Mass Spectrometry Based Proteomics
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Mod 03 Lecture Number 13

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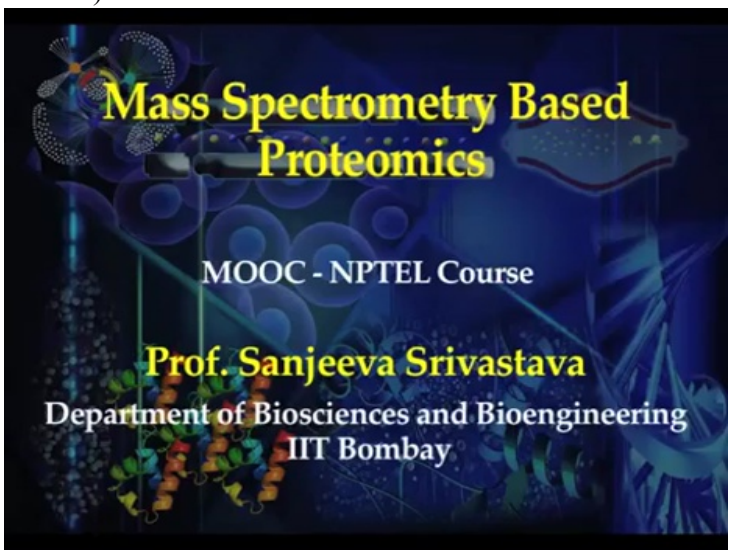

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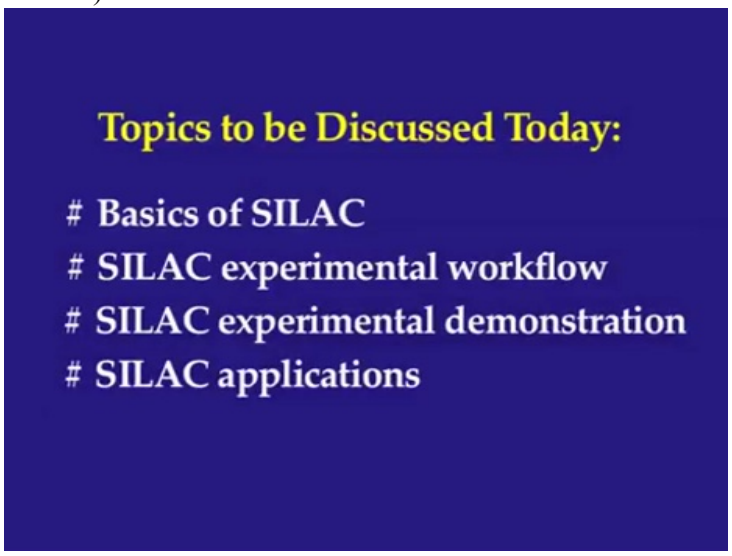

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In today's lecture, we will talk about …

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…quantitative proteomics, the Stable Isotope Labeling by Amino acids in Cell culture SILAC

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-markers. 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 advancements 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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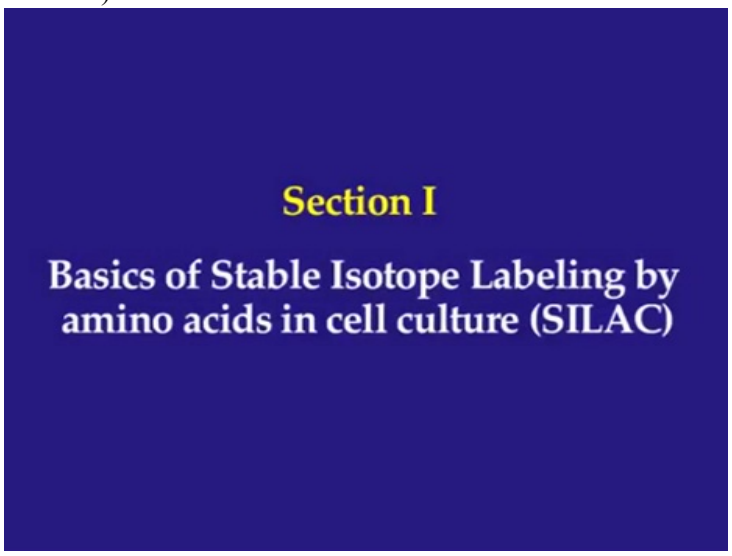

Now, let us discuss about Stable Isotope Labeling by Amino acid in Cell culture SILAC. So, the MS based quantitative proteomics is an increasingly popular approach to study changes in the protein abundance in various biological samples.

