Proteomics: Principles and Techniques

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Lecture No. # 24
Quantitative Proteomics: Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

Welcome to the proteomics course. In today's lecture, we will talk about quantitative proteomics. The stable isotope labeling by amino acids, in cell culture silica the complexity and dynamic nature of proteome present major technological challenges. Mass spectrometry advancements have improved the high-throughput identification and quantification of proteins. The mass spectrometers have advanced significantly and now offering opportunities to understand the human diseases and discover bio markets. MS has proven to be an extremely powerful tool to characterize the protein complexes; however, MS is not a quantitative technique to begin with. And peptide ionization efficiency is unpredictable.

So, usefulness of this data for the quantitative purpose remains limited. Many advancement in MS during the last decade have provided new ways for protein analysis and facilitated the study of proteomic analysis of various biological system. Advancement in MS include the development of highly sensitive mass spectrometers; fast scan rates; automation; nano flow liquid chromatography as well as new techniques and methods to quantify proteins. It is abundance for quantitative proteomic analysis.

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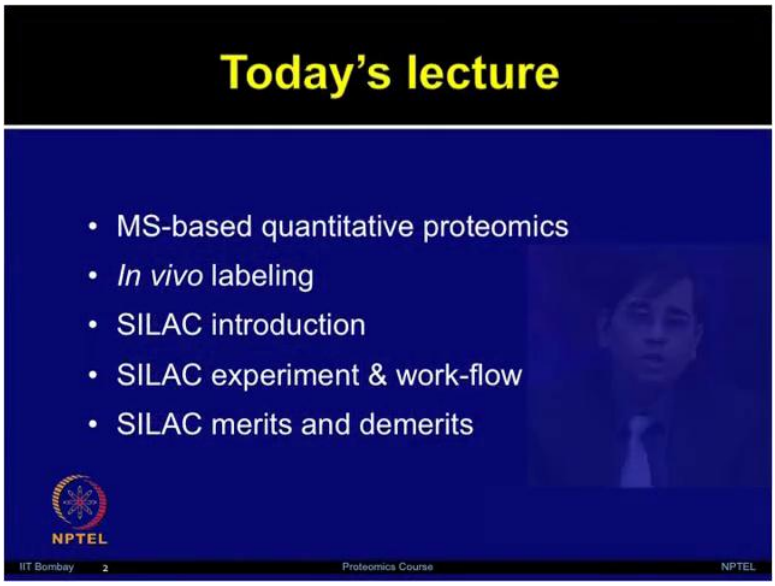

So, in today's lecture we will first discuss about the MS based quantitative proteomics in vivo labeling methods. I will introduce SILAC and then we will talk about the SILAC experiment and the step by step work-flow. We will then discuss the merits and demerits of SILAC method. So, let us start with the MS based quantitative proteomics. The protein labeling with stable isotopes are new and effective methods for quantitative proteome profiling using mass spec. These isotopic labels can be introduced in vivo or in vitro and provide relative abundance of proteins for proteomic analysis. The isotopically labeled peptides are chemically identical and they generate similar specific signal intensities in mass spectrometer. The relative levels of isotopically labeled peptides are determined by comparing the signal intensities of paired peptides.

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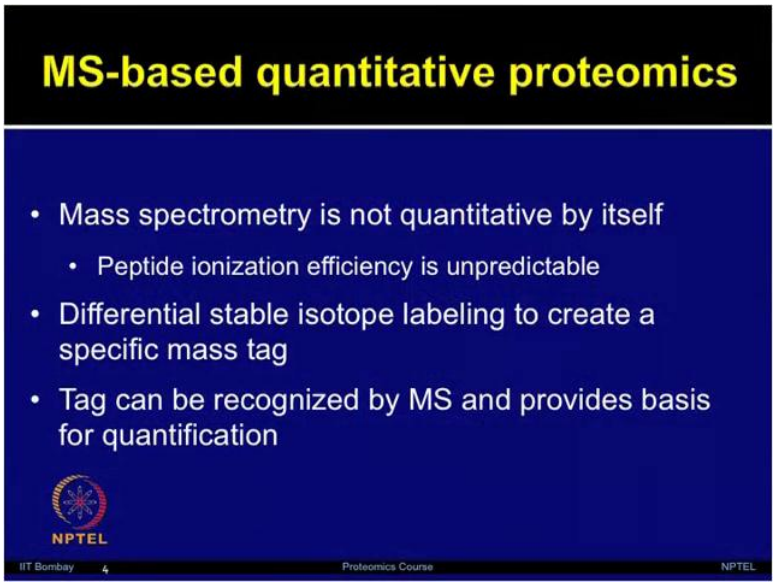

So, as we discussed mass spectrometer is not quantitative technique by itself due to the peptide ionization efficiency which is unpredictable. So, the differential stable isotope labeling is used to create a specific mass tags the different type of mass tags which are currently very promising for various quantitative proteomic applications including ICAT, ITRAQ and SILAC as the rest varies other tags. So, these tags can be recognized by MS and provide the basis for quantification.

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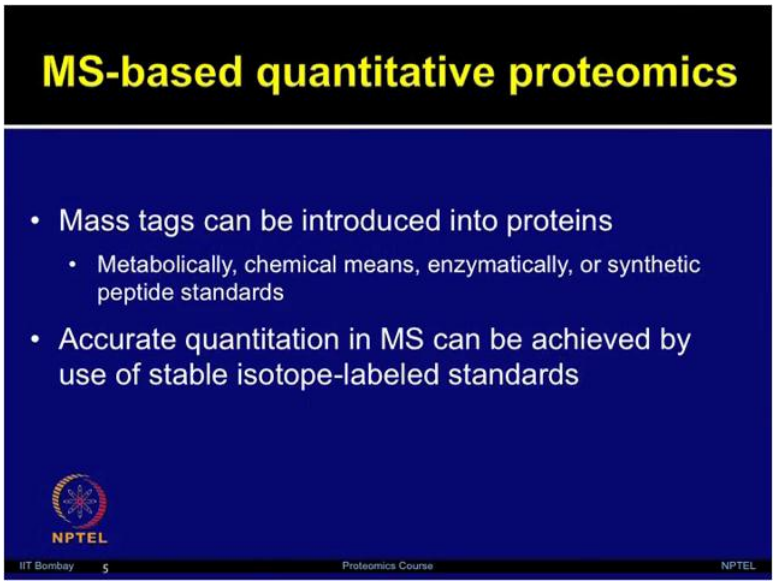

These mass tags can be introduced by various methods into the proteins such as metabolic labeling chemical means of labeling enzymatic methods or by using synthetic peptide standards. The accurate quantification in mass spec can be achieved by use of stable isotope labeled standards. So, there are different ways of quantitative proteomic analysis, in vitro and in vivo labeling methods. In today's lecture we will focus on in vivo labeling methods. So, most of the quantitative proteomic approaches by MS utilize isotopic labels as a reference for either the relative or absolute quantization. These labels can be introduced in vivo. For example, by growing an organism in a media enriched with specific isotopes. The labels are 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 and terminals are also developed. For comparative and quantitative proteomic analysis, the development of stable isotope tagging methods, can allow the quantification of relative levels of proteins. This differentially labeled peptides with a stable isotopes can be distinguished by characteristic mass shift in MS.

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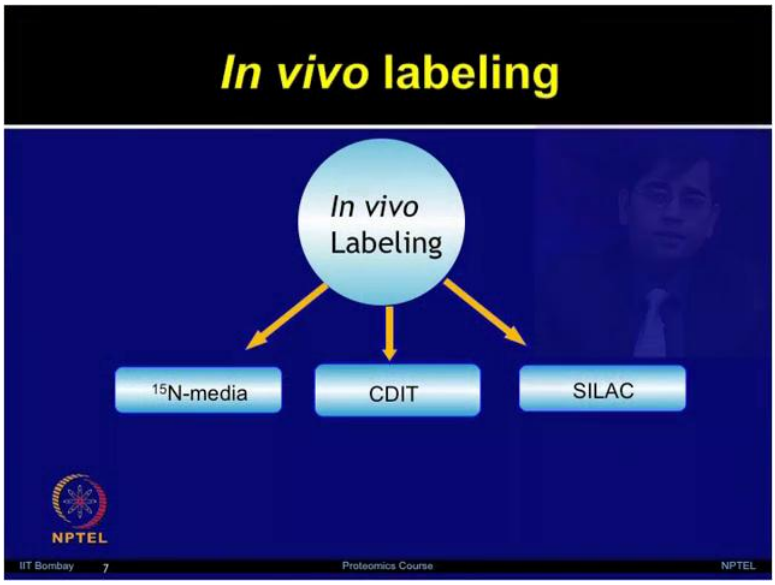

The different ways of In vivo labeling such as enrichment of ^{15}N-media; the culture derived isotope tags or CDIT stable isotope labeling by amino acids in cell culture or SILAC. Although we will discuss briefly about N-media method and culture derived isotope tags and then we will focus on mainly SILAC method for rest of the lecture.

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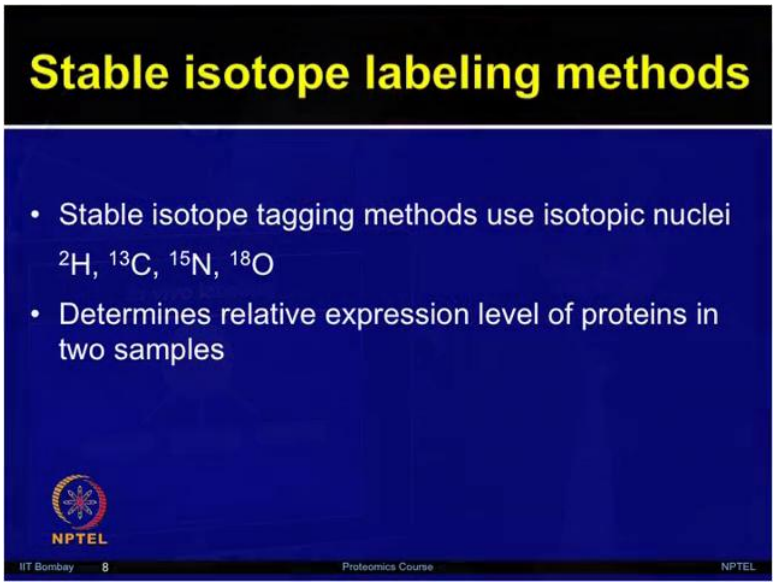

So, this stable isotope tagging methods use isotopic nuclei, such as two hydrogen, thirteen carbon, fifteen nitrogen and eighteen oxygen. These stable isotopes are incorporated in place of natural abundance isotope in heavy standard. By using this stable isotope labeling methods, one can determine the relative expression level of proteins in two samples.

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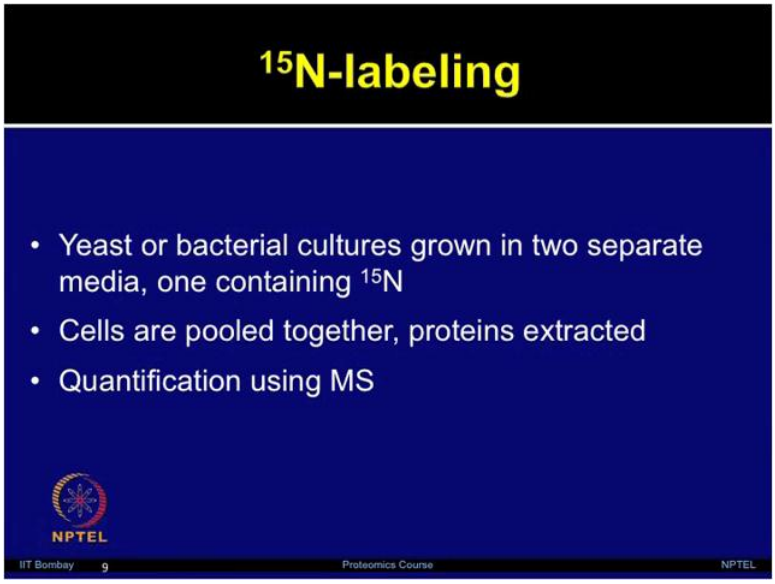

Let us briefly discuss about N labeling method. Say yeast or bacterial cultures which are grown in two separate media, one containing 15 nitrogen. The cells are pooled together; proteins can be extracted from these pooled cells and quantified by using mass spec.

