HANDOUT

LECTURE-24

QUANTITATIVE PROTEOMICS: STABLE ISOTOPE LABELING BY AMINO ACIDS IN CELL CULTURE (SILAC)

Slide 1:

In today's lecture we will talk about Quantitative proteomics: Stable isotope labeling by amino acids in cell culture (SILAC). The complexity and dynamic nature of proteome presents major technological challenges. Mass spectrometry advancements have improved the high-throughput identification and quantification of proteins and now offer opportunities to understand the human diseases and discover biomarkers.

Many advancements in MS during the last decade have provided new ways for protein analysis and facilitated the study of proteomic analysis of various biological systems. Advancements in MS include development of highly sensitive mass spectrometers, fast scan rates, automation, nano-flow liquid chromatography as well as new techniques and methods to quantify protein abundance for quantitative proteomic analysis.

MS has been proven to be an extremely powerful tool to analyze the protein complexes. However, it is not a quantitative technique to begin with and peptide ionization efficiency is unpredictable. Hence, usefulness of this application for quantitative purposes remains limited, and various strategies have arisen to address this lack.

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So in today's lecture we will first discuss:

- MS-based quantitative proteomics
- *In vivo* labeling methods,
- SILAC , with experimental workflow
- Merits and demerits of SILAC method.

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MS-based quantitative proteomics: Protein labeling with stable isotopes are new effective methods for quantitative proteome profiling using MS. These isotopic labels can be introduced *in vivo* or *in vitro* and provide information about the relative abundance of proteins for proteomic analysis. The isotopically labeled peptides are chemically identical and generate similar specific signal intensities in MS. The relative levels of isotopically labeled peptides are determined by comparing the signal intensities of the paired peptides.

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So as I discussed, MS is not a quantitative technique by itself due to the unpredictability of peptide ionization. Differential stable isotope labeling has been developed to address this problem, by creating a specific mass tag. Different types of mass tags including ICAT, ITRAQ and SILAC have been developed.

These tags can be recognized by MS and provide the basis for quantification.

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These mass tags can be introduced by various methods into the proteins such as metabolic labeling, chemicallabeling, enzymatic methods or by using synthetic peptide standards. Accurate quantification in MS can be achieved by the use of stable isotope-labeled standards.

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Both *in vitro* and *in vivo* labeling methods can be applied prior to quantitative proteomic analysis. In today's lecture we will focus on *in vivo* labeling methods. Most of the quantitative proteomics approaches by MS utilize isotopic labels as a reference for the relative or absolute quantification. The labels can be introduced *in vivo*, for eg., by growing an organism in a media enriched with specific isotopes. The labels can also introduced by performing tryptic digestion in presence of heavy water. Many methods using isotopically labeled reagents that react with specific amino acids or the protein N-terminals have also been developed. For comparative and quantitative proteomic

analysis, the development of stable isotope tagging methods can allow the quantification of relative levels of proteins. These differentially labeled peptides with stable isotopes can be distinguished by a characteristic mass shift in MS.

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There are different ways of *in vivo* labeling such as enrichment of ^{15}N media, culture derived isotope tags (CDIT) or SILAC. Although we will briefly discuss ^{15}N media and CDIT, we will focus on mainly the SILAC method for rest of the lecture.

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sSable isotope tagging methods use isotopic nuclei such as ^{2}H, ^{13}C, ^{15}N and ^{18}O. These stable isotopes are incorporated in place of natural abundant isotopes in heavy standard. This in turn increases the mass of the labeled species but keeps the chemical structure and hence other properties as similar to the analyte of interest as possible. One can determine the relative expression level of proteins in two samples by using the stable isotope labeling methods

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Let's briefly discuss about ^{15}N labeling methods. Yeast or bacterial cultures which are grown in two separate media, containing either^{15}N or the natural abundant isotope of nitrogen. The cells are pooled together and proteins are extracted from these pooled cells and quantified by using MS.

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Although ^{15}N labeling is an easy method, there are various disadvantages.

- The stable isotope ncorporation in your control and the treatment proteins could be unequal. Therefore the labeled and unlabeled peptides exhibit variable mass shift in MS spectra.
- The mammalian system shows very poor incorporation of these stable isotopes.
- Furthermore this method is difficult and expensive.

Due to these limitations researchers have explored other labeling methods.

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Now let's discuss CDIT. In this method, the cells which are cultured in the stable isotope-enriched medium are mixed with the tissue samples which serve as an internal standard. The synthetic unlabeled peptides can be used for the quantification of the corresponding proteins which are labeled with stable isotopes. The proteins are extracted and digested prior to the MS analysis. The ratio of distribution of the 2 isotopes can be determined by MS.