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 13 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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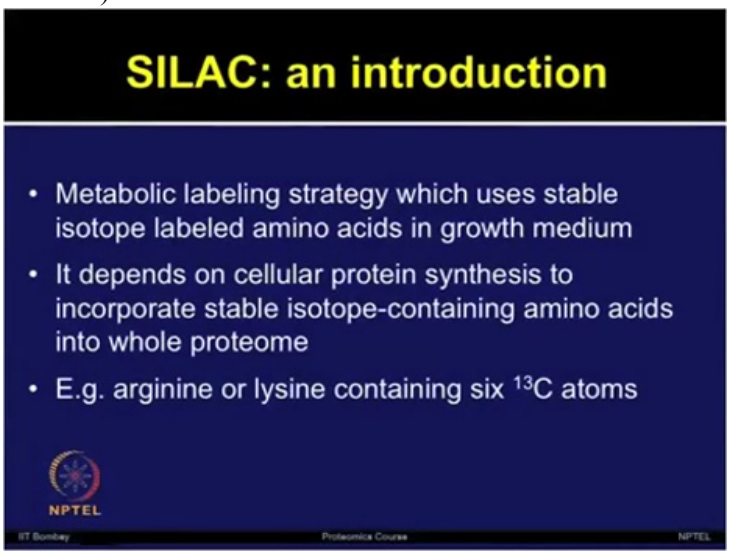

SILAC is a metabolic labeling strategy which uses the stable isotope labeled amino acids in the growth medium. This experiment depends on the cellular protein synthesis to incorporate the stable isotope containing amino acids into whole proteome. For example, arginine or lysine which contains six 13 Carbon atoms.

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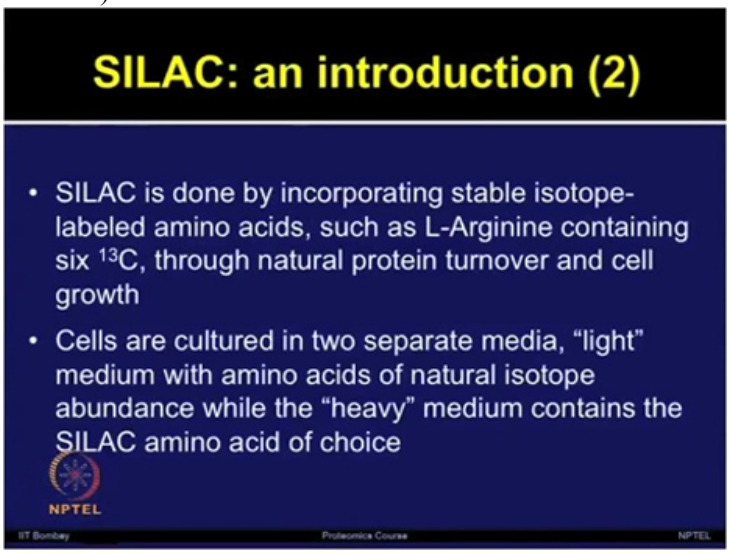

The SILAC experiments are performed by incorporating the stable, isotopically labeled amino acids such as l-arginine containing six 13 Carbon through the natural protein turnover and cell growth. The cells are cultured in two separate media for the light and the heavy forms.

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

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 Mass Spec can be used for determining the relative protein abundance by the intensity of light and the heavy peptides.

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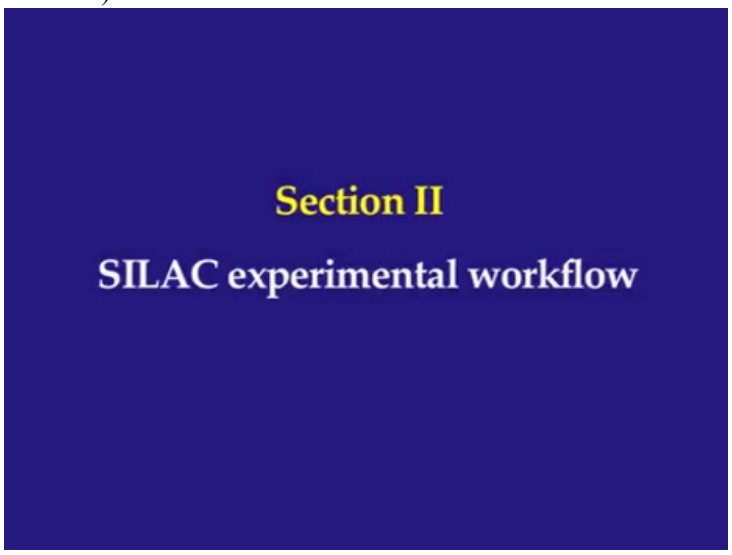

