REVIEW

Quantitative proteomics using SILAC: Principles, applications, and developments

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SILAC is based on direct addition of selected stable isotope amino acids into the cell culture medium, allowing superior quantitative analysis of the cellular proteome compared to other labeling methods. The great advantages of SILAC lie in its straight-forward implementation, quantitative accuracy, and reproducibility over chemical labeling or label-free quantification strategies, favoring its adoption for proteomic research. SILAC has been widely applied to characterize the proteomic changes between different biological samples, to investigate dynamic changes of protein PTMs, to distinguish specific interacting proteins in interaction proteomic analysis, and to analyze protein turnover in the proteome-wide scale. The present review summarizes the principles of SILAC technology, its applications in biological research, and the present state of this technology.

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1 Introduction

MS-based quantitative proteomics has become a powerful tool in biological research. Since MS is inherently nonquantitative, proteolytic peptides have different mass spectrometric responses due to their various physicochemical properties such as size, charge, hydrophobicity. Thus, it is infeasible to quantify the relative abundance changes between different experimental samples in the same MS analysis. To solve this problem, the stable isotopes are introduced into the proteins or peptides, allowing relative quantification of these molecules from different samples in the same MS analysis, which will reduce the variability from sample injection and ion suppression by MS instruments in different MS runs. Based on whether using stable isotope or the method employed for incorporating stable isotopes, MS-based quantitative proteomic methods are subdivided into three classes, namely chemical/enzymatic labeling, metabolic labeling, and label-free. Chemical/enzymatic labeling methods introduce the mass tags to the proteins or peptides, including ICAT [1], iTRAQ [2, 3], TMT [4], dimethyl labeling [5, 6], and ^{18}O labeling [7], while metabolic labeling methods utilize biological incorporation of stable isotope labels into proteins in living cells, including SILAC [8] and ^{15}N labeling [9]. In comparison, label-free methods conduct comparison without any labeling on the proteins or peptides, but by measuring ion intensity changes in chromatography or counting the number of fragment spectra identifying peptides of a given protein (spectrum counting) [10,11]. All of these MS-based quantification methods have their particular strengths and weaknesses (Table 1), therefore, quantitative method should be chosen according to types of sample and aim of experiments.

SILAC, which was first used in 2002 [8], is a metabolic labeling strategy employed in quantitative proteomics. Here, stable isotope-labeled amino acids are added to the growth medium of living cells, enabling the relative comparison of the cellular proteome of different states. Because only specific amino-acid residues are labeled with stable isotopes and used

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Abbreviations: ABC, activated B-cell; **DLBCL**, diffuse large B-cell lymphoma; **GCB**, germinal-center B-cell; **I-DIRT**, isotopic differentiation of interactions as random or targeted; **PPI**, protein–protein interaction; **pSILAC**, pulsed SILAC; **QUICK**, quantitative immunoprecipitation combined with knockdown; **TPP**, trans-proteomic pipeline

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Table 1. Characteristics and applications of MS-based quantitative methods

Quantification methods	Way to introduce label	Labeling level	Sample type	Number of conditions to be compared	Quantification accuracy	Quantitative proteome coverage	Linear dynamic range
Metabolic labeling							
SILAC	ex vivo, in vivo	Protein	Cells, expand to tissues or model organisms	Up to 5	+++	++	1–2 log
^{15}N	ex vivo, in vivo	Protein	Cells	2	+++	++	1–2 log
Chemical labeling							
ICAT	in vitro	Protein	Applicable to any sample (cells, animal, or human tissue samples)	2	++	++	2 log
iTRAQ	in vitro	Peptide		up to 4 or 8	++	++	2 log
TMT	in vitro	Peptide		up to 6 or 10	++	++	2 log
Dimethyl labeling	in vitro	Peptide		2–3	++	++	2 log
Enzymatic lebeling							
^{18}O labeling	in vitro	Peptide	Applicable to any sample	2	++	++	1–2 log
Label free							
Ion intensity	n.a.	n.a.	Applicable to any sample (cells, tissue samples)	No limit	+	+++	2–3 log
Spectrum counting	n.a.	n.a.		No limit	+	+++	2–3 log

n.a., not applicable; +, good; ++, very good; +++, excellent.

for quantification, this method has great advantages in quantitative applications over other metabolic labeling approaches such as ^{15}N labeling, which replaces all nitrogen atoms of the proteome. Here, quantification is based on the numbers of nitrogen atoms in the proteins, which greatly complicates data analysis.

SILAC is reported to be the most accurate quantitative MS method [11, 12]. The basis of this is that differentially treated samples can be combined at the level of intact cells or protein, namely at the very first step of the experimental workflow, and can be processed together to minimize experimental error or bias. However, a study shown that iTRAQ outperforms the method of SILAC in the number of protein identifications and analysis time [13]. A recent comparison of SILAC and dimethyl labeling shows that the two methods have comparable accuracy and quantitative dynamic range, but SILAC outweights dimethyl labeling in reproducibility [14]. From this point of view, SILAC is particularly suitable for studies with extensive sample processing, such as subcellular fractionation, affinity purification of protein complex or enrichment of peptides with PTMs [14, 15].

One of the few disadvantages of SILAC is the limited number of cellular states that can be compared, because of the limited labeling combinations available when using heavy labeled amino acids. However, the combination of several SILAC experiments with the same experimental state has allowed for investigation of nine-point dynamic signaling pathways [16]. More detailed information for this approach is provided in the section "Use of SILAC for multiplexed comparison." The advantage of chemical labeling over SILAC is that it can be used to analyze a wide range of samples, including cells, tissues, and body fluids. However, the development of spike-in SILAC [17] and super-SILAC [18] extends the application of SILAC to tissues and body fluids as well.

At present, SILAC is accepted as the best method available for quantitative proteomics in terms of easy implementation, quantitative reliability, and robustness [19, 20]. This review provides an overview of the SILAC technology, including its principle, development, and application in biological research.