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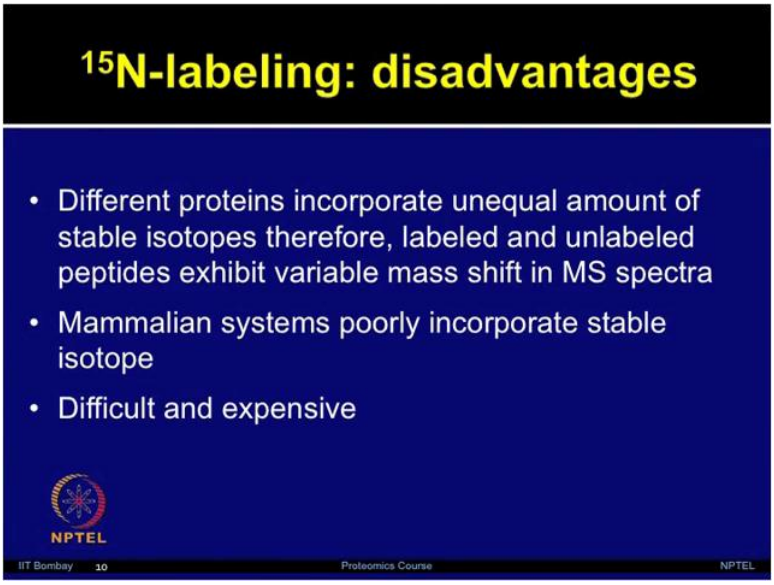

Although 15 N-labeling is easy method but there various disadvantages, the protein incorporation in your control and treatment could be unequal due to these stable isotopes therefore, the labeled and unlabeled peptides exhibit variable mass shift in the MS spectrum. The mammalian systems very poorly in corporate these stable isotopes. Furthermore this method is difficult and expensive. Due to these limitations researchers have tried other labeling methods as well.

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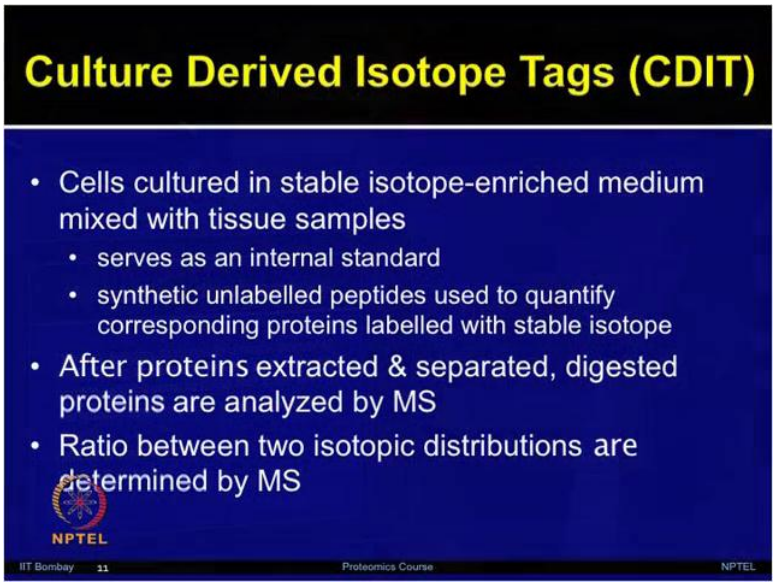

Now, let us discuss about culture derived isotope tags or CDIT. In this method, the cells which are cultured in the stable isotope in rich medium, they are mixed with the tissue, which serves as an internal standard. The synthetic unlabeled peptides can be used for the quantification of corresponding proteins which are labeled with stable isotopes. The proteins can be extracted and digested prior to the MS analysis. The ratio between two isotopic distributions can be determined by mass spec, after having discussed some of the less commonly used methods such as 15 N- media and CDIT.

Now, let us discuss about stable isotope labeling by amino acid in cell culture SILAC. So, the MS based quantitative proteomics is an increasingly popular approach to study changes in the protein abundance in various biological samples. SILAC, which is a metabolic labeling strategy to encode whole cellular proteome is one of the very widely used method for the quantitative proteomics. In SILAC method the cells are grown in a culture medium where natural form of an amino acid is replaced with a stable isotopic form such as, arginine bearing thirteen carbon atoms, incorporation of the heavy amino acid occurs through the cells grown protein synthesis and turnover. The SILAC method allows for light and heavy proteomes to be differentiated by the mass spec while avoiding any chemical derivatization and associated purification.

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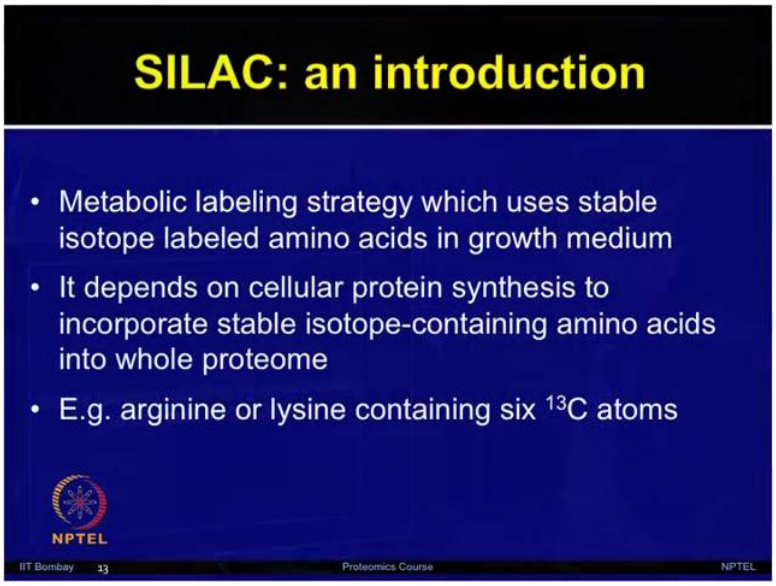

So, SILAC is a metabolic labeling strategy which uses the stable isotope labeled amino acids in the growth medium. This experiment depends on the cellular protein synthesis to incorporate the stable isotope containing amino acids into whole proteome.

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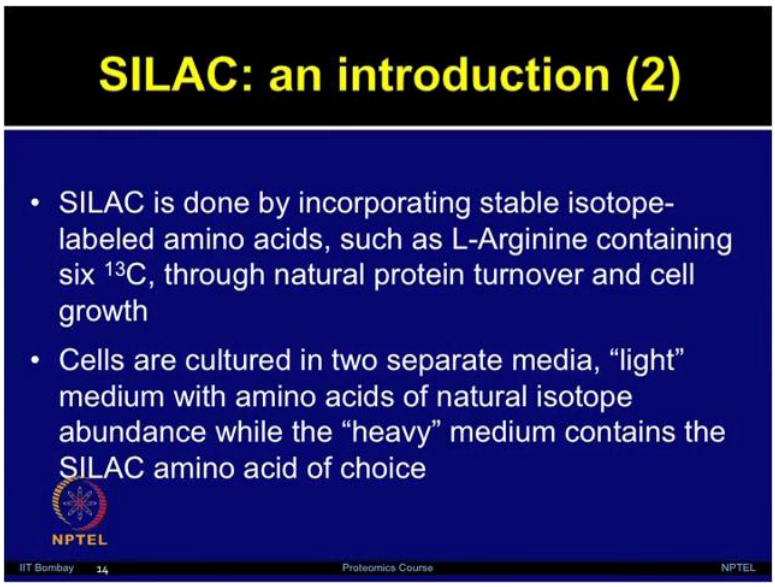

For example, arginine or lysine which contains 613 carbon atoms. The SILAC experiments are performed by incorporating the stable isotopically labeled amino acids, such as l-arginine containing six thirteen carbon through the natural protein turnover and cell growth. The cells are cultured in two separate media for the light and the heavy

forms. So, the light medium with amino acids of mutual isotope abundance whereas, the heavy medium contains the SILAC amino acids of choice, these are usually commercially available.

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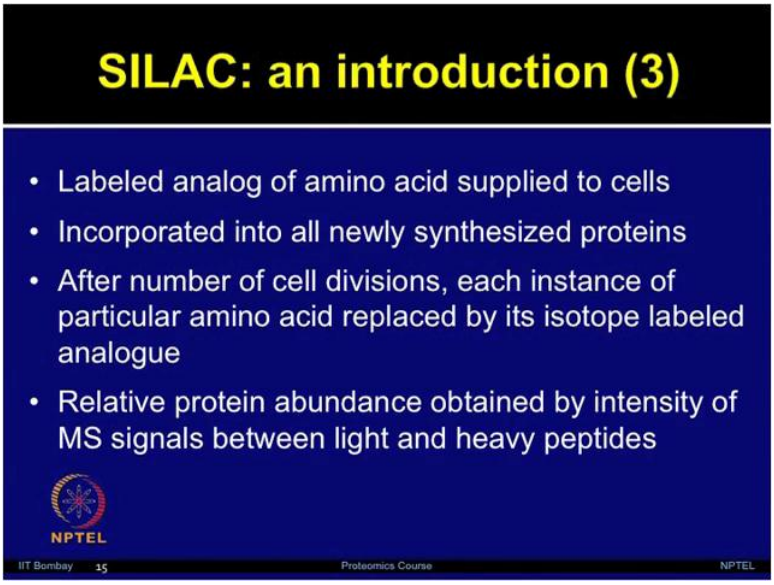

So, the labeled analog of amino acids, are supplied during the growth of these cells, which are incorporated during the protein synthesis in all the newly synthesized proteins. After number of cell divisions each instance of a particular amino acid is replaced by its isotope labeled analog and finally, the bias spec can be used for determining the relative protein abundance by the intensity of light and the heavy peptides. So, now we will discuss about SILAC experiment and the work-flow to perform a SILAC experiment. So, in the work-flow we will discuss the SILAC protocols and how to incorporate SILAC labels into any given experiment.

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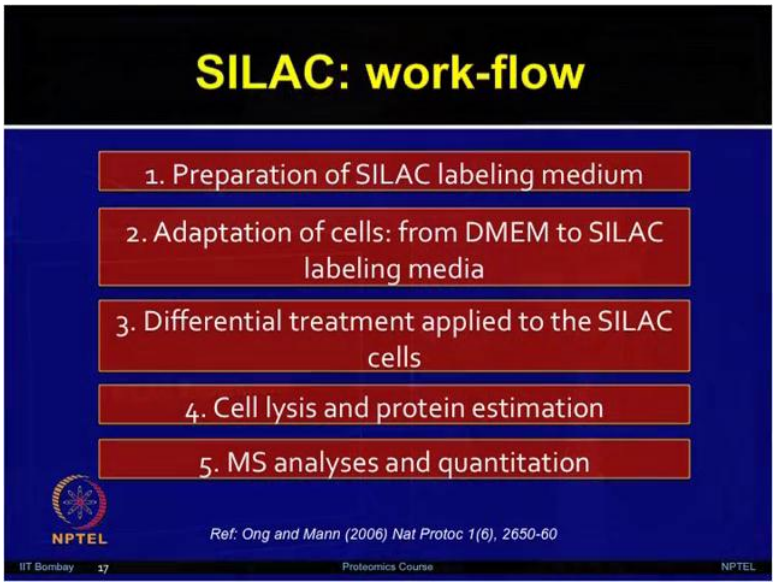

So, let us have a look on the work-flow for performing a SILAC experiment. First step is preparation of SILAC labeling medium; Second adaptation of cells from the normal DMEM medium to SILAC labeling media; Third the differential treatment application to the SILAC cells; Fourth cell lysis and protein estimation; Fifth MS analysis and quantification.

So, let us look at the work-flow stepwise. The first point: the preparation of SILAC labeling medium. So, in SILAC experiment any defined media with known sources of amino acids can be adapted for labeling. The amino acid for labeling can be left out from a media formulation to ensure that the light and heavy amino acid stocks used in the media preparation are only available source of amino acids in cells.

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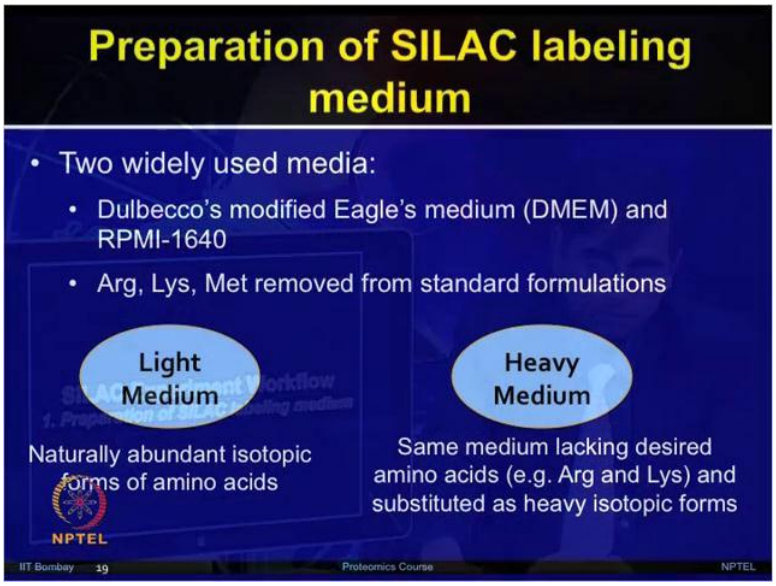

So, there're two widely used media one is the Dulbecco's modified eagle's medium DMEM and RPM-I 1640 the arginine lysine and methionine are removed from the standard formulations the light medium is the naturally abundant isotopic forms of amino acids whereas, the heavy medium is the same medium which lacks the desired amino acid. For example, the arginine and lysine and it can be substituted as the heavy isotopic forms.