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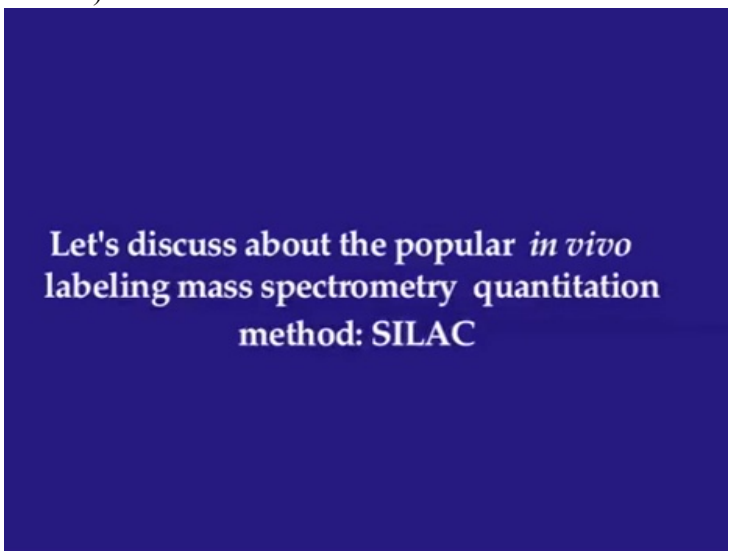

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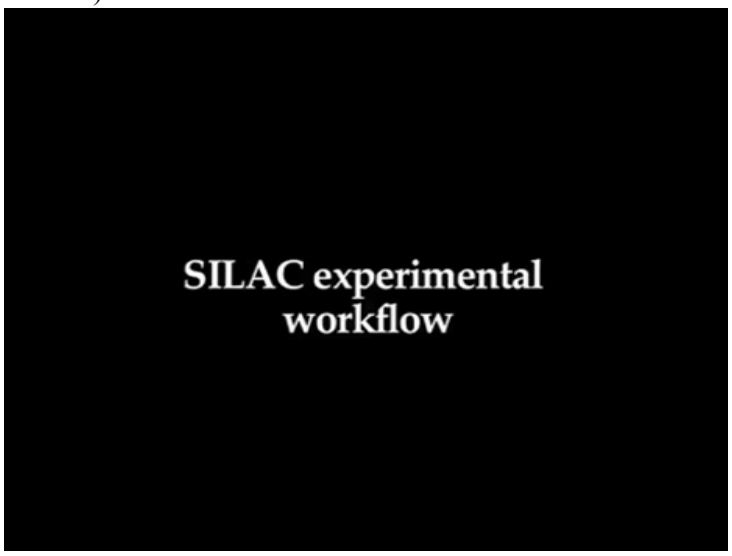

So, now we will discuss about SILAC experiment …

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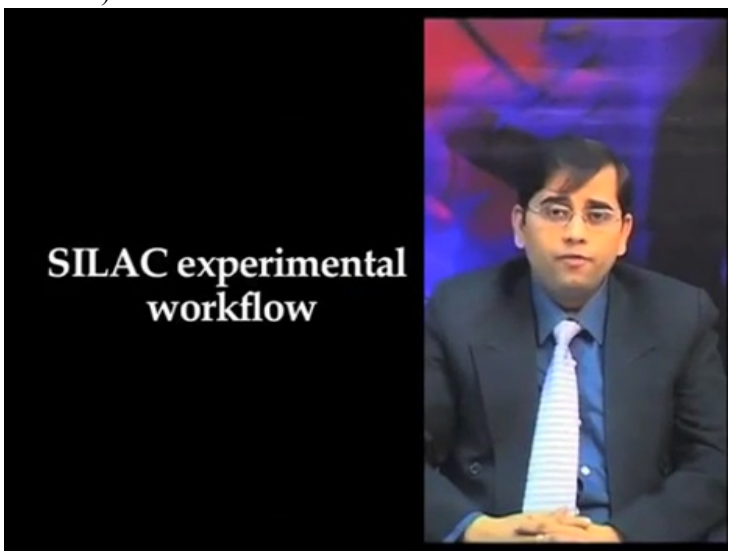