2 Principles of SILAC

2.1 Description of the SILAC method

The principle of SILAC is based on metabolically incorporating stable isotope labeled amino acids, such as ^{13}C or ^{15}N-labeled arginine or lysine, into the entire proteome during protein metabolism, specifically during the process of cell culturing. In SILAC, two populations of cells are grown in two different culture media, with the"light"medium containing amino acid(s) with the natural isotope, and the"heavy"medium containing stable isotope labeled amino acid(s). After a sufficient number of cell divisions, at least five cycles in mammalian cells [21], theoretically all the proteins from the cells cultured in heavy medium contain amino acids in the heavy state. However, the number of cell divisions required for complete labeling depends on the rate of protein synthesis, degradation, and turnover, therefore the

labeling efficiency should be carefully tested prior to quantification. After complete labeling (at least >95% labeling efficiency), the cell populations are experimentally manipulated and then equal amounts of labeled and unlabeled cells or protein extracts are mixed. The samples are then digested into peptides. Finally, the digested peptides are analyzed with LC-MS/MS. The quantification of SILAC is based on testing the ratio of introduced isotope-labeled peptides to unlabeled peptides. Thus, the signal intensities from light and heavy samples allow for quantitative comparison of their relative abundances in the mixture.

2.2 Choice of SILAC amino acids

Ideally, the SILAC amino acids should be amino acids essential for the survival of culture cells, which ensures the only source of the particular amino acid is from the culture medium. Leucine [8, 22], lysine [23], and methionine [24] are essential amino acids that have been used in SILAC.

Though arginine is not an essential amino acid, it has been shown to be essential for many cultured cell lines [25], and has been used successfully in SILAC labeling [26–28], despite the occurrence of metabolic conversion of arginine to proline. Tyrosine is another nonessential amino acid that has been used in SILAC. Heavy-labeled tyrosine was used to identify the substrates of tyrosine kinase [29] and to investigate the dynamics of tyrosine phosphorylation of proteins [30].

In the earlier SILAC studies, deuterium (^{2}H)-labeled leucine [8, 22] was selected as the labeled amino acid. However, a chromatographic shift during the reverse phase chromatography for the deuterium-labeled peptides compromised the accuracy of quantification [31–33]. Later, ^{13}C or ^{15}N-labeled amino acids were used, because these SILAC peptide pairs coelute during LC-MS/MS analysis. Now, more and more researchers use a combination of ^{13}C and ^{15}N-labeled arginine and lysine as labeled amino acids, since trypsin, the commonly used proteolytic enzyme in proteomics workflow, specifically cleaves at the carboxyl-termini of lysine and arginine residues [34]. Therefore, the combinations of trypsin digestion and SILAC labeling with lysine and arginine allows for quantitation of all tryptic peptides of a protein (except for the protein C terminus), ultimately resulting in improved coverage of overall proteomic quantification.

2.3 Metabolic conversion of arginine and proline in cell lines

Though arginine is commonly used in SILAC, metabolic conversion of arginine to proline via the arginase pathway has been observed in a number of cell types, such as Hela [26], HEK293T [35], and embryonic stem cells [36]. Such conversion has an impact on the accuracy of overall quantitation as mentioned above. The arginine-to-proline conversion usually occurs when heavy arginine is provided in the cell culture medium in excess, which complicates the quantitation of proline-containing peptides.

Different approaches have been developed to prevent or correct arginine-to-proline conversion. A widely used solution is to empirically determine the optimal arginine concentration to minimize its conversion to proline [37]. However, the reverse metabolic conversion of proline to arginine can also occur in low-arginine medium [37]. An alternative method to prevent the arginine-proline conversion is to supplement SILAC medium with unlabeled proline [35, 36]. Another described method is to replace $^{12}C^{14}N$ arginine with $^{12}C^{15}N$ arginine in the "light" medium, which allows the amount of converted proline to be normalized by quantifying the monoisotopic peak in the mass spectra [38]. Furthermore, several bioinformatics approaches have been developed to correct the SILAC ratios of proline-containing peptides [39,40]

2.4 Use of SILAC for multiplexed comparison

Most SILAC experiments compare two different cellular states. With three isotopically distinct forms of arginine and lysine available, however, comparison of three cell populations in a single experiment is now possible with the 3-plex SILAC [27, 41]. With the combination of different isotopic forms of arginine and lysine, SILAC can be used to compare up to five different cellular states in a single experiment (Table 2). 5-plex SILAC experiments can be performed with five isotopically distinct forms of arginine [42]. However, only the arginine-containing peptides are quantifiable, which underestimates the quantification efficiency of proteome. Now, 5-plex SILAC experiments can be carried out by combining two 3-plex SILAC experiments with the same experimental state [28, 43, 44] (Fig. 1).

2.5 SILAC workflow

The SILAC workflow comprises two principle phases, i.e. an adaptation phase and an experimental phase (Fig. 2). SILAC use dialyzed serum to grow the cells to avoid free amino acids that are present in the serum, however, some cell lines may not grow well in dialyzed media because of the loss of low-molecular-weight growth factors [45]. Supplemented with a small percentage of normal serum or purified growth factors in the growth media will potentially solve the problem [45,46]. Thus, cell lines should be tested before a SILAC-labeling experiment in the adaptation phase. During the adaptation phase, cells are grown in the unlabeled and labeled media until the heavy amino acids are fully incorporated into the cellular proteins. When the cells have been cultured for the duration of at least five cell divisions, a small fraction of the heavy cell population is harvested, lysed and proteins are digested into peptides. The degree of incorporated SILAC

Table 2. Recommended amino acids for SILAC experiments

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
2 Plex	$^{12}C/^{14}N$ light amino acids (K0/R0)	$^{13}C_6,^{15}N_2$-lysine+ $^{13}C_6,^{15}N_4$-arginine (K8/R10)			
3 Plex	$^{12}C/^{14}N$ light amino acids (K0/R0)	D_4-lysine/$^{13}C_6$-arginine (K4/R6)	$^{13}C_6,^{15}N_2$-lysine+ $^{13}C_6,^{15}N_4$-arginine (K8/R10)		
4 Plex	$^{12}C/^{14}N$ light amino acids (K0/R0)	D_4-lysine/$^{13}C_6$-arginine (K4/R6)	$^{13}C_6,^{15}N_2$-lysine+ $^{13}C_6,^{15}N_4$-arginine (K8/R10)	$^{13}C_6,^{15}N_2$, D_9-lysine+ $^{13}C_6,^{15}N_4$, D_7-arginine (K17/R17)	
5 Plex	$^{12}C_6,^{14}N_4$-arginine (R0)	$^{15}N_4$-Arginine (R4)	$^{13}C_6$-Arginine (R6)	$^{13}C_6,^{15}N_4$-arginine (R10)	$^{13}C_6,^{15}N_4$, D_7-arginine (R17)

amino acids is then evaluated by LC-MS/MS. The AUC of the MS peaks for the heavy and remaining light peptide pairs is used to evaluate the degree of labeling (Fig. 2A). To overcome the problem of incomplete incorporation of isotopic amino acids, several approaches have been developed either by dataset normalization with bioinformatic tools [47] or by SILAC label-swap replication experiment [48, 49].

