EXPERT REVIEWS

Super-SILAC: current trends and future perspectives

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Stable isotope labeling with amino acids in cell culture (SILAC) has risen as a powerful quantification technique in mass spectrometry (MS)–based proteomics in classical and modified forms. Previously, SILAC was limited to cultured cells because of the requirement of active protein synthesis; however, in recent years, it was expanded to model organisms and tissue samples. Specifically, the super-SILAC technique uses a mixture of SILAC-labeled cells as a spike-in standard for accurate quantification of unlabeled samples, thereby enabling quantification of human tissue samples. Here, we highlight the recent developments in super-SILAC and its application to the study of clinical samples, secretomes, post-translational modifications and organelle proteomes. Finally, we propose super-SILAC as a robust and accurate method that can be commercialized and applied to basic and clinical research.

KEYWORDS: biomarkers • cancer • clinical proteomics • mass spectrometry • quantitative proteomics • spike-in standard • stable isotope labeling • super-SILAC

Mass-spectrometry (MS)–based proteomics, in analogy to the term 'genomics', aims at the identification of the assortment of proteins expressed in a biological system, their interactions and modifications. However, given the high qualitative similarity between large varieties of cell types [1], accurate protein quantification is a requisite when applying proteomics to the study of biological processes. Inherently, MS data are not quantitative because of the differences in peptide chemical and physical properties. Quantitative comparison between samples can be achieved by the use of stable isotope labels that induce a mass shift, which is resolved by the MS, or by computational algorithms that compare peptide signals in independent LC-MS runs. These 'label-free' approaches, such as spectral counting and intensity-based quantification, reduce the experimental costs and simplify the procedure, but compromise the quantification accuracy [2]. However, recent computational advances have dramatically improved the accuracy of intensity-based nonisotope-labeled techniques, which can be readily used when large quantitative differences are expected [3]. The development of multiple techniques that use stable isotope-labeled compounds initiated the field of quantitative proteomics (TABLE 1). Differential isotope labeling of samples creates a mass difference between the peptides originating from the different samples, while it retains identical chemical properties. This mass offset can then be resolved by the mass spectrometer and can enable determination of their quantitative differences. Isotope Coded Affinity Tag (ICAT) was the first chemical labeling technique, based on cysteine tagging with deuterium-labeled biotinylated ICAT reagent [4]. However, the limited number of cysteines per protein resulted in a low number of protein identifications and quantification events. In the following years, alternative chemical labeling and metabolic labeling techniques were developed. The dimethyl labeling technique uses deuterium or heavy carbon formaldehyde to label peptide amino termini and to enable accurate MS-level quantification [5,6]. The nonisobaric mTRAQ reagent relies on N-hydroxysuccinimide ester chemistry. All primary amines are chemically labeled, thus separating peptides by a mass difference of 4 and 8 Da per tag. A recent study demonstrated that the high accuracy and depth can be achieved by this technique [7]. Isobaric tagging techniques, Tandem Mass Tags (TMT) and Isobaric Tag for Relative and Absolute Quantification (iTRAQ) provide MS/MS level separation between fragments of differentially labeled peptides and allow sample multiplexing [8,9]. These techniques undergo dynamic-range compression and reduced quantitative accuracy because of near-isobaric precursor

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Table 1. Advantages and disadvantages of mass spectrometry–based proteomics quantification techniques.

Labeling technique	Name	Description	Advantages	Disadvantages	Ref.
Label-free	Label free quantification	*In silico* comparison of independent LC-MS runs	Simple Cost effective	No multiplexing Reduced quantitative accuracy	[2,3]
In vitro chemical labeling	ICAT	Cysteine labeling with deuterated, biotinylated tag	Reduced complexity Sample multiplexing (up to two plex) Applicable to any sample type	Limited number of cysteines per protein results in low coverage Expensive reagents Retention time shift of deuterated peptides Sample handling inaccuracy	[4]
	Dimethyl labeling	Heavy formaldehyde label of primary amines	Sample multiplexing (up to three plex) Applicable to any sample type	Retention time shift of deuterated peptides Sample handling inaccuracy	[5,6]
	mTRAQ	Nonisobaric tag of primary amines	Sample multiplexing (up to six plex) Applicable to any sample type	Sample handling inaccuracy	[7]
	TMT iTRAQ	Isobaric tag of primary amines	Sample multiplexing (up to 10 plex) Applicable to any sample type	Dynamic range compression (can be overcome with advanced MS techniques) Sample handling inaccuracy	[8–11]
In vivo metabolic labeling	SILAC	Heavy amino acids metabolic labeling	Sample multiplexing (up to five plex) Applicable to model organisms Spike-in reference standard for nonlabeled samples	Not applicable to human tissues Increases sample complexity	[12–29]
	Super-SILAC	Mixture of different cell lines labeled with heavy amino acids as a spike-in standard	Broad application to human tissues Long-term storage Reduced sample processing Cost effective	Limited to biological samples with representative cell lines No multiplexing	[30–39]