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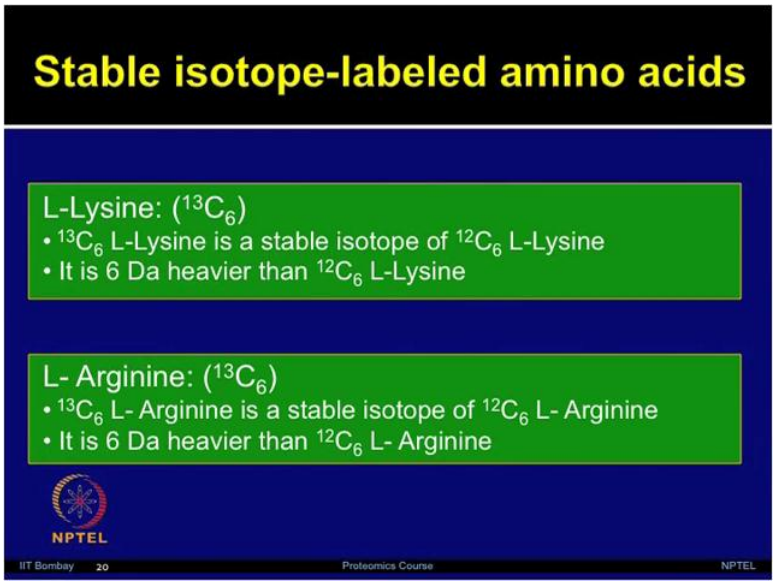

So, here I am providing you one example of stable isotope labeled amino acids. Thirteen carbon 6 L-lysine is a stable isotope of 12 carbon 6 L-lysine, it gives 6 dalton difference in the mass spec as compared to the twelve carbon 6 L-lysine. The 13 carbon 6 L-arginine is another stable isotope of 12 carbon 6 L-arginine form. It is again 6 dalton heavier than the twelve carbon 6 L-arginine.

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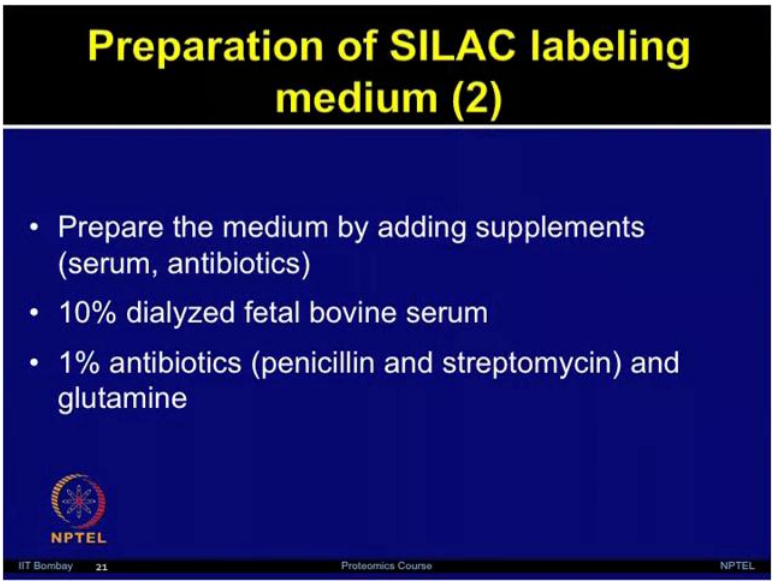

So, for a preparation of SILAC labeling media one need to add various supplements such as serum antibiotics. So, a recommended percentage for the serum could be ten percent dialyzed fetal bovine serum and one percent of antibiotic and glutamine, but these percentage can be optimized depending upon type of cell cultures. In the SILAC experimental work-flow lets discuss about second point, the adaptation of cells from the DMEM media to the SILAC labeling media this cells need to be adapted due to the slight differences in media formulations.

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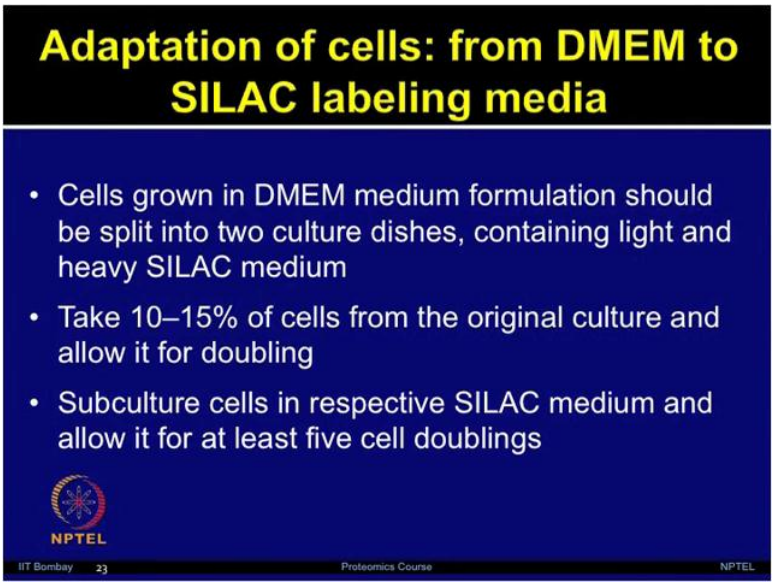

So, the cells are grown in DMEM medium it should be first split into two culture dishes, containing light and heavy SILAC medium. So, first take out ten to fifteen percent of cells from the original flask, the original culture and allow it for doubling then sub culture cells in respective SILAC medium the light and the heavy medium and allow it for at least five to six cell doublings. I will discuss that, what is the significance of this doubling process in the next couple of slides.

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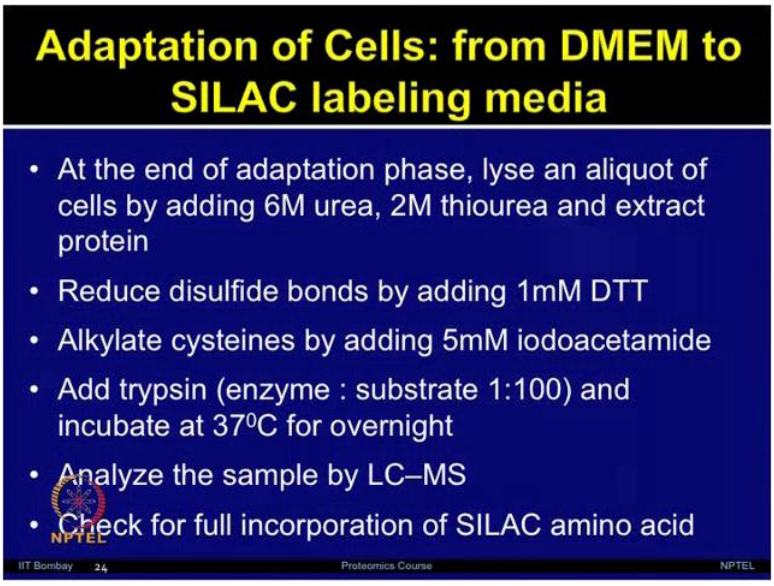

So, for the adaptation of cells from DMEM to SILAC labeling media, at the end of this adaptation phase lies the cultures by adding 6 molar urea, 2 molar thiourea and then extract the protein reduce disulphide bonds by adding one millimolar of dithiothreitol then add 5 millimolar of iodoacetamide to alkylate cysteine residues, add trypsin overnight for digestion with the enzyme and substrate ratio for one to hundred then these samples can be analyzed by LC-MS or LC MS/MS, but first thing one need to ensure that the SILAC labels are fully incorporated.

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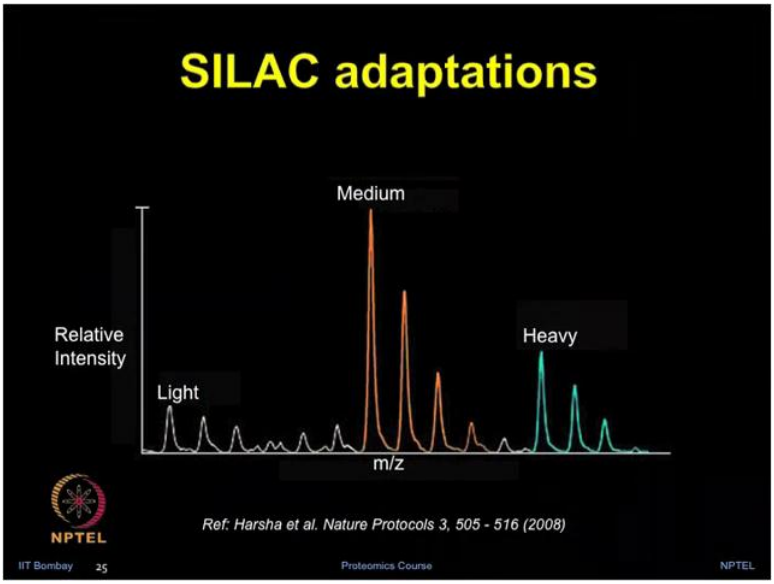

So, adaptation of cell lines in heavy medium is the first step in SILAC based experiments; cells are adapted in heavy medium for at least five or six doublings to be fully labeled. The digested protein samples can then further be analyzed by mass spec, these are representative spectra showing m by z ratio and the relative intensity of light medium and heavy forms so in SILAC experimental work-flow.

Let us discuss the third point, the differential treatment application to the SILAC cells. So, differential response between the control and experimental cell populations can be introduced by treatment of drug growth factors to one cell population. Proteins from cells adapted to the light and heavy SILAC media can be analyzed and distinguished by mass spec.

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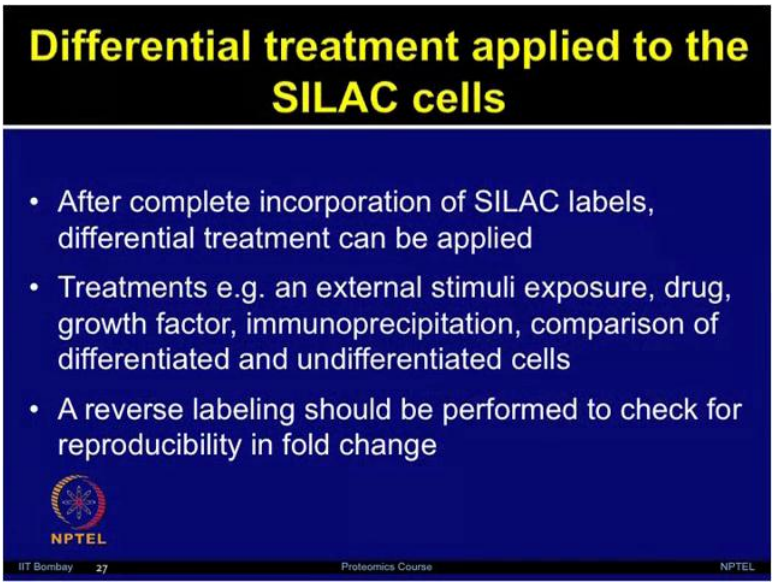

So, once the SILAC labels are incorporated in the cells in the cell culture then differential treatment can be applied. This treatment could be the external stimuli exposure drug treatment, growth factors, immunoprecipitation, the comparison of differentiated and undifferentiated cells or it could be some other treatment. While we are doing this labeling it is very good idea to replicate the experiment to repeat the experiment to ensure that the fold changes are uniform. And also a reverse labeling should be performed to check for the reproducibility because if reverse labeling is also showing similar trend then it means experiment is unbias due to any labeling issue. During SILAC experiments one also need to check for the arginine to proline conversion.