During the experimental phase (Fig. 2B), and after the full incorporation of heavy amino acids has been confirmed, the two cell populations are subjected to different treatments according to the aim of the study, and then combined equally prior to subsequent optional subcellular organelle purification, cell lysis, protein extraction, and protein digestion. Then samples are analyzed with LC-MS/MS to identify and quantify the ratios of heavy peptides to light peptides. Most laboratories use the Orbitrap-based mass spectrometers such as linear ion-trap Orbitrap (LTQ-OrbitrapVelos) [50] or quadrupole Orbitrap (Q-Exactive) [51, 52] to perform the SILAC MS analysis. The advantages of these instruments are their high resolving power, high mass accuracy, high dynamic range, and high sequencing speed.

After obtaining high-quality MS data, the identification and quantification of peptides/proteins is accomplished via database search software, such as the commercial software Proteome discovery or freely available software tools such as MaxQuant [53, 54], Census [40], Trans-proteomic pipeline (TPP) [55], and pQuant [56]. MaxQuant is a computational proteomic platform that provides a complete data analysis workflow from raw MS files to output tables, which contain detailed information about identified proteins (peptides) and relative changes in abundance [54]. MaxQuant is by far the most popular software for the analysis of SILAC data, as it was first developed for such analysis. In addition to SILAC data, now label-free quantification and most standard labeling techniques such as iTRAQ, TMT, and dimethyl labeling are supported in MaxQuant. Besides MaxQuant, other quantitative software tools also have specificity and features (Table 3), for example, Census [39, 57] and pQuant [56] can handle the data generated from ^{15}N labeling, which is not included in MaxQuant analysis. TPP can support not only the CID (collision-induced dissociation) type of MS/MS data but also the ETD (electron transfer dissociation) type of MS/MS data [55]. pQuant improves quantification by minimizing the interference of coeluting ions of similar m/z values [56].

Finally, annotation databases such as GO [58], KEGG [59], STRING [60], or bioinformatic tools such as GoMiner [61], cytoscape [62], DAVID [63] are applied to transfer the proteomic protein list into meaningful results and biological insights [64].

3 Applications of SILAC

3.1 Expression proteomics

The most popular application of SILAC is to characterize global changes in protein expression between different biological samples, so called expression proteomics [65, 66]. The SILAC technique has been successfully applied to compare protein expression changes during cell differentiation, such

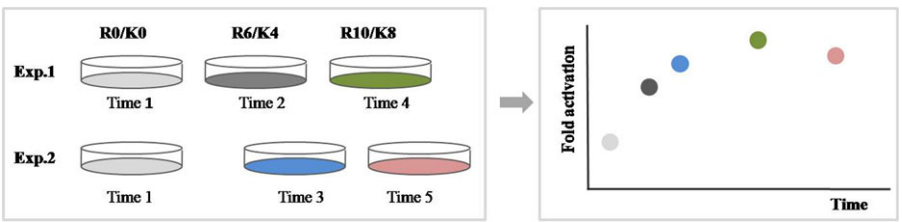

Figure 1. 5-plex SILAC profiling with two 3-plex SILAC experiments. 5-plex SILAC experiments can be carried out by combining two 3-plex SILAC experiments of one identical experimental state.

