

LECTURE-17

SILAC Clinical Applications

HANDOUT

PREAMBLE

The use of SILAC has been restricted to culturable cells only. This restricts its usage in clinical proteomics because of the time it takes to arrive at a result. Nevertheless, because of its high reproducibility and efficiency, it is widely used in clinical research. The most important application of SILAC remains in the field of studying the dynamic protein concentration change in tumor progression. The subtle changes in protein concentration can be detected with utmost confidence using SILAC-based approaches and hence can be used for quantitative analysis. Different applications of SILAC are discussed in details in the following lecture.

OUTLINE OF LECTURE

1. SILAC – An introduction
2. Work-flow of SILAC
3. Clinical applications of SILAC
 - a) In tumor progression
 - b) Identifying substrates for various kinases
 - c) Protein-protein Interaction
 - d) Functional analysis
 - e) Multiplexed comparison of cellular states

- f) Protein turnover study
 - g) Post-translational modification
4. Conclusion

1. SILAC – AN INTRODUCTION

Stable isotope labeling of amino acids in cell culture (SILAC), is a metabolic method of incorporating isotopically labeled amino acids in proteins. Labeled amino acids are provided in the cell culture media in appropriate concentration. The cells while growing take up the labeled amino acid and hence label the proteins. To ensure that the entire proteome has been labeled, keeping in mind the natural turnover number of proteins and the cell divisions, the cells are allowed to divide for at least five generations. Usually it is lysine or arginine that is isotopically labeled with ^{13}C , although other options like labeling the amino acid with ^{15}N are also available. Labeling lysine or arginine with ^{13}C , increases the molecular weight by 6 Da per lysine or arginine. Hence the shift in the MS peak by m/z of 6 Da denotes the peak for the labeled peptide. The ratio in the abundance of the light peak vs. the heavy peak is a measure of differential expression of that protein, subject to a particular external stimuli (Fig 1).

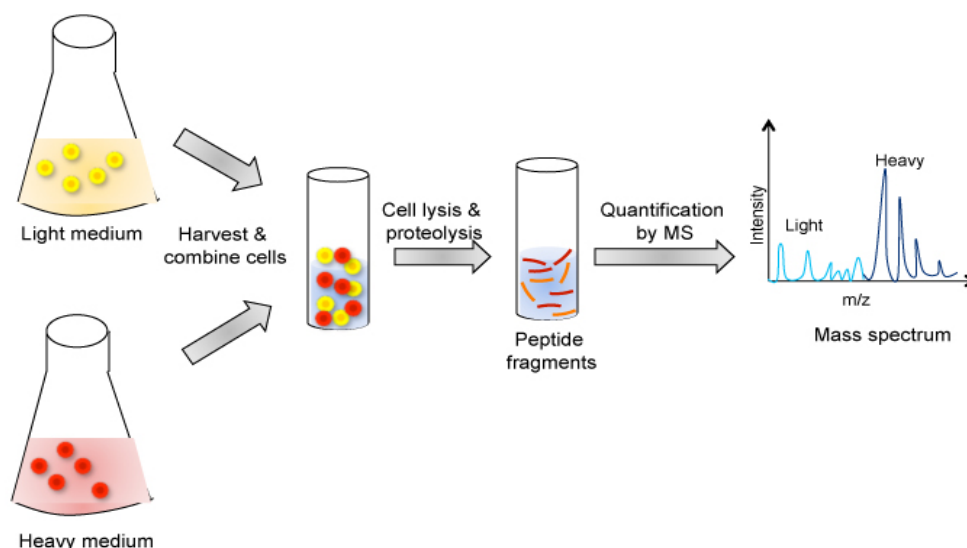
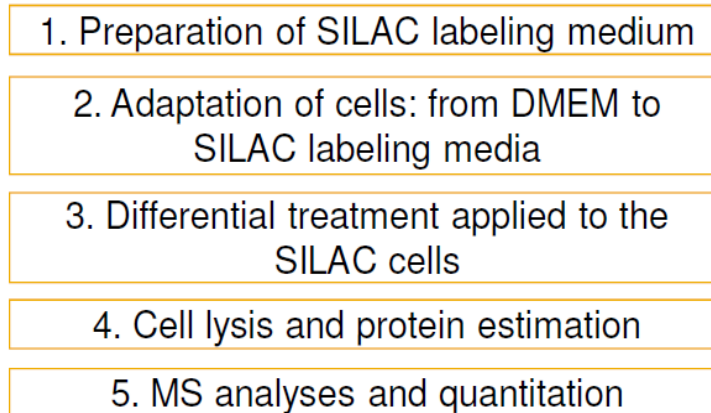


