15N metabolic labeling as a tool to study nutrient induced signaling processes in plants

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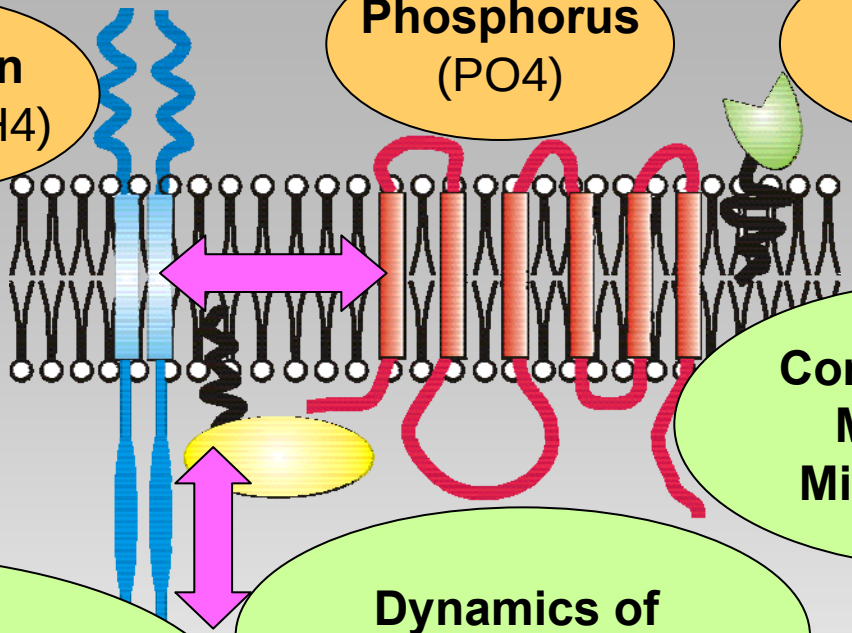
Membrane proteins and nutrient signaling



Nitrogen (NO3, NH4)

Phosphorus (PO4)

Carbon (sucrose)

Uptake across membrane

Composition of Membrane Microdomains

Dynamics of Phosphorylation

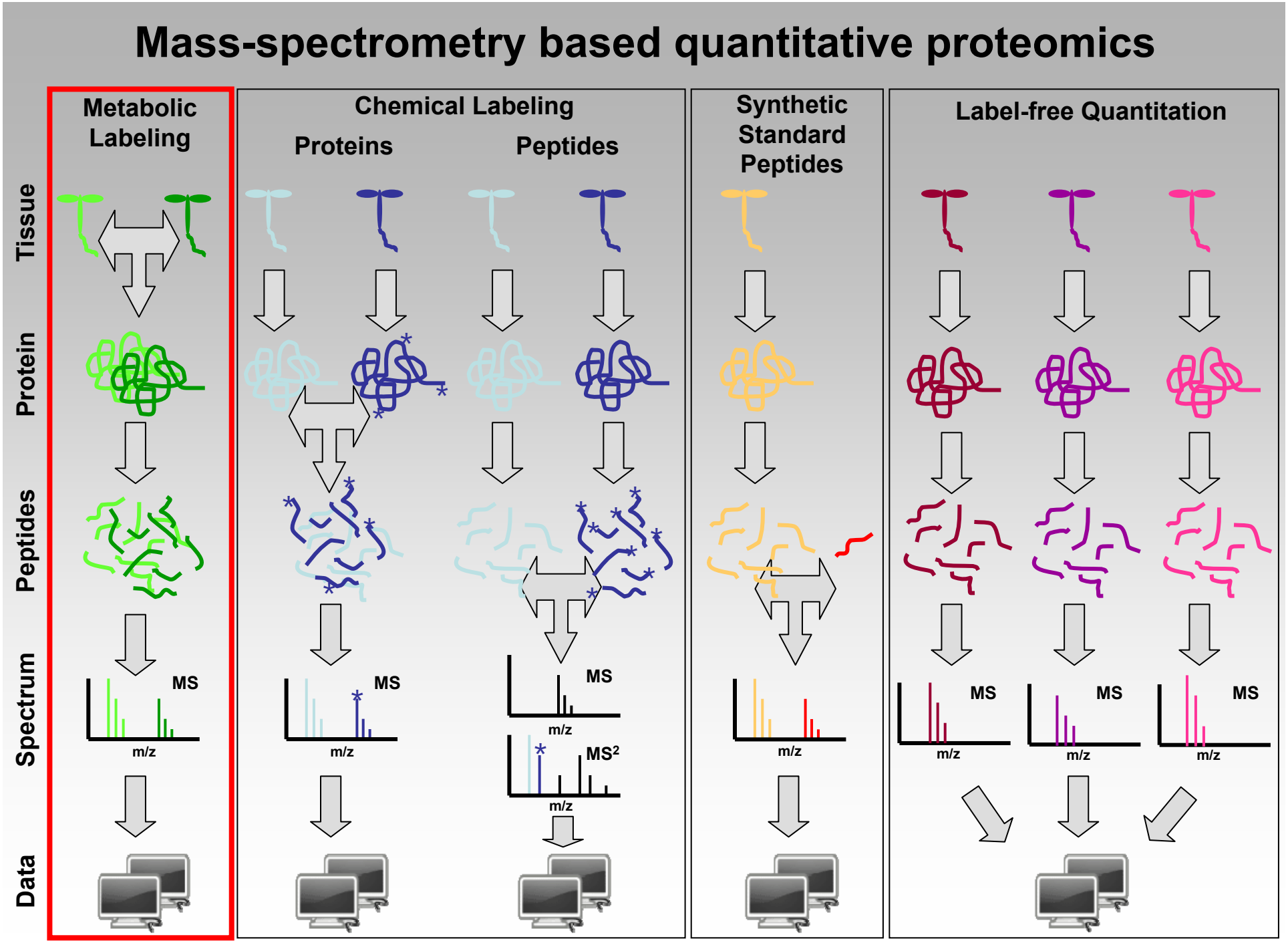
Phosphorylation-Dependent Protein-Protein Interactions

„Signaling Network“

Recognition / signal transduction

Regulation of transcription

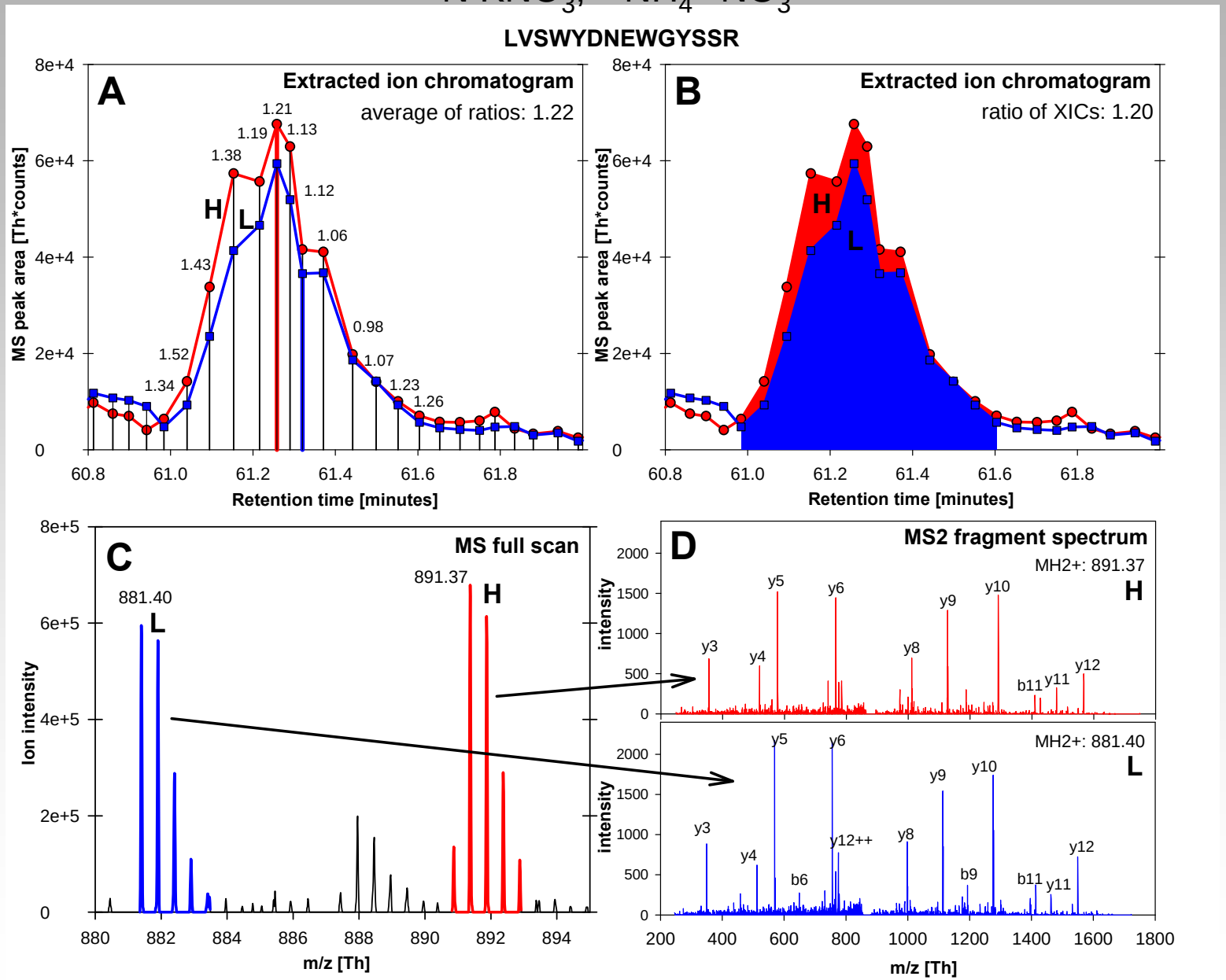
Mass-spectrometry based quantitative proteomics