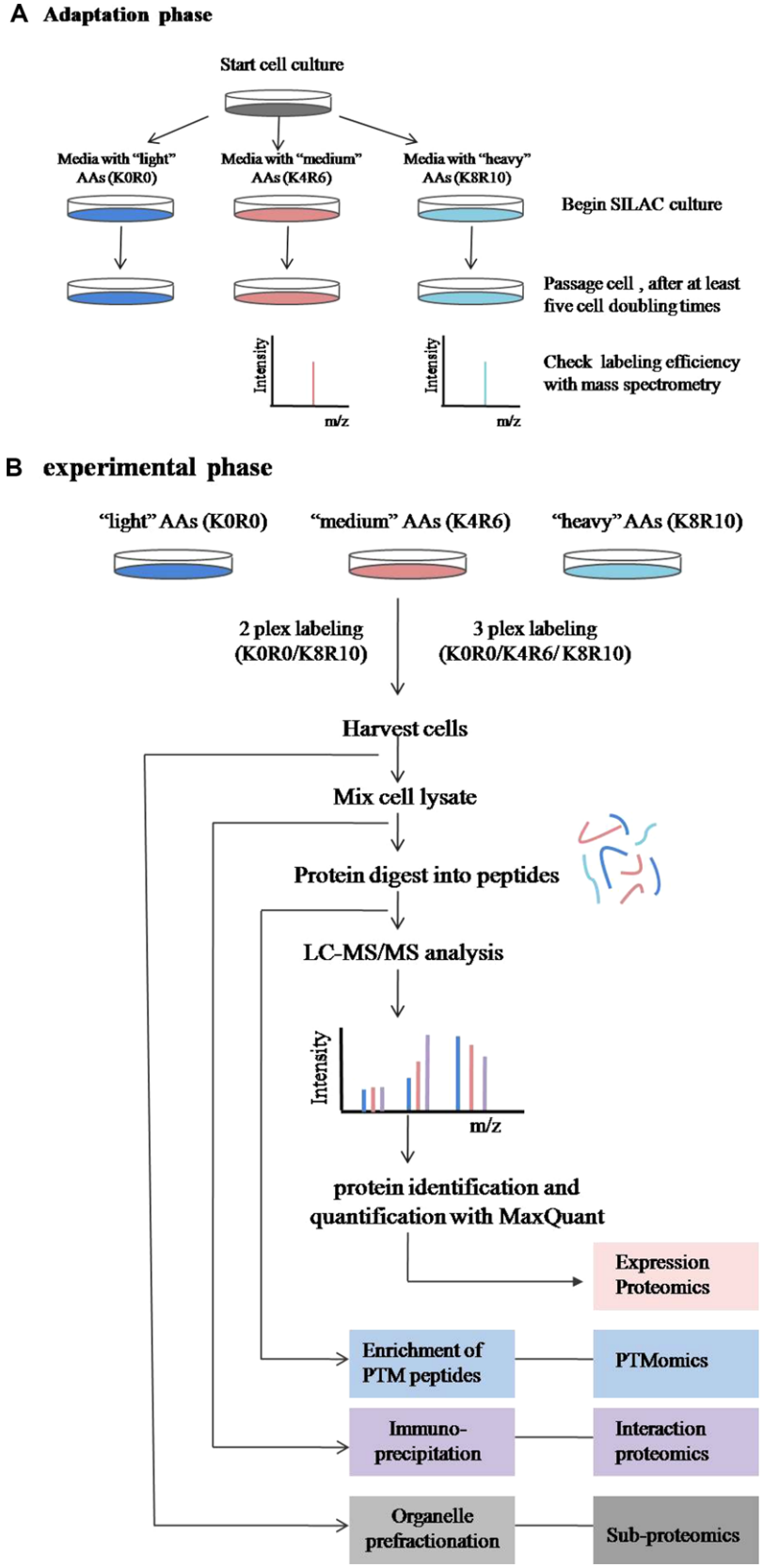

Figure 2. Workflow for quantitative proteomic experiments using SILAC. The SILAC experiment consists of two phases: an adaptation phase (A) and an experimental phase (B). (A) During the adaptation phase, cells are grown in light and heavy SILAC media for several cell divisions until full incorporation of the heavy amino acids in the growing cells. The degree of SILAC amino acids incorporation can be evaluated by MS analysis. Depending on the study design, a triple strategy using light, medium, and heavy labeling can be used. (B) During the experimental phase, after the full incorporation of SILAC amino acids was confirmed, the cells populations are experimentally manipulated. Subsequently, the cells populations or protein lysates are mixed depending on the study. For sub-proteome analysis, cells populations are combined for organelle prefractionation; and for expression proteomics, interaction proteomics or PTMomics, the extracted protein lysates are mixed. After digestion of the SILAC-labeled proteins into peptides, peptides are then analyzed with LC-MS/MS. The identification and quantification of peptides is accomplished with quantitation software such as MaxQuant. In case of investigating protein–protein interaction, protein complexes are immuno-precipitated from the mixture of SILAC-labeled cell lysates. For PTMomics analysis, SILAC labeling peptides are subject to a fractionation and an enrichment step to improve identification of PTM-peptides. Figure 2B courtesy of Prof. G. Giamas [217], adapted with slight modifications.

Table 3. Features of different quantitative proteomics software tools

Software	Types	Supported quantitative data types	Features	References
Proteome discovery	Commercial software from Thermo Fisher Scientific	SILAC, iTRAQ, TMT, dimethyl labeling, label-free	Integrates all different steps in quantitative proteomics experiment into a single automated workflow. It supports multiple database search algorithms (SEQUEST, Z-Core, MASCOT, etc.) and multiple dissociation techniques (CID, HCD, ETD) for more comprehensive analyses.	Proteome discoverer user guide
MaxQuant	Freely available	SILAC, iTRAQ, TMT, dimethyl labeling, label-free	The most popular software for the analysis of SILAC data	[53, 54]
Census	Freely available	SILAC, iTRAQ, label-free, TMT, ^{18}O labeling, ^{15}N labeling	Support low-resolution MS data	[39, 57]
TPP	Freely available	SILAC, iTRAQ, ICAT	Support both CID and ETD type of MS/MS data	[55]
pQuant	Freely available	SILAC, ^{15}N labeling	Minimizing the interference of coeluting ions of similar *m/z* values	[56]

ETD, electron transfer dissociation; CID, collision-induced dissociation; HCD, higher energy collisional dissociation.

as muscle cell differentiation [8, 46] and adipocyte differentiation [35], or after application of different biological treatments [67–69], to gain novel insights into the role of differential proteins in specific biological processes.

The greatest advantage of SILAC over other chemical labeling methods is that metabolically labeled cell populations can be combined early before the subsequent subproteome fractionation or enrichment of PTM-modified peptides, thus it is suitable for comparing protein expression in the subcellular organelles or particular cell compartments. One of the interesting applications of SILAC is the study of the secretome [70], which is the sum of all proteins released into the extracellular environment by a specific cell or cell type [71]. Secreted proteins, including cytokines, interleukins, growth factors, hormones, and others, all function as key messengers to coordinate body homeostasis [70]. Cancer cells also secrete proteins or protein fragments into body fluids such as blood or urine, and these peptides can be used as biomarkers [72]. SILAC was previously applied for comparisons of secreted proteins in different cancer cells, such as gastric epithelial cells [73], pancreatic cancer cells [74], esophageal squamous cell carcinoma cells [75], malignant glioblastoma cell lines [76], and colorectal cancer cells with different metastatic capacity [77]. Though these studies provided a list of secreted proteins, they did not include any information about their secretion rates, differential secretion between cancers cells and other normal cancer cell types, a fact that limits the use of these secreted proteins in clinical diagnostics.

Other interesting targets are membrane proteins, as they are prime candidates for potential biomarkers, especially in cancer diagnostics, but also as drug targets. In one study, SILAC was combined with a membrane purification technique to find differentially expressed membrane proteins between normal and malignant breast cancer cells [78]. In other studies, this combination was applied to compare proteins of cancer cells with different metastatic capabilities [79–81].