ICAT: Isotope coded affinity tag; iTRAQ: Isobaric tag for relative and absolute quantification; MS: Mass spectrometry; SILAC: Stable isotope labeling with amino acids in cell culture; TMT: Tandem mass tags.

interference. However, this limitation was addressed by eliminating interference using the MS3 triple-stage MS [10] or gas-phase purification [11]. These approaches together with recent advances in mass spectrometric techniques now increase the quantitative accuracy of these labeling techniques.

Metabolic labeling techniques are based on the introduction of desired heavy labels during protein synthesis in living cells. These were initiated with ^{15}N labeling, and later developed to heavy amino acid labeling, with the Stable isotope labeling with amino acids in cell culture (SILAC) technique [12]. In SILAC, media deficient of essential amino acids is supplemented with their heavy counterparts (synthesized with ^{13}C, ^{15}N or ^{2}H) that would be incorporated into the proteome of cells through cell proliferation and protein turnover. Differentially labeled samples are combined at an early sample preparation stage, thereby reducing sample handling errors and significantly improving quantification accuracy. Using light, medium and heavy labels of the amino acids for different cell states, SILAC allows samples to be triplexed and analyzed together in a single MS run. The most widely used SILAC amino acids are lysine $^{2}H_4$ or $^{13}C_6{}^{15}N_2$ (Lys 4 or 8, respectively) and arginine $^{13}C_6$ or $^{13}C_6{}^{15}N_4$ (Arg6 or 10, respectively). The use of trypsin as the site-specific protease ensures that all except the carboxy-terminal peptides are labeled and increases the protein quantification accuracy. The ease of application of SILAC to various biological systems has led to its widespread acceptance as the method of choice and has now emerged as the gold standard in proteomic quantification [13].

Since its introduction, multiple variations of SILAC have been developed, aiming to increase its multiplexing, to analyze protein translation and turnover rates, and to investigate cell–cell communication. Multiplexing up to five samples, with various amino acid combinations, has been demonstrated for proteomic and phosphoproteomic profiling [14,15]. To increase multiplexing further, without increasing spectral complexity, Coon and colleagues conceived the Neucode SILAC technique, which uses ultra-high mass resolution of the MS acquisition (>200,000) to distinguish peptides based on subtle mass differences, resulting from variations in nuclear binding energies in stable isotopes [16]. Labeling with differentially labeled amino

acids enabled up to six-plex quantification in a single isotope cluster, without the added complexity [16,17]. Analysis of protein translation and turnover rates using SILAC was achieved with the development of the pulsed-SILAC and dynamic-SILAC techniques, respectively, which are based on labeling for defined periods of time [18,19]. Trans-SILAC and Cell Type–specific labeling using amino acid precursors (CTAP) use SILAC to study cell–cell communication using differentially labeled cocultures [20–22]. Despite the vast applications of SILAC, because of the requirement of active protein translation, until recently it was limited to cell culture systems, which can be readily controlled. In addition, the standard double- or triple-SILAC, and to a higher extent five-plex SILAC, increase the complexity of the sample because of the addition of heavy peptides, which may result in reduced identification rates in the analysis. Nevertheless, the fidelity of SILAC was described over a decade ago and has since been applied in its native and modified versions showing its applicability to a large variety of systems and biological applications.