Schulze WX Usadel B (2010) Annual Reviews of Plant Biology 61: 491-516

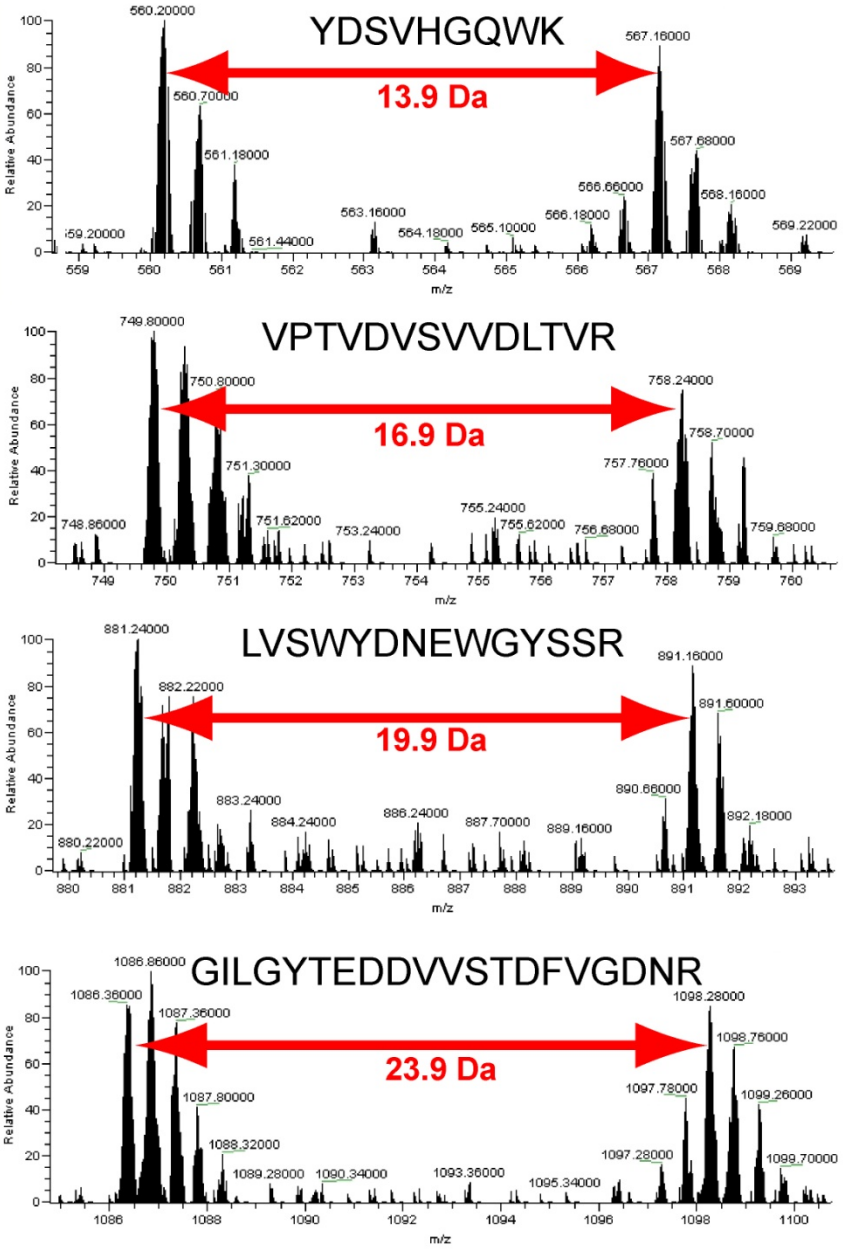
^{15}N-labeling: Data extraction workflow

$^{15}N\ KNO_3$, $^{15}NH_4{}^{15}NO_3$



Schulze WX, Usadel B (2010) Annual Review of Plant Biology 61: 491-516

Mass-shifts of ^{15}N-labeled peptides

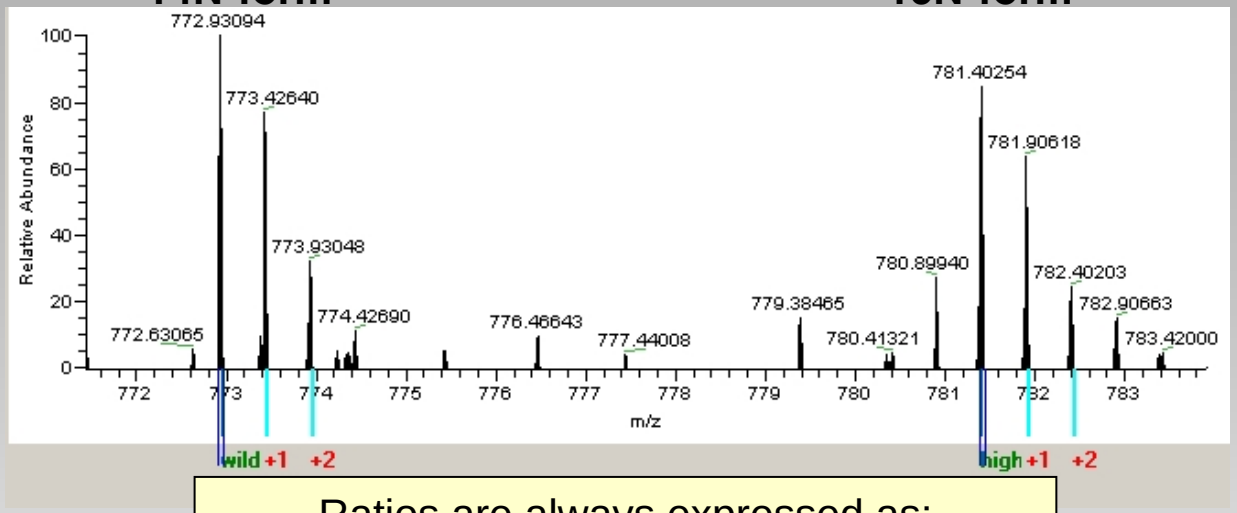


- Mass shift is dependent on the amino acid sequence.
- Identification of the peptide is necessary before the peptide pairs of labeled and unlabeled form can be quantified.

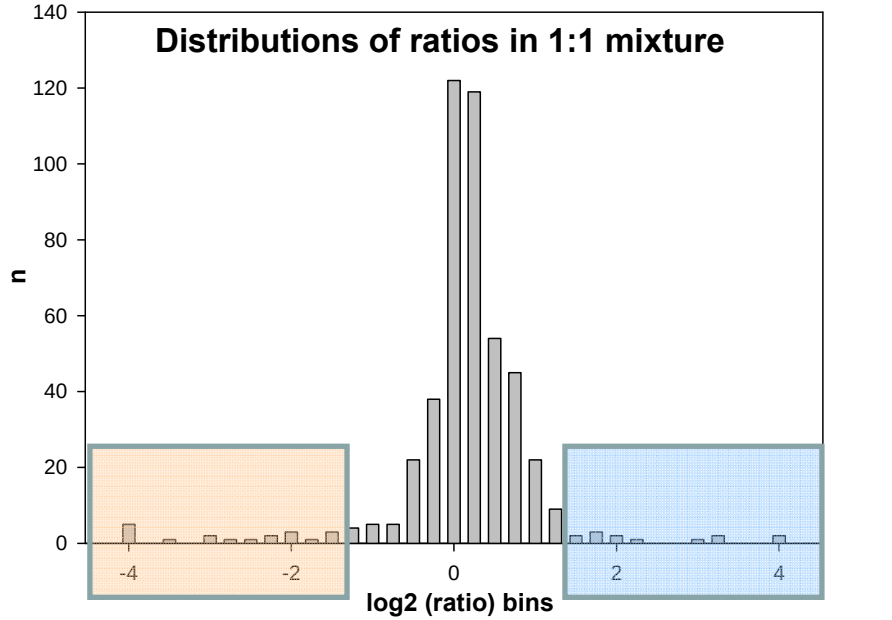
Engelsberger WR, et al (2006) Plant Methods 2(14): 1-11

^{15}N-labeling: The basics

14N-form **15N-form**



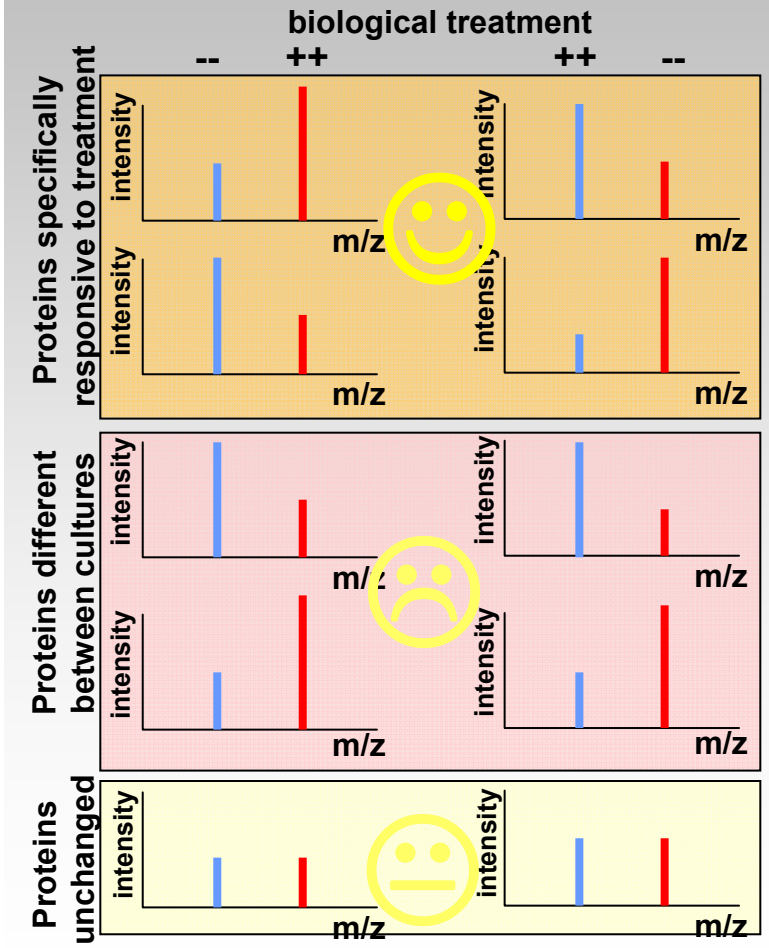
Ratios are always expressed as:
heavy/light
ratio = Intensity$_{\mathbf{15N}\text{-form}}$/Intensity$_{\mathbf{14N}\text{-form}}$