SILAC was also applied to identify differentially expressed proteins in organelles, such as nucleus [82, 83], nucleolus [28, 84] or β-cell insulin secretory granules [85]. Our laboratory focuses on the study of mitochondrial function, an essential organelle that plays important roles in cell life and death [86]. Previously, our lab investigated the changes of mitochondrial protein expression in pancreatic INS-1β cells in response to chronic hyperglycemia stimulation, and presented insightful new details on the effect of glucotoxicity on β-cell mitochondria [87]. Recently, we used SILAC to compare the expression of mitochondrial protein in the ovarian cancer cell line OVCAR8 and its doxorubicin-resistant cell line NCI/ADR_RES, and found that 122 mitochondrial proteins displayed significant changes in the NCI/ADR_RES cells. A subsequent functional study using RNA interference (RNAi) technology verified that the mitochondrial protein TOP1MT is involved in doxorubicin resistance in NCI/ADR_RES cells. Besides mitochondrial protein expression, mitochondrial morphology, localization, and function were also found to be changed in NCI/ADR_RES cells relative to OVCAR8. Together, these data indicate that mitochondria present a potential therapeutic target to overcome doxorubicin resistance in ovarian cancer cells [88].

SILAC was also applied to identify the genuine components in cellular compartment that cannot be isolated in their pure form, such as lipid rafts [22]. After two populations

of cells were SILAC-labeled, the heavy amino acid-labeled population was kept untreated, while the light amino acid-labeled population was treated with cholesterol-disrupting drugs to break up lipid rafts. Subsequently, the untreated and treated cells were combined and the low-density detergent-resistant fraction was isolated. The genuine components in the lipid raft exhibited specific quantitative changes, while the ratios of the nonspecific proteins showed little change between the two samples.

In conclusion, the combination of SILAC with different purification techniques has provided valuable information about the role of "subproteomes" in the cell.

3.2 Dynamic changes of protein PTMs

PTMs are known to play crucial roles in the regulation of protein function. PTMs can directly affect protein structure, localization, activity, and interactions with other proteins [89]. Many PTMs act as regulatory switches for various signaling pathways. Therefore, characterization of these modifications and their dynamic changes can provide valuable information for deciphering the mechanism of a specific signal transduction pathway. The tremendous progress of MS technology in the past decade made it possible to finally profile PTMs at a whole-proteome scale.

Protein phosphorylation, one of the most widespread and important protein PTMs in the cells, plays a key role in almost all aspects of cellular activity/function, such as signal transduction, differentiation, proliferation, and metabolism [90]. It is estimated that approximately one-thirds of all proteins in eukaryotic cells are phosphorylated at any given time [91, 92]. Traditionally, ^{32}P-labeling was coupled to 1D- and 2D-gels or Western blotting with phosphosite-specific antibodies to quantify the relative changes in protein phosphorylation, however, this method was not suitable for the identification of novel phosphoproteins. Moreover, this method cannot identify the precise localization of a phosphorylation site. However, this information is very important for cell studies, because different phosphosites in the same protein could be differentially regulated in different signaling pathways. In addition to the detection of phosphorylation sites, quantitative studies of the dynamic changes of phosphorylation events are also important for understanding cell signaling pathways. In this regard, it is critically important to quantify the relative abundance of signaling molecules and their phosphorylation sites. SILAC coupled with MS allows for accurate, global, and site-specific quantitation of protein phosphorylation in the whole proteome [41, 43, 93]. Recent development of proteomic technologies, including phosphopeptide enrichment techniques, high-accuracy MS technology, and the associated bioinformatics tools enable quantification of >30 000 phosphorylation sites in a single-cell type [16].

For large-scale phosphoproteomic analysis, enrichment of phosphopeptides is indispensable because of the low abundance and low ionization efficiency of phosphopeptides. Because of this fact, the signal intensities of phosphopeptides are easily suppressed by the abundant, nonphosphorylated peptide ions [94]. Several techniques have been developed and optimized for enrichment of phosphoproteins [95] and phosphopeptides [96]. The most popular techniques to-date are affinity chromatography-based phosphopeptide enrichment technologies such as IMAC [97,98] and titanium dioxide (TiO_2) [99, 100]. Combined with peptide fractionation techniques such as SCX [43, 101] or hydrophilic interaction liquid chromatography [102,103], these phosphopeptide enrichment methods allow for the identification of several thousand serine/threonine phosphorylation sites. For instance, Gruhlerand coworkers used SILAC and IMAC for phosphopeptide enrichment to quantify phosphorylation changes of proteins in G-protein-coupled receptor signaling pathways in response to pheromone signals in yeast [104]. Similar strategies have been successfully used in other proteomic studies [105, 106].

Thanks to its high affinity and selectivity, enrichment of phosphopeptides with TiO_2 has been widely used for investigating dynamic signaling pathways. More recently, Humphrey and coworkers used this technique to analyze dynamic changes in protein phosphorylation in adipocytes, following exposure to insulin and identified 37 248 phosphorylation sites, making it the largest phosphoproteome reported for a single cell type to date [16]. This strategy has also been used efficiently to study the dynamics of the EGF signaling pathway [27, 43, 44] as well as DNA damage response [107], to elucidate cellular events underlying the human embryonic stem cell differentiation [93], to compare the phosphorylation levels upon EGF stimulation and EGF combined with different kinase inhibitors [108], to investigate the phosphoproteome changes following knockdown of a specific phosphatase by RNA interference in the Drosophila cells [109] or by overexpression of a dominant mutant PIK3CA in breast epithelial cells [110]. Together, these global and dynamic phosphoproteome analyses have opened new perspectives in studying complex biological signaling networks.

However, the low occurrence of tyrosine phosphorylation compared to serine/threonine phosphorylation makes it difficult to be identified. Immunoprecipitation with a high-affinity anti-phosphotyrosine antibody helps to improve the coverage of tyrosine phosphorylation sites [27, 111–113].

In addition, significant progress has been made in the global and dynamic analysis of other PTMs, including acetylation [114–116], glycosylation [117–119], ubiquitination [120–122], methylation [24,123,124], and palmitoylation [125]. Several studies even investigated the cross-talk of several PTMs at the global level [126–129], suggesting an interplay among PTMs in the regulation of cell activities [130].

3.3 Interaction proteomics (interactomics)

Most proteins in cells do not function alone, but perform particular cellular activities through interacting with other specific proteins, or through forming protein complexes.