…and the workflow to perform a SILAC experiment So, in the workflow we will discuss the SILAC protocols and how to incorporate SILAC labels into any given experiment

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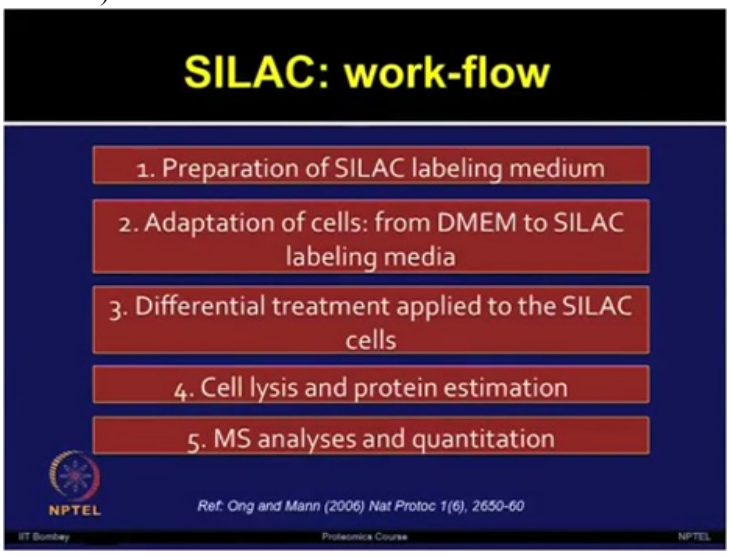

So, let us have a look on the workflow for performing a SILAC experiment. First step is preparation of SILAC labeling medium; second, adaptation of cells from the normal DMEM medium to SILAC labeling media; third, the differential treatment application to the SILAC cells; fourth, cell lysis and protein estimation; fifth, MS analysis and quantification.

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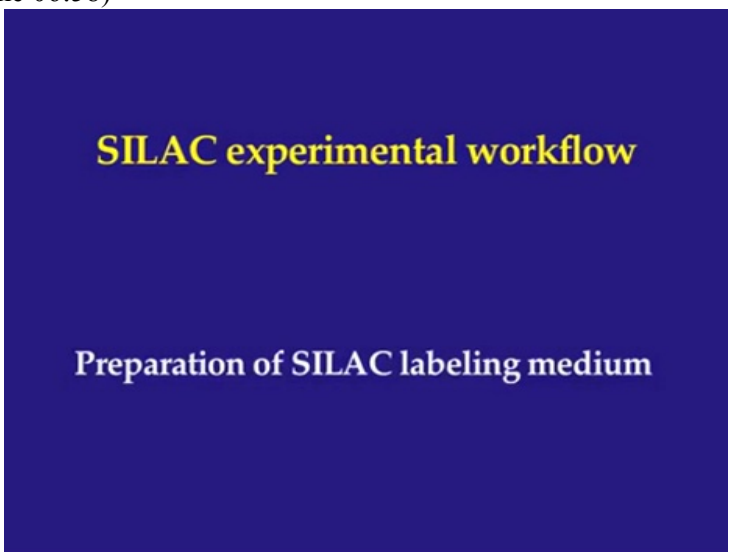

So, let us look at the work-flow stepwise. The first point…

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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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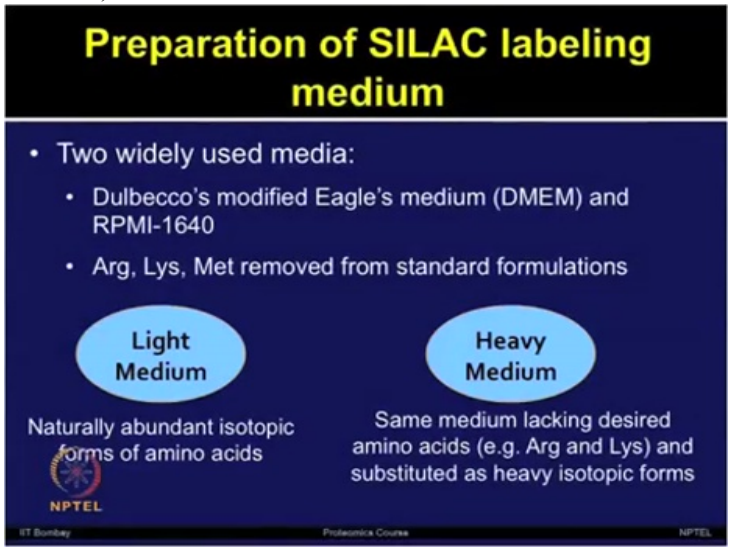

So, there're two widely used media. One is the Dulbecco's Modified Eagle's Medium DMEM and RPMI-1640. The arginine, lysine and methionine are removed from the standard formulations.