Fig 1. An overview of SILAC experiment

2. WORK-FLOW OF SILAC

Details of SILAC work-flow was described in previous lecture. Schematically the entire workflow of SILAC can be represented by the following flowchart:



3. CLINICAL APPLICATIONS OF SILAC

SILAC is very efficient and reproducible method of quantitative proteomics using mass spectrometry based approach. The ability for multiplexing has increased the ability to handle more number of samples in a high-throughput fashion. However, the biggest disadvantage of SILAC is its restriction to culturable cells only. Many cell lines like HeLa, C127, HEK293 etc. can be readily used for SILAC. However, critical factor is formulating the media for each selective cell line and then optimizing the growth formulations for each cell lines.

SILAC finds its best application in the field of clinical proteomics, where the dynamic change in the concentration of proteins can be studied at each level, for example, at various stages of glioma tissue samples. Many other studies involving post-translational modification, membrane protein dynamics, protein-protein interactions, can also be studied in great details using SILAC (Fig 2), which cannot be done using other quantitative proteomic approaches.

Illustration: SILAC application - quantification of haploid versus diploid yeast

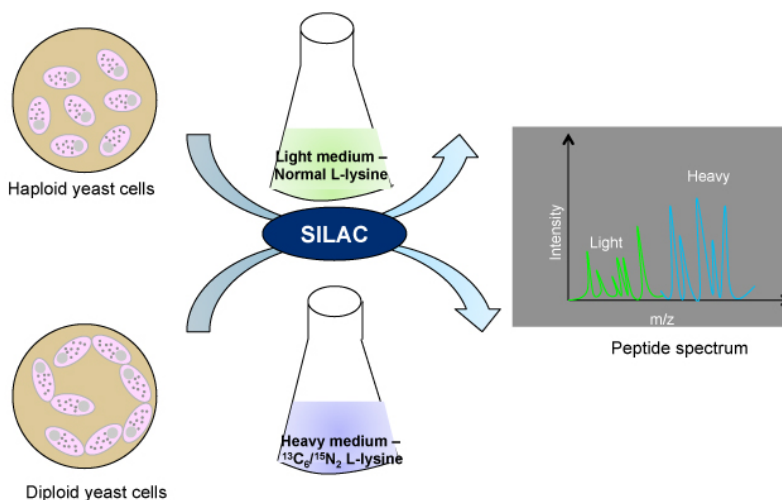


Fig 2. Quantitative comparison of haploid vs diploid yeast using SILAC

SILAC is a useful quantitative approach that has found applications for several proteomic studies. The authors determined fold change of peptide pairs between haploid and diploid yeast cells using SILAC. Labeled lysine residues were used to grow the diploid yeast cells while haploid cells were grown in normal lysine medium. The cultures were mixed, proteins extracted and analyzed by LC-MS/MS. Protein ratios between haploid and diploid cells were determined with high accuracy. Comparison revealed that 97.3% of the proteome changes less than 50% in abundance.

Illustration: Interactivity - SILAC Mouse for Quantitative Proteomics

Kruger et al. tracked the incorporation of lysine-6 into the mouse proteome over 4 weeks by providing a C-13 containing lysine diet. Their development, growth and behaviour were observed in addition to sampling various blood proteins. The labeled mice were found to develop normally. Average lysine-6 incorporation over 4 weeks in human serum albumin and hemoglobin is depicted in the graphs. What inference can be drawn from the difference in lysine-6 incorporation between human serum albumin and hemoglobin in the mouse proteome?

(a) IN TUMOR PROGRESSION

Tumor metastasis has been the interest of researchers. Tumors are classified into various stages as per their progression. However, progression from one stage to the other is so subtle that it is hardly possible to detect the preliminary stages and hence diagnosis becomes a big challenge. SILAC based studies have greatly revolutionized the ability to detect the subtle changes in protein expressions in tumors, which directly reflect their progression.

Gieger et al, have identified 8750 proteins from estrogen receptor negative breast cancer cell lines and also quantified 7800 proteins of them using SILAC method. The approach involved both MS analysis and validation using immuno-histochemistry. The results correlated with the global loss of tissue architecture and the change in metabolism in the cancerous cells. High levels of protein named IDH2 and CRABP2 and low levels of SEC14L2 were considered as prognostic markers for breast cancer survival.