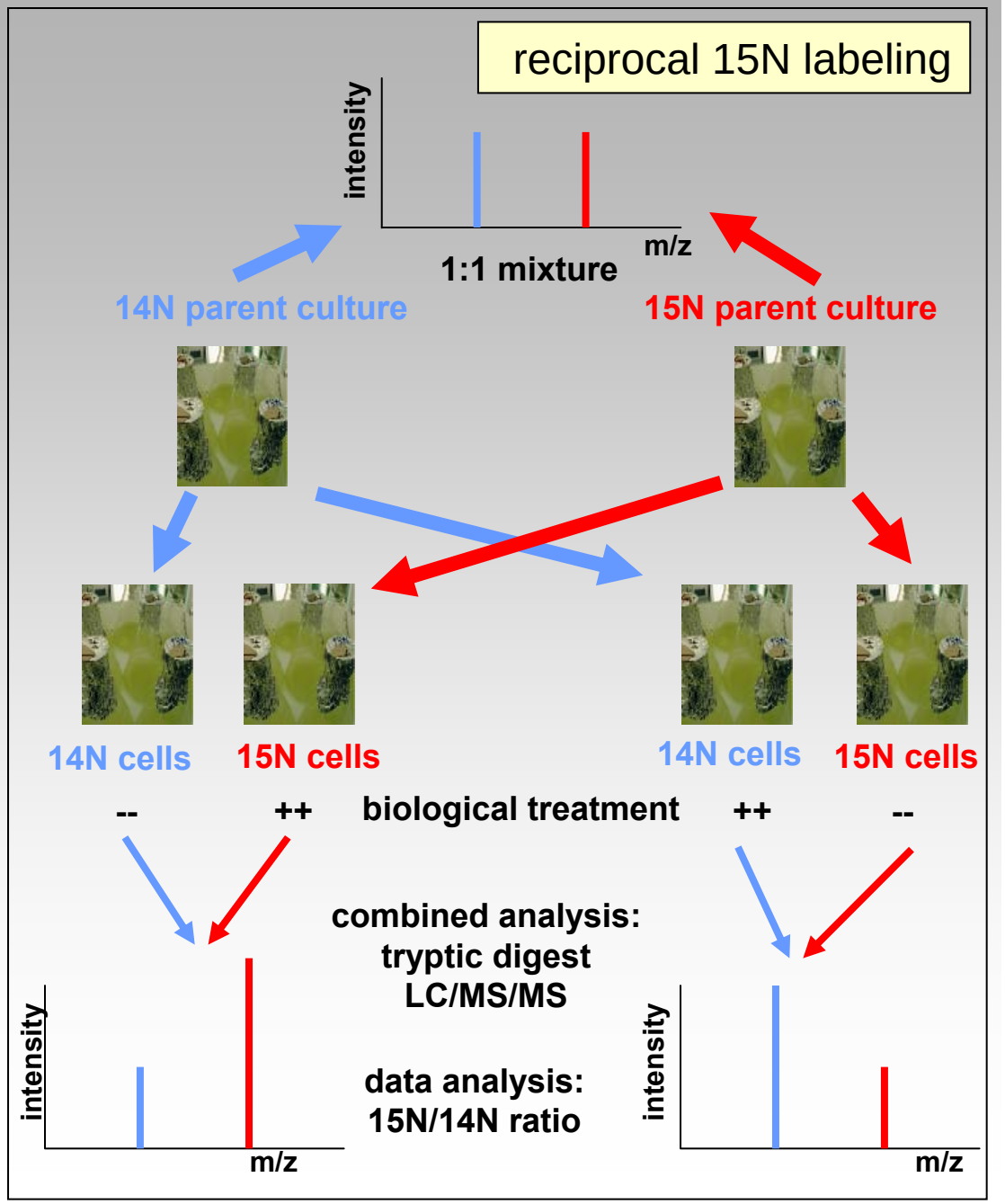


^{15}N-labeling: Reciprocal experimental setup

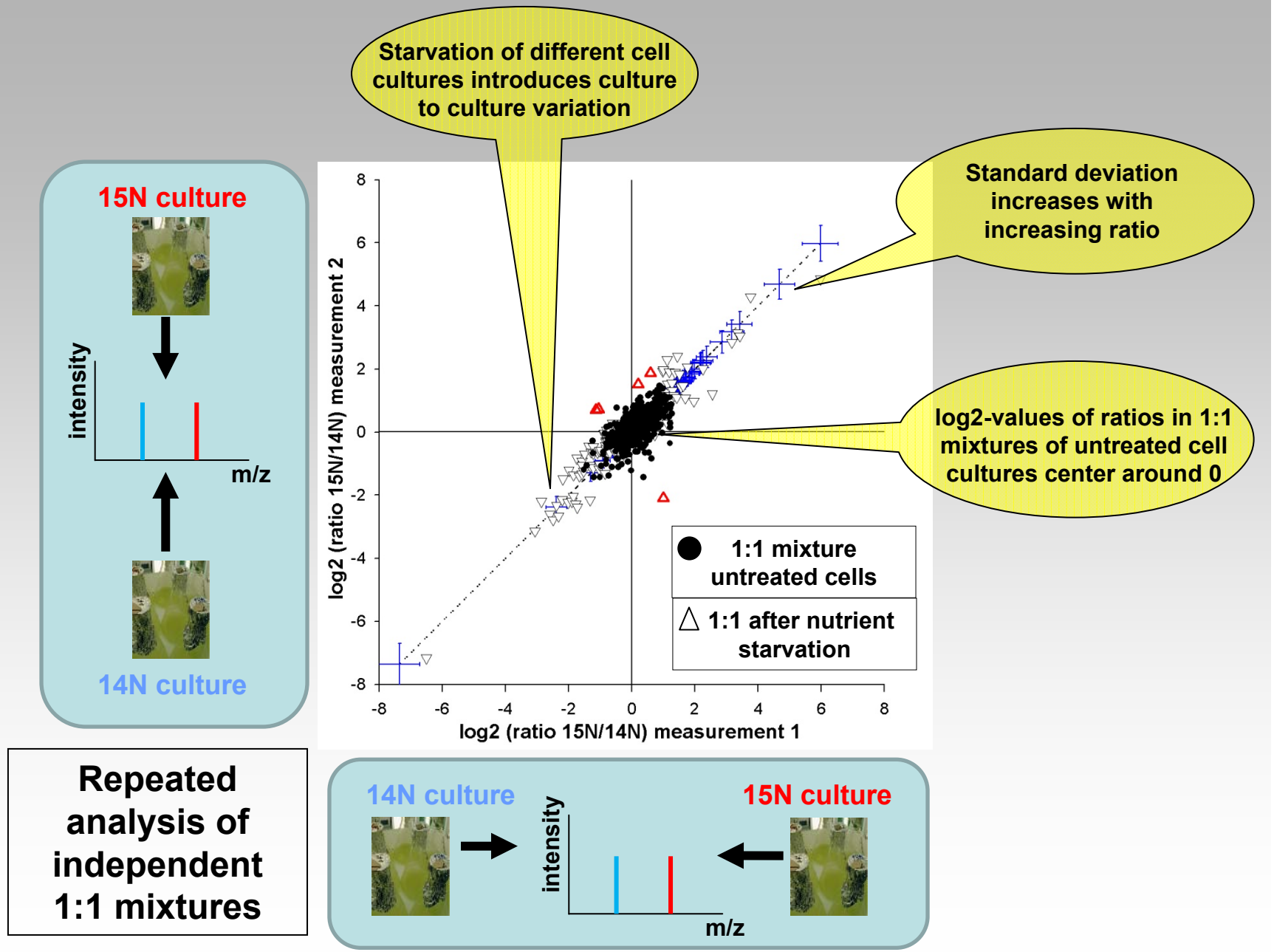
We need a strategy to distinguish:

Responses to biological treatment
and
Treatment-independent differences



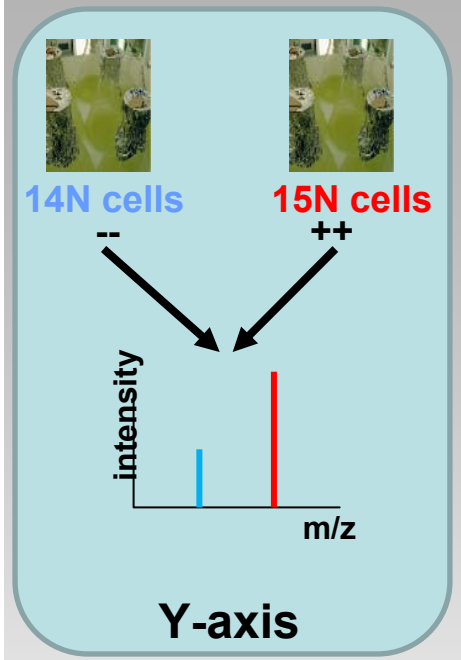


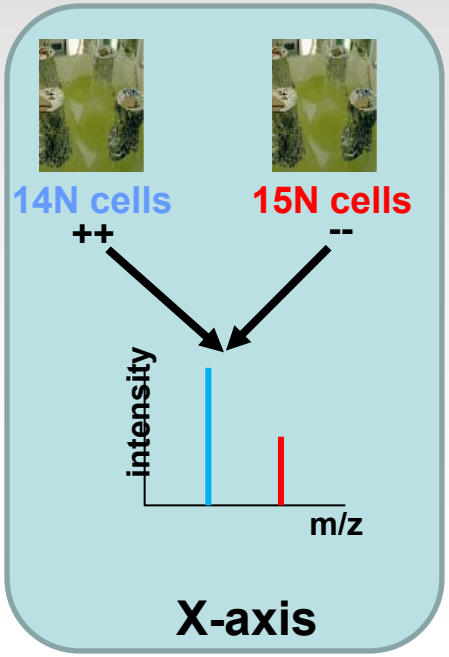
Reciprocal experimental setup: Definition of variation

