Problem Session

2021. 8.28.

Aoi Takeuchi

Stable isotope labeling by amino acids in cell culture (SILAC) is a metabolic labeling strategy that uses stable isotope-labeled amino acids in growth medium to encode cellular proteomes for quantitative analysis. Please read the description below and answer the problems.

Description of method

SILAC labels cellular proteomes depending on cellular protein synthesis to incorporate stable isotope-containing amino acids, such as arginine or lysine containing six ^{13}C atoms, into whole proteomes. The SILAC experiment consists of two distinct phases—(a) an adaptation phase and (b) an experimental phase.

(a) Two populations of cells are grown in two separate medium formulations, the light medium containing the amino acid with the natural isotope abundance and the heavy medium containing the SILAC amino acid. During the adaptation phase, cells are grown in light and heavy SILAC media until the heavy cells have fully incorporated the heavy amino acids. This allows the two SILAC cell pools to be fully distinguishable by MS (black dot and red star in **Figure 1a**, indicating light and heavy SILAC peptides, respectively).

(b) In the second phase, the two cell populations are differentially treated, inducing changes in the proteome. The light and heavy cell populations are mixed and subjected to trypsin digestion to give peptides as a single pool. The sample is analyzed by MS for protein identification and quantification. Protein abundances are determined from the relative MS signal intensities.

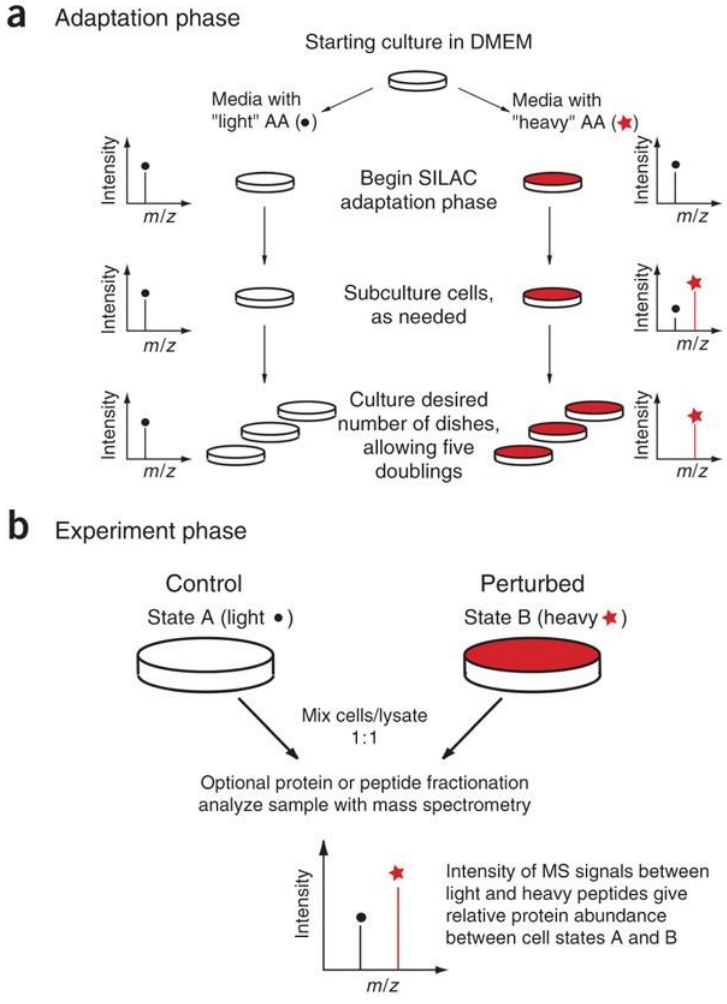


Figure 1. Two-phase of SILAC experiment workflow—(a) an adaptation phase and (b) an experimental phase.

Problem

Human embryonic stem cells (hESCs) were cultured and analyzed in the following SILAC protocol (**Figure 2**). As a result, two digested phosphopeptides **A**, and **B** were identified, sequenced, and quantified (**Table 1**). Although the light and heavy samples were mixed in a 1:1 protein ratio, the resulting MS abundance ratios were significantly unequal to 1 (**Figure 3a** for phosphopeptide **A**, and **Figure 4a** for phosphopeptide **B**).

A simple adjustment of the conventional SILAC method could improve quantification accuracy. In fact, the MS abundance ratios in the modified protocol resulted in nearly 1 (**Figure 3b** for phosphopeptide **A**, and **Figure 4b** for phosphopeptide **B**).

(1) Please explain why MS abundance ratios exhibited poor quantification accuracy in the conventional protocol.
(2) Please explain how the SILAC method was adjusted for the better quantification accuracy.
(3) Please assign all the mono-isotopic peaks of phosphopeptide **B** in **Figure 4**.

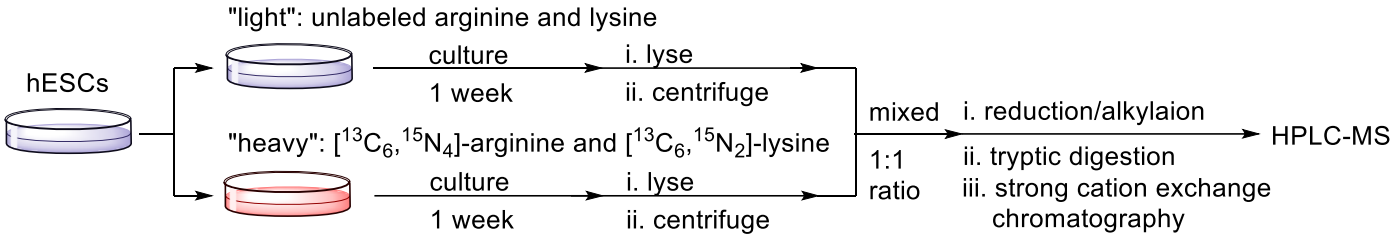


Figure 2. conventional SILAC protocol. cell line: hESCs (line HES-2) grown on mouse embryonic fibroblast feeder cells. medium: Dulbecco's Modified Eagle Medium (DMEM) supplemented with dialyzed fetal calf serum containing unlabeled/labeled amino acids.

Table 1. the identified sequences of digested phosphopeptides

phosphopeptide	sequence
A	p(SSGS)PYGGGYGSGGGSGGYGSR
B	STPFIVPp(SS)PTEQEGR

Note: Lower case “p” means that one of the Ser residues inside the following bracket was phosphorylated.

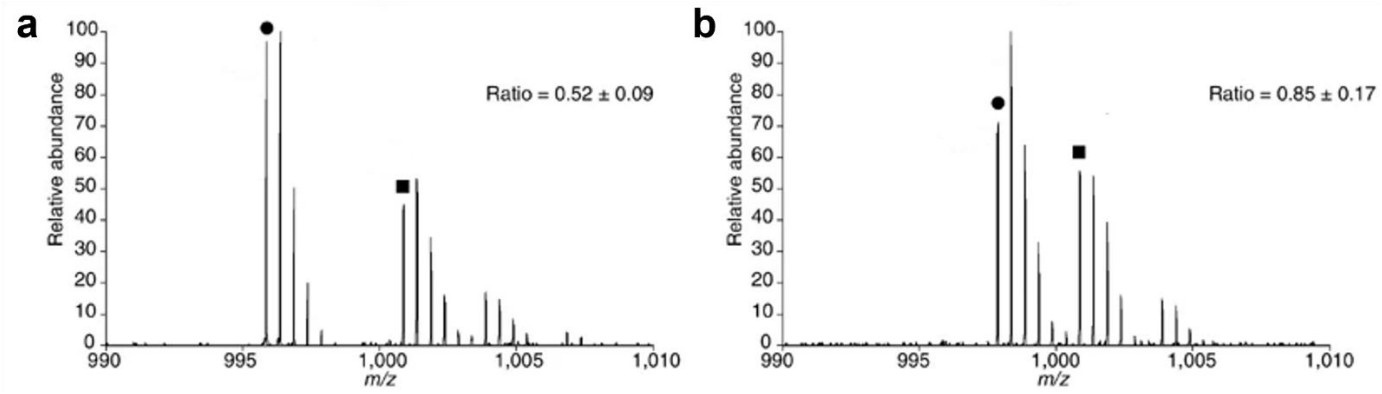


Figure 3. MS spectrum of a fraction containing phophopeptide **A** in (a) the conventional protocol and (b) the modified protocol (*m*/*z* = 990-1010). Abundance ratios were calculated from spectral intensities of the mono-isotopic peaks of light (●) and heavy (■) phosphopeptide pairs.