Protein–protein interactions (PPIs) play key regulatory roles in various physiological processes, such as signal transduction, cell growth and proliferation, DNA replication, transcription and translation, and metabolism [131]. Therefore, identification of PPIs at a global level will provide important insights into the regulation of cellular processes. In the past, genetic methods such as the yeast two-hybrid (Y2H) were used for comprehensive investigation of PPIs. However, such techniques suffer from high rates of false-positive and false-negative signals, mainly because the assay is performed under nonphysiological conditions [132]. With a considerable progress of MS technology, as well as the development of tagging methods such as tandem affinity purification [133], it has recently been possible to reveal a proteome-wide PPI network in yeast [134, 135].

Though affinity purification coupled with MS has become one of the most effective approaches to study PPIs [136], the selectivity, and specificity of the method is compromised by the nonspecific binding of proteins to the antibodies or carrier beads [137, 138]. However, in combination with quantitative proteomic strategy such as ICAT [139, 140] or SILAC [141], specifically interacting proteins can be efficiently distinguished from nonspecific background proteins. The abundance of specific interaction partners purified from the bait sample is significantly higher than the one from the control sample, resulting in quantified ratios much higher than 1. In contrast, the abundance of nonspecific background proteins should be comparable from both the bait and the control sample, resulting in their ratios being close to 1 (Fig. 3).

To investigate the exogenous PPIs, the bait protein is expressed in the cells following transient transfection with an expression vector, the protein complexes are then pull-downed with tag-specific antibody. To discriminate the specific interactors, a SILAC-based method called I-DIRT (isotopic differentiation of interactions as random or targeted) was used (Fig. 3A). In I-DIRT technique [142], cells containing affinity-tagged proteins are grown in light medium and wild-type cells are grown in heavy medium. Protein complexes are then immunoaffinity-purified from the equal-mass mixture of the light and heavy cell lysates. Then, the protein complexes are eluted, digested, and analyzed with LC-MS/MS. In MS, specific interacting partners recognized as having only or predominantly light isotopic peaks, while nonspecific interaction partners have SILAC ratios close to 1. This strategy has been successfully applied for the analysis of the interactome of the adaptor protein Odin involved in the growth factor signaling pathway [143] and integrin-linked kinase [144], for determining the insulin-dependent interactions of proteins with GLUT4 [145]. Finally, SILAC has also been applied to define membrane protein complexes in yeast [146].

When combined with RNAi, SILAC can also be applied to detect endogenous PPIs (Fig. 3B). After metabolic labeling of cells using the SILAC method, the protein of interest is knocked down by RNAi in either light or heavy sample. The cell lysates are then incubated with an immobilized antibody against the protein of interest. Subsequently, the precipitated proteins are combined, eluted, digested, and finally analyzed by MS. The target protein and its partners are expected to be more abundant in the untreated cells relative to knockdown cells. In contrast, contamination proteins would be present in similar amounts in both untreated and knockdown cells, thus specific interactors and contaminations can be clearly discriminated. This method was termed QUICK (quantitative immunoprecipitation combined with knockdown) [147]. QUICK assesses interactions between untagged endogenous proteins at their normal cellular levels, and it can therefore identify protein–protein interactions with very high confidence. Selbach and coworkers used this method first to identify interaction partners of β-catenin and Cbl [147]. It has also been applied successfully in multiple myeloma cells for identification of interacting partners of 14-3-3ζ [148] and Stat3 [149], but also to investigate the role of BAG3 in human [150], and to study the molecular functions of Drosophila ISWI, an ATP-hydrolyzing motor present in different chromatin remodeling complexes [151].

In addition, SILAC can be used to identify the components of inducible protein complexes that are formed upon activation of signaling pathways (Fig. 3C). Blagoev and coworkers first used SILAC to investigate the EGFR pathway in HeLa cells, by identifying specific interactors of the SH2 domain present in the signaling adapter protein Grb2, following EGF stimulation [141]. Also, three-plex SILAC was applied to determine stimulus-specific interactions between proteins of the wnt pathway [152].

In cells, the interactions between proteins is usually not permanent and static (stable PPIs), but mostly transient (dynamic PPIs). In the original PAM (purification after mixing)-SILAC procedure, purification of protein complexes was carried out after mixing the light and heavy cell lysates, and heavy-light exchange of dynamic interaction partners in the protein complexes can occur during incubation. Thus, the ratios in relative abundance of these dynamic interactors were similar to those background proteins, which made it difficult to effectively distinguish the specific but dynamic interactors from background proteins based on their SILAC ratios [153]. The MAP (mixing after purification)-SILAC approach [154, 155] solves this problem by mixing the purified samples after purification. It has been applied successfully in the identification of the dynamic interactors in 26S proteasome complexes [155], COP9 signalosome complexes [156], and TBP transcription complexes [157].

Besides protein–protein interactions, the SILAC approach has been applied to the study of protein–bait interactions using different molecular baits, such as peptides [48, 158, 159], DNA [160, 161], RNA [162, 163], small molecules, or drugs [164, 165].

3.4 Protein turnover

Conventional proteomics compares the difference in protein expression between two or more different states or