(b) IDENTIFYING SUBSTRATES FOR VARIOUS KINASES

Kinases are enzymes which phosphorylate specific residues of proteins, especially tyrosine or serine/threonine. They play a very important role in cell signaling by modulating the strength of signal. Identifying substrates for kinases is another application of SILAC, which was first demonstrated by Ibarrola et al. Identification of tyrosine kinase substrates by pulling down using anti-phosphotyrosine antibodies limits the number of substrates and also provides erroneous results because of the basal level of phosphorylation. The approach by Ibarolla et al, involved growing the ligand induced cells in heavier carbon containing media and then enriching the phosphotyrosine proteins by pulling down using anti-phosphotyrosine antibodies and doing MS analysis. SILAC thus offers an excellent platform for studying cell signaling as well. Thus using MS approach, not only the level of phosphorylation, but also the site for phosphorylation can be determined.

(c) PROTEIN-PROTEIN INTERACTION

To study protein-protein interaction, SILAC approaches are usually combined with affinity-based purification to select the strong interacting partners. Blagoev et al identified 228 proteins of which 28 were selectively up-regulated upon Epidermal growth factor (EGF) stimulation. Upon phosphorylation, the epidermal growth factor receptor (EGFR) binds to the Grb2 protein, which again binds to several proteins downstream. Using SILAC, many such signaling proteins were identified, like plectin, cytokeleton network and Histone H3. EGFR signaling pathway up-regulation by mutation is the cause of many cancers like lung and anal cancers. It is extremely difficult to suppress a mutation at the genetic level. However, a complete knowledge about the interacting partners of the receptor can help in developing competitive inhibitors for the receptor, for shutting down the signaling cascade and hence in diagnosis.

(d) FUNCTIONAL ANALYSIS

The first functional proteome analysis of the lipid rafts was done quantitatively using SILAC by Foster et al. Due to the extreme hydrophobicities of the membrane proteins, it is often difficult to isolate them and hence quantify them. However, strategically labeling them can lead to their identification and quantification.

Foster et al, used cholesterol disrupting drugs to treat unlabeled cells and then compared the membrane proteome by dissolving the lipid rafts. The treated cells lost majority of the protein, associated with the cholesterol while the untreated cells retained them and hence identified. The analysis of such proteins provided an idea about their function in the cholesterol domain, mainly acting as signaling molecules.

(e) MULTIPLEXED COMPARISON OF CELLULAR STATES

At the general level, SILAC can also be used to study the global proteome changes, subject to external stimuli. Ong et al, studied the differentiation of muscle cells from progenitor cells and reported up-regulation of glyceraldehydes-3-phosphate and fibronectin during the process of differentiation. Andersen et al demonstrated using SILAC, the global proteome change in the nucleolus subject to different growth conditions. Such a detailed study, gives an overview of the mechanistic processes, which lead to cell division.

(f) PROTEIN TURNOVER STUDY

SILAC can also be used to study the dynamic turnover number of proteins. By studying the shift in MS spectrum of the digested peptides over different time course, an idea about the relative turn over number of a protein can be calculated. This study becomes important mainly for genetic engineering, where a particular protein of interest might be induced and its presence over a longer period of time is necessary for some processes of the cell.

(g) POST-TRANSLATIONAL MODIFICATION

Post-translational modification appears to be an extremely important phenomenon in proteomics. Certain proteins are made functional by certain modifications such as phosphorylation, glycosylation, methylation etc. Phosphorylation appears to be extremely important in terms of signal transduction. SILAC based approach of studying post-translational modification involves both the usage of mass spectrometry as well as affinity purification to identify the post-translated proteins.

SILAC based approach cannot only detect the change in the level of the post-translated proteins, but also can determine the site of modification. This becomes important in designing structural analogs of enzymes, which act upon these proteins for signal transduction. For example, receptor tyrosine kinases are activated by phosphorylating specific tyrosine residues of proteins. Designing structural analogs of these proteins will inactivate either the kinase or will inactivate the next downstream molecule thereby disrupting the signal.

CONCLUSION

SILAC finds enormous applications in the field of clinical proteomics, despite its limitation to culturable cells. Some of the applications are described in this lecture. The time consuming nature of SILAC experiment makes its use in clinical proteomics restricted for fundamental research. Nonetheless, its contribution in the clinical proteomics and life sciences has been phenomenal and new advancement of super-SILAC will further accelerate this approach for the quantitative proteomics applications.

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