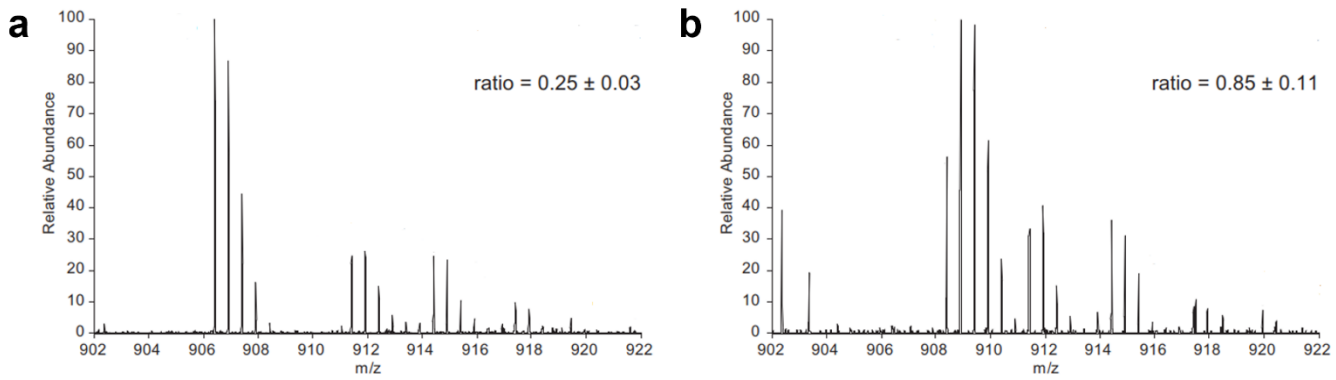


Figure 4. MS spectrum of a fraction containing phophopeptide **B** in (a) the conventional protocol and (b) the modified protocol (*m*/*z* = 902-922). Abundance ratios were calculated from spectral intensities of the mono-isotopic peaks of light and heavy phosphopeptide pairs.

Reference

Figure 1 was cited from; Ong, S. E.; Mann, M. *Nat. Protoc.* **2006**, *1*, 2650.

Problem Session

2021. 8.28.

Aoi Takeuchi

Topic: Mass spectrometry (MS)-based quantitative proteomics

Contents:

I. MS-based quantitative proteomics
II. Stable isotope labeling by amino acids in cell culture (SILAC)
III. Solution for the problem

I. MS-based quantitative proteomics

Quantitative data acquisition is essential for proteomics, the global analysis of gene expression at the protein level in a cell, providing insights into the biochemical state of the relevant cell or tissue. It requires a reproducible, sensitive and selective method, which provides a linear response over a wide range of concentrations.

While quantitative proteomics has its origins in the technology of two-dimensional gel electrophoresis recording differences in the staining pattern of proteins derived from two states of cell populations or tissues[1], mass spectrometry (MS)-based methods have emerged as the method of choice for the confident and near-exhaustive identification and quantification of the proteins contained in a biological sample over the past two decades[2]. They have significantly contributed to the unraveling of cellular signaling networks[3], elucidation of the dynamics of protein–protein interactions in different cellular states[4], and pharmacological evaluation[5].

For accurate quantifying proteins in MS-based systems, several unique stable labelling strategies have been developed[6] (**Table 1**, **Figure 1**). Each approach utilizes stable isotopes, which are incorporated into proteins or peptides of different samples. Stable isotopes change the masses of similar peptide fragments in one sample compared to the other, without any other effect on the biophysical and chemical properties of peptides or proteins. These small mass differences in peptide fragments are detected in MS, and relative protein abundances can be calculated based on the peak intensities for the distinguishable *m/z* values.

Table 1. Approaches of MS-based quantitative proteomic analyses

	(a) Metabolic labeling	(b) Chemical labeling
how to label	culture in medium with labeled amino acids	react different mass tags with peptides
pros.	・labeling in the early stage of preparation →minimize error in quantification	・compatible with many biological samples ・compare many samples at one analysis
cons.	・restricted to labeling proteins in cell culture	・labeling in the later stage of preparation →lower accuracy and sensitivity
example	SILAC[7]	ICAT[8], iTRAQ[9], TMT[10]
	(c) Enzymatic labeling	(d) Label-free
how to label	digest proteins in the presence of ^{18}O-water	compare spectral counts of unlabeled samples
pros.	・versatile method ・low cost	・identify and quantify peptides at one analysis ・compare unlimited number of samples
cons.	・variable enrichment efficiency of ^{18}O[11]	・poor quantitative accuracy
example	^{18}O-labeling[12]	normalized spectral index (SI_N)[13]