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After having discussed some of the less commonly methods used such as ^{15}N media and CDIT, now let's discuss SILAC. The MS-based quantitative proteomics is an increasingly popular approach to study the changes in protein abundance in various biological samples. SILAC, which is a metabolic labeling strategy to encode the whole cellular proteome is one of the very widely used methods for the quantitative proteomics. In the SILAC methodology, the cells are grown in a culture medium where natural form of an amino acid is replaced with a stable isotopic form such as argenine bearing ^{13}C atoms. Incorporation of the heavy amino acid occurs through the cells grown protein synthesis and turn over. The SILAC method allows for light and heavy proteomes to be differentiated by MS while avoiding any chemical derivitization and associated purification.

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SILAC is a metabolic labeling strategy which uses stable isotope labeled amino acids in the growth medium. This experiment depends on the cellular protein synthesis to incorporate the stable isotope containing amino acids into whole proteome, for eg., argenine or lysine which contains six ^{13}C atoms.

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The SILAC experiments are performed by incorporating the stable isotopically labeled amino acids such as L-Argenine containing six ^{13}C through the natural protein turnover

and cell growth. The cells are cultured in 2 separate media for the light and the heavy forms. So the light medium contains amino acids composed of naturally occurring isotopes whereas the heavy medium contains the SILAC medium amino acids of choice. These are commercially available.

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So the labeled analogues of these amino acids are supplied during the growth of these cells which are incorporated during the protein synthesis in all the newly synthesized proteins. After number of cell divisions, certain amino acids are completely replaced by their heavy isotope containing counterparts and finally MS can be used for determining the relative protein abundance by the intensity of light and heavy peptide spectra..

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Now we will discuss about SILAC experiment and the workflow to perform the experiment. In the workflow, we will discuss the SILAC protocols and how to incorporate SILAC labels into any given experiment.

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So let's have a look on the workflow for performing a SILAC experiment.

- The first step is preparing SILAC labeling medium.
- Second, adaptation of cells from the normal DMEM medium to SILAC labeling media.
- Third, the differential treatment application to the SILAC cells.
- Fourth, cell lysis and protein estimation.
- Fifth, MS analysis and quantification.

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So let's look at the workflow stepwise.

1) The preparation of SILAC labeling medium:. So in SILAC experiment, a defined media with known sources of amino acids can be adapted for labeling. The amino acid for labeling can be left out from the media formulation to ensure that the light and heavy

amino acid stocks used in the media preparation are the only available source of amino acids in cells.

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So there are two widely used media. One is the Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640. The argenine, lysine and methionine are removed from the standard formulations. The light medium is naturally abundant isotopic forms of amino acids whereas the heavy medium is the same medium which lacks the desired amino acids (for eg. argenine and lysine) and they can be substituted as the heavy isotopic forms.

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Here I am providing you an example of stable isotope-labeled amino acids. $^{13}C_6$ is a stable isotope of $^{12}C_6$ L-Lysine. It gives 6 Da difference in the MS as compared to $^{12}C_6$ L-Lysine. $^{13}C_6$ is another isotope of $^{12}C_6$ L-Arginine form. It is again 6 Da heavier than $^{12}C_6$ L-Arginine.

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For the preparation of SILAC labeling media, one needs to add certain supplements such as serum, antibiotics. The recommended percentage for serum would be 10% dialyzed fetal bovine serum and 1% of antibiotic and glutamine. But these percentages can be optimized depending upon the type of cell cultures.

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In the SILAC experimental workflow, let's discuss the second point.

The cells need to be adapted due to slight differences in media formulations.

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2. The adaptation of cells from DMEM to SILAC labeling media: If the cells are grown in DMEM medium, it should be first split into two culture dishes containing light and heavy SILAC medium. So first, take out 10-15% of cells from the original culture and allow doubling. Then subculture the cells in respective light and heavy media, and

allowing atleast five to six cell doublings. I'll discuss the significance of this doubling process in the next slides.

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So, for the adaptation of cells from DMEM to SILAC labeling media, at the end of this adaptation phase lyse the cultures by adding 6 M Urea, 2 M thiourea and then extract the proteins, reduce disulfide bonds by adding 1 mM of dithio tritol (DTT). Then add 5 mM of iodoacetamide (IAA) to alkylate the cysteine residues. Add trypsin for digestion, overnight at 37°C, with the enzyme and substrate ratio of 1:100. Then these samples can be analyzed by LC-MS or LC-MS/MS. However one needs to ensure that SILAC amino acids are fully incorporated.