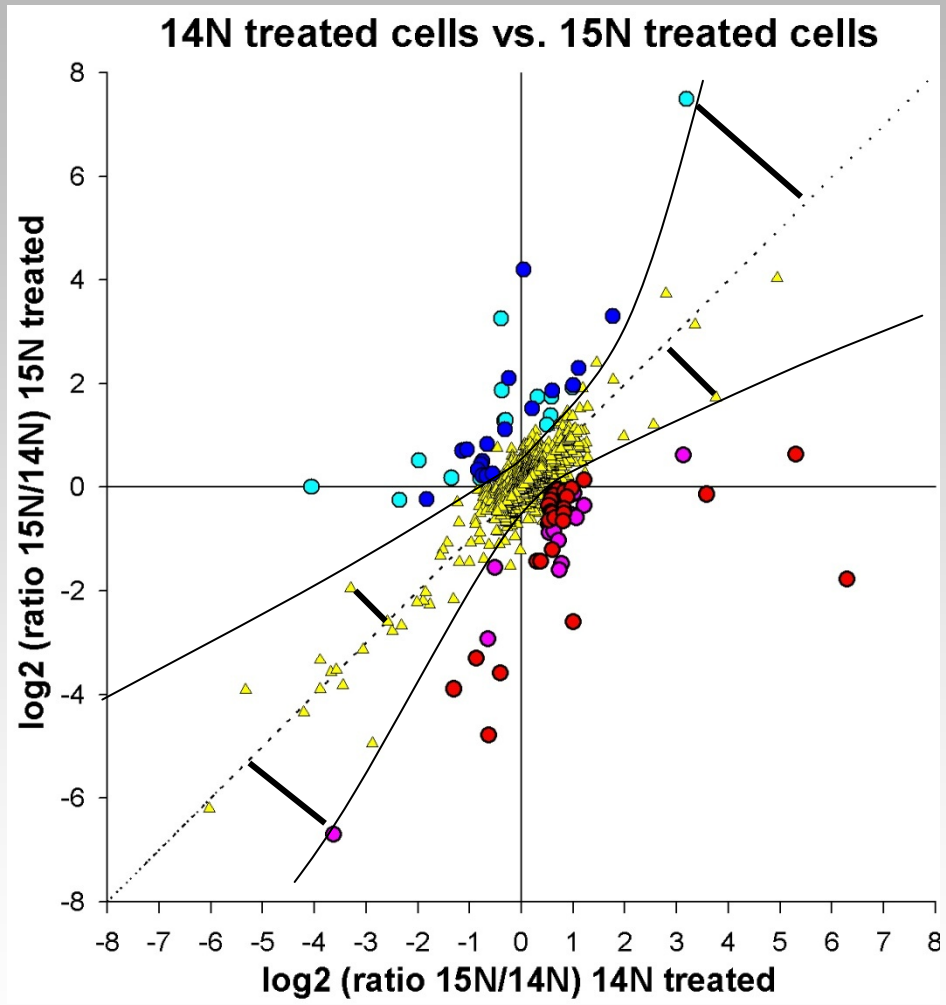


Kierszniowska S, et al (2009) Proteomics 9: 1916-1924

Reciprocal experimental setup: Data analysis





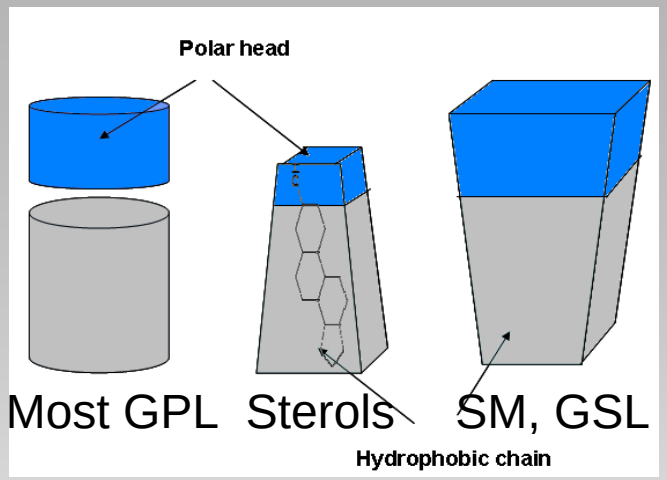


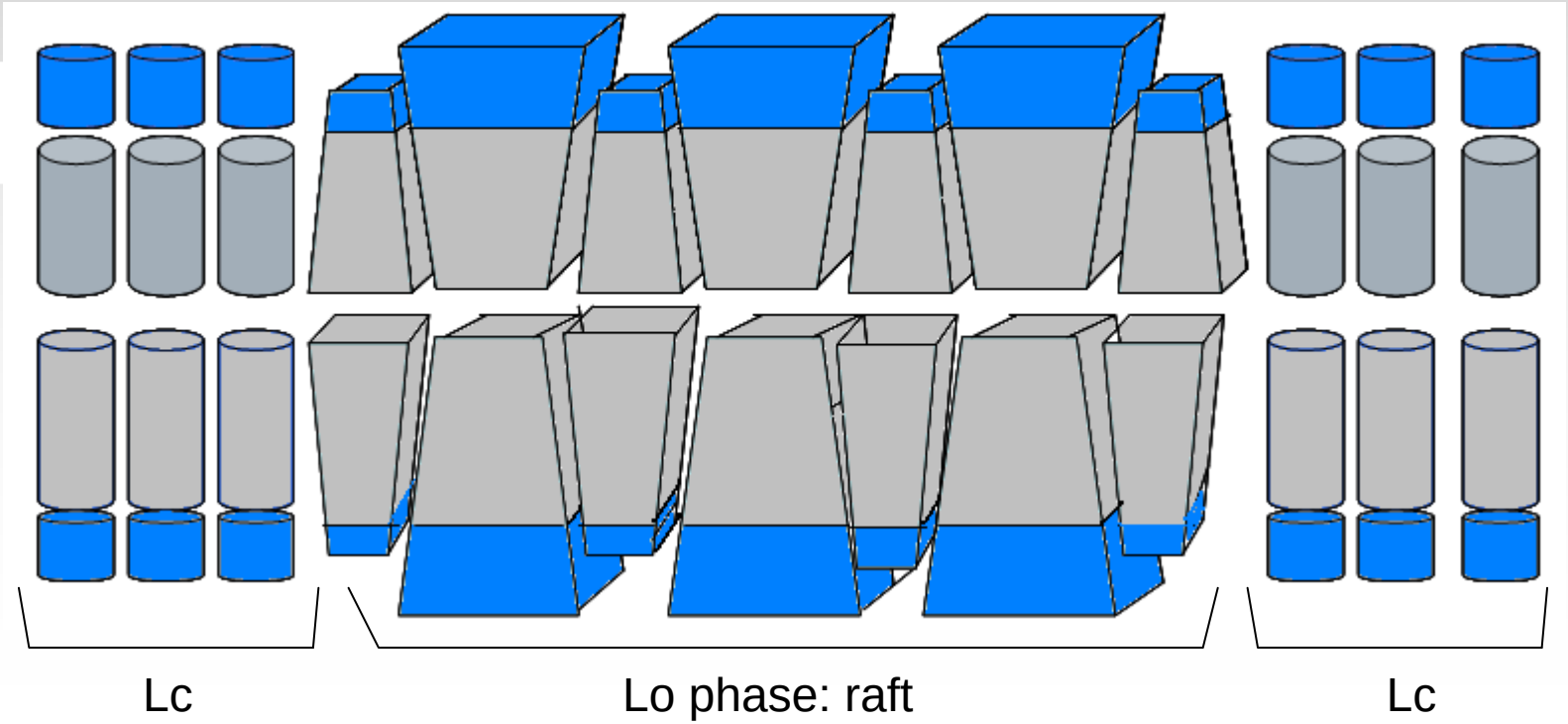
- ✓ Distance to diagonal is used as a measure for 'responsiveness' and to define significance.
- ✓ Each data point has a specific p-value.

Kierszniowska S, et al (2009) Proteomics 9: 1916-1924

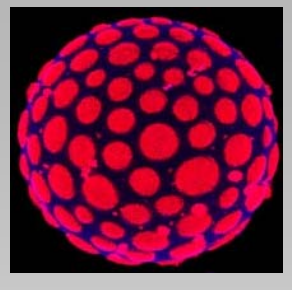
Dynamic changes in plasma membrane microdomain composition

Basis of sterol enriched microdomain



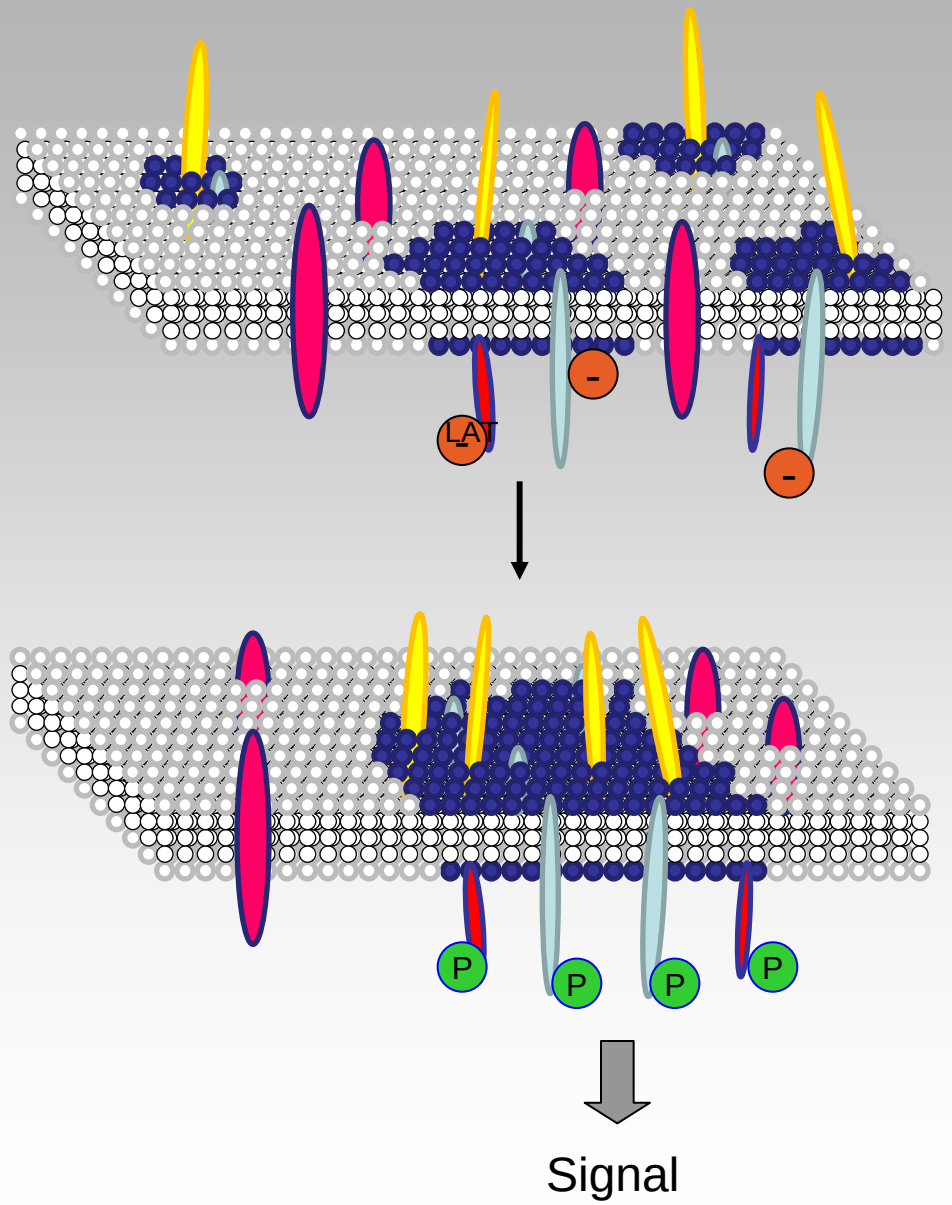


Membrane microdomains and signaling



Artificial membrane vesicle with distinct sterol-rich domains

Baumgart T, et al. (2003) Nature 425: 821-824

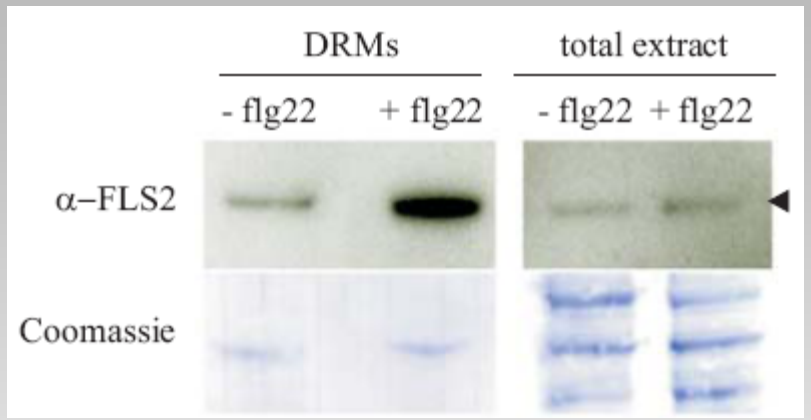


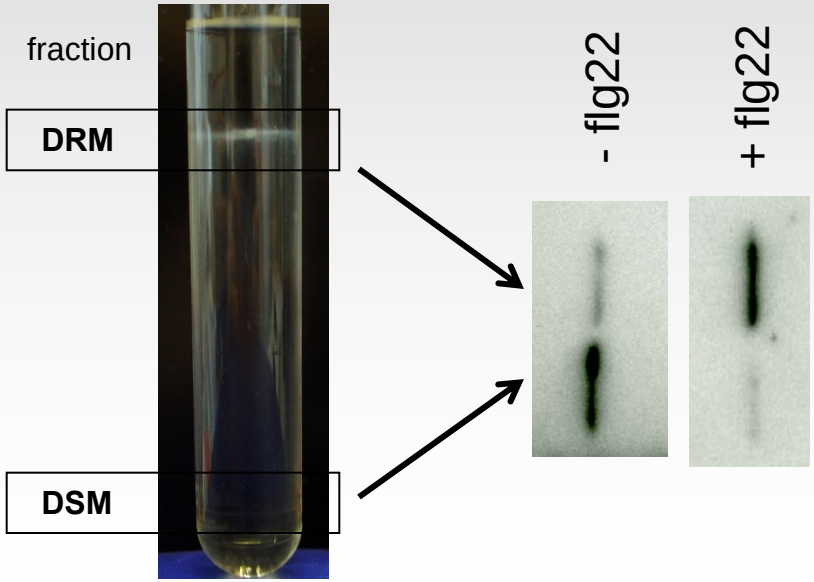
Evidence from mammalian cells and yeast:

- **Membrane microdomain composition can change with environmental stimuli and with protein modification**
- **Only indirect indications in plants**

Enrichment of receptor FLS2 in DRM

Cells were stimulated with flg22 for 10 min and DRMs were prepared

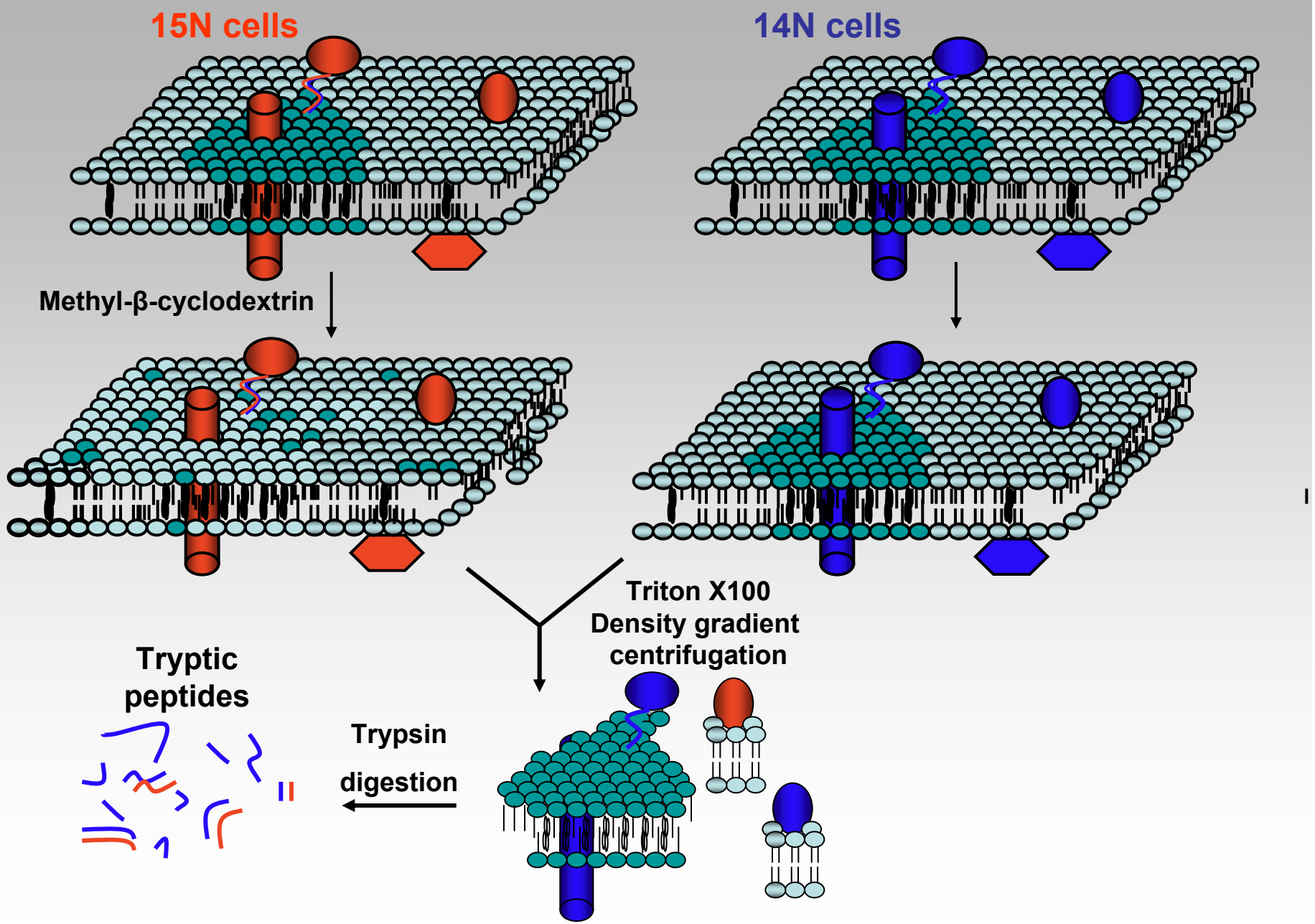




- FLS2 is recruited to DRM upon flg22 stimulation
- In stimulated cells FLS2 receptor is enriched in DRM fraction
- In non-stimulated cells FLS2 receptor is enriched in DSM fraction

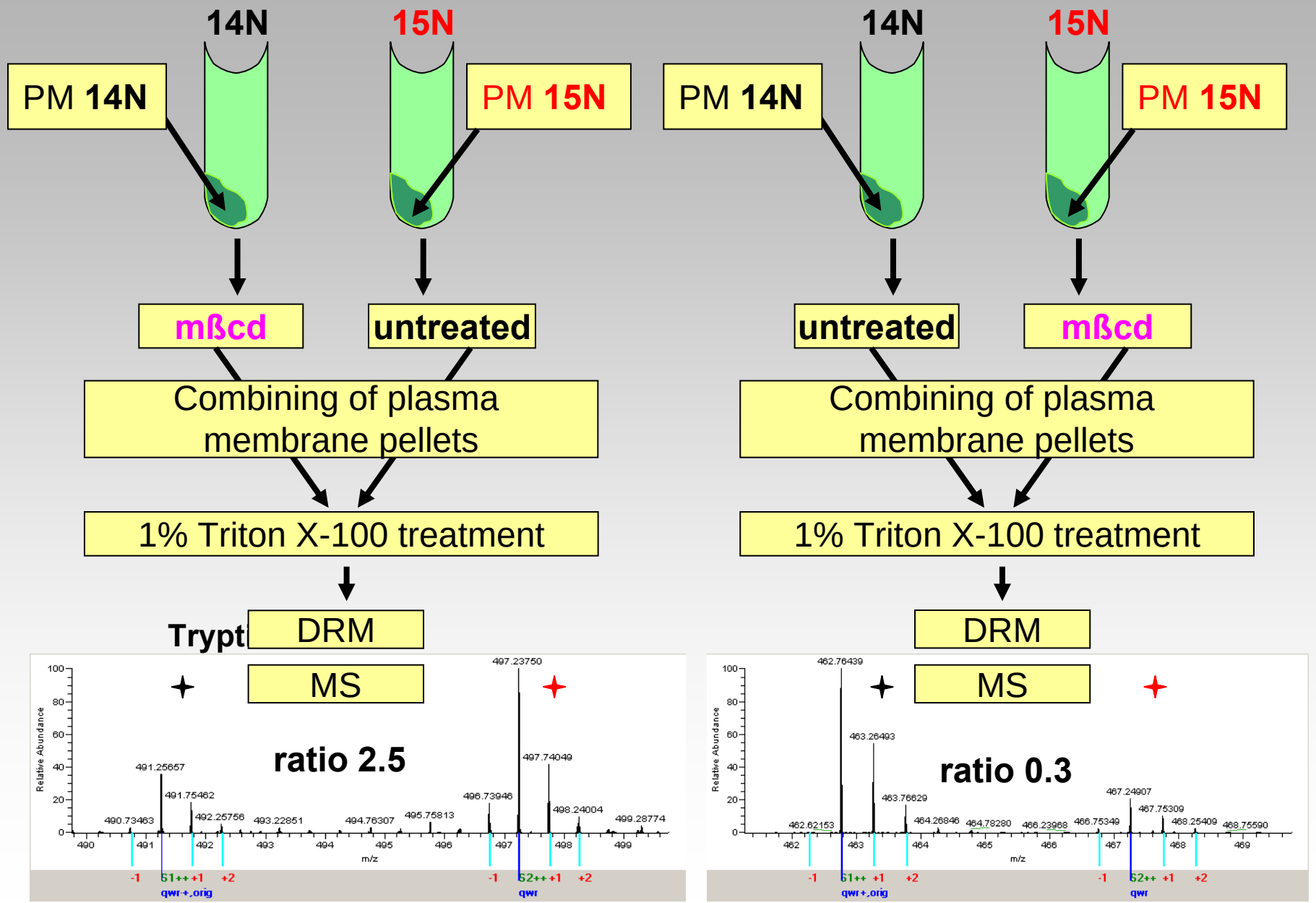
Keinath N, et al (2010) submitted

Characterisation of DRM with sterol disrupting reagent



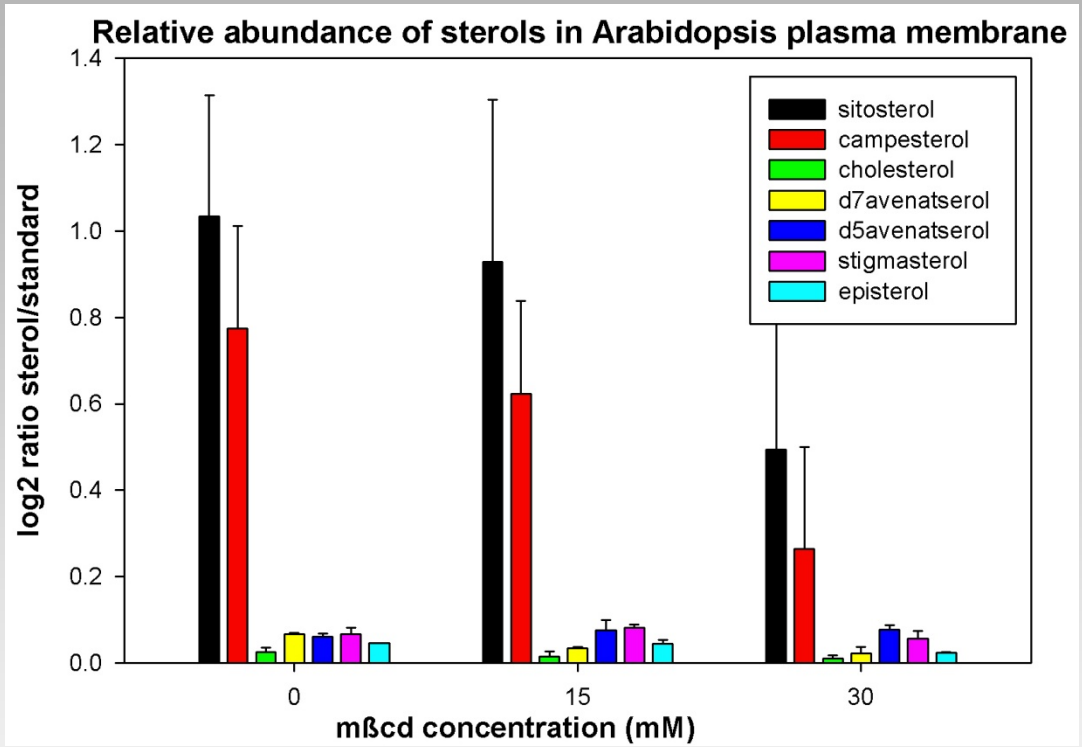
Foster LJ et al. (2003) Proceedings of the National Academy of Sciences of the USA 100: 5813-5816