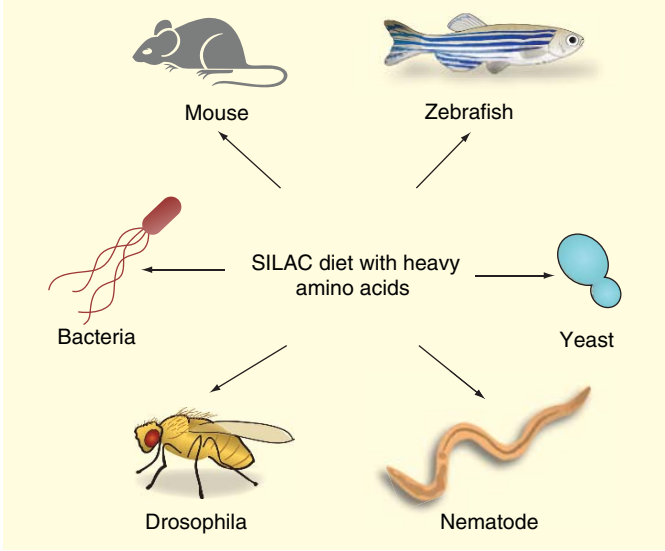

Figure 1. Stable isotope labeling of model organisms with a heavy amino acid-containing SILAC diet.

SILAC as a spike-in standard

To expand the applicability of SILAC to diverse sample types, a distinct SILAC strategy uses it to serve merely as a reference for quantification. Contrary to the typical SILAC experiments, here the standard is prepared separately while the experimental samples are unlabeled. Using this approach, these unlabeled samples are quantified against the heavy standard that is common to all the samples in the experiment. The peptide fold changes between samples can then be generated by calculating the ratio of ratios. One of the pioneering studies that used SILAC as a spike-in standard implemented metabolically labeled Neuro2A cell line as a reference standard to analyze unlabeled mouse brain [23]. Since then, this approach has been used for relative quantification of proteomes and their modifications, and for absolute quantification of selected proteins. In 'absolute-SILAC', endogenous or recombinant proteins purified from SILAC-labeled cells can be used to determine the absolute amounts in unrelated samples. This approach was first demonstrated for absolute quantification of the proteins MBP and Grb2 [24] and was further expanded to multiplexed absolute quantification [25]. Spike-in SILAC for proteome quantification was also expanded to cell culture systems, additional model organisms and human tissues.

The use of SILAC as an internal standard offers multiple advantages over classical SILAC or chemical labeling approaches: first, the use of the SILAC-labeled sample as a reference increases the applicability to samples that cannot be metabolically labeled, such as tissue samples, primary cells and body fluids. Second, the separation between the labeling of the standard and the experiment relieves the necessity to label cells as a part of the experimental workflow. Furthermore, the stability of SILAC, when compared with chemical labels, enables long-term storage of a standard and use of the same reference in multiple experiments. Finally, the use of dialyzed serum, which is necessary for complete SILAC labeling, often modifies the cellular proteome when compared with the standard serum. Although this effect is negligible in the comparison of cell populations grown in the same conditions (only distinguished by the amino acid mass), it becomes significant when compared with other biological experiments that are performed in the normal conditions. Using SILAC as a standard dismisses these problems, because the experimental samples are grown under normal conditions. Thus, the use of SILAC as a spike-in standard provides important advantages that broaden the applicability of SILAC-based quantification.

Application of SILAC to tissue analysis

One of the main limitations of SILAC was its inapplicability to tissue samples owing to their complex composition. SILAC-based quantitative proteomics of tissues from model organisms was achieved by labeling whole organisms and using their tissues as internal standards. The development of special diets that contain heavy amino acids and support normal growth was the first prerequisite for establishment of SILAC organisms. The SILAC mouse was the first model organism to be fully labeled [26] and was followed by the labeling of Drosophila [27], nematode [28] and zebrafish Figure 1 [29]. Complete labeling of model organisms requires labeling of one (zebrafish) or two (Drosophila, nematode and mouse) generations, which is both costly and time-consuming and as a result hampers repeated generation of labeled organisms for each experimental condition, each strain or transgenic organism. SILAC as a spike-in standard overcomes this necessity, because it enables the use of one strain for the quantification of all others, as long as they share the same protein sequences. Finally, combination of multiple tissues can serve as a standard for a large number of distinct tissues and enables quantitative comparison of protein expression in the entire organism [30].