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Cells are adapted in heavy medium for atleast 5 or 6 doublings to be fully labeled. The digested proteins samples can further be analyzed by MS. These are representative spectra showing m/z ratio and the relative intensity of light, medium and heavy forms.

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So in SILAC experimental workflow let's discuss the third point,

3. Differential treatment application to the SILAC cells:. Changes in in the proteome between the control and experimental populations being studied can be introduced at this point, by treatment with drugs or growth factors to one cell population. For example, cells grown in regular media receive control or placebo-treatment, while cells grown in the heavy media receive a drug treatment. Proteins from the cells adapted to the light and heavy SILAC media can be analyzed and distinguished by MS.

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Once the SILAC labels are incorporated in the cell culture then differential treatment can be applied. This treatment could be the external stimuli exposure, drug treatment, growth factors, immunoprecipitation, and the comparison of differentiated and undifferentiated cells or it could be some other thing. While we are doing this labeling it

is a very good idea to repeat the experiment to ensure that the fold changes are uniform and a reverse labeling should also be performed to check reproducibility of findings. If reverse labeling also shows similar trends, then it means that experiment is unbiased due to any labeling issue.

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During SILAC experiments, one also need to check for argenine-to-proline conversion and this could be manually adjusted for in the experimental conditions. One may need to reduce the argenine concentration or add proline to the medium to overcome such issues. There are some software's that can be used for calculate the argenine-to-proline conversion.

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In the SILAC experimental workflow, let's discuss the fourth point.

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4. **Cell lysis and protein estimation:** So once the cells have incorporated the labels, they can be harvested using any standard protocols.. One needs to obtain a small aliquot of each cell lysate and estimate the protein concentration. We have talked different methods of doing protein quantification earlier. One can use Bradford assay or some other type of protein quantification assays. After the protein concentration determination, the protein concentration should be normalized prior to mixing both heavy and light lysates. Because we want to do quantification before mixing mixing both cell cultures, it is very important we are staring with equal protein amount in both light and heavy forms. This process can be done by normalizing by diluting the cell lysates with lysis buffer.

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In the SILAC experimental workflow the last and most important point is mass spectrometric analysis.

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5. **MS analysis, protein identification and quantification.** As we discussed earlier, prior to the MS analysis, one needs to do DTT treatment to cleave the disulfide bonds, IAA for alkylation and trypsinization for protein digestion. These treatments should be performed and then the combined digested mixture and desalted peptides can be further used. The desalting can be performed by using C^{18} columns. By using the raw MS data files, extract sequence-specific MS/MS peak list. Then it can be used for the identification of peptides and proteins using various databases.

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The quantification of SILAC-labeled heavy and light peptide pairs:. The peptides, for eg. containing heavy argenine are 6 Da heavier than the normal ones. The fold abundance ratios can be determined by different methods. Two methods are suggested to calculate the ratio of the intensities of each peptide from individual MS spectrum or the ion chromatograms of light and heavy peptides eluted from reversed-phase columns. These can then be used to determine the ratio of area under curve.

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In the MS spectra, the pairs of chemically identical peptides incorporating heavy and light isotpes can be differentiated due to their mass difference. The ratio of peak intensities of such peptide pairs demonstrates the population ratio for 2 proteins. Here, I have shown the light and heavy form separation by6 Da.

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Having discussed the workflow for performing SILAC experiments, let's have an overview of the protocol. In SILAC, two different populations can be grown in DMEM media containing $^{13}C_6$ stable-labeled form of argenine in the place of normal or light argenine. After allowing 5 or 6 doublings in each protein, all incorporated argenine is of heavy form now. The cells can be combined and further lysed prior to further proteolytic steps.

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So continuing into the protocol, once we have combined the heavy and light populations then these can be separated on SDS-PAGE gel and each of these bands can be further digested by adding trypsin. After digestion of isolated proteins to peptides by trypsin the argenine containing peptides will be 6 Da heavier than their light counterparts which can be analyzed by using MS.

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Now I will show you an animation of SILAC.

Animation 1

So let's discuss the SILAC method.

SILAC is a simple method for *in vivo* incorporation of a label into proteins for quantitative proteomic purposes. Two groups of cells are cultured in media that are identical in all respects except that one contains a heavy, isotopic analog of an essential amino acid while the other contains the normal light amino acid. The essential amino acids which are obtained from the cell culture medium are incorporated into the corresponding newly synthesized proteins during cell growth and replication. The medium containing the heavy amino acids will give rise to heavy, isotopic proteins. After a number of cell divisions, all instances of the particular amino acid will be replaced by its isotopic analog. The grown cells are then combined together and harvested. Centrifugation of the mixture will result in the pelleting of cells which can then be used for further analysis.