Characterisation of DRM with sterol disrupting reagent



Foster LJ et al. (2003) Proceedings of the National Academy of Sciences of the USA 100: 5813-5816

Mßcd removes sterols from DRM

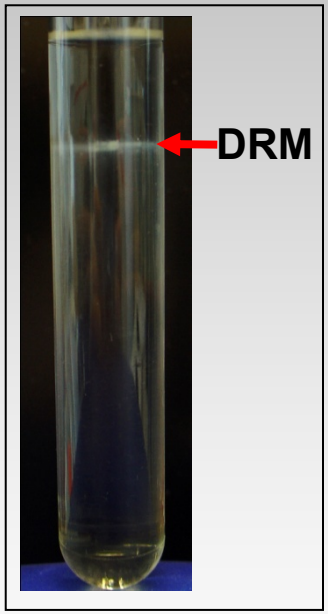


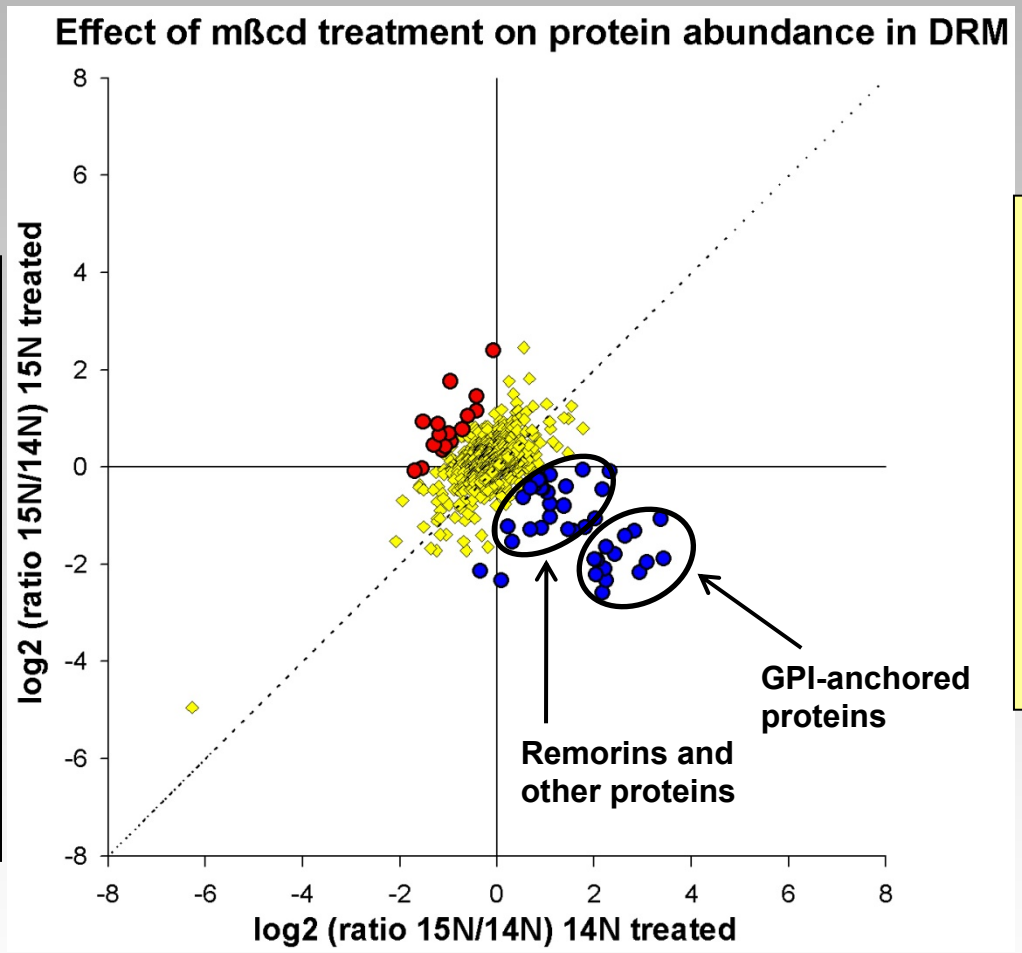
- Sitosterol and campesterol are the most abundant sterols in plant DRM.
- Major sterol are removed from DRM preparations by mßcd.

Kierszniowska S, et al (2009) Molecular and Cellular Proteomics 8(4): 612-623

Definition of plant Lipid Rafts

Centrifugation of Triton-X-100 treated plasma membrane in sucrose step gradient (250000g, 18h at 4°C) after treatment with mßcd

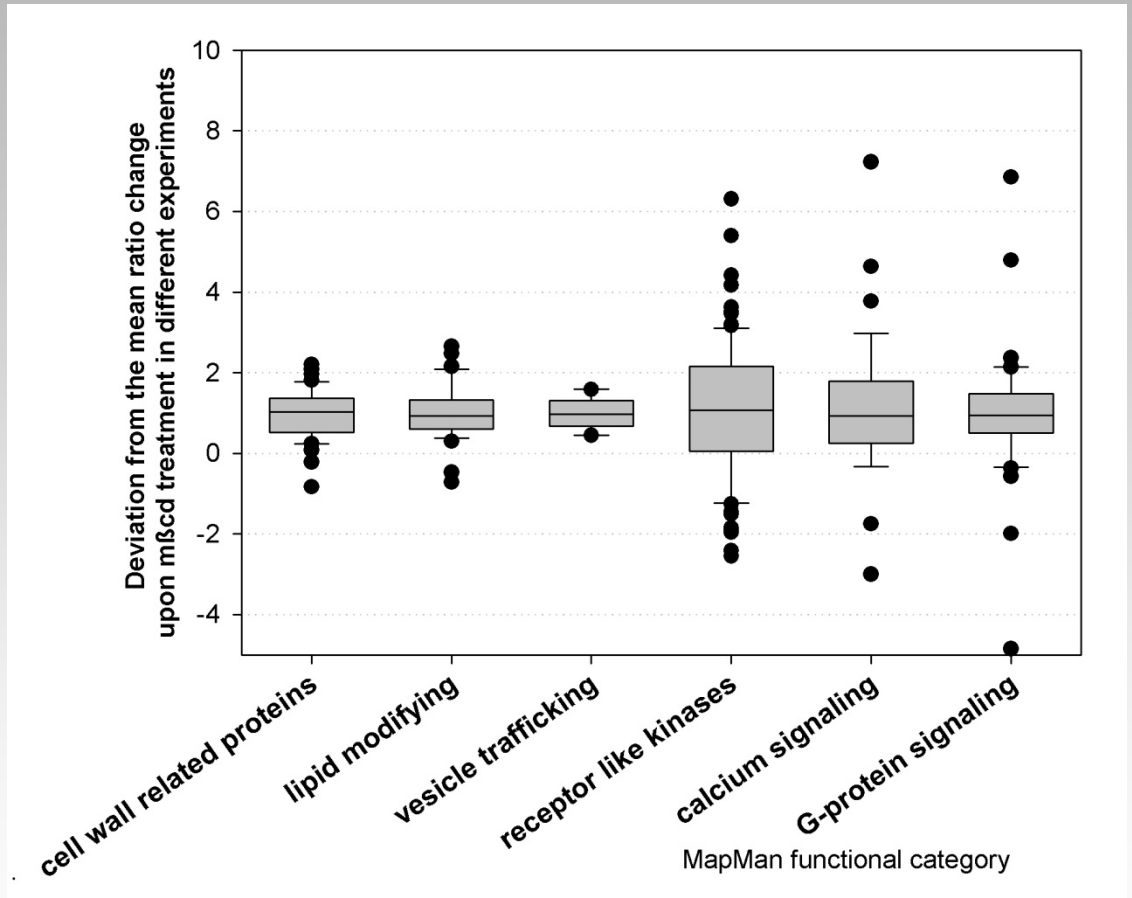




- 8% of all data points (n=465) are considered as significantly depleted from DRM by mßcd.
- Among the 'depleted' proteins are cell wall proteins, proteins for steroid metabolism, and signaling proteins.

Kierszniowska S, et al (2009) Molecular and Cellular Proteomics 8(4): 612-623

Constant and variable components of DRM



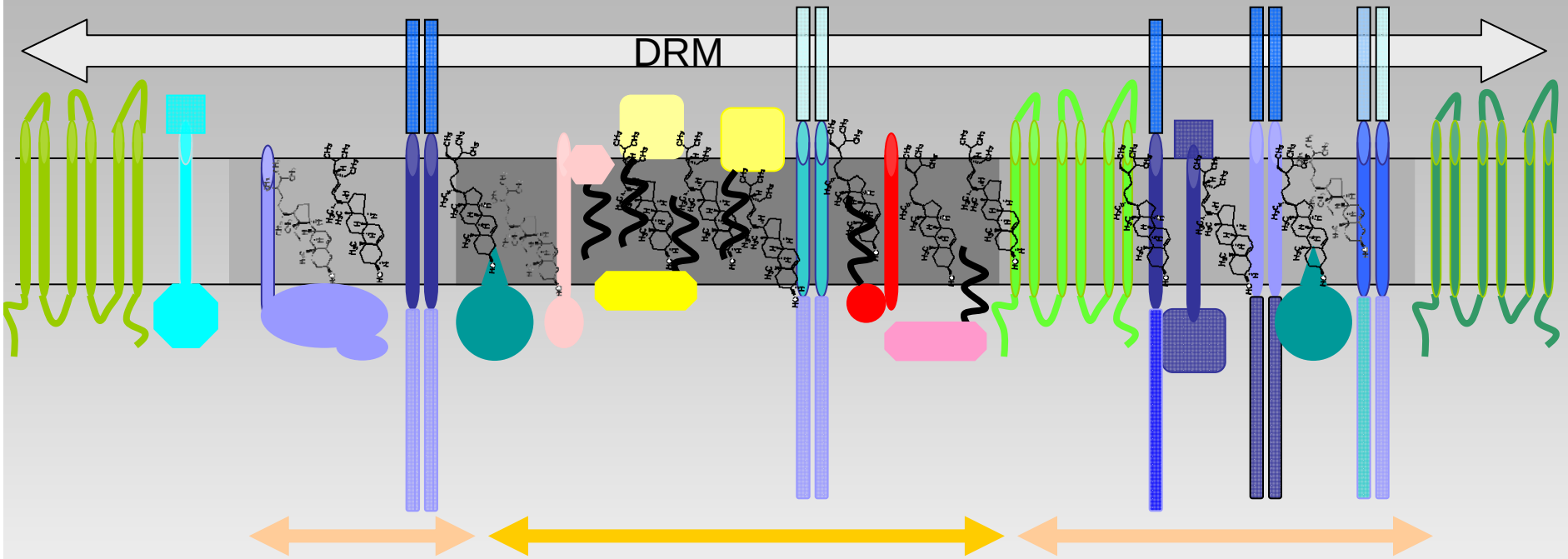
- Cell wall proteis, lipid modifying proteins and proteins with functions in vesicle trafficking are reproducibly found as sterol-dependent proteins.
- Signaling proteins display are stronger experiment-to-experiment variation and stonger variation between proteins.