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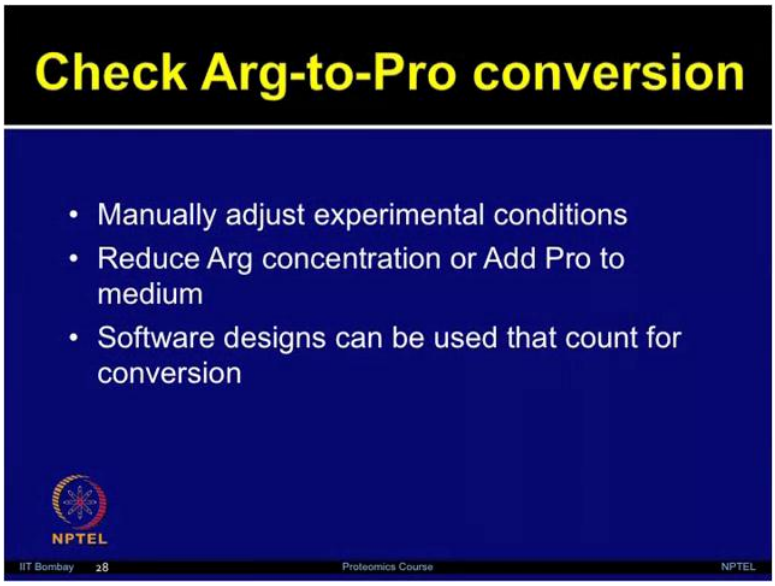

And this could be manually adjusted for the experimental conditions. One may need to reduce the the arginine concentration or add proline to the medium to overcome such issues. Now, there are some softwares which are designed which can be used for counting for these arginine to proline conversion and then experimental conditions can be adjusted accordingly. In the SILAC experimental about flow let us discuss fourth point cell lysis and protein estimation.

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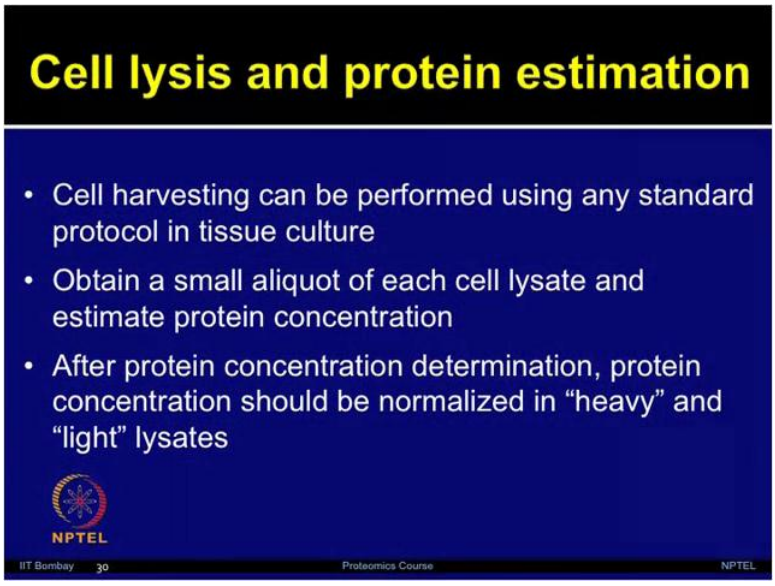

So, once the cells have incorporated the labels. Now, these cells can be harvested by

using any standard protocols which one uses in the tissue culture one need to obtain a small aliquot of each cell lysate and estimate protein quantification. We have talked various 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 the quantification later on. So, prior to mixing the both the cell cultures it is very important that we are starting with the equal protein amount in both light and heavy forms. So, this process can be done by normalizing by diluting the cell lysates with lysis buffer. In the SILAC experimental work flow the last and most important point is MS analysis and protein identification and quantification.

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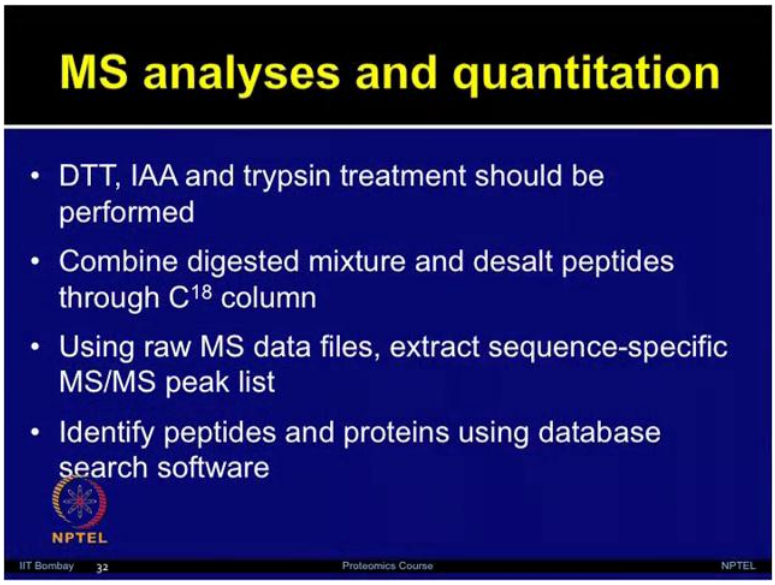

So, as we discussed earlier for prior to MS analysis one need to do dithiothreitol treatment to the disulphide bonds, iodoacetamide treatment for alkylation and trypsinization for protein digestion. These treatments should be performed and then the combine digested mixture and desalted peptides can be further used. The desalting can be performing by using C 18 columns. So, by using raw MS data files extract the sequence specific MS by MS peak list and then it can used for the identification of peptides and proteins using various database research.

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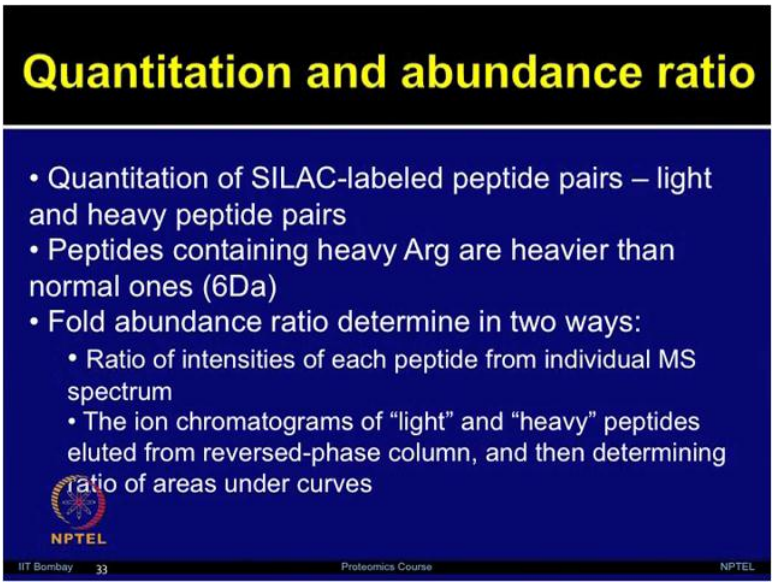

The quantification of SILAC labeled peptide pairs which are light and the heavy peptide pairs. So, these peptides containing for example, heavy arginine are heavier than 6 dalton of the normal once. So, these four abundance ratios can be determine in different methods. Here the two base are suggested to calculate the ratio of the intensities of each peptide from individual MS spectrum harm the ion chromatograms of light and heavy peptides eluted from the reversed phase columns and then it can be used to determine the ratio of areas curves.

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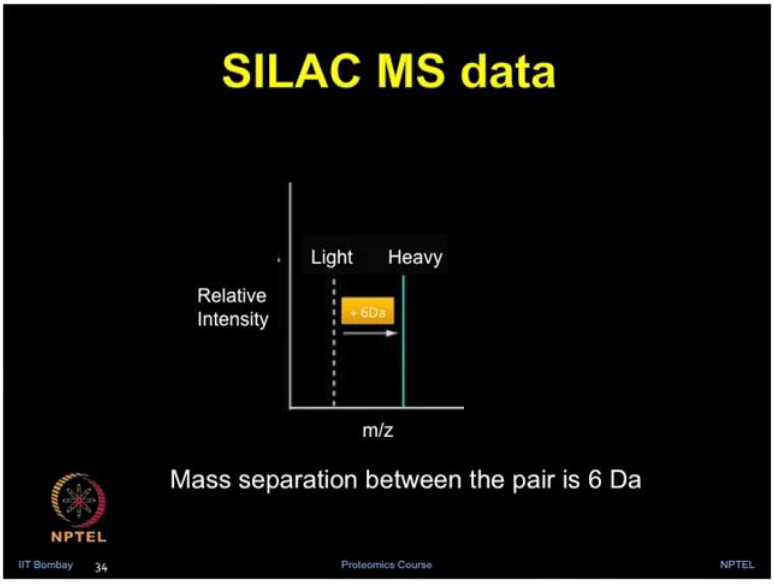

So, in this MS spectrum the pairs of chemically identical peptides of different stable isotopes can be differentiated due to their mass difference. The ratio of peak intensities for such peptide pairs, demonstrate the population ratio for two proteins. So, here as shown the light and heavy form separation with the six dalton difference. So, having discussed the work flow for performing a SILAC experiments let us have an over view of the protocol.

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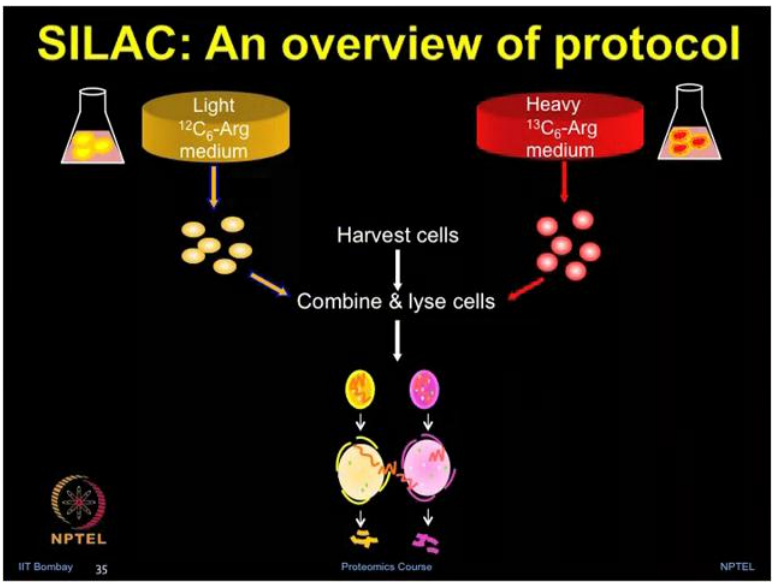

So, in SILAC two different cell populations can be grown in DMEM media containing 13 C 6 stable labeled form of arginine in place of normal or light arginine. After allowing for 5 or 6 doublings in each protein the arginine is heavy form now, these cells can be combined and further lysed prior to further proteolytic steps.

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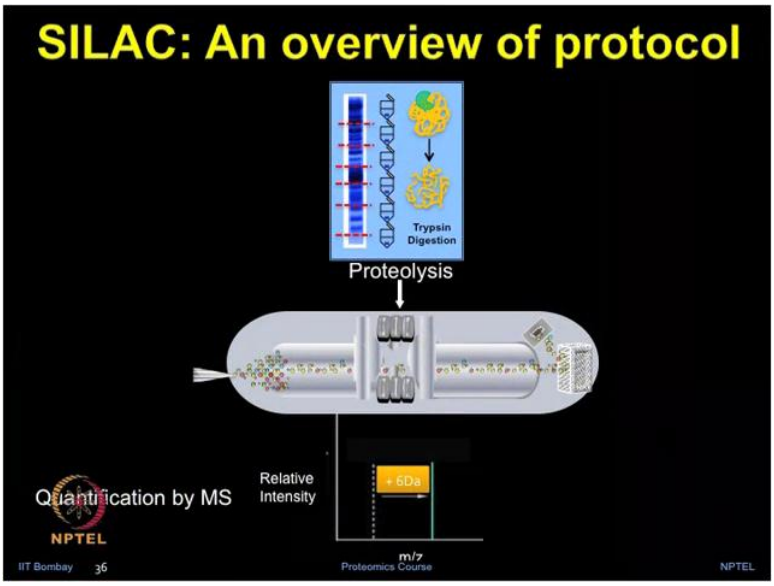

So, continuing into the protocol once we have combined these heavy and light populations then this can be separated on the SDS page gel and each of these bands can further be digested by adding trypsin. So, after digestion of isolated proteins to peptides by trypsin the arginine containing peptides will be 6 dalton heavier than their light counter parts which can be analyzed by using mass spec.