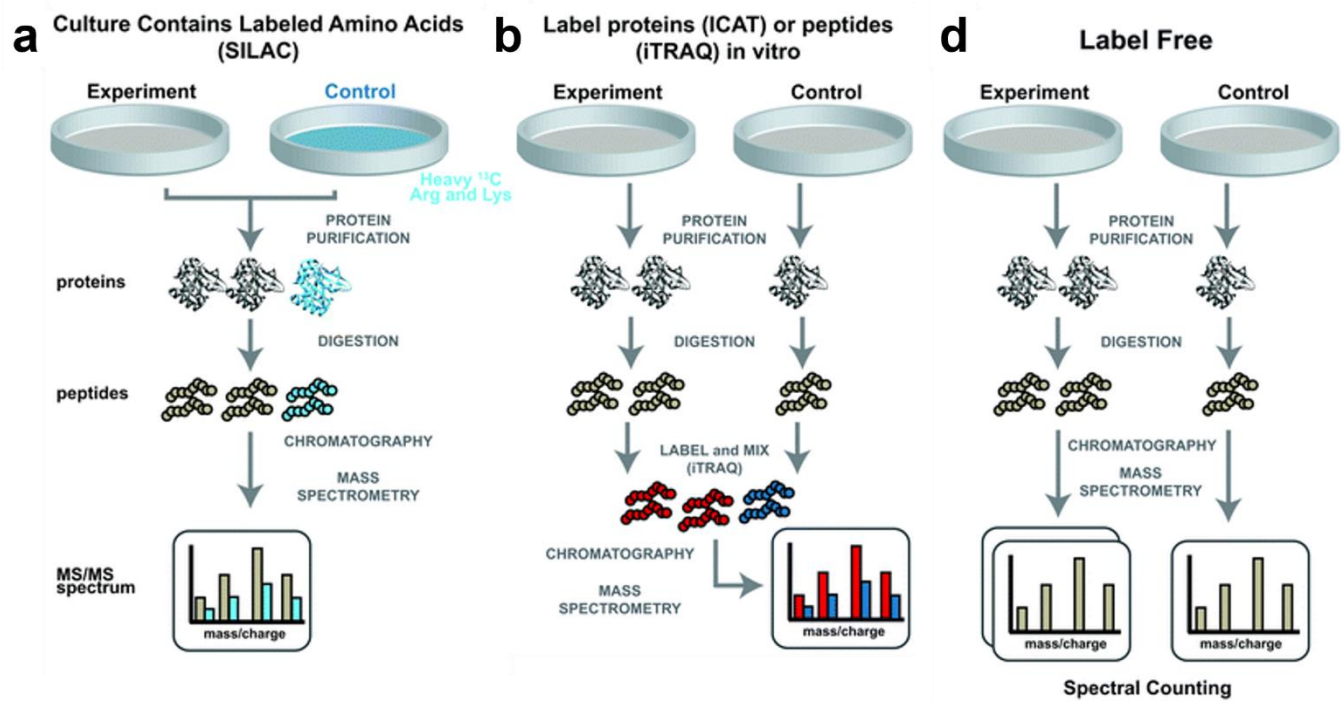


Figure 1. Approaches of MS-based quantitative proteomic analyses[6]. (a) metabolic labeling, (b) chemical labeling, and (d) label-free method. A figure is omitted for (c) enzymatic labeling.

II. Stable isotope labeling by amino acids in cell culture (SILAC)

The concept of stable isotope labeling by amino acids in cell culture (SILAC) was reported in 2002 for the first time by Mann's group[7]. As the general workflow of SILAC is described in **Description of method**, other supplementary information about the protocol is noted below. For the labeling strategy, arginine and lysine are typically applied as labeled amino acids because trypsin and lys-C are common proteolytic enzymes in proteomics workflow[14]. As they are very aggressive yet specific proteases that cleave at the carboxyl-termini of lysine and arginine residues[15] (lys-C cleaves only those of lysine), resulting digested peptides contain one labeled residue for each, enabling easy detection of residue-specific mass shift on MS spectrum[16] (**Figure 2**).

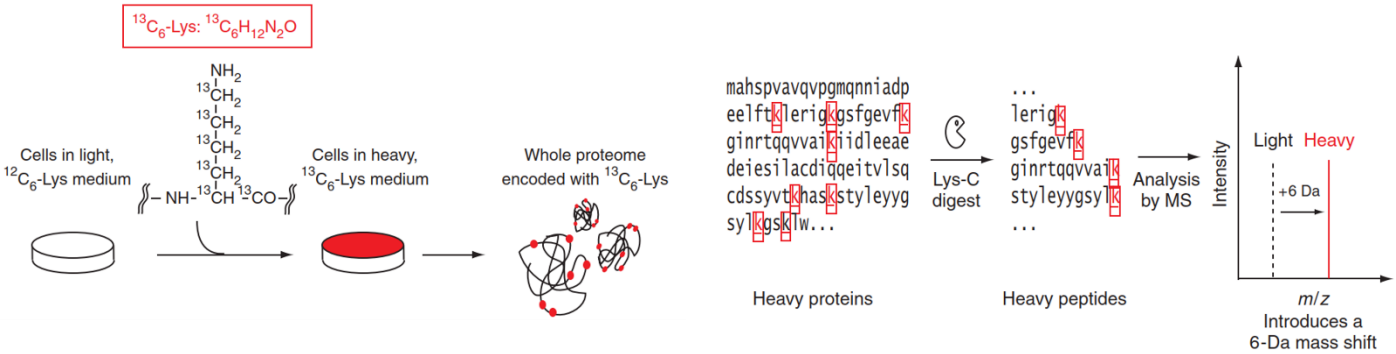


Figure 2. Encoding quantitative information into whole proteomes with [$^{13}C_6$]-lysine[17]. Digesting proteins with lys-C results in peptides bearing [$^{13}C_6$]-lysine on carboxyl-terminal and the residue-specific mass shift of 6 Da from the light peptide, thereby distinguishing the two forms for quantification by MS.

Quantitative analysis of proteomes using metabolic labeling requires stoichiometric incorporation of the label into an organism's proteins without metabolic scrambling. In this problem session, one of elegant arrangements in SILAC experimental design was picked up, and it is aimed to understand possible biochemical reactions in vivo and how to avoid loss of quantification accuracy concerning metabolic reactions.

III. Solution for the problem

At the very beginning, the information in **Figure 3** should be well-organized. The exact mass of phosphopeptide **A** is calculated from its sequence.

phosphopeptide **A**: p(SSGS)PYGGGYGSGGGSGGYGSR (exact mass: 1989.75)

As **A** contains one arginine and no lysine residues which were labeled through SILAC protocol, 10 Da peak shift should be observed between light and heavy **A** because of [$^{13}C_6$, $^{15}N_4$]-arginine. For sake of simplicity, isotopic labeling state of a peptide is depicted as ^{N}X, where N is the shifted mass number by isotope labeling and X is one-letter-code of a labeled amino acid residue. Accordingly, ^{0}R stands for the light **A** and ^{10}R stands for the heavy **A** in this case.

In fact, mass-to-charge ratio (*m/z*) of mono-isotopic peak ● in **Figure 3a** corresponds to light **A** $[M(^{0}R)+2H]^{2+}$ = 995.88, and that of ■ corresponds to heavy **A** $[M(^{10}R)+2H]^{2+}$ = 1000.88. Note that the observed MS peaks of **A** in **Figure 3** were double charged ions judging from the gaps between isotopic peaks.

The main issue of this problem is the low ratio of MS peak intensity of ■ to that of ● although the light and heavy samples were mixed in a 1:1 protein ratio, exhibited poor quantification accuracy in **Figure 3a**. The ratio of MS peak intensity of ■ to that of ● improved from 0.52 ± 0.09 to 0.85 ± 0.17 by a simple adjustment (**Figure 3b**). It should be noted that +4 Da peak shift (*m/z* = 995.88→997.88) was observed in mono-isotopic peak ● by this adjustment, suggesting that the adjustment is related to isotopic labeling strategy. It is also to be mentioned that another mono-isotopic peak *m/z* = 1003.88 (marked as ▲) was observed in both MS spectrum, and the isotopic distribution of peak ● was different between **Figure 3a** and **3b**.