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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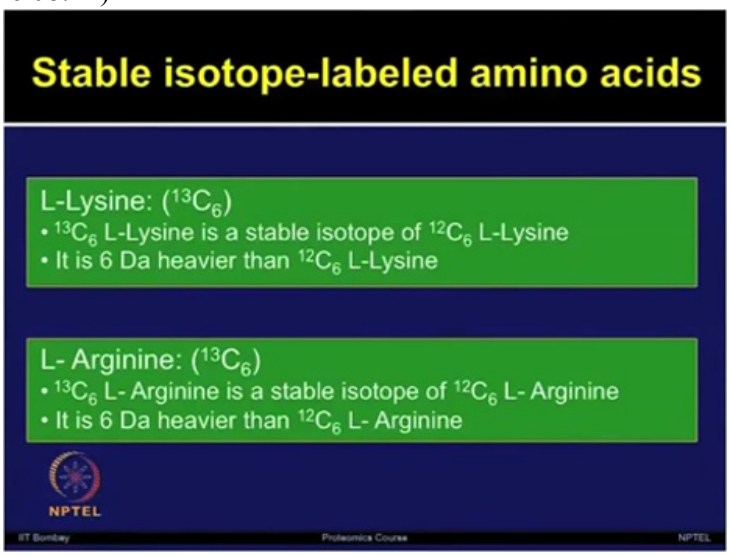

Here I am providing you one example of stable isotope labeled amino acids. 13 Carbon 6 L-lysine is a stable isotope of 12 carbon 6 L-lysine. It gives 6 Dalton difference in the mass spec as compared to the 12 carbon 6 L-lysine.

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 12 carbon 6 L-arginine.

So, for a preparation of SILAC labeling media, one need to add various supplements such as serum, antibiotics.

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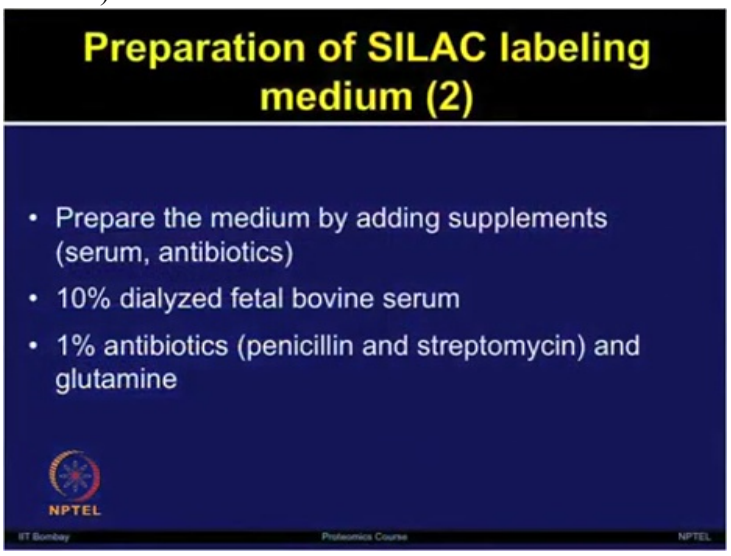

So, a recommended percentage for the serum could be 10% dialyzed fetal bovine serum and 1% of antibiotic and glutamine, but these percentages can be optimized depending upon type of cell cultures.

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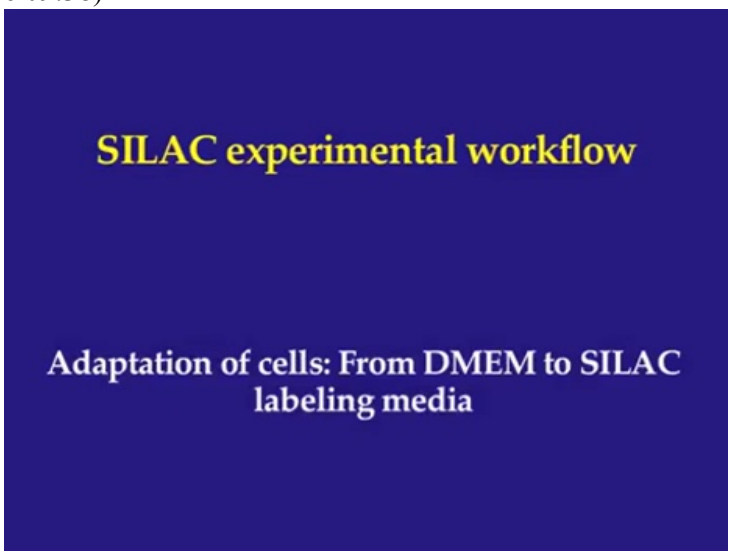