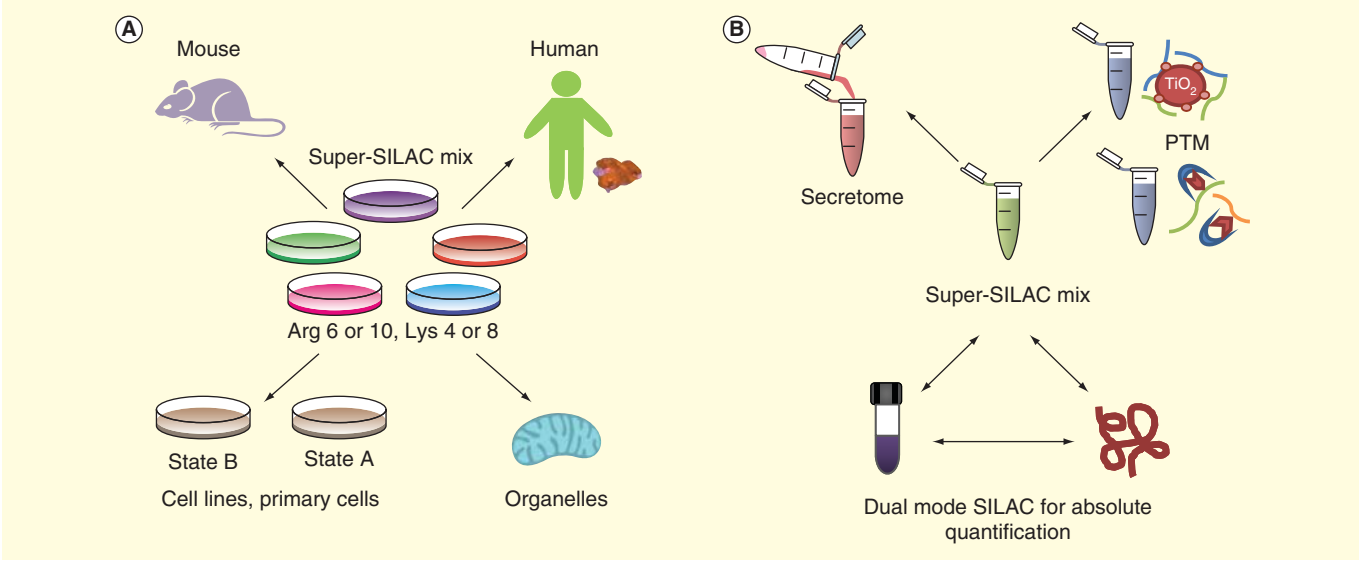

Figure 2. Super-SILAC applications. (A) Sample types analyzed with super-SILAC mix as a spike-in standard: non-labeled cell lines, tissues and organelles. **(B)** Proteomic workflows with super-SILAC quantification: Secretomes, post translational modifications (PTM) and dual mode SILAC approach that enables relative and absolute quantification of candidate biomarkers.

The super-SILAC approach for human tissue quantification

Despite the expansion of SILAC to model organisms, quantification of human tissues can be done only relative to cell lines, but single cell lines do not adequately represent the complexity and heterogeneity of the tissues. To expand SILAC to human tumor samples, we developed the super-SILAC mix as a spike-in standard [31]. Super-SILAC is defined as a mixture of more than two labeled cell lines that are representative of the experimental sample. Initially, super-SILAC was developed for the quantification of human breast tumor samples and astrocytoma [31] and was later expanded to additional tumor types and distinct proteomic workflows.

The super-SILAC mix is an integration of cell cultures that differ in origin and cell states and encompass the complexity of the tissue sample. The design and development of the super-SILAC mix are crucial to the quality of the internal standard. The main parameters that determine standard quality are the ratios toward the target tissue, the coverage of the tissue proteome and the number of orphan peptides that do not have a heavy SILAC partner [32–34]. Low SILAC ratios (less than fivefold) ensure accurate quantification, and high coverage ensures representation of biologically relevant proteins. The design of a typical super-SILAC for tumor quantification involves the selection of three–seven cell lines representing the tissue type, preferably cell lines that are diverse from each other but are similar to the tissue. Selecting fewer cell lines may lead to under-representation of tissue proteins and using too many cell lines tend to dilute out one another [34]. An initial label-free analysis of each of the cell lines, followed by principal component analysis, could be useful in determining their inclusion in the mix. In most cases, a ratio of 1:1 between the cell lines is appropriate, but other combinations can be computationally designed. The cell lines chosen are labeled and prepared separately with the desired heavy amino acids but digested and processed along with the light samples. The super-SILAC mix can be prepared in advance, stored for long periods and combined in appropriate ratios with the sample just before the preparation for MS analysis (Table 1). This enables potential commercialization of super-SILAC mixes for distinct tissues. Moreover, the use of the same standard during long periods allows retrospective comparisons with samples previously analyzed with the same standard.