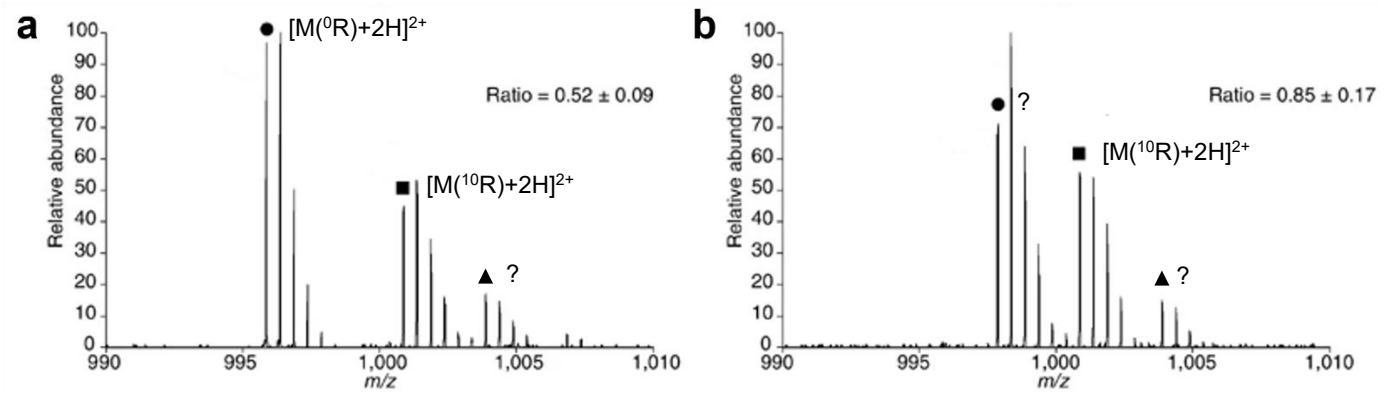


Figure 3. MS spectrum of a fraction containing phosphopeptide **A** in (a) the conventional protocol and (b) the modified protocol. Abundance ratios were calculated from spectral intensities of the mono-isotopic peaks of light (●) and heavy (■) phosphopeptide pairs. While the peaks ● and ■ correspond to $[M(^{0}R)+2H]^{2+}$ and $[M(^{10}R)+2H]^{2+}$, respectively in **Figure 3a**, the peak ▲ is not theoretically accountable so far. By adjusting the isotopic labeling strategy, the ratio of MS peak intensity of ■ to ● improved to nearly 1 in **Figure 3b**.

(1) Please explain why MS abundance ratios exhibited poor quantification accuracy in the conventional protocol.

As the “light” dish and “heavy” dish were treated with no difference except amino acid components of medium, it is probable that the metabolism of isotopically labeled amino acids in the cell is responsible for the different behavior in MS spectrum.

➢ Gluconeogenesis and amino acid catabolism

Amino acids are synthesized by all living organisms. They are built from other amino acids or from different metabolites, and undergo various catabolic (degradative) processes as well. All amino acids except leucine and lysine can be degraded to form metabolic intermediates whose carbon skeletons can be converted to glucose via gluconeogenesis. These amino acids are therefore called 'glucogenic'. On the other hand, amino acids whose degradaton forms intermediates that can be converted to ketone bodies (acetoacetate, β-hydroxybutyrate or acetone) are called 'ketogenic'. Lysine and leucine are purely ketogenic, whereas phenylalanine, isoleucine, threonine, tyrosine and tryptophan are both glucogenic and ketogenic (**Figure 4**)[18].