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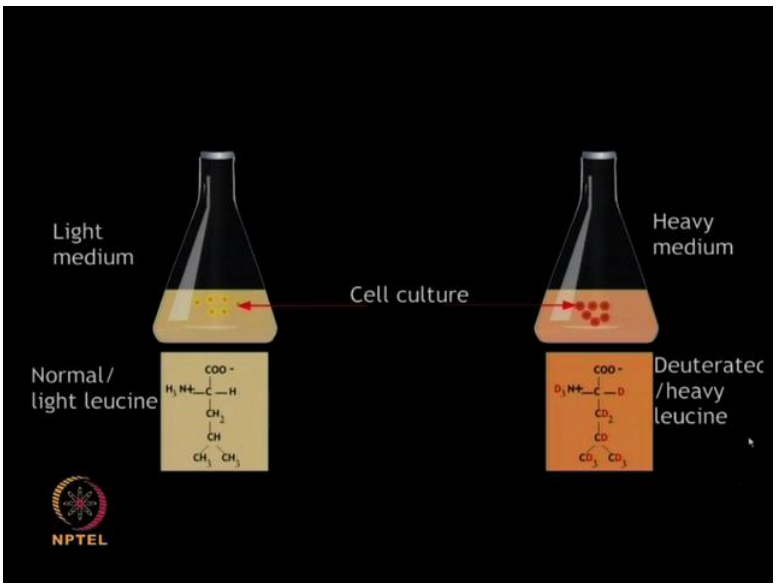

So, now I will show you an animation of a stable isotope labeling by amino acids in cell culture or SILAC. So, let us discuss about SILAC method. SILAC is a simple method for

in vivo incorporation of a label into proteins for the quantitative proteomics purposes. In SILAC method two group of cells are cultured in media that are identical in all aspects except that one contains a heavy medium, a heavy isotopic analog of an essential amino acid while the other contains the normal or light amino acid.

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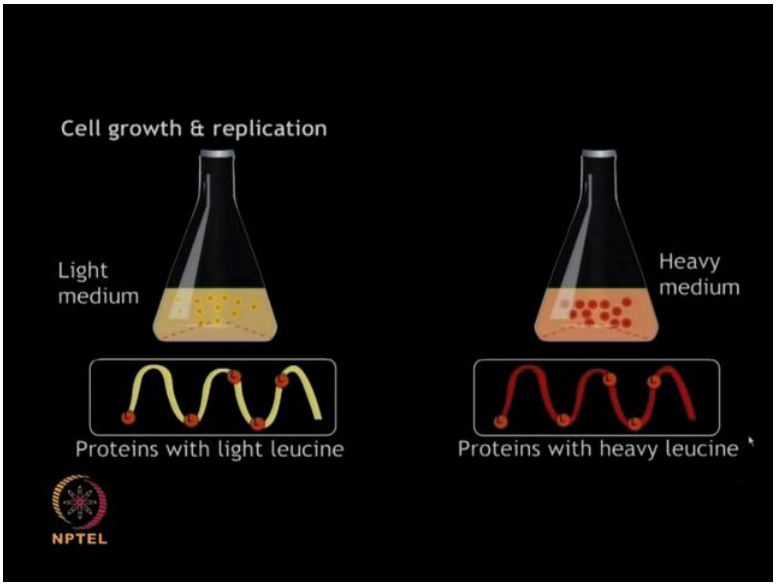

The essential amino acids which are obtain from the cell culture medium are incorporated into the corresponding newly synthesize proteins during growth and replication.

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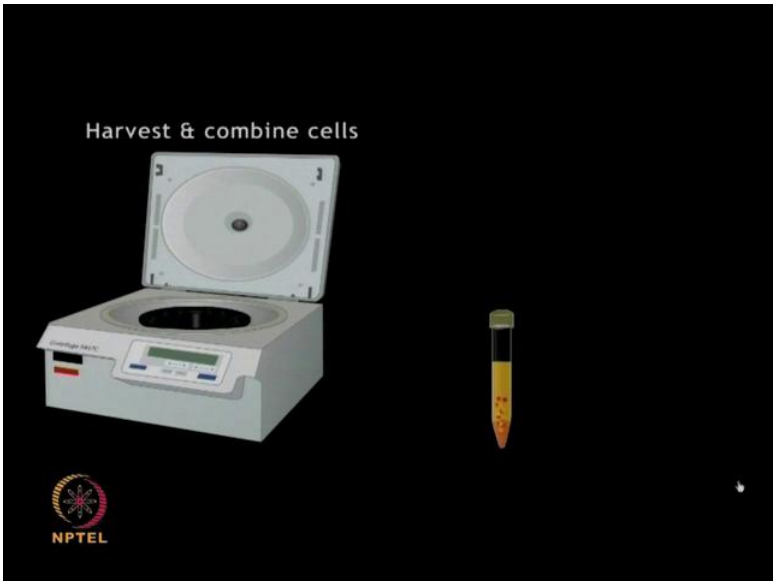

The medium containing the heavy amino acids will give rise to heavy isotropic proteins. After a number of cell divisions all the instances of particular amino acids will be replaced by the isotropic analog. The grown cells can be combined together and harvested; centrifugation of the mixture will result in pelleting of cells which can then be use for further analysis.

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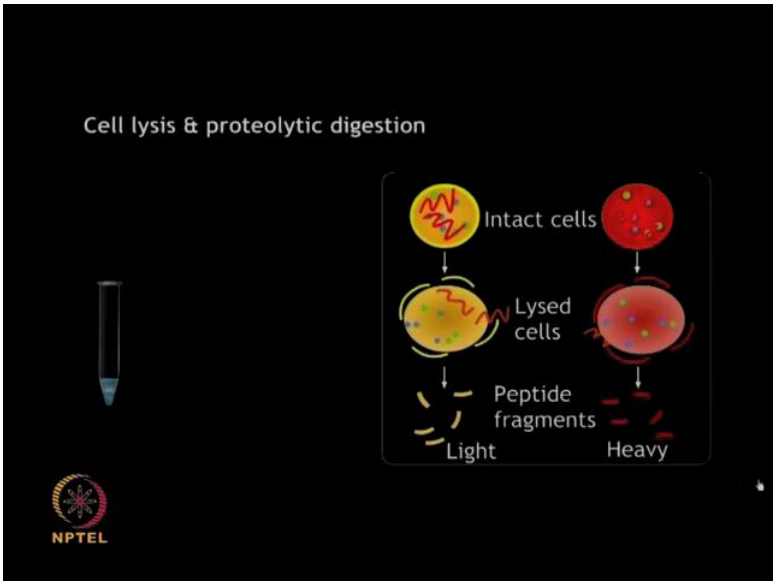

The grown cells are then lysed using a suitable lysis buffer and proteins are cleaved by using a proteolytic enzyme such as trypsin. This results in a mixture of light and heavy peptide fragments which can be quantified further by using mass spec.

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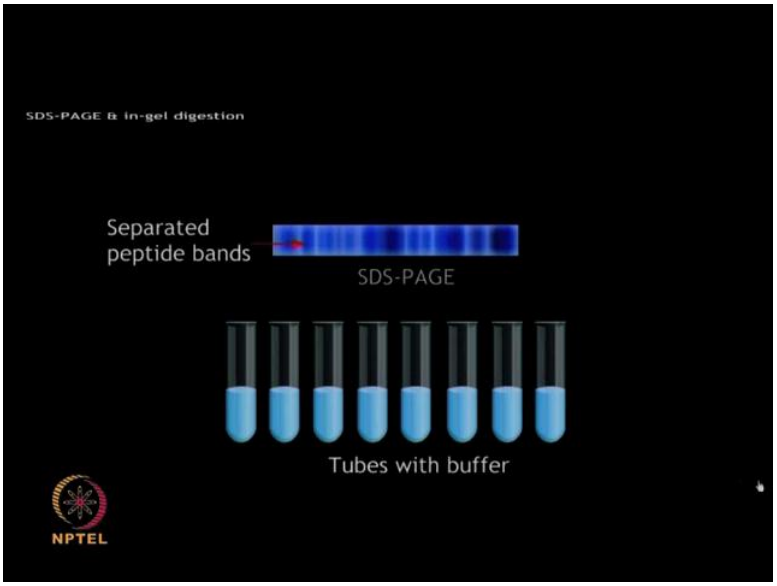

The complex mixture of peptide fragments is further separated by SDS page to simplify this analysis. Each band of the SDS page gel is cut out and redissolved in a suitable buffer solution. These simplified peptide fragments can then further be used for mass spec analysis.

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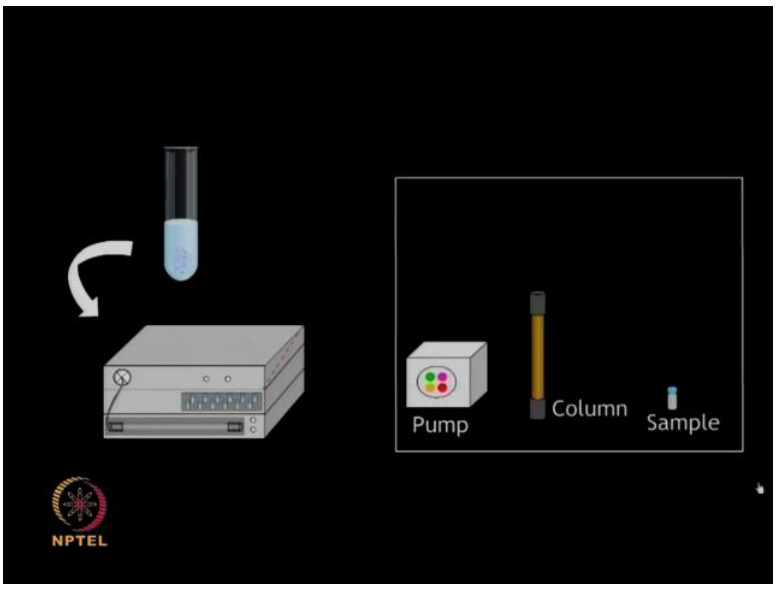

Further purification can be carried out by liquid chromatography. Where in the sample is passed through the column containing a pack stationary phase matrix that selectively adsorbs only certain analyte molecules. The reverse phase and strong cation exchange

chromatography are the most commonly used methods these eluted fractions are further characterized by MS.

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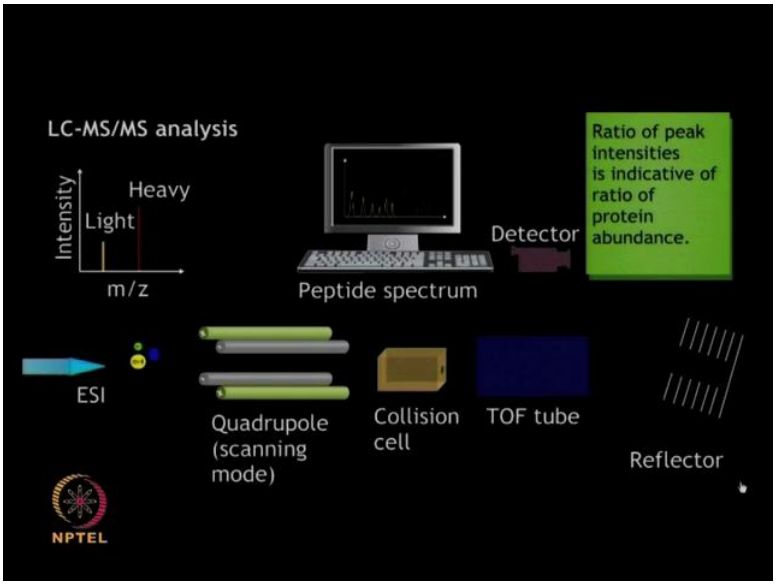

The purified peptide fragments are analyzed by MS/MS peptides containing the heavy amino acid show higher m by z as compare to the corresponding peptide fragments. The pairs of identical peptides can be differentiated due to the mass difference and ratio of peak intensities can be correlated to the corresponding protein abundance.

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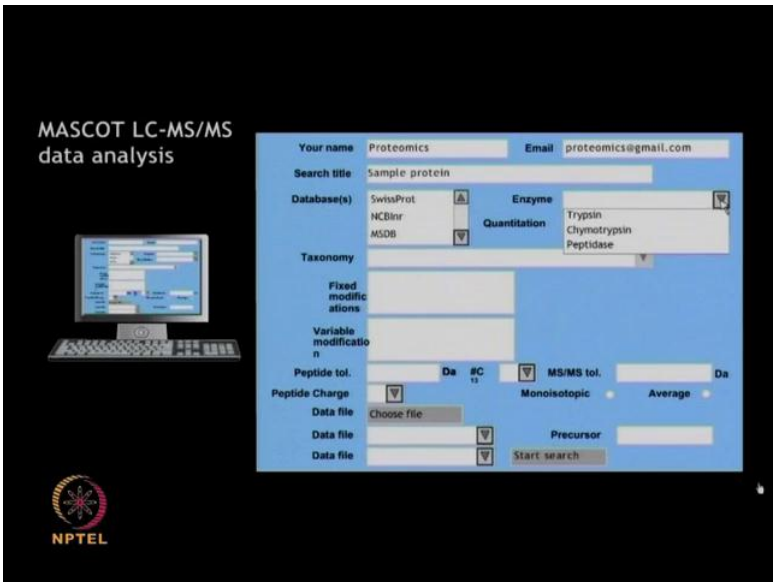

The data obtained from tend a mass spectrometry following SILAC experiment can be

analyzed by means of mascot search engines. The MS/MS data analysis share, where requires input from the user regarding the experimental parameters used such as enzyme cleavage, protein name, modifications instrument used, peptide charge etc and the desired such criteria like taxonomy, peptide tolerance, etcetera. The commonly used protein databases against which the MS information is processed to retrieve sequence data include NCBI, MSTB and sis prot. The data file generated from MS is uploaded and the search is carried out.