Applications of super-SILAC

Since the first super-SILAC publication, this approach has expanded to additional tumor types and models. Deeb *et al.* developed a novel super-SILAC standard for lymphoma, which enabled accurate quantification and classification of diffuse B-cell lymphomas [35]. This work further provided the rationale and workflow for the construction of new super-SILAC mixes that initially analyzes the cells with a label-free approach, and based on these analyses selects the cell line combination, which provides highest diversity that represents the complexity of the experimental system. Lund *et al.* produced a super-SILAC standard to study a progression model of breast cancer and used the human cell-line–based standard for the protein quantification of human-tumors in a mouse xenograft model [36]. Other studies combined super-SILAC with analysis of cellular subproteomes, such as organelles, secreted proteins and post-translationally modified proteins (Figure 2). Accurate quantification of differentially expressed mitochondrial proteins from brown and white

adipocytes has been performed using an internal standard consisting of enriched mitochondrial fractions from heavy metabolically labeled 3T3-L1 and from brown adipocytes as a spike-in standard [37]. Phosphoproteomics super-SILAC was performed in the study of insulin-dependent signaling in mouse liver. Monetti *et al.* metabolically labeled mouse hepatoma cells and treated one half of the cell population with insulin to obtain full coverage of both insulin-dependent and insulin-independent phosphosites in the standard [38]. This mix was then spiked as a reference into protein samples extracted from liver of mice treated with insulin, followed by phosphopeptide enrichment and global phosphoproteomic profiling. Using this approach, more than 10,000 phosphosites were quantified, thereby providing the largest *in vivo* phosphoproteome dataset at that time. Super-SILAC was further applied to the quantification of secreted, glycosylated peptides. Most often secreted proteins are glycosylated as a protection mechanism against proteolytic degradation and can be specifically captured by lectins. Boersema *et al.* examined the glycoproteome isolated from supernatants of 11 different cell lines of breast cancer, representing different stages of disease progression. A super-SILAC mix combining the conditioned medium from three SILAC-labeled breast cancer cell lines was used as an internal standard. The N-glycosylated peptides were selectively separated using lectins and allowed accurate quantification of previously known glycoproteins and potential biomarkers and further expanded the potential use of super-SILAC also for plasma protein quantification [39]. Overall, super-SILAC is a simple, robust and cost-effective technique that provides a large variety of analytical applications. We propose that future commercialization of super-SILAC mixes for distinct purposes will further broaden its application to basic research and to the clinic.

Expert commentary & five-year view

Accurate quantification of proteomic changes occurring at a global level allows for the selection of unique biomarkers that are significantly changing between normal and diseased states. A stable, cost-effective standard that covers the clinically relevant proteome can be used to quantify thousands of proteins in single LC-MS runs rather than examining single biomarkers in individual assays. Such a standard can be commercialized, standardized and applied to a large number of clinical tests. Furthermore, the relative quantification provided by analyzing the clinical samples together with the standard can be expanded to absolute quantification of biomarkers. We envision a dual-mode SILAC quantification, which is composed of two quantification steps. First, the super-SILAC is used for relative quantification of the clinical samples; second, individual biomarkers, with known absolute amounts, are quantified relative to that same standard. Using these two simple steps, the absolute protein amounts can be accurately determined in the clinical samples even retrospectively. Overall, the advantages of super-SILAC make it an ideal approach for basic and clinical research from the identification of protein changes, to absolute quantification of biomarkers. In the future, we envision that super-SILAC will be applied as a general, economical and reproducible method for MS-based proteomics in clinical diagnostics.

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Key issues

- The super-stable isotope labeling with amino acids in cell culture (SILAC) mix as a spike in standard is a robust technique that allows accurate quantification of proteins from primary cells, body fluids and tissues, which cannot be metabolically labeled.
- The nonlabeled samples are treated as light labeled and quantified with respect to the heavy standard common to all samples in the experiment.
- In combination with specific enrichment techniques, super-SILAC can be applied to the study of post-translational modifications, such as phosphorylations and glycosylations.
- Dual-mode super-SILAC enables relative quantification of several samples and retrospective absolute quantification of specific proteins.
- In the future, super-SILAC can be commercialized for a broad range of tumor types, thereby increasing its application in clinical diagnostics.

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