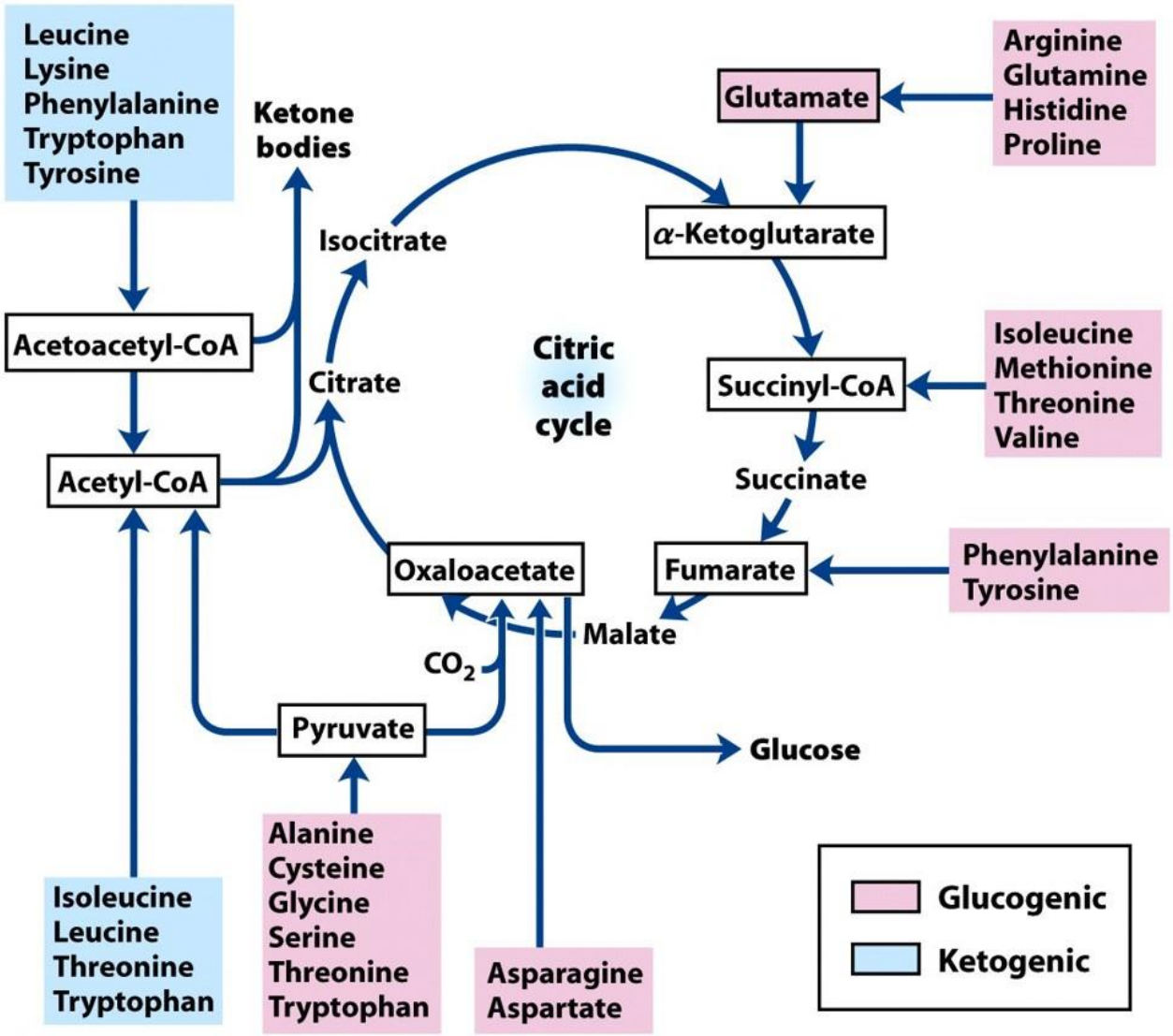
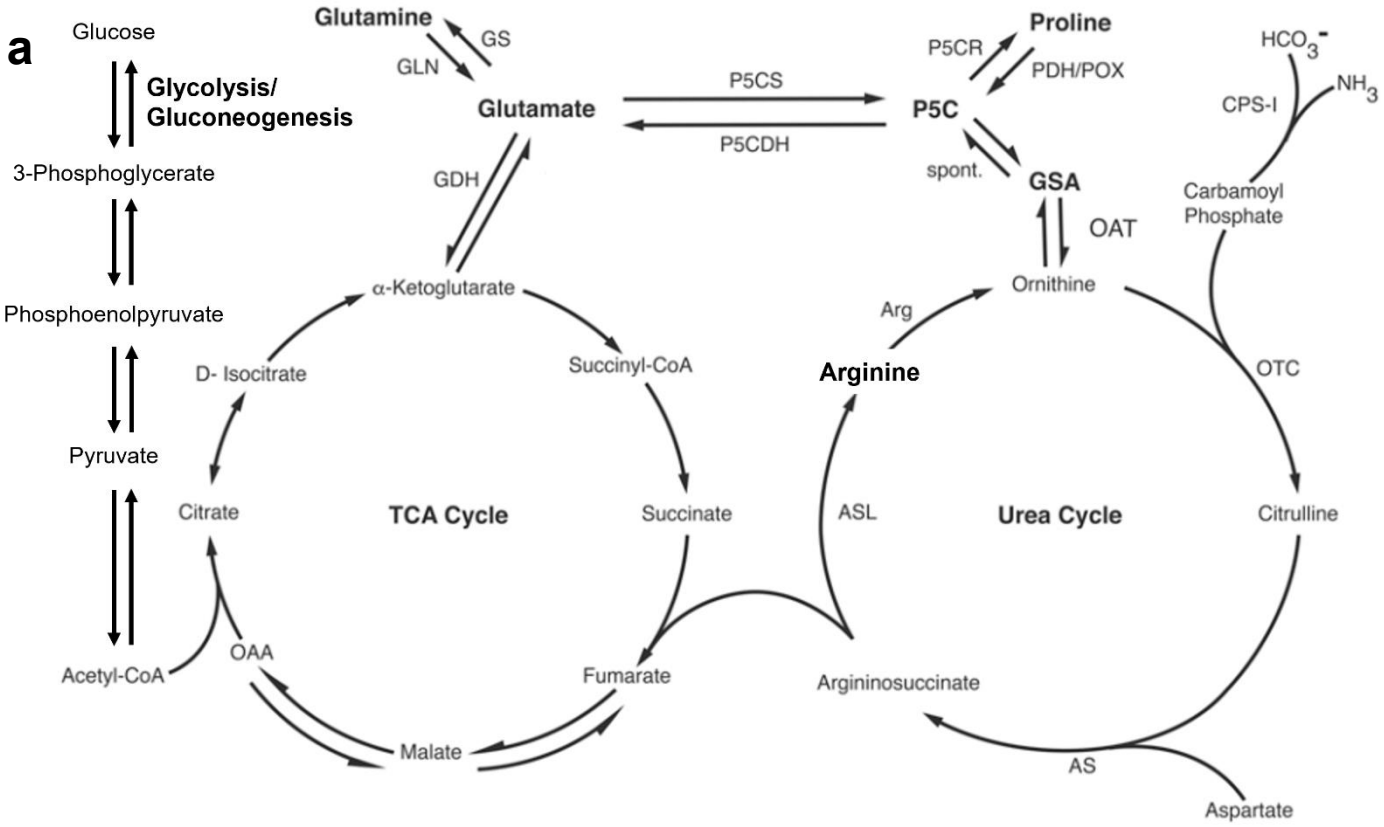
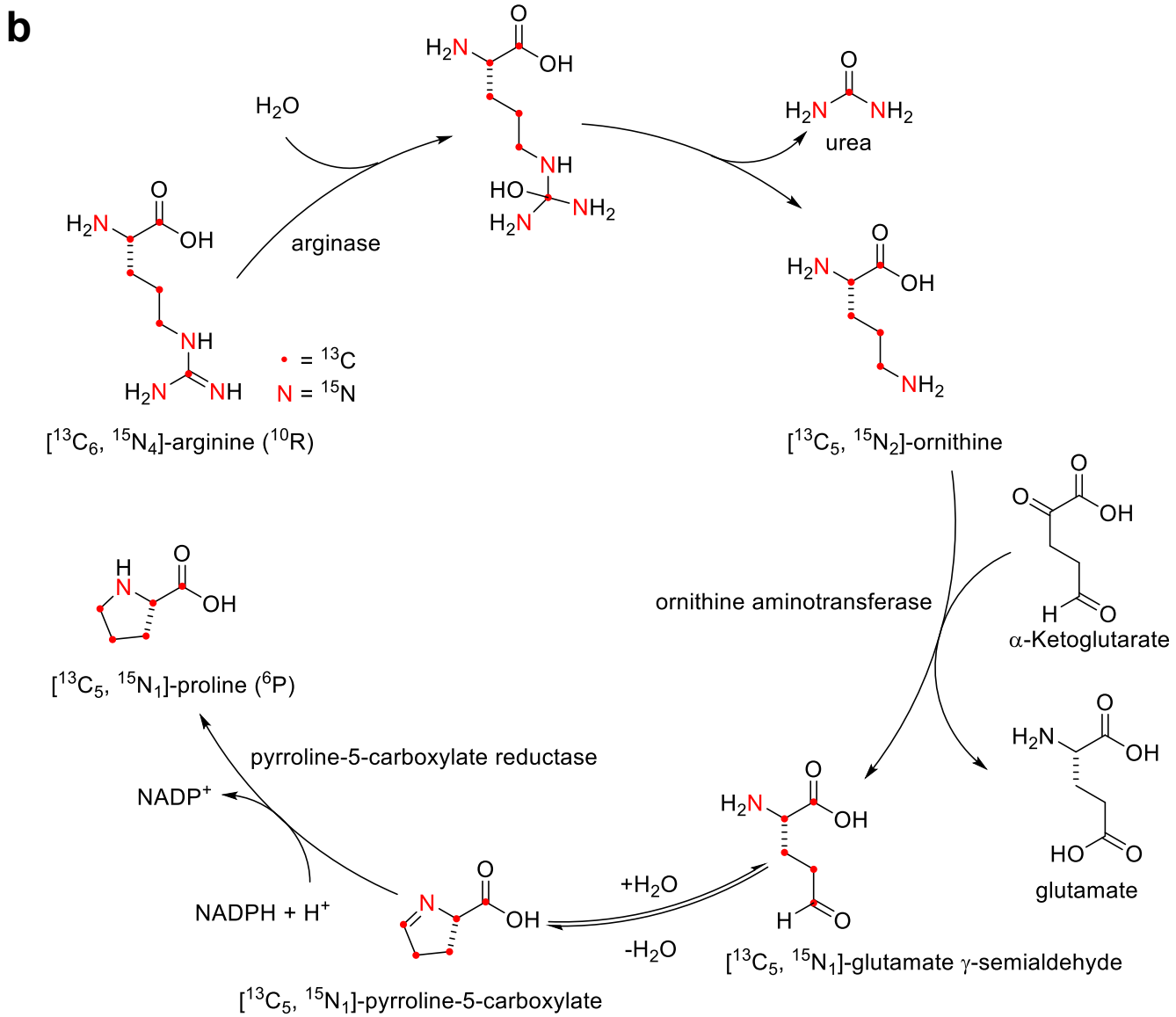


Figure 4. Simplified scheme of amino acid catabolism to building blocks of central metabolism[19]. All amino acids are catabolized into seven metabolites in white boxes.

➢ Arginine-proline metabolic conversion

Among various catabolic pathways of amino acids, arginine, proline, and glutamate metabolism are functional links between the TCA cycle and urea cycle, the process in which toxic ammonia is converted to less toxic urea[20] (**Scheme 1a**). It is often the case that the quantitative accuracy of SILAC is compromised by the metabolic conversion of arginine to proline in eukaryotes[21]. As a result, and [$^{13}C_6$,$^{15}N_4$]-arginine becomes [$^{13}C_5$,$^{15}N_1$]-proline (**Scheme 1b**). This results in the generation of multiple satellite peaks for all proline-containing tryptic peptides in the labeled state, which hampers accurate quantitation[19] (**Figure 5**).