So, often discussing the SILAC technique and watching this animation of this entire process. Now, let us discuss about advantages and disadvantages of SILAC method and compare it with some other tagging methods so, as you have discussed and realized that SILAC method is very simple and robust and it labels the entire proteome without chemical derivatization and less sample handling steps. The label samples are mixed at the stage of whole cells which make SILAC approach ideally suited for the quantitative proteomics experiment because there will be less handling variation manual i t facts for each population separately studies such as the sub proteome analysis. For example, the cellular organelles or complex purification protocols, this can also be performed with very good quantitative accuracy by using these methods.

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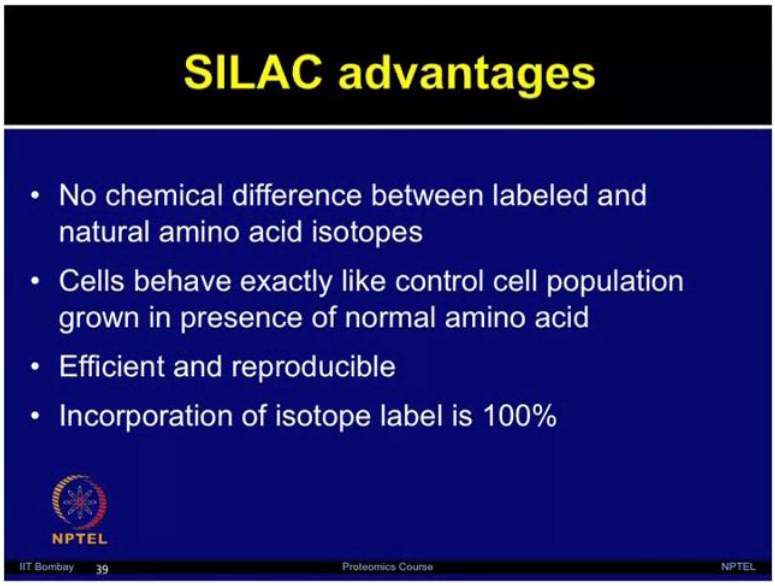

So, let us discuss about some of the advantages of using SILAC method. In SILAC no chemical differences observed between the labeled and natural amino acid isotopes. The

cells are grown in the tissue culture medium and they behave exactly like the control cell population grown in presence of the normal amino acids.

So, this method is very efficient and very reproducible. It has been observed that the isotope labels are hundred percent incorporated and one need to ensure that by doing the doubling for different generation. And after 5 or 6 generation it has been observed that it is hundred percent incorporated. So, samples can be mixed prior to the processing before doing the mass spec analysis.

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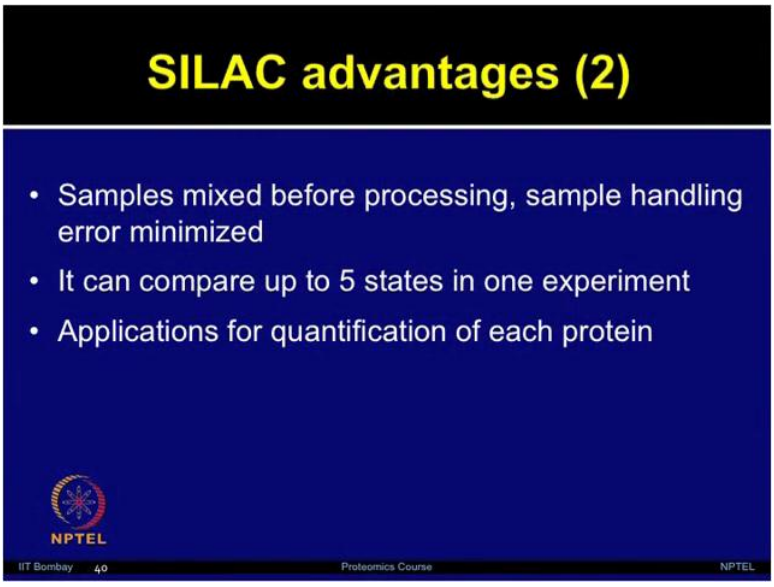

So, this method eliminates some of the handling errors. Now, let us SILAC experiments have demonstrated that it can provide the five flexing capability to compare five different states in one experiment. So, there are various applications of quantification of proteins by using SILAC method which we will discuss in next few slides. All though there are many advantages of using SILAC, but there are some disadvantages due to the inherent nature of this method. SILAC is applicable for only culture cells it cannot be use for tissue or body fluids.

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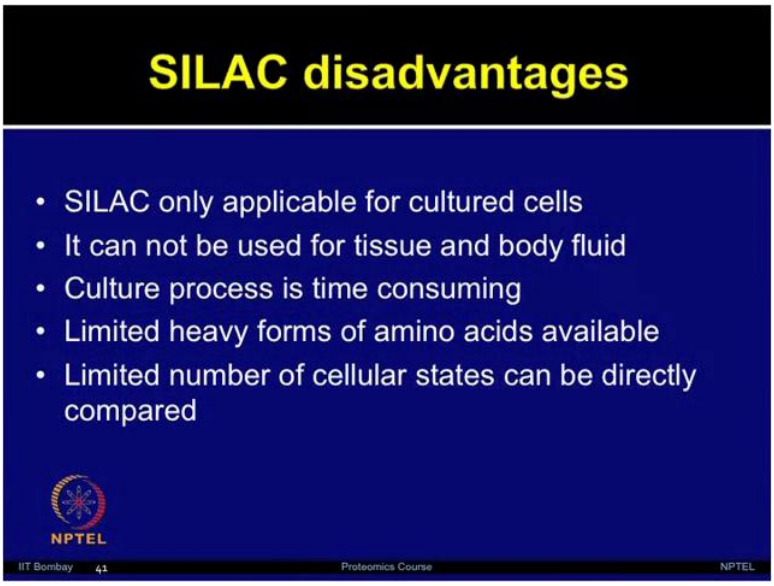

So, that is one of the major limitations of this method. The tissue culture process is always very time consuming and it requires very meticulous and efficient work. Then there are few forms of available heavy isotopes, the heavy forms amino acids which are available, these are commercially available, but there are very limited forms of these heavy amino acids due to this, only limited states can be compared by using SILAC. The metabolic conversion of arginine to proline this one of the commonly observed issue in the SILAC experiments which results in the triptych peptides containing heavy prolines.

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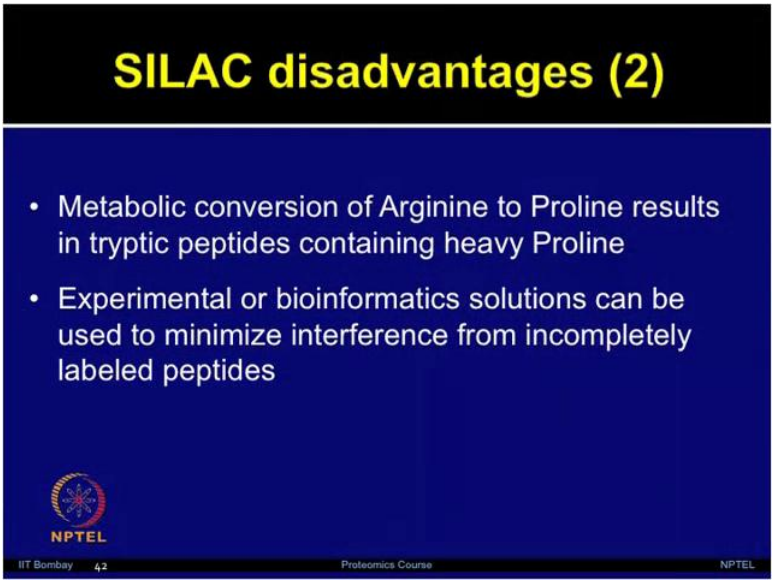

Now, there are various experimental or informatics solutions in place which can be used to reduce the interference from in completely labeled peptides.

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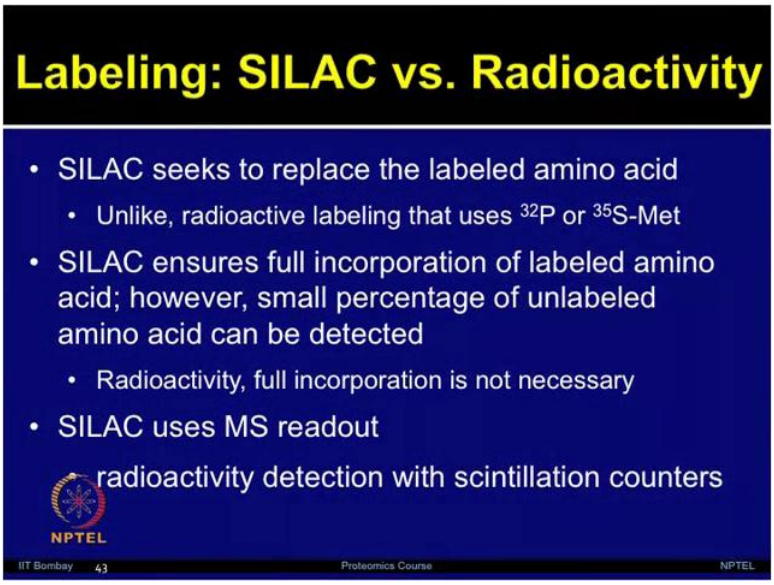

So, we will discuss about how SILAC compares with the radio activity labeling methods the SILAC method six to replace the labeled amino acid which is unlike the radioactive labeling; which uses 32P or 35S methionine. The SILAC method ensures that labeled amino acids are fully incorporated in the cells.

However, the small percentage of unlabeled amino acids can be deducted. In the radio activity base labeling the full incorporation is not necessary. In SILAC one can read out the signals by using mass spectrometers whereas the radio activity detection is possible by using scintillation counters or by registering the films for the signal intensity. Let us now, discuss few applications of SILAC briefly. The SILAC method is very promising for any cell line.

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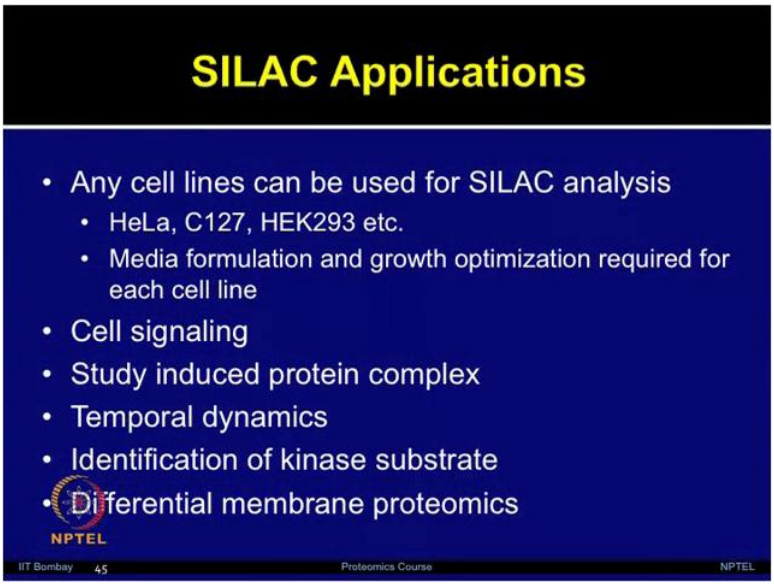

So, this method can be applied for any cell lines whether it is heLa, cell C127, HEK293 or different type of cell lines people have shown. However, the media formulation and the growth optimization is required individually for each cell line. SILAC applications have been demonstrated in different applications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

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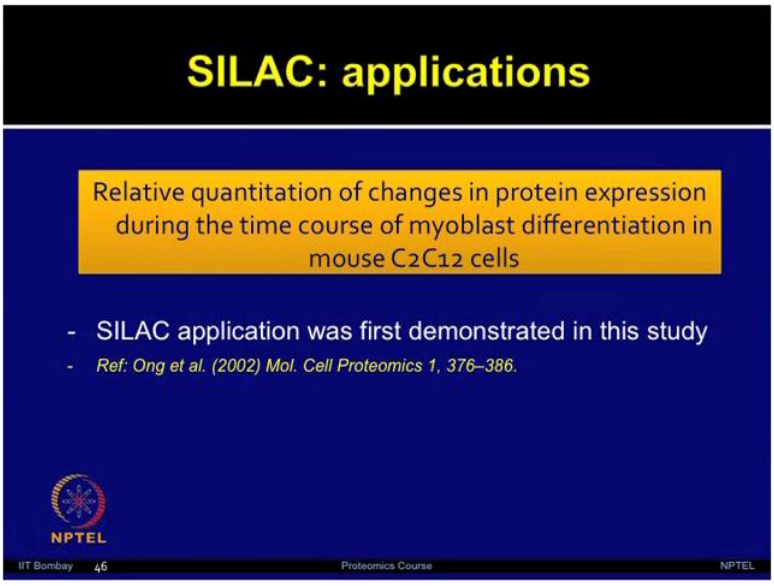

So, there are various applications. We will have a look on the some applications now. So,

Ong et al to al in 2002 publish a paper and molecular cell proteomics which was the first SILAC application demonstrated where they used the relative quantation of changes in protein expression during the time course of myoblast differentiation in mouse cells.