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In the SILAC experimental workflow lets discuss about second point, the adaptation of cells from the DMEM media to the SILAC labeling media. These cells need to be adapted due to the slight differences in media formulations.

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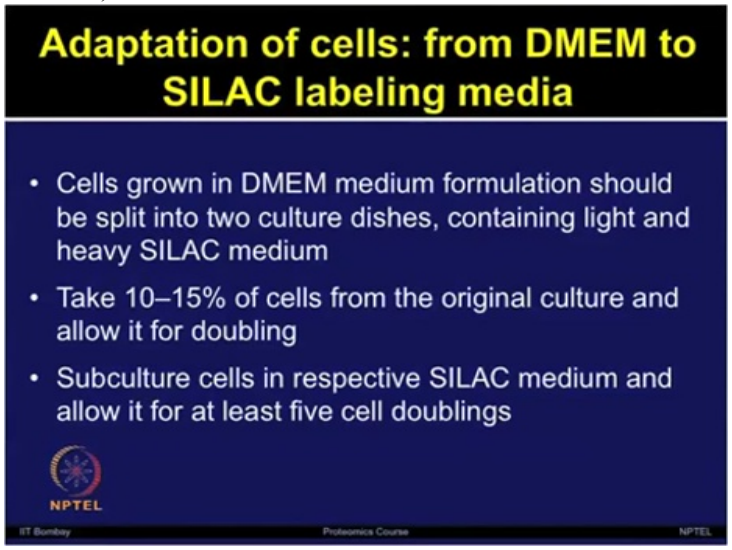

So, the cells are grown in DMEM medium it should be first split into two culture dishes containing light and heavy SILAC medium.

So, first take out 10 to 15% of cells from the original flask, the original culture and allow it for doubling. Then subculture cells in respective SILAC medium, the light and the heavy medium and allow it for at least 5 to 6 cell doublings.

I will discuss that, what is the significance of this doubling process in the next couple of slides

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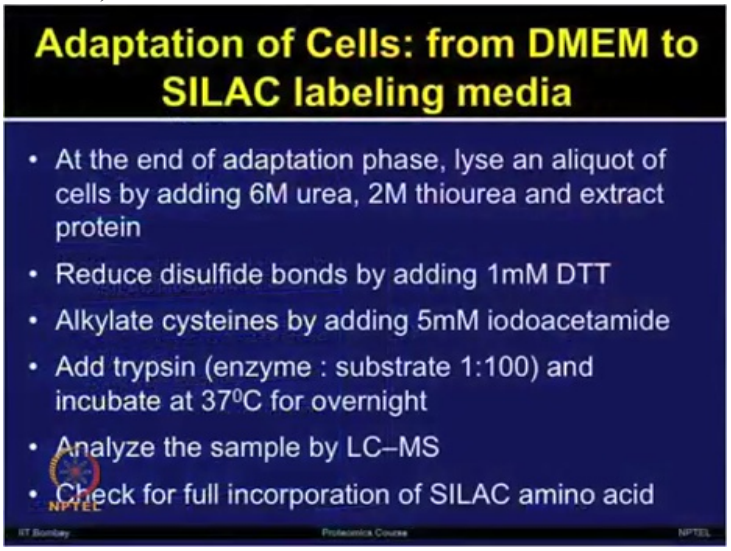

So, for the adaptation of cells from DMEM to SILAC labeling media, at the end of this adaptation phase lies the cultures by adding 6 molar urea, 2 molar thiourea and then extract the protein. Reduce disulphide bonds by adding one milli molar of dithiothreitol.

Then add 5 milli molar of iodoacetamide to alkylate cysteine residues. Add trypsin overnight for digestion with the enzyme and substrate ratio for 1 to 100.

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.

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 5 or 6 doublings to be fully labeled.

