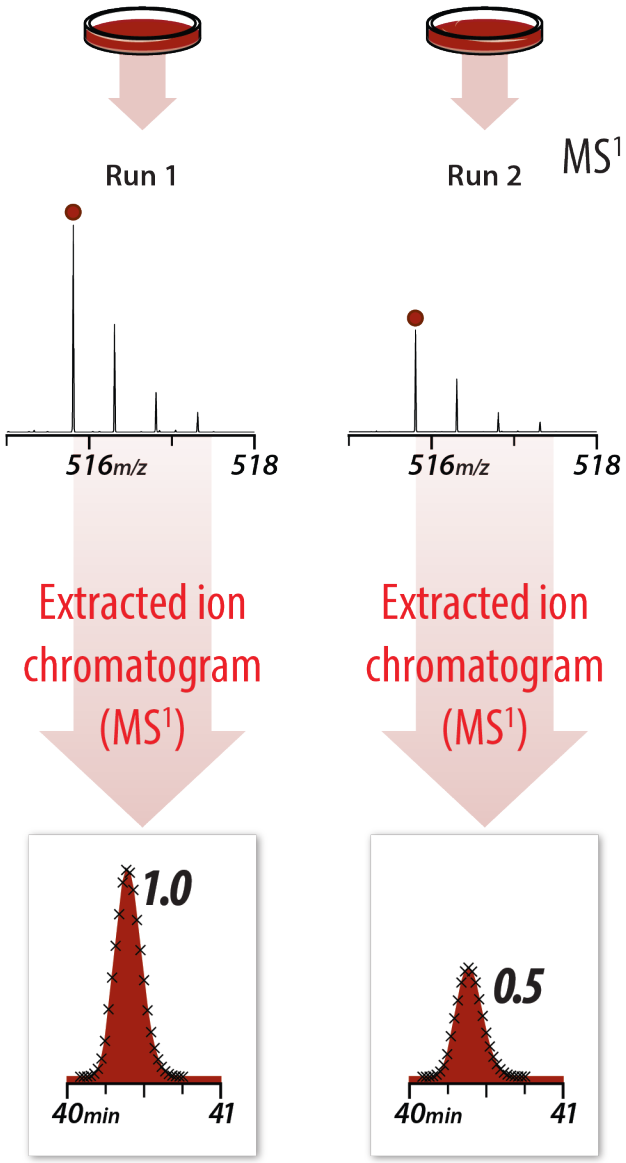
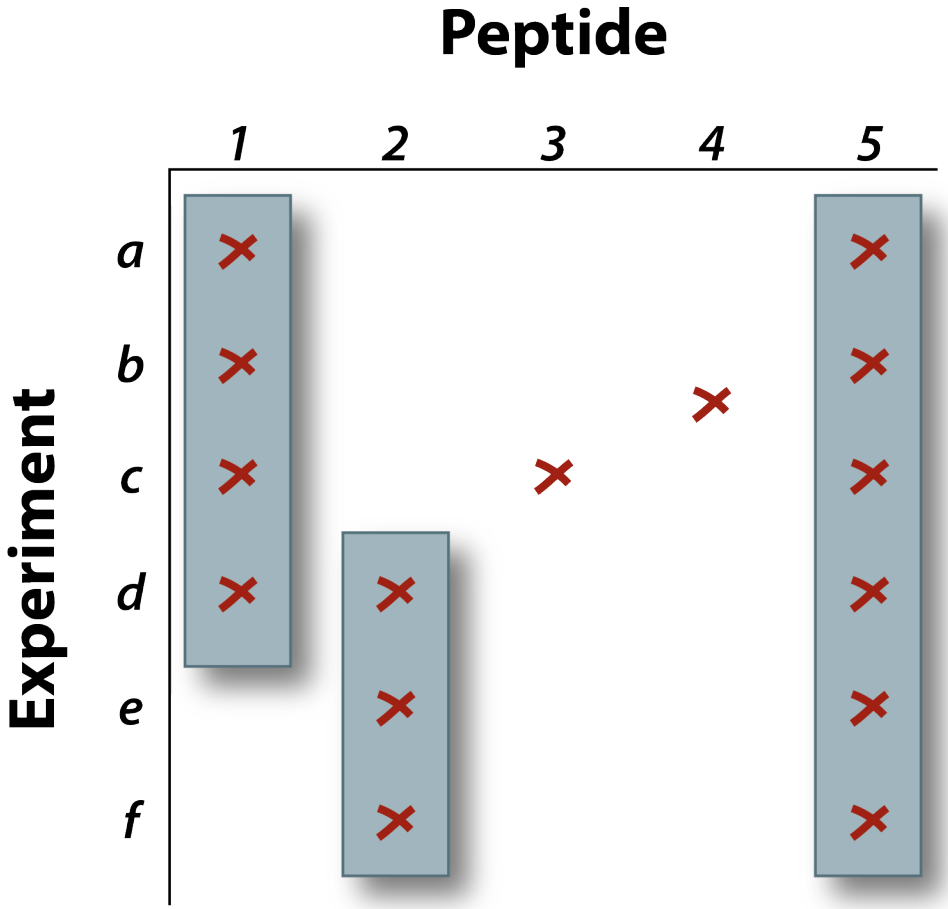


