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Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) A Primer

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What is SILAC?

Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) was developed to monitor the relative abundance of proteins by mass spectrometry[1]. This method works on the premise that cell treatment with light (^{12}C and/or ^{14}N) and heavy isotope (^{13}C and/or ^{15}N) labeled amino acids gives rise to two almost identical proteomes, which, under the same cell culture conditions, differ only in their masses. Deuterium is used to a lesser extent as deuterated compounds are often resolved from the non-deuterated compounds by reversed-phase liquid chromatography. This adversely affects quantitation when performing LC/MS experiments. Due to this substitution, a mass increment is observed in the mass spectra for each peptide comprising of at least one of the heavy isotope-labeled amino acids (e.g. 10 Da for $^{13}C_6$,$^{15}N_4$-Arg). The advantages of this method over alternative derivatization-based labeling techniques (such as Isotope-Coded Affinity Tag, ICAT™), is that the incorporation of light and heavy isotopes takes place in the proteome of living cells before a given biological experiment (e.g. stimulating cells with a cytokine). Thus, it is possible to combine the cells directly after harvesting them for subsequent purification steps and analysis. This ensures maximum reproducibility and minimum sample variation with regard to the protein level.

How does SILAC work?

The basis of SILAC is the incorporation of a stable isotope containing amino acid into the whole proteome. A typical SILAC experiment is designed in a differential manner, thus allowing the comparison of different cellular states such as stimulated vs. non-stimulated, or as various time points under identical biological conditions. As the two isotopically-labeled amino acids are essentially chemically identical, their incorporation does not interfere with normal cell growth while leading to proteins/peptides that are distinguishable by mass and thus, are ideal for mass spectrometric analysis. By choosing the right heavy amino acids, it is possible to multiplex up to three different conditions (e.g. Arg; $^{13}C_6$-Arg; $^{13}C_6$,$^{15}N_4$-Arg). The SILAC samples are then subjected to enzymatic digestion and LC/MS analysis (in a typical bottom-up proteomics approach). The protein quantification is therefore carried out on the peptide level by comparing the peak height or area of the corresponding doublets i.e. peptides which have the same amino acid composition and sequence but different masses.

The complete incorporation of the heavy isotope is achieved even for proteins with a low turn-over after five doublings. This is sufficient to exclude any partially-labeled artifacts for MS-based quantification[1].

In order to obtain sufficient incorporation of the heavy isotope, a typical SILAC experiment is divided into two stages. In the first stage, the cells are fed with the stable isotope-labeled amino acids. To ensure the exclusive incorporation of the heavy isotopic-labeled amino acid, the following points have to be addressed:

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A) Ideally, the substituted amino acid should be essential to guarantee that the cell relies on an external source of this amino acid. The most frequently used essential amino acids are leucine[1] lysine and methionine. In addition to these essential amino acids, arginine has often and successfully been applied to SILAC experiments despite the fact that it is a nonessential amino acid[2]; the availability of exogenous arginine is probably responsible for a down-regulation of arginine biosynthesis. The combined use of e.g. lysine and arginine in conjunction with tryptic digestion lead to a complete labeling of all tryptic peptides (except for the C-terminal peptide). The comprehensive coverage is obtained through the specificity of trypsin to cleave C-terminal to lysine and arginine.

B) Cells have to be grown in the presence of dialyzed serum to minimize the contamination of non heavy isotope-labeled amino acids.

C) The use of heavy arginine was reported to lead to partial labeling of proline through metabolic conversion. This conversion results in multiple satellite peaks for all proline-containing tryptic peptides in the heavy state, which in turn affects the accuracy of quantitation. Recently, Krijgsveld *et al*. reported an experimental strategy to correct for this artifact. By using [$^{15}N_4$]-arginine in combination with light lysine in the light condition and [$^{13}C_6$,$^{15}N_4$]-arginine in combination with [$^{13}C_6$,$^{15}N_2$]-lysine in the heavy condition, heavy proline will be formed at the same rate under both conditions (that is, [$^{15}N_1$]-proline and [$^{13}C_5$,$^{15}N_1$]-proline, respectively), thus providing an internal correction for arginine conversion.[3]

Advantages of SILAC

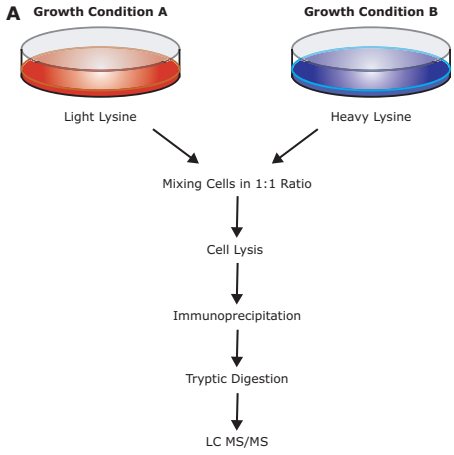
- No *in vitro* labeling steps are necessary.
- Both amino acids share the same physico-chemical properties.
- No differences in the labeling eficiency are expected.
- Compared with metabolic labeling, using heavy amino acids is sequence specific and results in a constant mass shift.
- The introduction of labeled amino acids leads to an excellent prediction of mass-labeled peptides.
- The detection of several labeled peptides derived from the same protein enables better statistics to quantify the protein level and therefore better confidence in the measurements.[1]