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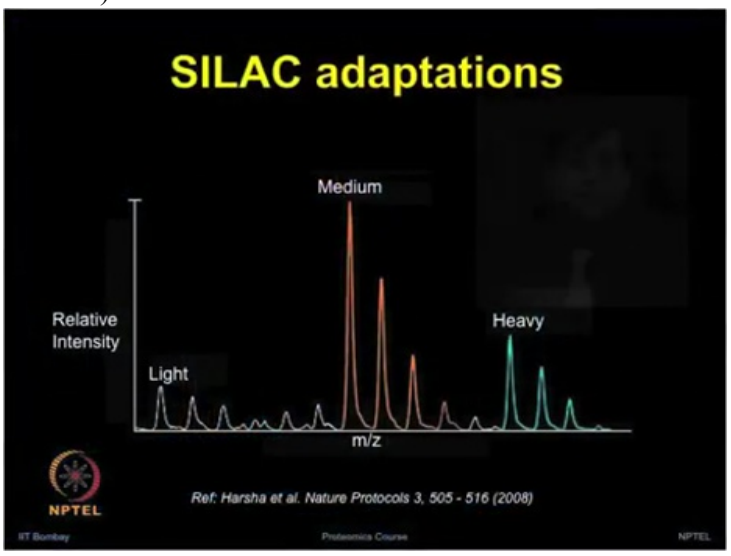

The digested protein samples can then further be analyzed by mass spec. These are representative spectra showing m by z ratio and the relative intensity of light, medium and heavy forms

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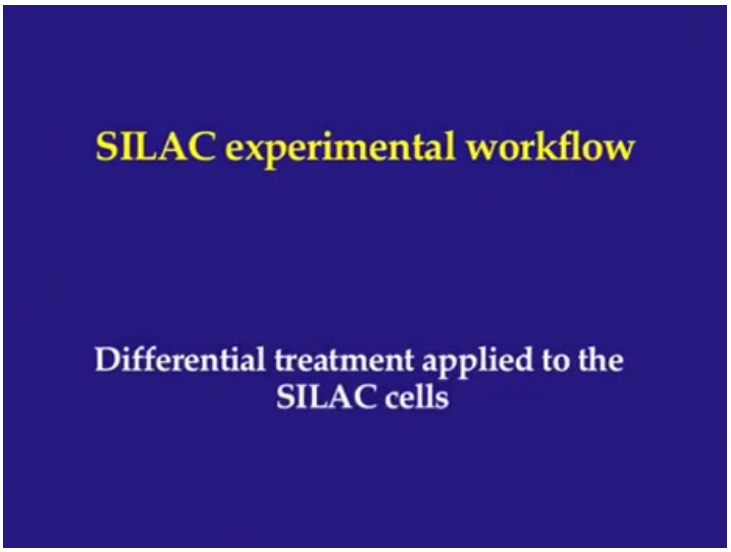

In SILAC experimental work-flow, let us discuss the third point…

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…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.

So, once the SILAC labels are incorporated in the cells in the cell culture, then differential treatment can be applied.

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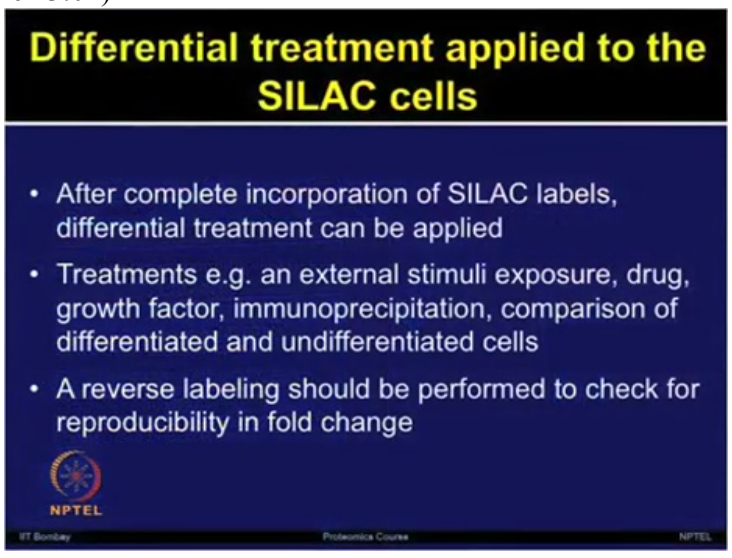

These treatment could be the external stimuli exposure, drug treatment, growth factors, immuno-precipitation, the comparison of differentiated and undifferentiated cells or it could be some other treatment.

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 unbiased due to any labeling issue.