✓ **Indications for 'constant' and 'variable' components of sterol-rich membrane domains.**

Kierszniowska S, et al (2009) Molecular and Cellular Proteomics 8(4): 612-623

Plasma membrane DRM and Lipid Rafts

➢ DRM and 'Lipid Rafts' are not the same

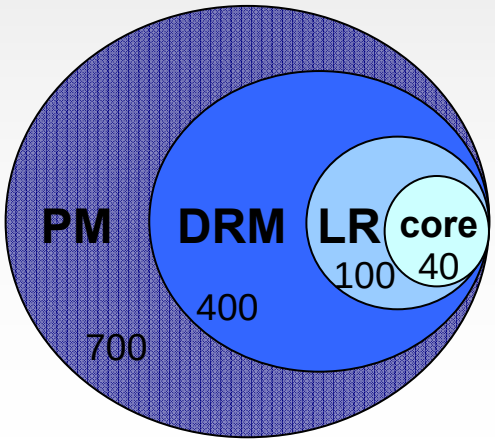


'Lipid Raft' core region

- GPI-anchored proteins
- Cell wall proteins, lipid modification
- Constant component

'Lipid Raft' variable region

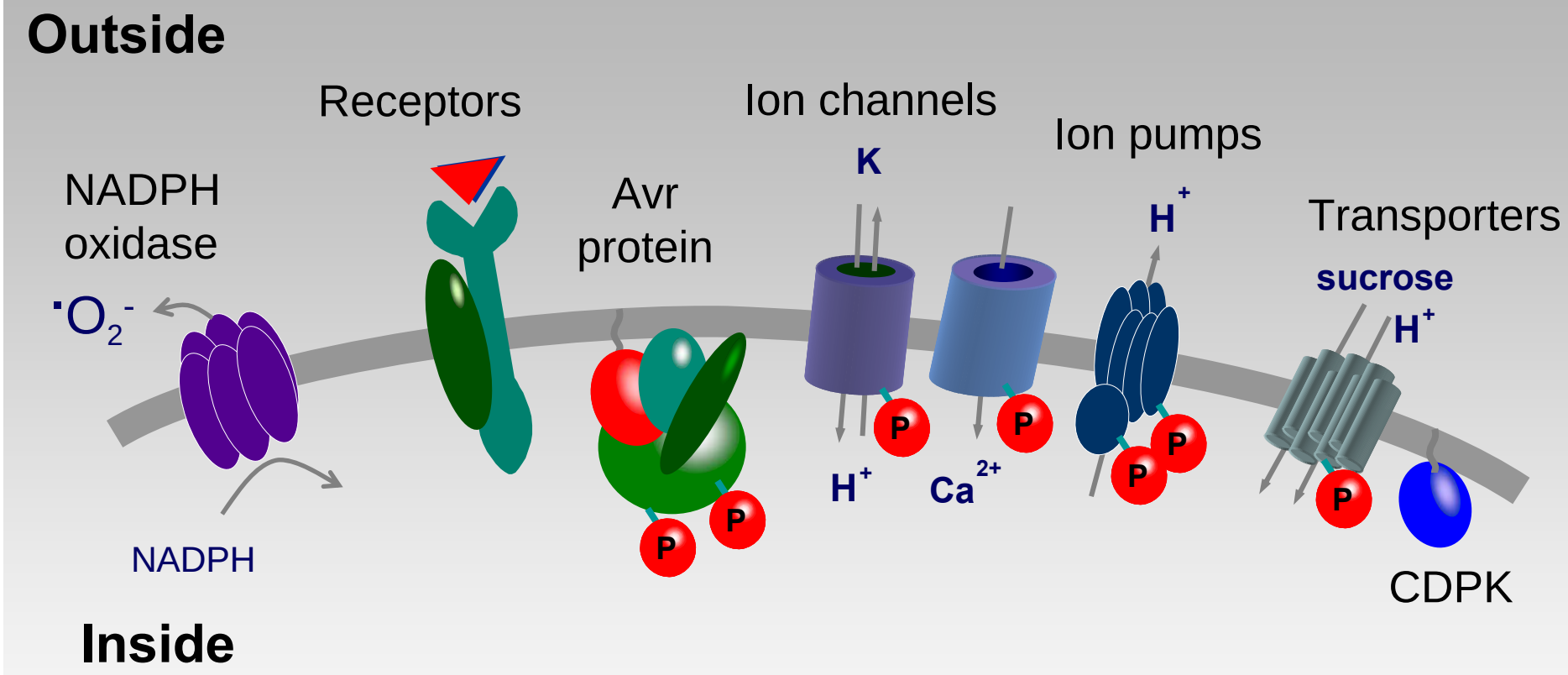
- Single-TM proteins
- Signaling proteins (RLK, calcium, G-protein)
- Variable component



Kierszniowska S, et al (2009) Molecular and Cellular Proteomics 8(4): 612-623

Dynamic changes in protein phosphorylation

Membrane proteins and phosphorylation

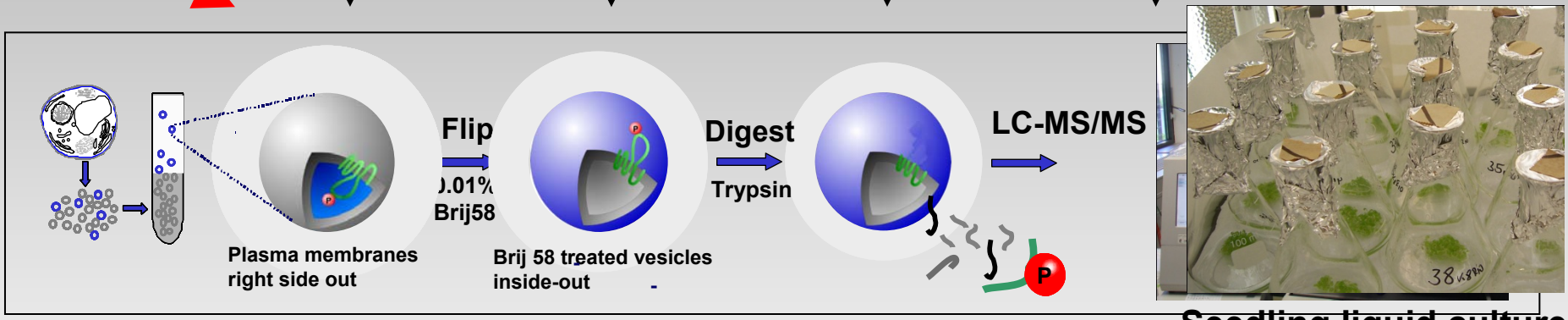


- Membrane proteins are phosphorylated on the cytosolic side.

Experimental setup: nutrient starvation and resupply

starved | 3 min | 5 min | 10 min | 30 min

15N → 14N



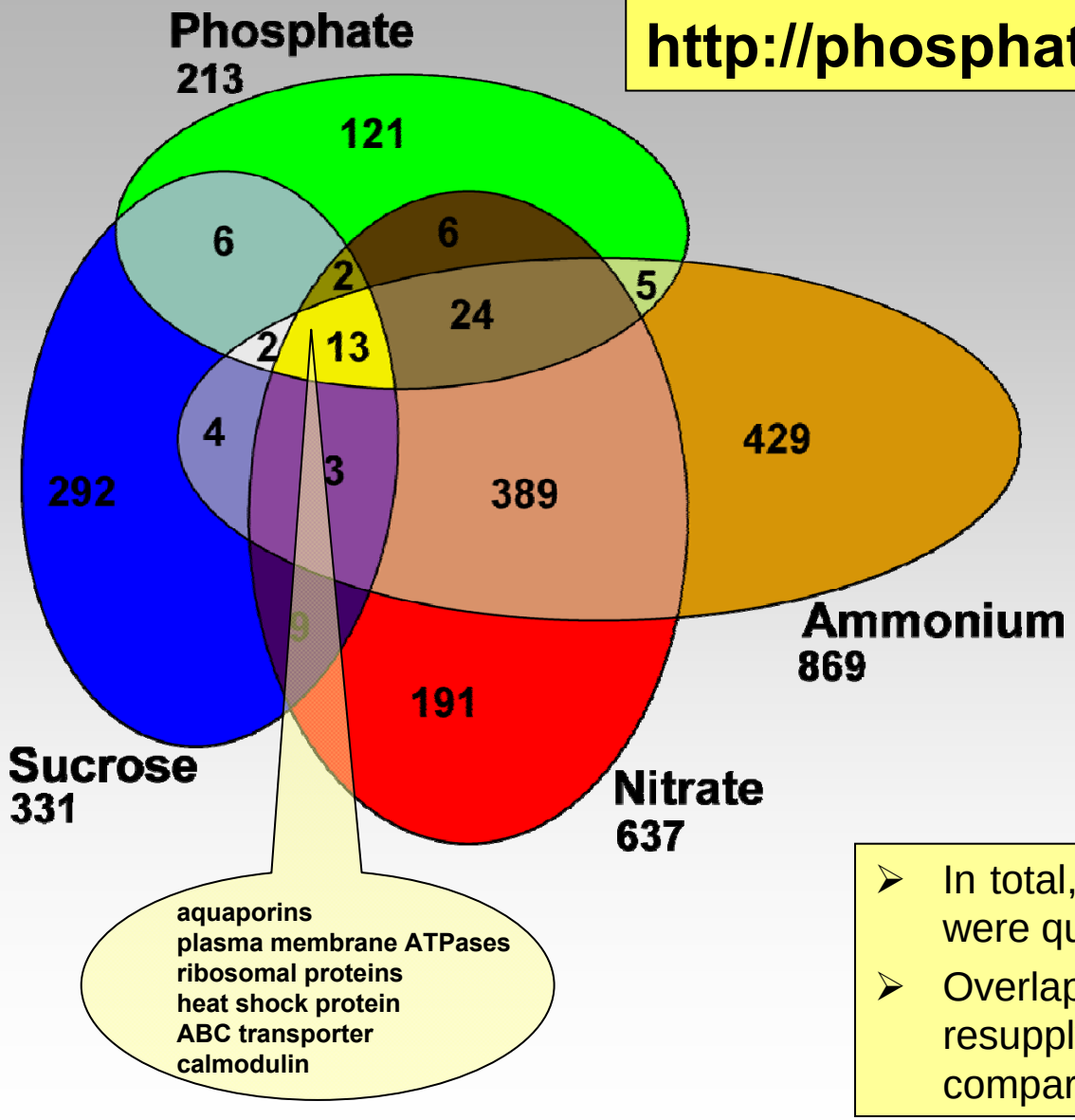
Seedling liquid culture

analysis by LC-MS/MS with neutral loss scans / MSA

display of relative changes in phosphorylation
label-free quantitation – protein correlation profiling