a
Glucose
Glycolysis/
Gluconeogenesis
3-Phosphoglycerate
Phosphoenolpyruvate
Pyruvate
Acetyl-CoA
Citrate
D- Isocitrate
α-Ketoglutarate
Succinyl-CoA
Succinate
Fumarate
Malate
OAA
TCA Cycle
Glutamine
GS
GLN
Glutamate
GDH
P5CS
P5CDH
Proline
P5CR
PDH/POX
P5C
spont.
GSA
OAT
Ornithine
Arg
Arginine
ASL
Argininosuccinate
AS
Aspartate
Urea Cycle
OTC
Citrulline
Carbamoyl
Phosphate
CPS-I
HCO3-
NH3


b
H2O
arginase
urea
• = 13C
N = 15N
[13C6, 15N4]-arginine (10R)
[13C5, 15N2]-ornithine
ornithine aminotransferase
α-Ketoglutarate
glutamate
[13C5, 15N1]-proline (6P)
pyrroline-5-carboxylate reductase
NADP+
NADPH + H+
+H2O
-H2O
[13C5, 15N1]-pyrroline-5-carboxylate
[13C5, 15N1]-glutamate γ-semialdehyde

Scheme 1. (a) Arginine, proline and glutamate metabolism pathways linking between the TCA and urea cycles. Abbreviations: Arg, arginase; AS, argininosuccinate; ASL, argininosuccinate lyase; CPS-I, carbamoyl phosphate synthetase I; CoA, coenzyme A; GDH, glutamate dehydrogenase; GLN, glutaminase; GS, glutamine synthase; GSA, glutamate-γ-semialdehyde; OAA, oxaloacetic acid; OAT, ornithine aminotransferase; OTC, ornithine transcarbamylase; PDH, proline dehydrogenase; POX, proline oxidase; P5C, pyrroline-5-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P5CS, P5C synthase; spont., spontaneous; TCA, tricarboxylic acid. (b) Metabolic pathway of arginine to proline with isotope labeling by ^{13}C and ^{15}N.

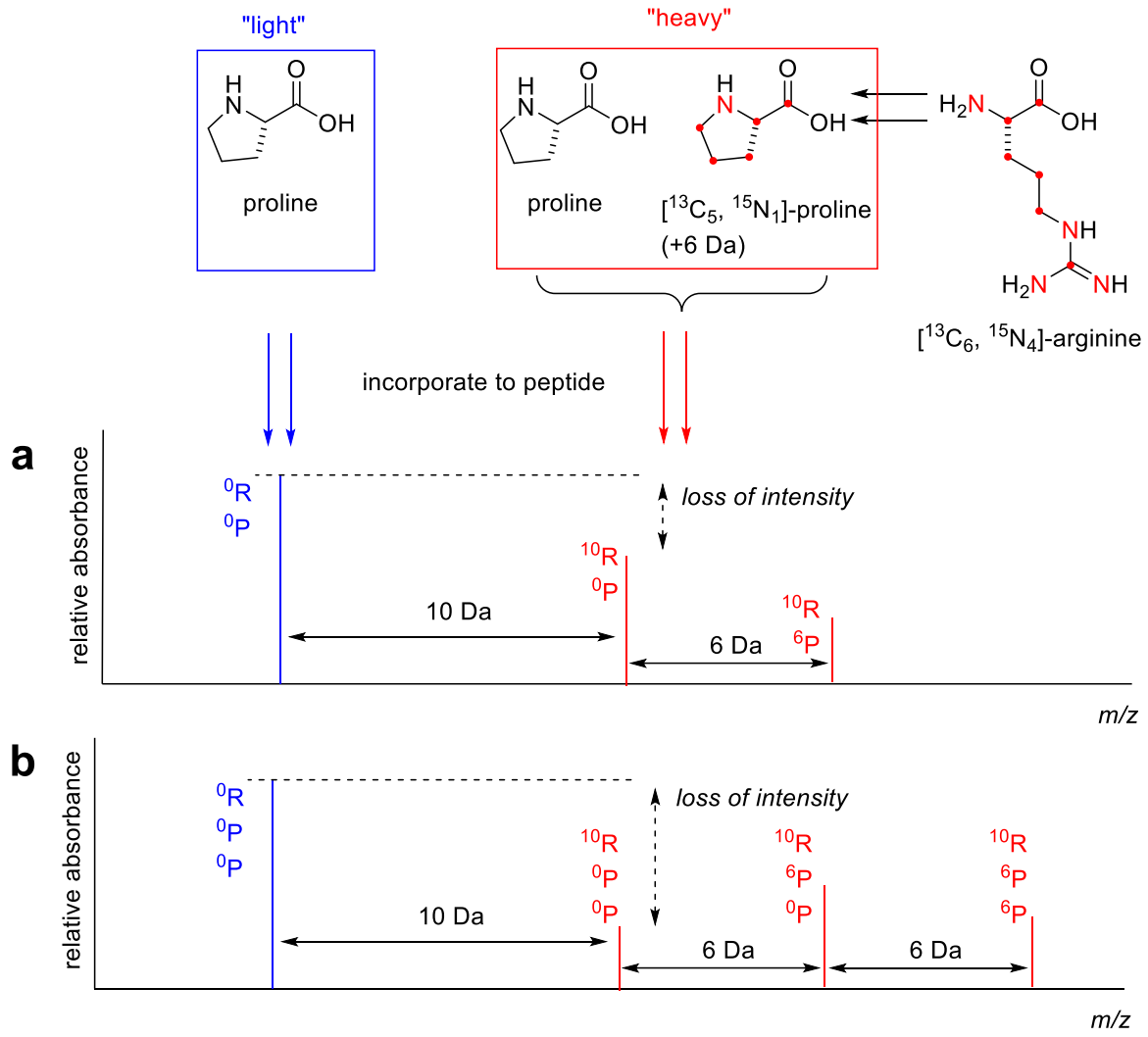


Figure 5. Theoretical mass spectra of peptides having (a) one or (b) two proline residues as they would appear in a tryptic digest of unlabeled proteins mixed in a 1:1 ratio with proteins derived from cells cultured in medium containing [$^{13}C_6$,$^{15}N_4$]-arginine.

As a result of arginine-proline conversion, one additional peak would appear in MS spectra of phosphopeptide **A**. This corresponds to $^{10}R^{6}P$: an isotopologue of **A** to whose sequence [$^{13}C_5$, $^{15}N_1$]-proline was incorporated. This rationalizes the existence of peak ▲, which is shifted in +6 Da from peak ■($^{10}R^{0}P$) in **Figure 3**.

As comparing **Figure 5a** and **5b**, the more proline residues a target peptide has, the greater the loss of intensity would become. In fact, MS peak abundance ratio of phosphopeptide **B** (three prolines) was lower than that of **A** (one proline) in the conventional protocol.

(2) Please explain how the SILAC method was adjusted for the better quantification accuracy.

Several modifications to improve quantification accuracy have been developed. A widely used simple solution is to reduce the arginine concentration[21] to minimize conversion to proline as seen in some commercially available SILAC kits; however, this is not applicable to every cell type as it negatively affects cell behavior by introducing a stress condition and reduces growth rates especially of fast-growing cells[22]. In fact, human embryonic stem cells (hESCs) have particularly strict culture requirements; small changes in medium composition or environment including arginine starvation may initiate differentiation or cell death.

Alternative solutions include manual or mathematical corrections for all proline-containing peptides, adding the contribution from the heavy proline to the heavy arginine peak[14,21]. This, however, can substantially reduce accuracy, particularly for low-intensity ion signals, and is laborious for large data sets.

Krijgsveld's group reported a simple and effective adjustment in isotopic labeling strategy[23], which realizes highly accurate quantification and may be useful for cell types in which arginine starvation is not a viable option. The principle is to utilize an isotopically labeled arginine in "light" conditions as well. [$^{15}N_4$]-arginine is used in combination with unlabeled lysine in the "light" conditions while [$^{13}C_6$,$^{15}N_4$]-arginine is used in combination with [$^{13}C_6$,$^{15}N_2$]-lysine in the "heavy" conditions. Labeled prolines ([$^{15}N_1$]-proline and [$^{13}C_5$,$^{15}N_1$]-proline) will be formed at the same rate under both "light" and "heavy" conditions, thus providing an internal correction for arginine conversion (**Figure 6**).