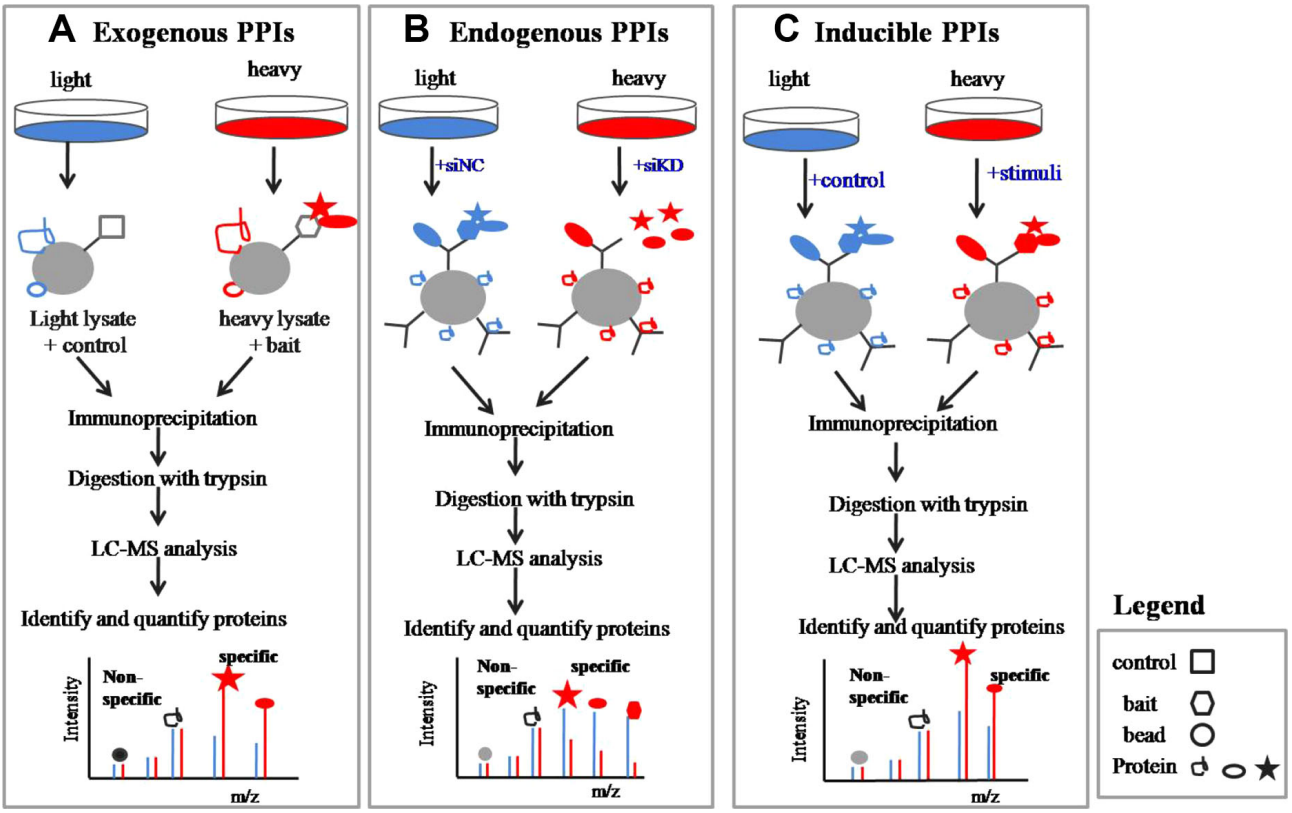

Figure 3. Quantitative interaction proteomics with SILAC. SILAC-based quantitative proteomics can be used to identify the specifically interaction proteins in investigating (A) exogenous PPIs, (B) endogenous PPIs, or (C) inducible PPIs. (A) To investigate the exogenous protein complex, wild-type cells or cells expressed affinity-tagged proteins are grown in light or heavy medium. Then protein complexes are immunoaffinity-purified from the mixture of the light and heavy cell lysates. (B)To study an endogenous protein complex, the protein of interest is knocked down by RNAi in cells grown in light or heavy medium. Then protein complexes are immuno-precipitated with the interested antibody from the mixture of the light and heavy cell lysates. (C) In the case of inducible PPIs, protein complexes are induced by specific stimulation in cells grown in light or heavy medium, and then immuno-affinity purified from the mixture of the light and heavy cell lysates. After getting the protein complexes, proteins were digested into peptides and analyzed with LC-MS/MS. The specific interactors or nonspecific background proteins can be distinguished by their SILAC ratios. However, these PAM methods that mix light and heavy cell lysates before coIP cannot distinguish the specific but dynamic interactors. This problem can be solved by mixing purified samples after coIP (MAP methods). Figure 3 courtesy of Prof. S. E. Ong [19], adapted with modifications.

conditions. However, it does not provide information about the dynamics of protein expression, as the protein abundance is determined by the balance between protein synthesis and degradation. Therefore, protein turnover creates an additional dimension for proteomic studies [166]. Earlier studies of protein turnover relied on detecting the incorporation of radiolabeled amino acids into newly translated proteins and either analyzed bulk protein turnover, or else turnover of individual proteins [167]. The development of pulsed SILAC (pSILAC), allows the determination of the turnover rates of large numbers of proteins in a single experiment [168–172].

pSILAC is a recently developed modified version of the SILAC approach. In pSILAC, cells are first cultured in medium with the normal light (L) amino acids. Then, cells are transferred to culture medium containing middle (M) or heavy (H) labeled amino acids for a certain period of time. From this point time on, cells are pulse-labeled since all newly synthesized proteins incorporate either the H or the M amino acids. Subsequently, samples are combined and analyzed together. The abundance ratio of H/M reflects changes in protein production. pSILAC has been employed to study the global protein turnover in yeast [166] and in the nucleus of cultured cells [169]. pSILAC was also used for global analysis of the dynamic expression of MHC in human cancer cells [168], protein translation [170, 173], protein degradation [171], PTMs [174], and also for investigating the impact of chemical inhibitors on protein synthesis [175]. Furthermore, pSILAC was applied to assess the dynamics of secreted protein during osteoblast differentiation [176] and macrophage activation [177]. In a comprehensive study, Boisvert and coworkers combined pulse-labeling and subcellular organelle purification strategy to characterize the expression, localization, synthesis, degradation, and turnover rates of human proteins in different subcellular organelles, and provided a

system-wide overview of the dynamics of cultured human cells [172].

Another important application of pSILAC is to quantify protein expression changes induced by miRNAs [178–181], which are small noncoding RNAs that regulate gene expression. To investigate the impact of overexpression or knockdown of miRNA on the protein translation, Selbach and coworkers first applied pSILAC to investigate the impact of five human miRNAs on protein synthesis. After induction of miRNA expression, cells were pulse-labeled with isotope-labeled amino acids, which were incorporated into all newly synthesized proteins. Subsequent MS analysis allowed detecting changes in protein production [178]. Recently, Kaller and coworkers investigated the targets of miR-34a, itself a transcriptional target of the p53 tumor suppressor protein [181]. In summary, pSILAC combined with MS-based proteomics is a powerful strategy to find the targets of miRNAs and to provide more information about the function of miRNAs.