Johansson F, et al. (1995) Plant Journal 7: 165-173
Stensballe A, et al. (2001) Proteomics 1: 207-222
Palmgren M, et. al. (1990) Biochem Biophys Acta 1021: 133-140
Nühse T, et al. (2003) Molecular and Cellular Proteomics 2 (11): 1234-1243

Nutrient-responsive phosphorylation sites



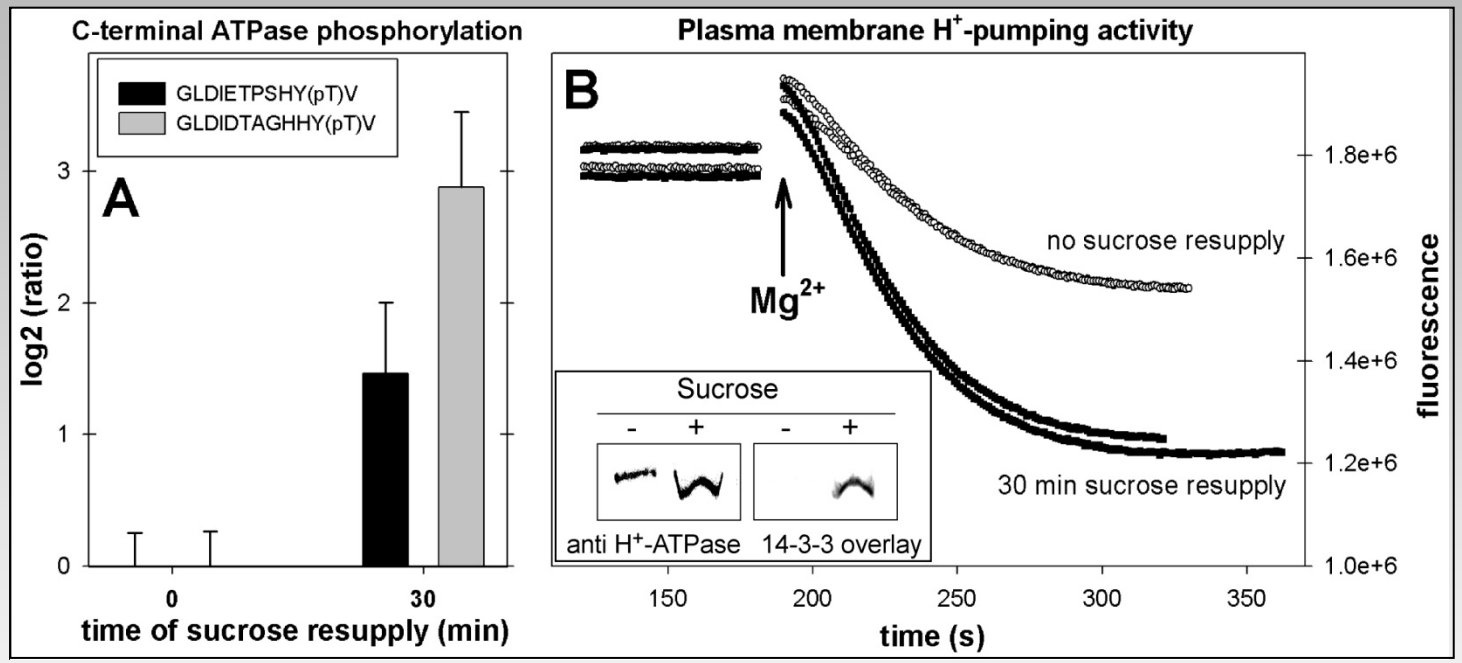
http://phosphat.mpimp-golm.mpg.de

PhosPhAt
(Start application)

- In total, **1500** different peptide sequences were quantified.
- Overlap between nitrate and ammonium resupply after nitrogen starvation is larger compared to other nutrient responses.

Engelsberger WR, et al. (2010), submitted

Phosphorylation responses: Verification



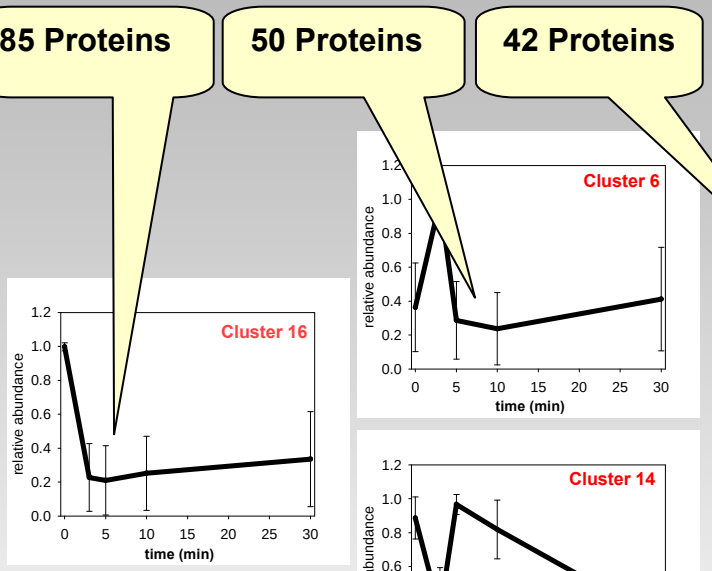
Verification:

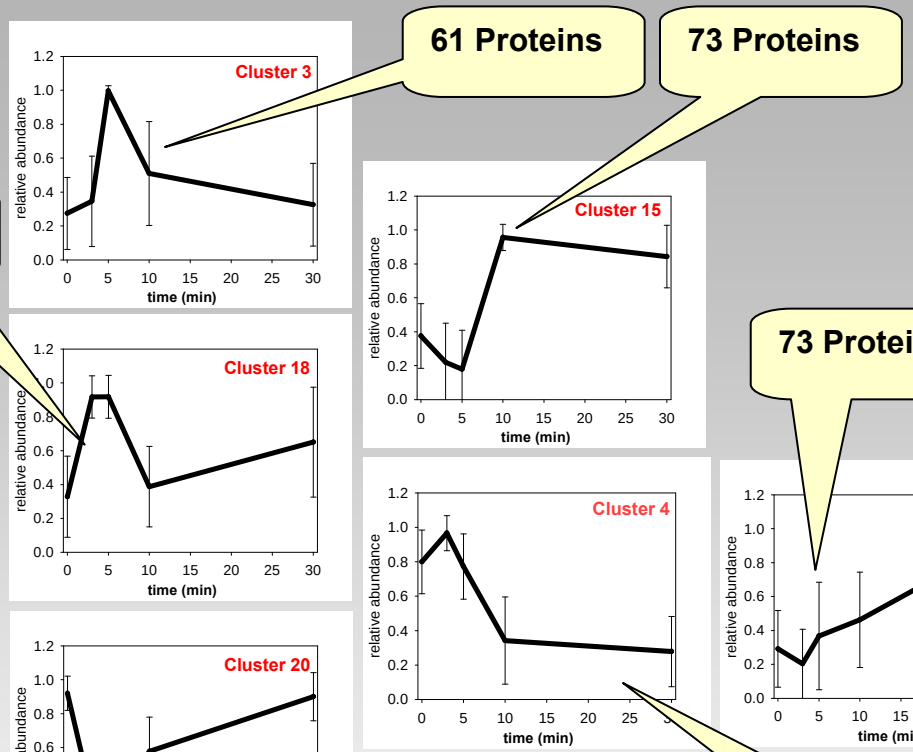
- ✓ Changes in phosphorylation of H+-ATPase AHA1 and AHA2 correspond to changes in ATPase activity upon sucorse resupply after starvation.

Niittylä T, et al (2007) Molecular and Cellular Proteomics 6(10): 1711-1726

Nutrient-dependent response classes

k-means clustering of time course responses for all treatments



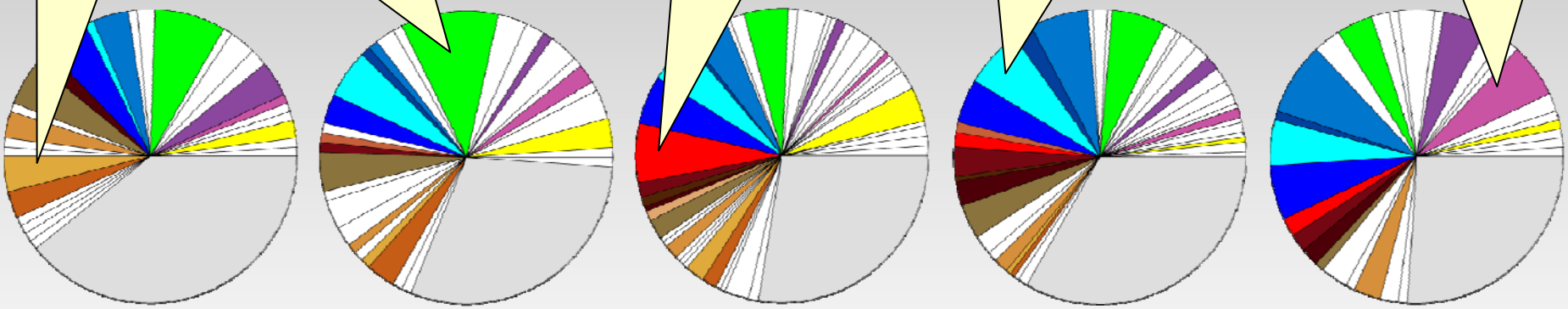


starved → 3 minutes → 5 minutes → 10 minutes → 30 minutes

Engelsberger WR, et al. (2010), submitted

Protein functions represented in response classes

starved	3 minutes	5 minutes	10 minutes	30 minutes
over-represented: • transport p- and v-ATPases • transport aquaporins • cell organization • unknowns • TFs: MADS	**over-represented:** • TFs: SBP, PWWP •transport aquaporins • RNA processing • lipid metabolism steroids, glycolipids • protein degradation • secondary metabolsim	**over-represented:** • signaling (RLK, kinases) • phosphatases • protein synthesis • transport aquaporins • transport other • lipid metabolsim (steroids)	**over-represented:** • protein degradation • protein synthesis • signaling (calcium, G-protein) • transport p- and v-ATPases	**over-represented:** • protein synthesis • unknowns • central metabolism • hormone metabolism • vesicle transport • transport p- and v-ATPases



- **transport**
- **cell organizatioin**
- **signaling**
- **transcription factors**
- **protein degradation/synthesis**
- **GPI-anchored plasma membrane**
- **glycolysis & metabolism**
- **hormone metabolism**

- **Early responses (up to 5 min)** involves GPI-anchored membrane proteins, receptor kinases, cell organization, transcription factors.
- **Late responses (after 5 min)** involve protein degradation and synthesis, second messenger signaling and metabolism.

Engelsberger WR, et al. (2010), submitted