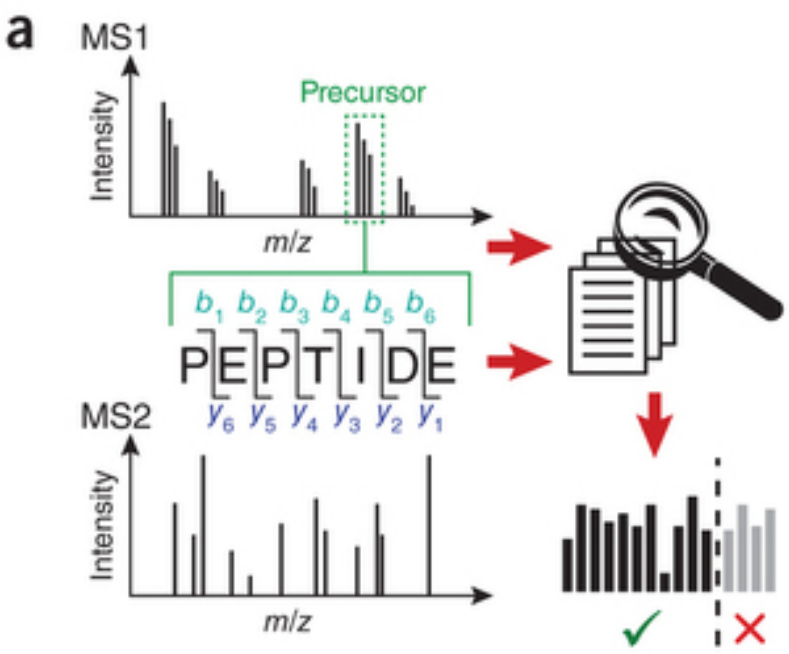


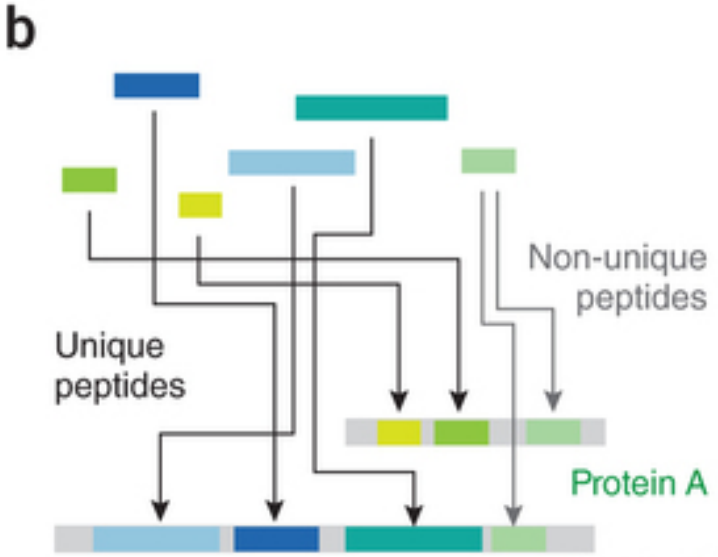
Label free quantitation AUC

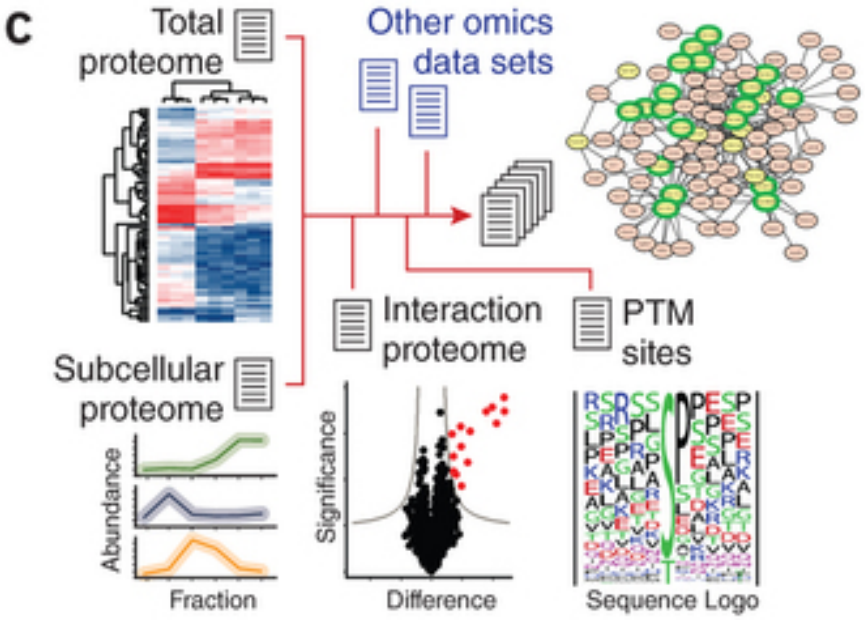




What proteins did we identify? MaxQuant software

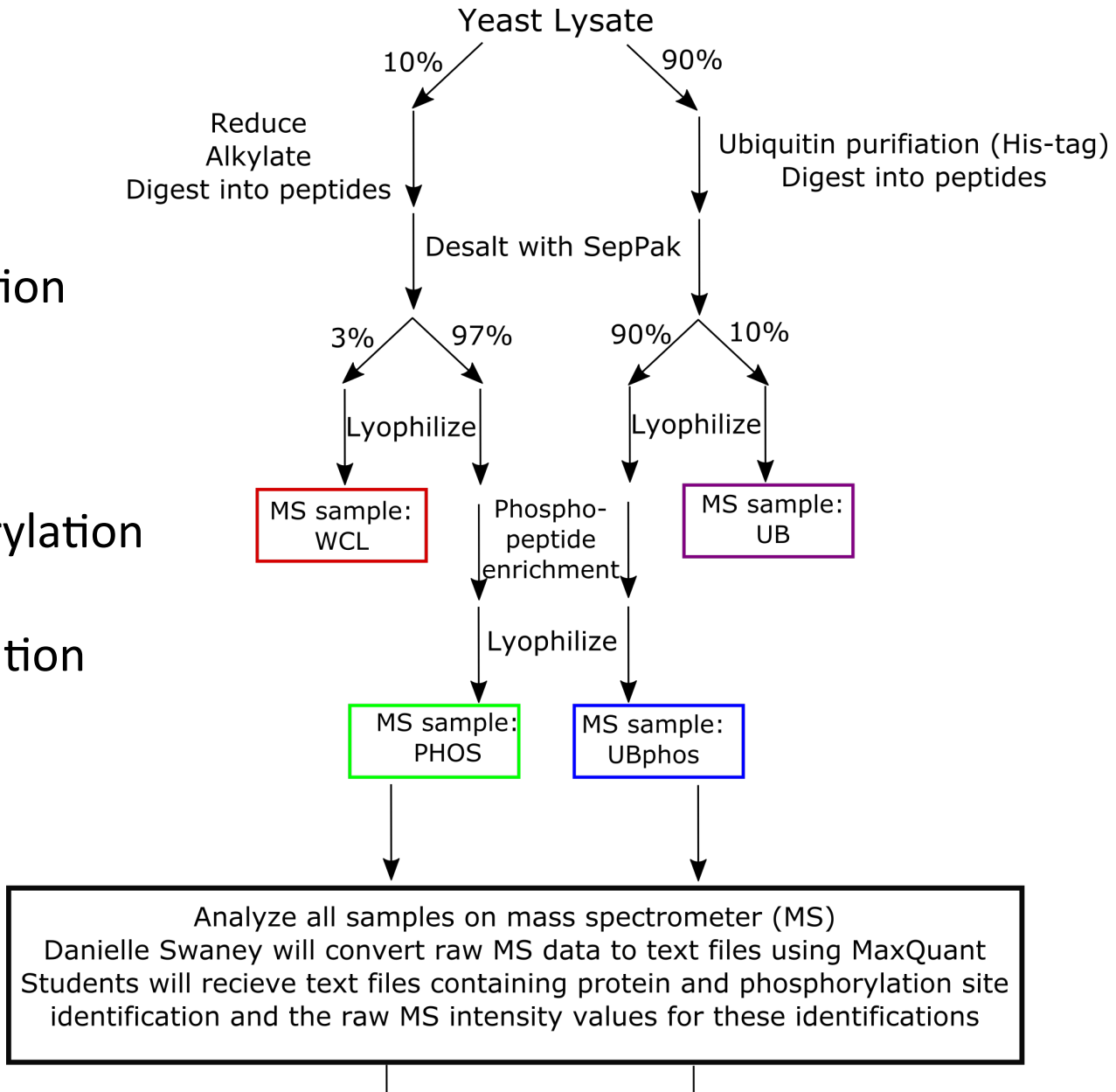






- Conditions:
  - Control
  - Kinase KO
  - Chemical perturbation

- Measurements:
  - Ubiquitin
  - Ubiquitin phosphorylation
  - Global proteome
  - Global phosphorylation



PRO TIPS

- **You are purifying a MINORITY population from a complex mixture.**
- **Focus on REMOVING as much of what you don't what from the sample as possible**
- **Worry less about maintaining 100% of your analyte of interest.**
- **Understand where your sample is.**