The grown cells are then lysed using a suitable lysis buffer and the proteins degraded using a proteolytic enzyme like trypsin. This results in a mixture of light and heavy peptide fragments which can be quantified suitably by MS. The complex mixture of peptide fragments is further separated by SDS-PAGE to simplify the analysis. Each band of the gel is cut out and re-dissolved in a suitable buffer solution. These simplified peptide fragments are then used for further analysis.

Further purification is carried out by liquid chromatography wherein the sample is passed through a column containing a packed stationary phase matrix that selectively adsorbs only certain analyte molecules. Reverse phase and strong cation exchange chromatography are the most commonly used. The eluted fractions are further characterized by MS. The purified peptide fragments are then analyzed by MS/MS. Peptides containing the heavy amino acid show higher m/z than the corresponding light peptide fragments. The pairs of identical peptides can be differentiated due to the mass difference and the ratio of peak intensities can be correlated to the corresponding protein abundance.

The MS/MS data analysis shareware has some extra inputs such as Quantitation, MS/MS tolerance, peptide charge, instrument etc. in addition to the fields for PMF. They require inputs from the user regarding the experimental parameters used such as enzyme cleavage, protein name, modifications etc. and the desired search criteria like taxonomy, peptide tolerance etc. Commonly used protein databases against which the MS information is processed to retrieve sequence data include NCBI, MSDB and SwissProt. The data file generated from MS is uploaded and the search carried out.

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After discussing the SILAC technique and watching this animation of this entire process now let's discuss about advantages and disadvantages of the SILAC method and compare it with some other tagging methods. As we have discussed and realized that SILAC method is very simple and robust and it labels the entire proteome without chemical derivitization and with fewer sample handling steps. The labeled samples are mixed at the stage of whole cells which makes SILAC approach ideally suited for the quantitative proteomic experiment because there will be fewer handling variations and manual artifacts for each population separately. Studies such as the subproteome analyis, for eg. the cellular organelles or complex purification protocols, can be performed with very good quantitative accuracy by using these methods.

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So let's discuss about some of the advantages of using SILAC method. In SILAC no chemical difference is observed between labeled and natural amino acid isotopes. The cells are grown in tissue culture medium and they behave exactly like the control cell population grown in the presence of normal amino acids. So this method is very efficient and reproducible. After 5 or 6 generations it has been observed that heavy isotope incorporation is at a 100%.

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The samples can be mixed prior to the MS analysis. This method eliminates some of the handling errors. SILAC experiments have demonstrated that they have 5-plexing capability to compare 5 different states in one experiment.

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Although there are many advantages of SILAC, there are some disadvantages due to the inherent nature of this method. SILAC is applicable only to the culture cells. It cannot be used for tissue or body fluids. That is one of the major limitations of this method. The tissue culture process is always time consuming. There are very limited forms of heavy amino acids available. Due to this limited number of states can be compared using SILAC.

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The metabolic conversion of argenine to proline is one of the commonly observed issues in SILAC experiments which results in tryptic peptides containing heavy proline. Now there are various experimental and biological solutions which can be used to reduce the interference from incompletely labeled peptides.

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Let's compare SILAC with radioactivity labeling methods. The SILAC methods seeks to replace the labeled amino acid which is unlike the radioactive labeling that uses ^{32}P or ^{35}S-Methionine. SILAC ensures that the labeled amino acids are fully incorporated in the cells; however, small percentage of labeled amino acids can be detected. In the

radioactive-based labeling the full incorporation is not necessary. In SILAC one readout the signals by using MS, whereas the radioactive detection is possible using scintillation counters.

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Let's now discuss few applications of SILAC briefly.

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The SILAC method is very promising for any cell line. So this method can be applied for any cell line whether it is HeLa cell, C127, HEK293, etc. However, the media formulation and the growth optimization is required individually for each cell line. SILAC applications have been demonstrated in different applications such as cell signaling, studying the induced protein complexes, studying temporal dynamics, identification of kinase substrates and studying differential membrane proteomics.

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Ong et al. published a paper in 2002 which was the first demonstration of SILAC application where they used the relative quantification of changes in protein expression during the time course of myoblast differentiation in mouse C_2C_{12} cells.

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Researchers have reported various unique metabolic-labeling strategies; for example, identification of tyrosine kinase substrates using ^{13}C tyrosine. Labeling that incorporates heavy methyl groups which can shed light on methylation sites in proteins..