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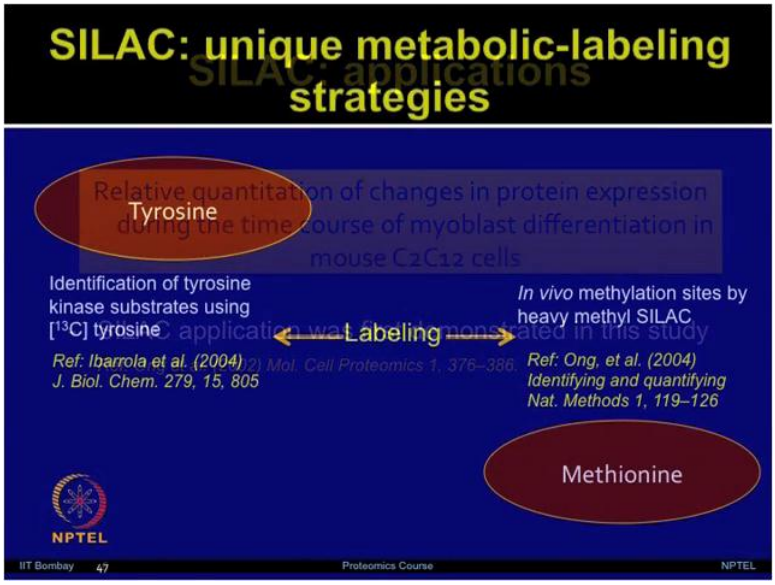

Researchers have reported various unique metabolic labeling strategies for example, by using tyrosine the identification of tyrosine in kinase substrate using ^{13}C tyrosine. Labeling is also performed by using methionine, the in vivo methylation sites by heavy methyl SILAC.

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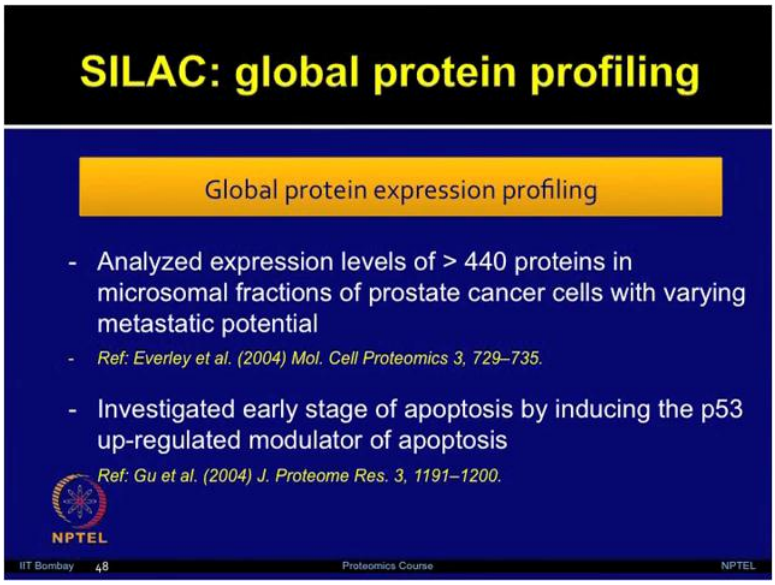

The numerous studies based on the global protein expression profiling using SILAC method. I am just highlighting some of the very earlier studies which set up the path for performing these protein expression profiling. So, study by Everley et al in 2004 analyzed the expression level of more than four hundred forty proteins in the microsomal fractions of prostate cancer cells with varying metastatic potential. Another study by Gu at al investigated the early stage of apoptosis by inducing p fifty three up regulated modulator of apoptosis. SILAC has also been used for functional assays to study the protein protein interaction As. study by blagoev et al used the differential labeling of proteins in EGF stimulated versus unstimulated cells.

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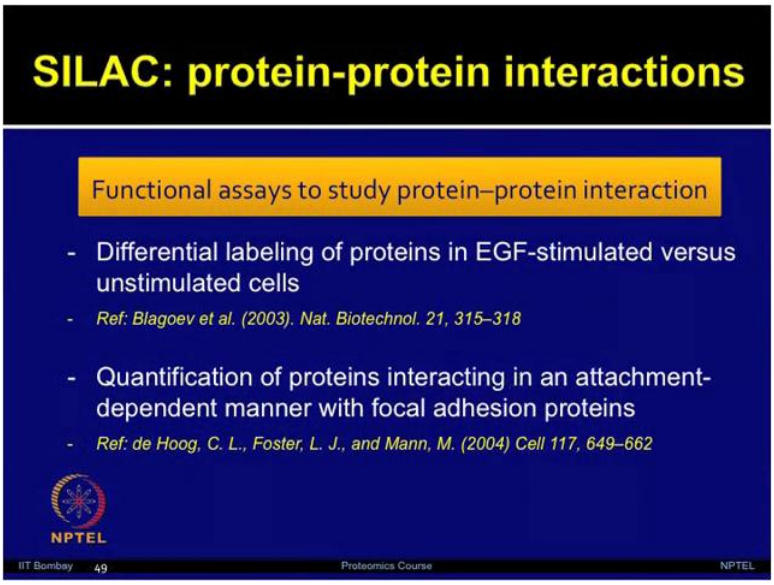

A study by de Hoog, et al did quantification of proteins interacting in an attachment dependent manner with focal adhesion proteins. These are the just few examples of studying the functional assays and performing protein interactions using SILAC the identification of proteins, which are enriched in specific cellular structures.

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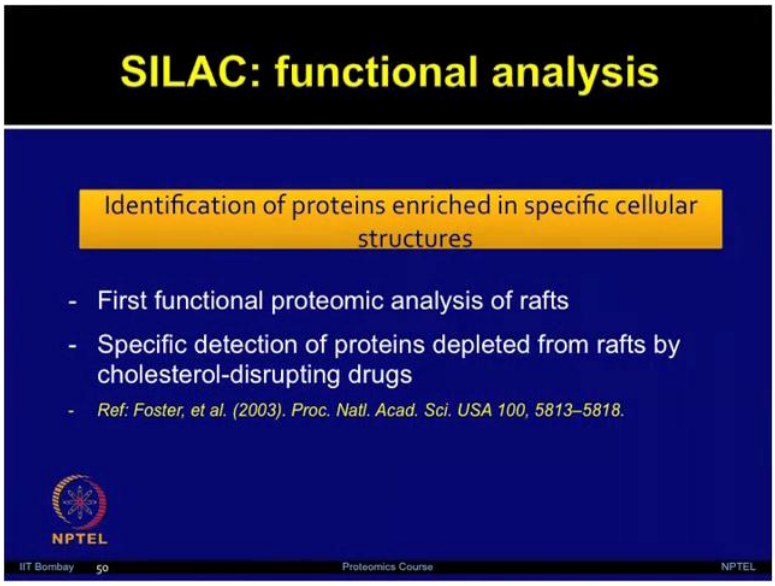

A study by Foster et al used the first functional proteome analysis of rafts. And they showed the specific detection of proteins depleted from the rafts by cholesterol disrupting drugs.

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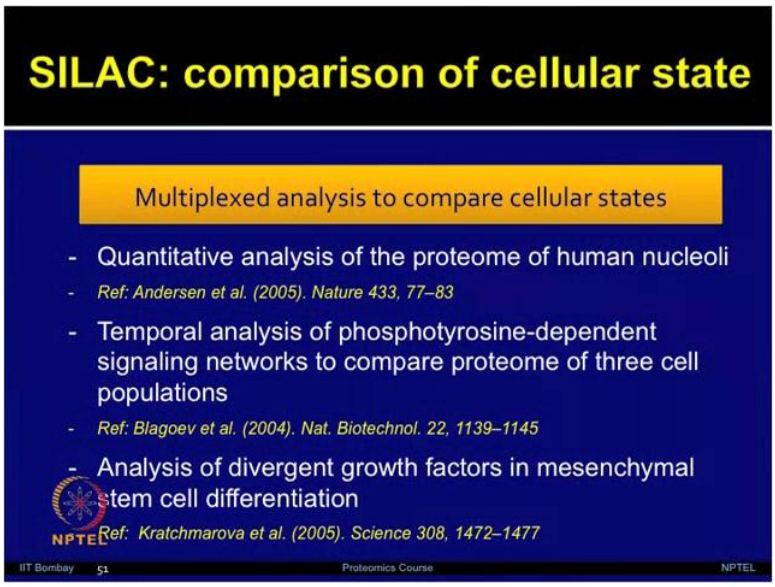

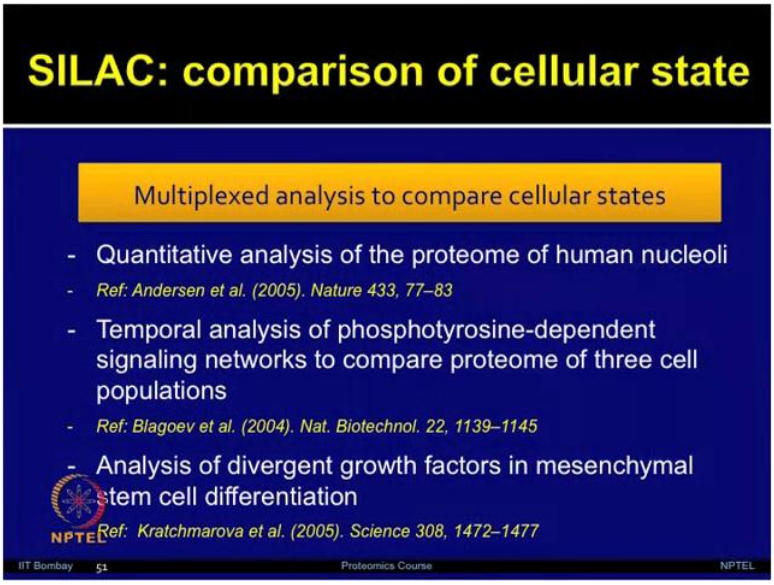

SILAC has been widely used for multiplex analysis to compare the cellular states. Andersen et al showed the quantitative analysis of proteome of human nucleoli. Blagoev et al performed a temporal analysis of the phosphotyrosine dependent signaling networks to compare the proteome of three cell populations. Kratchmarova et al analyzed the divergent growth factors in mesenchymal system cell differentiation. These are just few examples of multiplex analysis.

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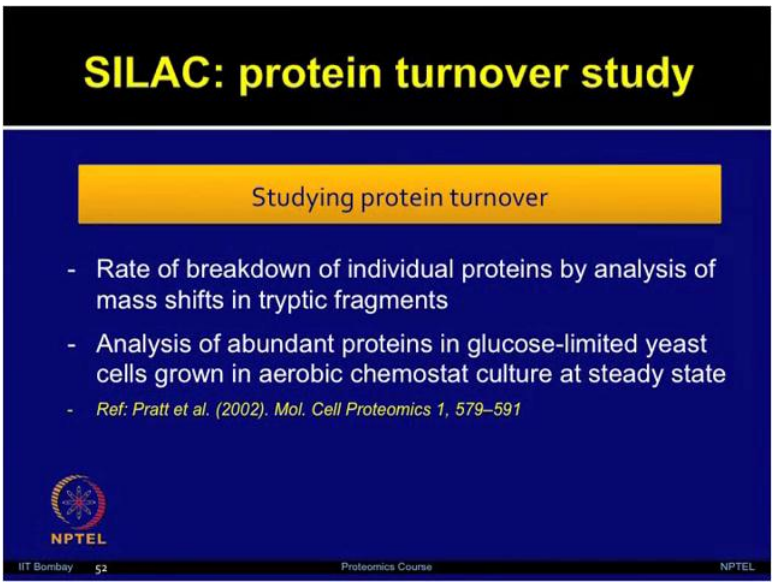

Now, if you look into literature there are many studies which have used the SILAC method of comparison of cellular states. SILAC method has also been use to study the protein turnover. Study by Pratt et al use the rate of breakdown of the individual proteins

by analysis of the mass shift in tryptic peptide fragments. The analysis of the abundant proteins in glucose limited yeast cells which were grown in aerobic chemostat cultures at steady state was performed by using SILAC method. SILAC has been used for identification and quantitation of protein post translation modifications. Study by Ibarrola et al identified and quantitated phosphorylation sites.