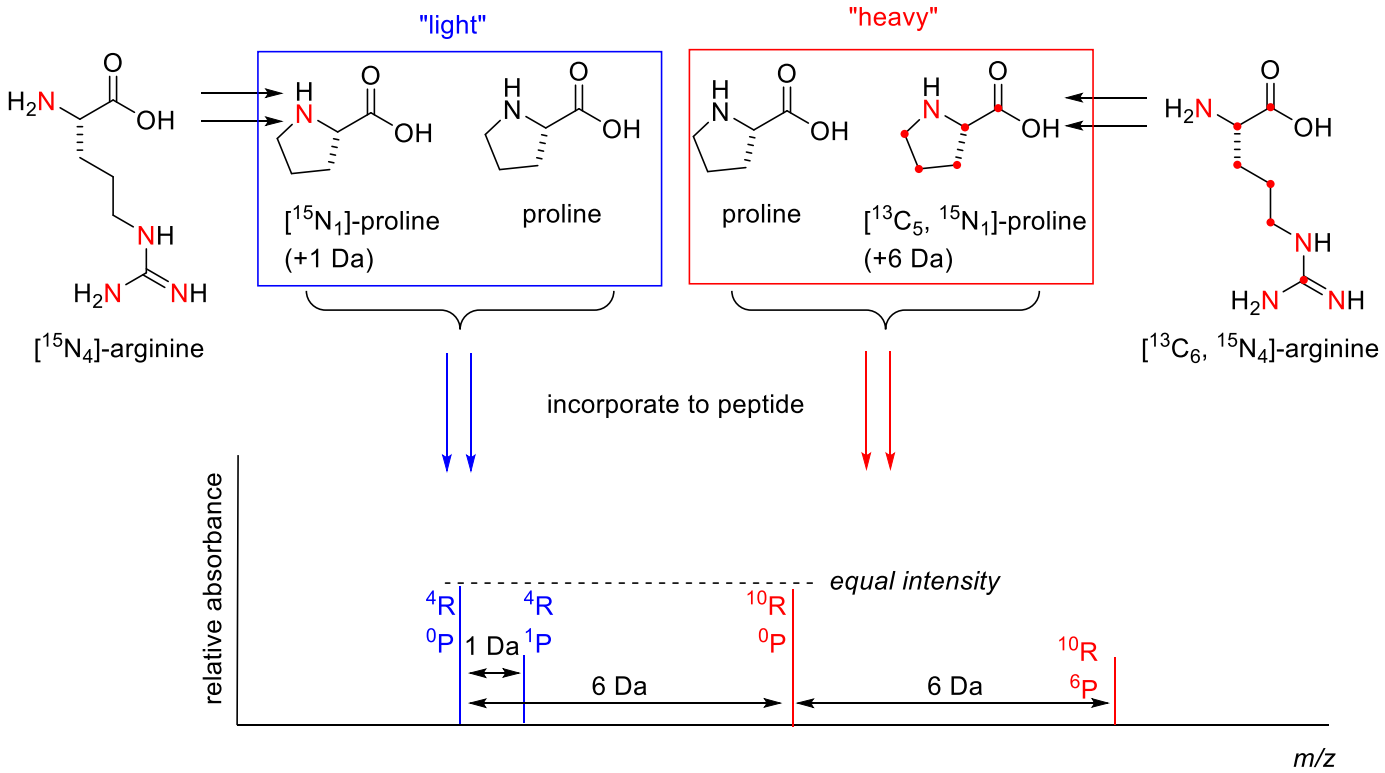


Figure 6. Theoretical mono-isotopic peaks of a proline-containing peptide labeled with [$^{15}N_4$]-arginine mixed in a 1:1 ratio with the same peptide labeled with [$^{13}C_6$,$^{15}N_4$]-arginine. As labeled proline will be formed at the same rate, the mono-isotopic peak intensity of $^4R^0P$ and $^{10}R^0P$ should be equal, exhibiting accurate quantification.

As a result, the mono-isotopic peak of a proline-containing peptide in the "light" conditions shifts in +4 Da ($^0R^0P$ → $^4R^0P$). Although it is possible to use arginine labeled in other manners such as [$^{13}C_6$]-arginine instead of [$^{15}N_4$]-arginine for substitution from nonlabeled arginine in the "light" conditions, they are unlikely to account for +4 Da peak shift (*m/z* = 995.88→997.88) of the mono-isotopic peak ● from **Figure 3a** to **3b**.

Unlike the theoretical mass spectra in **Figure 6**, the MS peak of $[^4R^1P+2H]^{2+}$ overlaps with that of $[^4R^0P+2H]^{2+}$ in the actual MS measurement because each peptide has isotopic distribution ranging from 0 to +3 Da of its mono-isotopic peak due to natural abundance of heavy isotope atoms. That is why the isotopic distribution of peak ● was different between **Figure 3a** and **3b**. The relative intensity of the most abundant peak of ● got higher in **Figure 3b** due to the overlap of peaks of $[^4R^0P+2H]^{2+}$ and $[^4R^1P+2H]^{2+}$. Peak assignment of the mono-isotopic peaks of phosphopeptide **A** in MS spectrum is described in **Figure 7**.

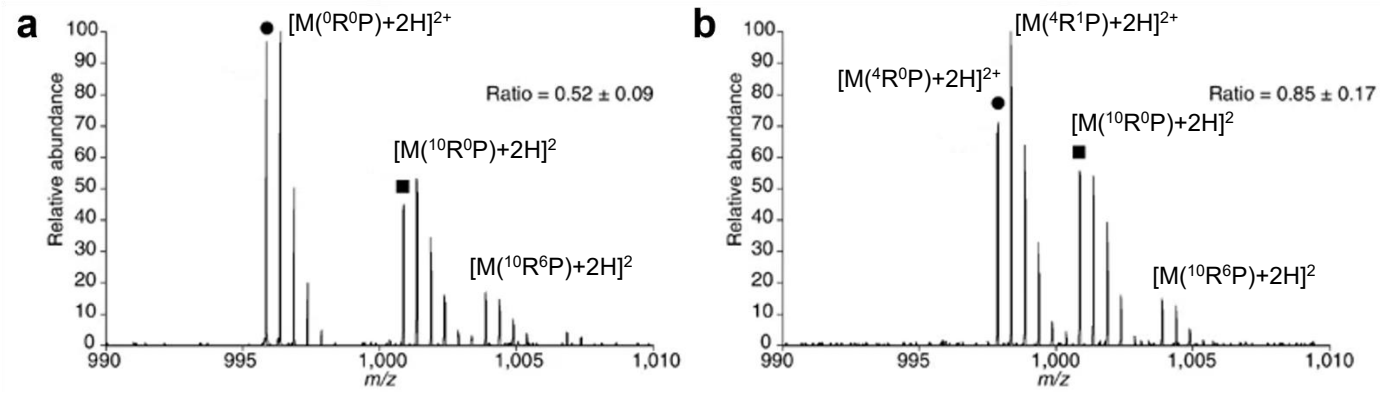


Figure 7. Peak-assigned MS spectrum of a fraction containing phophopeptide **A** in (a) the conventional protocol and (b) the modified protocol. Abundance ratios were calculated from spectral intensities of the mono-isotopic peaks of light (●) and heavy (■) phosphopeptide pairs.