4 Developments of SILAC

Though the SILAC method was originally developed for labeling mammalian cell lines, it has been expanded to almost all types of model organisms from bacteria to mammalian [182], including bacteria *Escherichia coli* [183, 184] and *Bacillus subtilis* [185, 186], yeast [104, 187, 188], single-celled protozoan *Trypanosoma brucei* [189], higher plant model *Arabidopsis thaliana* [190], *Drosophila melanogaster* [191, 192], *Caenorhabditis elegans* [193, 194], Zebrafish *Danio rerio* [195, 196], as well as mouse [197]. Though these model organisms can be metabolically labeled with SILAC methods, the ways to incorporate isotopic labels into organisms are quite different: mammalian and *Arabidopsis thaliana* cells incorporate stable isotopic amino acids by supplementing them in the growth media, while unicellular organism such as bacteria and yeast are typically auxotrophic for specific amino acids, ensuring that all of these amino acids are replaced by their counterpart labeled amino acids. However, complex organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans*, Zebrafish, and mouse incorporate stable labeling by feeding with SILAC-labeled *Escherichia coli* or yeast, or a customed SILAC diet [198].

The SILAC mouse model has become a powerful tool for systematic analysis of disease model mice [199] as well as knockout mice [200, 201], thereby improving our understanding of the protein functions in vivo and advancing our knowledge of pathogenic processes.

One of the shortcomings of SILAC is the fact that it cannot be applied to the study of tissues or body fluids, which greatly limits its clinical application. However, the development of spike-in SILAC [17] and super-SILAC [18] partially solved the problem. During spike-in SILAC, the cells are SILAC-labeled, and the proteins extracted from the SILAC cells are spiked into different experimental samples, which are then processed and analyzed together. In the MS analysis, the spike-in SILAC sample is the heavy population, with the light population being the real sample. Here, SILAC samples are served as internal references, to which the experimental samples are compared. Since the internal standard is identical, the relative quantification between different samples is feasible and the fold changes between different samples are the "ratio of ratios" [17]. Ishihama and coworkers first applied these cell culture-derived reference standards for mouse-brain quantitative proteomics [202]. Therefore, this strategy is now becoming increasingly popular for SILAC experiments. For instance, Pan and coworkers compared the changes in proteome changes between mouse primary hepatocytes and a mouse liver cell line by SILAC labeling the Hepa1-6 cell line [203]. A similar strategy was applied to compare in vivo the mitochondrial proteome between brown and white fat cells by using the SILAC-labeled mitochondrial proteins as internal references [204]. Combined with phosphoproteomic analysis, spike-in SILAC was also applied to investigate the in vivo signaling pathway. For instance, Monetti and coworkers used the SILAC-labeled liver Hepa1-6cells as an internal standard to analyze the quantitative phosphoproteome of mouse liver in response to insulin, and provided detailed in vivo phosphorylation information for liver tissue [205].

Though spike-in SILAC allows quantification of samples from tissues or body fluids, it introduces added variability to the analysis. Moreover, the internal standard used in the spike-in method, either cell lines or proteins, does not always contain all the proteins that are present in the analytical sample. The development of a so-called super-SILAC method [206] solved this problem, at least partially. During super-SILAC, a SILAC standard is generated by labeling more than two cell lines that are representative of the experimental sample (a super-set of cell lines) [18] or a labeled model organism [207]. Geiger and coworkers first used this technique for quantitative analysis of breast cancer tissue, with the SILAC-labeled protein mix from five different cell lines, all of which were derived from the same tissue [18]. Recently, this method was applied to the quantification of the proteomic differences between synaptic and nonsynaptic mitochondria in the mouse brain [208]. Combined with different protein PTM enrichment techniques, super-SILAC has been applied to compare the levels of phosphorylation [209] and glycosylation [119, 210] in disease, thus providing insights into the regulation of signaling pathway during pathogenesis.

Super-SILAC has tremendous potential in clinical application. It has been used to distinguish histological subtypes of cancers, namely by comparing their protein expression patterns and identifying significant differences [211]. For instance, Deeb and coworkers investigated two histologically indistinguishable subtypes of diffuse large B-cell lymphoma (DLBCL), namely activated B-cell-like (ABC) and germinal-center B-cell-like (GCB) subtypes. They developed a super-SILAC mix, which is constructed by combining cell lysates from B-cell lymphoma cell lines. The super-SILAC mix was then combined with cell lysates from ABC-DLBCL and

GCB-DLBCL cell lines, and analyzed via MS. PCA of the data obtained by super-SILAC allowed the classification of two cancer subtypes [211]. Furthermore, super-SILAC combined with N-glycoproteome technology allowed even better differentiation of those two cancer types [210]. Super-SILAC has also been applied to differentiate two major histological subtypes of non-small cell lung cancer, namely adenocarcinoma and squamous cell carcinoma [212] or to study the study a progression model of breast cancer [213]. In summary, super-SILAC combined with MS analysis can be used in the clinic as a reliable and rapid way to diagnose cancer subtypes. Future applications of super-SILAC in clinical samples will improve our understanding of disease, and hold the promise for discovering new biomarkers for diagnosis of disease [206, 214].

SILAC-based proteomics is still under development. Recently, a protein quantification method called NeuCode (neutron encoding) was developed by integrating the SILAC and isobaric tagging methods for multiplexed proteome quantification by high-resolution MS [215, 216]. The application of NeuCode SILAC labeling in yeast cells allowed the monitoring of time-resolved responses of five signaling mutants in a single 18-plex experiment [216]. This new strategy will expand the scale of comparative proteome analysis, allowing for its application in addressing important questions in the fields of biology.

5 Conclusion

SILAC is a simple, robust, and powerful approach in MS-based quantitative proteomics. Compared with other chemical labeling or label-free quantitative proteomic techniques, SILAC has the advantage of quantitative accuracy and reproducibility. SILAC coupled with LC-MS/MS has been widely applied to characterize protein changes between different samples, to investigate the dynamics of protein PTMs in response to stimuli, to distinguish specific interacting proteins in the protein–protein interaction networks, and to analyze cellular protein turnover in the proteome-wide scale. The development of spike-in SILAC and super-SILAC technology allows the application of SILAC technology in the clinic. In conclusion, SILAC has become an important tool in functional proteomics research to answer important questions in diverse areas of biomedical research.

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