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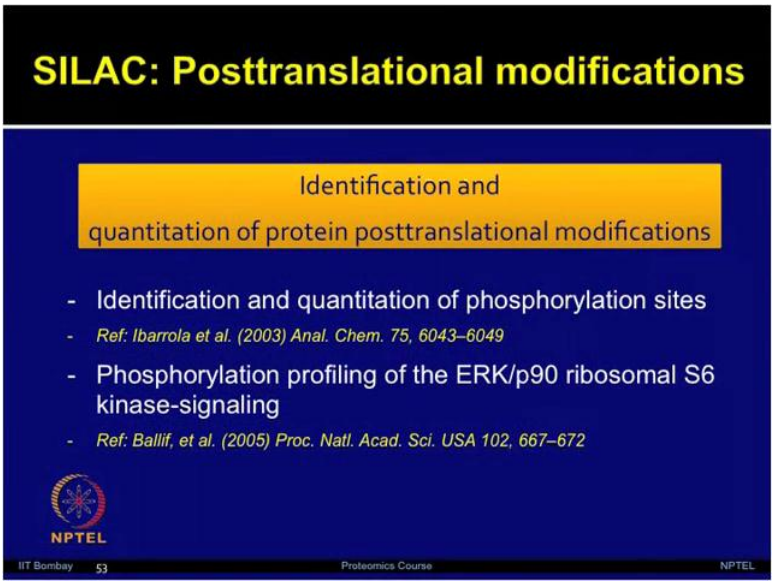

Another study by Ballif et al also identified quantitated the phosphorylation sites. So, there are many studies which have used SILAC method for studying post translational modification.

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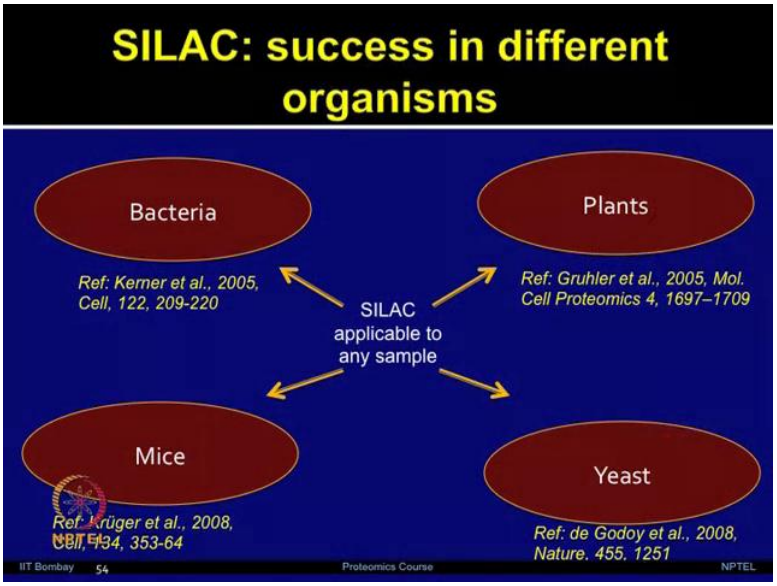

So, interestingly now SILAC method has been used in different organism. In bacteria in yeast these were the more commonly used SILAC methods due to the growth in the cell culture, but there are some studies on arabidopsis in the plants as well as in the mice which has shown that SILAC can be applied to the wide variety of the organisms.

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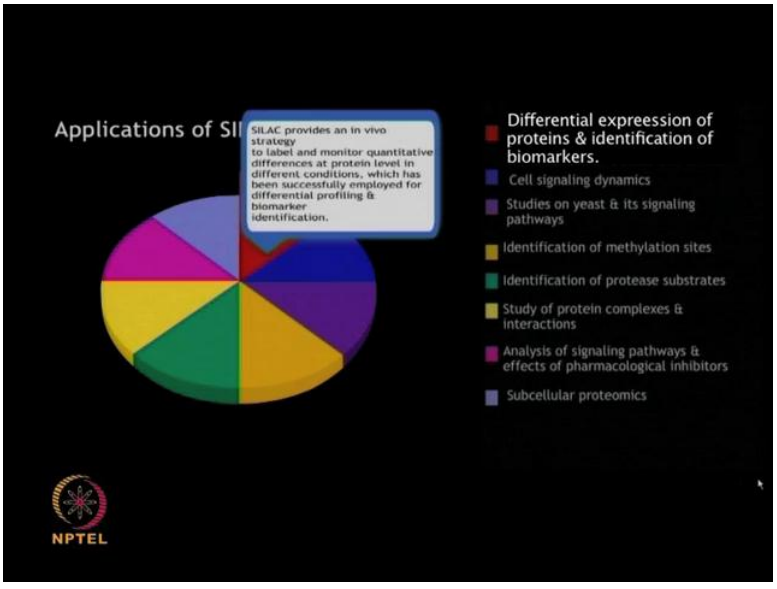

So, SILAC applications are straight forward and it only requires some initial attention to the cell culture conditions. Let us discuss about the SILAC applications in an animation. Let us now discuss the applications of SILAC. SILAC is a very useful quantitative

approach which has found application for several proteomic studies. SILAC provides an in vivo strategy to label and monitor the quantitative differences at protein level in various conditions which has been successfully employed for differential profiling and biomarker identification.

The temporal dynamics of cell signaling pathways which transmit the information through various post translational modifications, most commonly the reversible phosphorylation have been efficiently studied by using SILAC method coupled with mass spec. The quantitative proteomic studies using SILAC have been carried out with the yeast; which is a model system for eukaryotic cells to provide insight into various biological processes.

Methylation, which is one of the very common p t m's having various biological significant roles is successfully studied using isotopically labeled methionine residues. One of the recent applications of SILAC include the identification of protease substrates using differentially labeled bacterial cell cultures. The cellular functions are mediated by several protein complexes which interact with one another. SILAC has been applied for the quantitative determination of such complexes and their interacting protein partners.

The signaling pathways which involve kinases employed in cell growth and differentiation play a major role in cancer development and progression. These pathways and effects of various inhibitors have been successfully studies by using SILAC. SILAC allows for the labeling and monitoring of dynamically changing proteome of sub cellular organelles; which are involved in various activities during apoptosis in cells.

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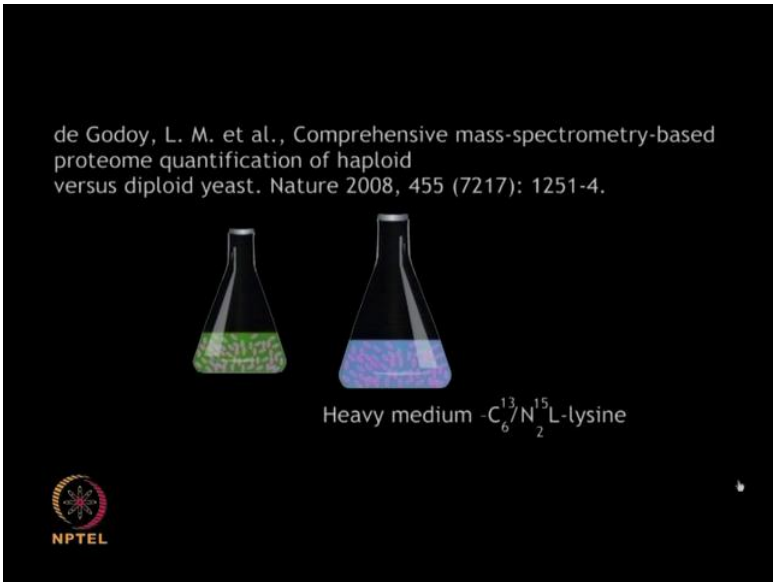

Now, let us take the case study from Godoy and colleagues which determine the fold change of a peptide pairs between haploid and diploid yeast cells. The study was published in nature 2008. A comprehensive mass spectrometry based proteome quantification of haploid versus diploid yeast.

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The labeled lysine residues where use to grow the diploid yeast cells while the haploid cells were grown in normal lysine medium. The cultures were mixed proteins extracted and analyzed by LC MS/MS.

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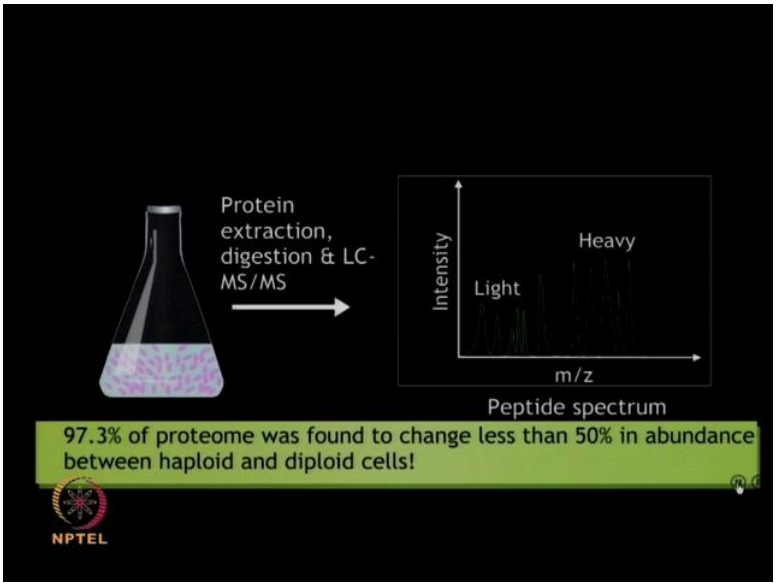

The protein ratio between the haploid and diploid cells, were determined with very high accuracy. The comparison reveled that ninety seven point three percent of the proteome changes less than fifty percent in abundance between haploid and diploid yeast cells. So, after discussing about different type of applications of SILAC method now let us summaries what we have studied in this lecture, so in a SILAC in a typical experiment the control and treated cell.

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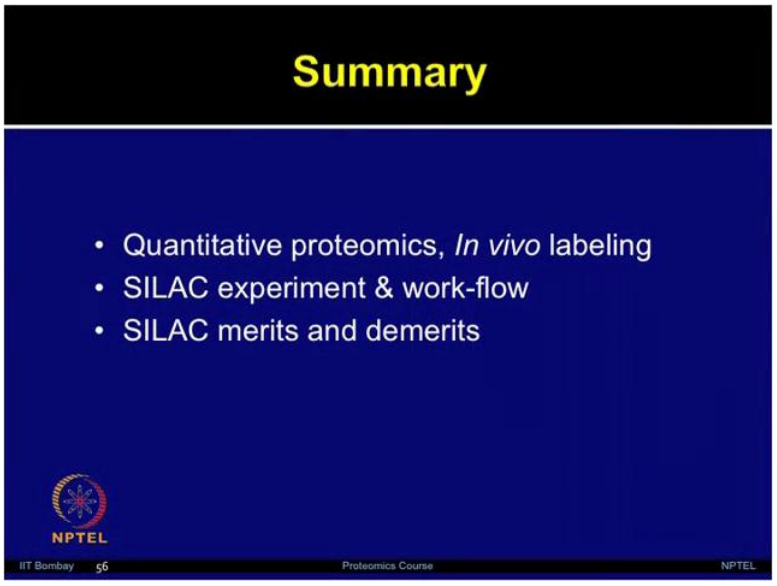

The cell lines are grown in different media; one was enriched with the lite form and other with the heavy isotope containing amino acids. The peptides from each heavy analyte forms are mixed in one to one ratio and proteins were extracted purified and digested. The peptides were analyzed by LC MS/MS method the signal intensity of labeled and unlabeled peptides, was able to provide the quantitative information.

So, the limitations include its limitation of using only the cell culture and the metabolic conversion of argentine to proline, but overall SILAC is very promising technique and its applications are published in several papers. I hope by today's lecture you are able to appreciate different type of In vivo labeling methods. The SILAC experiment and the stepwise workflow of performing a SILAC experiment the merits and demerits of SILAC method thank you.