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There are numerous studies based on the global protein expression profiling using SILAC method. I am just highlighting some of the early studies which set the path for performing these protein expression profiling. So the study by Everley et al. in 2004 analyzed the expression levels of more than 440 proteins in the microsomal fractions of prostate cancer cells with varying metastatic potential. Another study by Gu et al.

investigated the early stage of apoptosis by inducing the p53 up-regulated modulator of apoptosis.

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SILAC has also been used for functional assays to study the protein-protein interactions. A study by Blagoev et al. differentially labeled proteins in EGF-stimulated versus unstimulated cells. Another study by de Hoog et al performed. quantification of proteins interacting in an attachment-dependent manner with focal adhesion proteins. These are just examples of studying the functional assays and performing protein interactions using SILAC.

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SILAC can also be used for the identification of proteins which are enriched in specific cellular structures. A study by Foster et al. used the first proteomic analysis of rafts and they showed that the specific detection of proteins depleted from the rafts by cholesterol-disrupting drugs.

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SILAC has been widely used for multiplex analysis to compare the cellular states. Anderson et al. showed the quantitative analysis of the proteome of human nucleoli. Blagoev et al. performed a temporal analysis of phosphotyrosine-dependent signaling networks to compare the proteome of three cell populations. Kratchmarova et al. analyzed the divergent growth factors in mesenchymal stem cell differentiation. These are just few examples of multiplex analysis. Now if you look into literature, there are many studies which have used SILAC method for comparison of cellular states.

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The SILAC method has also been used to study the protein turnover. A study by Pratt et al. used the rate of breakdown of individual proteins by analysis of mass shifts in tryptic peptide fragments. The analysis of abundant protein in glucose-limited yeast cells which were grown in aerobic chemostat culture at steady state was performed by using SILAC method.

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SILAC has been used for identification and quantification of protein posttranslational modifications. A study by Ibarrola et al. identified and quantified phosphorylation sites. Another study by Ballif et al. also identified and quantified the phosphorylation sites. There are many studies which have used SILAC method for studying posttranslational modifications.

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Interestingly, the SILAC method has been used in different organisms and not just with single cells such as Bacteria and yeast.There are some studies on Arabidopsis, a plant, as well as in mice that have shown that the SILAC procedure can be adapted for a wide variety of organisms.

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So SILAC applications are straightforward and it only requires some initial attention to the cell culture conditions. Let's discuss the SILAC applications in an animation.

Animation 2

- SILAC is a useful quantitative approach that has found applications for several proteomic studies.
- SILAC provides an in vivo strategy to label and monitor quantitative differences at protein level in different conditions, which has been successfully employed for differential profiling & biomarker identification.
- Temporal dynamics of cell signaling pathways that transmit information through various PTMs, most commonly reversible phosphorylation, have been efficiently studied by SILAC coupled with MS.
- Quantitative proteomic studies using SILAC have been carried out with yeast, which serves as a model organism for eukaryotic cells in providing insights into biological processes.

- Methylation, which is one of the most common PTMs having various biological roles, has been successfully studied using isotopically labeled methionine residues.
- One of the more recent applications of SILAC include the identification of protease substrates using differentially labeled bacterial cell cultures.
- Cellular functions are mediated by several protein complexes that interact with one other.
- SILAC has been applied for quantitative determination of such complexes and their interacting protein partners.
- Signaling pathways involving kinases are employed in cell growth and differentiation which play a major role in cancer development and progression.
- These pathways and effects of inhibitors on them have been studied using SILAC. SILAC allows for labeling and monitoring of dynamically changing proteomes of sub-cellular organelles which are involved in several activities during apoptosis in cells.

Now let's take a case study from de Godoy and colleagues which determined the 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.

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After discussing about different types of applications of SILAC method, now let's summarize what we have studied in this lecture. In a typical SILAC experiment, control and treated cell lines are grown in different media. One was enriched with light form and another with heavy isotope containing amino acids. The peptides from heavy and light form are mixed in 1:1 ratio and proteins were extracted, purified and digested. The peptides were analyzed by LC-MS/MS. The signal intensity of labeled and unlabeled

peptides was able to provide the quantitative information. There are some limitations of SILAC method. The limitations include its usage only in the cell culture and the metabolic conversion of argenine to proline. But overall SILAC is a very promising technique and its applications are published in several papers. I hope today's lecture has been useful in illustrating the different types of *in vivo* labeling methods, the SILAC experiment and the stepwise workflow of performing a SILAC experiment, the merits and demerits of the SILAC method.