(3) Please assign all the mono-isotopic peaks of phosphopeptide **B**.

The exact mass of phosphopeptide **B** is calculated from its sequence.

phosphopeptide **B**: STPFIVPp(SS)PTEQEGR (exact mass: 1810.81)

MS peaks of **B** observed in the problem sheet are double charged ions. In addition to one arginine and no lysine residues which were labeled through SILAC protocol, **B** contains three proline residues which can be partially labeled due to the metabolic conversion from arginine. As a result, four possible isotopologues would be generated in the "heavy" conditions ($^{10}R^0P^0P^0P$, $^{10}R^6P^0P^0P$, $^{10}R^6P^6P^0P$, $^{10}R^6P^6P^6P$). Other four isotopologues would be generated in the "light" conditions in the modified protocol as well ($^4R^0P^0P^0P$, $^4R^1P^0P^0P$, $^4R^1P^1P^0P$, $^4R^1P^1P^1P$). Thus, all the theoretical isotopologues are listed in **Table 2**, and described as MS spectrum in **Figure 8**. These predictions well match with the real MS spectrum. Peak assignment of the mono-isotopic peaks of phosphopeptide **B** in MS spectrum is described in **Figure 9**.

Table 2. Theoretical isotopologues of phosphopeptide **B** generated in SILAC protocols

	conventional protocol		modified protocol	
	species	theoretical *m/z*	species	theoretical *m/z*
"light" conditions	$[M(^0R^0P^0P^0P)+2H]^{2+}$	906.41	$[M(^4R^0P^0P^0P)+2H]^{2+}$	908.41
			$[M(^4R^1P^0P^0P)+2H]^{2+}$	908.91
			$[M(^4R^1P^1P^0P)+2H]^{2+}$	909.41
			$[M(^4R^1P^1P^1P)+2H]^{2+}$	909.91
"heavy" conditions	$[M(^{10}R^0P^0P^0P)+2H]^{2+}$	911.41	$[M(^{10}R^0P^0P^0P)+2H]^{2+}$	911.41
	$[M(^{10}R^6P^0P^0P)+2H]^{2+}$	914.41	$[M(^{10}R^6P^0P^0P)+2H]^{2+}$	914.41
	$[M(^{10}R^6P^6P^0P)+2H]^{2+}$	917.41	$[M(^{10}R^6P^6P^0P)+2H]^{2+}$	917.41
	$[M(^{10}R^6P^6P^6P)+2H]^{2+}$	920.41	$[M(^{10}R^6P^6P^6P)+2H]^{2+}$	920.41

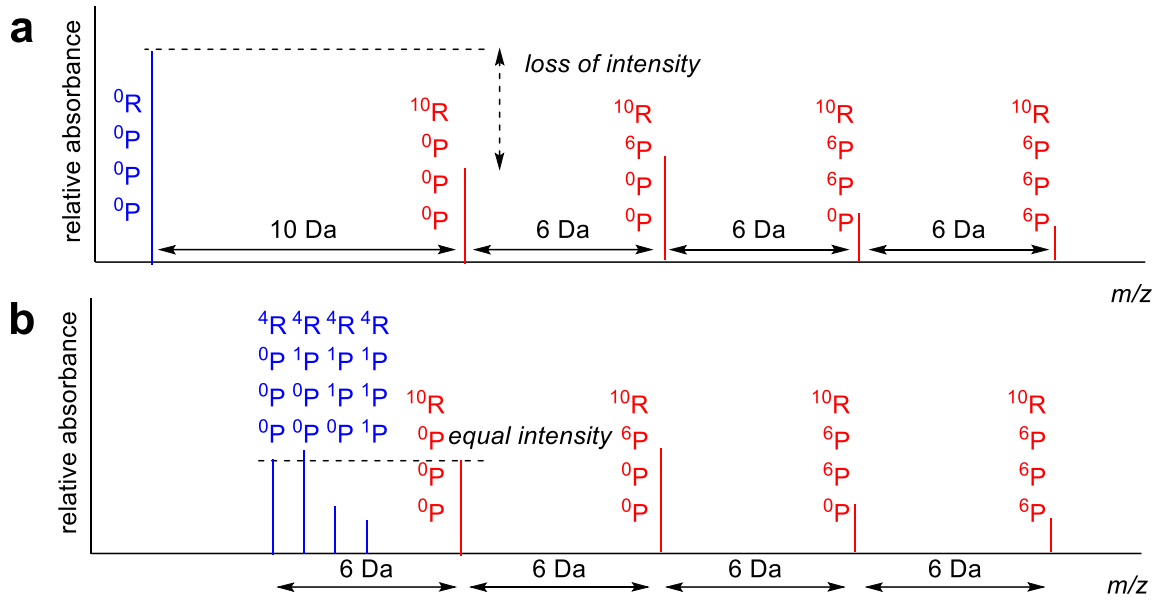


Figure 8. Theoretical mono-isotopic peaks of phosphopeptide **B** labeled with [$^{13}C_6$,$^{15}N_4$]-arginine mixed in a 1:1 ratio with (a) nonlabeled **B** and (b) the same peptide labeled with [$^{15}N_4$]-arginine. The situations in **Figure 7a** correspond to the conventional protocol and those in **Figure 7b** correspond to the adjusted protocol.

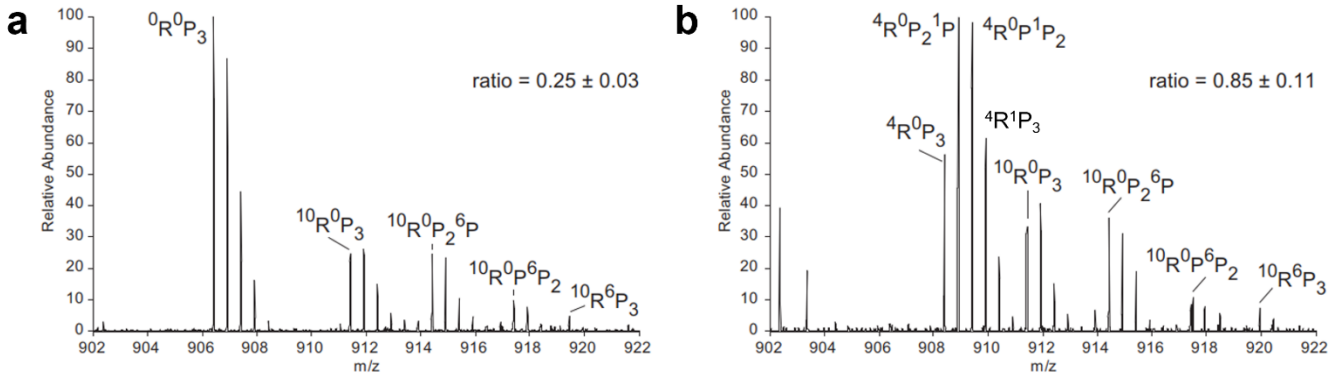


Figure 9. Peak-assigned MS spectrum of a fraction containing phophopeptide **B** in (a) the conventional protocol and (b) the modified protocol. All peaks are observed as double charged ions of proton adducts. Abundance ratios were calculated from spectral intensities of the mono-isotopic peaks of light and heavy phosphopeptide pairs.

Answer

(1) Because of arginine-proline metabolic conversion during cell culture

(2) Use [$^{15}N_4$]-arginine for the “light” conditions instead of nonlabeled arginine

(3) See **Figure 9**

References

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