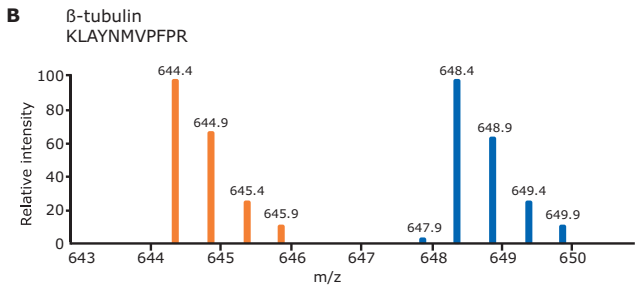
Shortcomings of SILAC

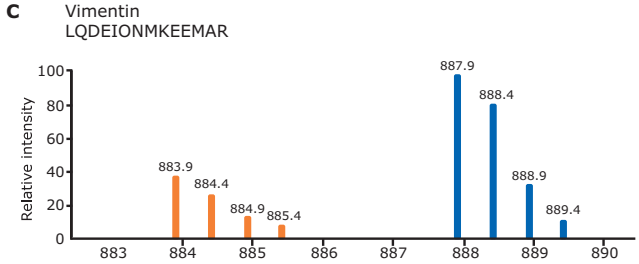
- Division of the ion current in LC/MS experiments in two signals.
- SILAC is limited to cell culture and labeling of whole organisms (such as C. elegans and D. melanogaster).[14]
- Increase of the sample complexity due to the duplets.
- The multiplexing is limited to 3 different conditions.
- The dialyzed FBS might have an influence on the cell fitness.

Figure A shows a SILAC workflow, where cells from two different cell stages are grown in light lysine and heavy lysine ([$^{13}C_6$,$^{15}N_2$]-lysine) containing media. The cell lysates generated under the two different conditions were combined in a 1:1 ratio prior to co-immunoprecipitating the interactors of Aurora-B kinase.

Mass spectrometry data from two different proteins are shown in Figure B and C. Tubulin did not show a significant difference between the light and heavy forms (ratio ~1.0) whereas vimentin is clearly more abundant in heavy labeled, stage B cells (ratio ~2.5) indicating that the association of vimentin to this protein complex is cell cycle dependent.







Examples for SILAC Applications

SILAC has been widely used to compare proteomes of different cell populations such as cells with and without cytokine stimulation, RNAi knock-down cells vs. wild type or disease vs. normal cells (for details see review Mann, 2006[4]).

Everley *et al*. used the SILAC technology to compare the protein composition of two prostate cancer cell lines, which differ in their metastatic potential. This differential set up aimed to correlate the protein changes with the different metastasis ability of these cell lines.[5]

Another example of a differential SILAC set up was used to determine the cytosolic interaction partners of all four ErbB receptor family members.[6] Due to the usage of stable isotope-labeled amino acids, it was possible to distinguish between false positive and bait specific interactions, both of which are easily detected by mass spectrometry. Any protein that shows a ratio of 1 between controls (i.e. unstimulated or only tag expressing cells) and the real sample (stimulated or bait tagged expressing cells) can be assigned as background protein. Whereas, proteins that specifically interact with the bait will show a ratio significantly different from 1.[7,8] Recently, Wang *et al*. pointed out that specific but dynamic interactors may not be distinguished from the background proteins. Dynamic interactors result in an equilibrium between two isotopic-labeled forms bound to the bait due to the fast on/off rates, so the ratio would be close to 1. Thus, protein purifications, both before and after mixing the cell lysates, are advisable.[9]

In addition to the determination of protein levels, SILAC approaches are well suited for monitoring changes in post-translational modifications. Examples for these applications include the measurement of changes in protein phosphorylation and methylation.

The utility of SILAC approach in the study of phosphorylation dynamics was demonstrated by Olsen *et al*., who examined phosphorylation dynamics in response to EGF (epidermal growth factor) by using three different arginine isotopes to label cells. This approach facilitated the comparison of three different time points upon EGF stimulation. They reported the temporal profiles of more than 6,500 phosphorylation sites upon growth factor stimulation.[10]

Another example for the use of SILAC for the quantification of protein modifications was presented by Ong *et al*.. They reported a “heavy methyl SILAC” strategy where methylation sites were directly labeled by growing cells under light and heavy methionine conditions. This approach provided more confidence in detection and quantification of protein methylation since the methylated peptides were present in pairs separated by the mass difference of the labeled methyl groups. Using this strategy, it was possible to describe 59 unique methylation sites on 33 different proteins in HeLa cells.[11]

The use of SILAC for more comprehensive quantitation of several protein modifications was recently demonstrated by Bonenfant *et al*. and Vermeulen *et al*. Both groups used SILAC for the study of various histone modifications. While Bonenfant *et al*. used the SILAC approach for a comprehensive analysis of the dynamics of histone modifications (i.e. acetylation, methylation and phosphorylation) changes through cell cycle[12], Vermeulen *et al*. took a SILAC based histone peptide pull-down approach to screen specific interactors of histone H3 trimethylated on Lys-4 (H3K4me3). They showed that basal transcription factor TFIID specifically binds to H3K4me3. Using triple SILAC pull-down assays, they further showed that H3dimethylation on Arg-2 inhibits TFIID binding to H3K4me3, whereas acetylation facilitates this interaction.[13]

Summary

In summary, SILAC has proved to be a powerful method to quantify the relative differential changes in protein complexes. Due to the fact that the isotopic labels are introduced very early during normal cell growth, SILAC has the great advantage of carrying all the steps from purification to data analysis together with the proper internal control.

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