During SILAC experiments, one also need to check for the arginine to proline conversion. And this could be manually adjusted …

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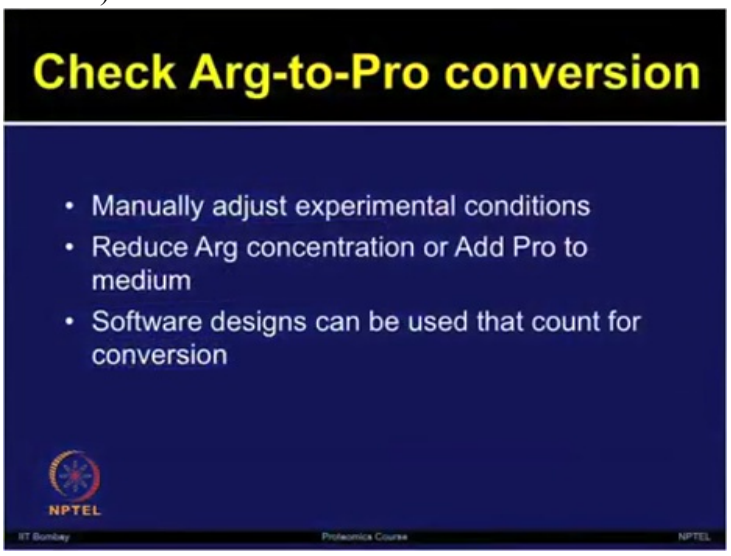

…for the experimental conditions

One may need to reduce 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.

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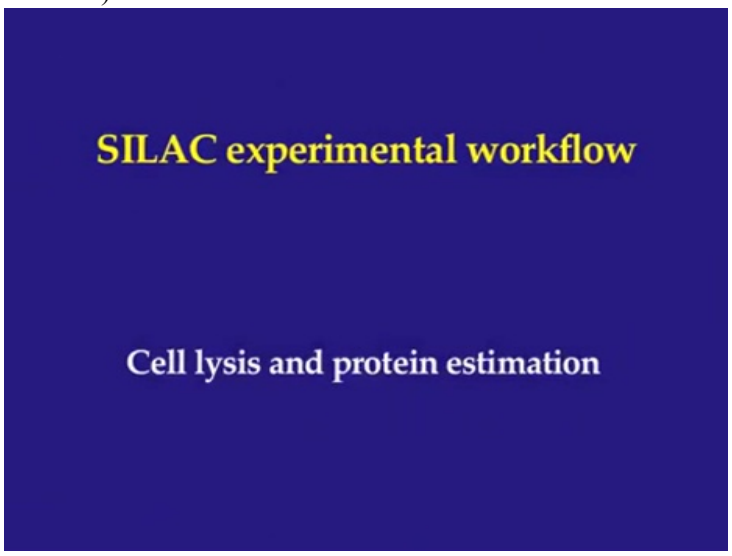

In the SILAC experimental workflow we will discuss fourth point, cell lysis and protein estimation. So, once the cells have incorporated the labels, now these cells can be harvested…

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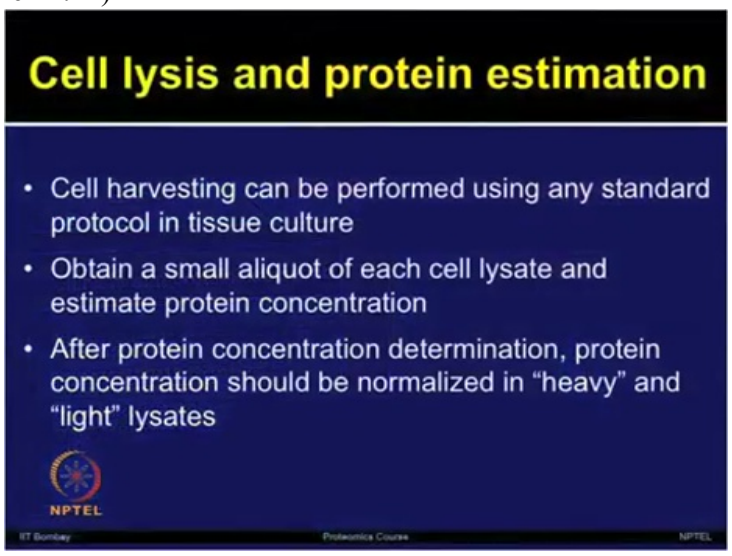

… by using any standard protocols which one uses in the tissue culture

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.

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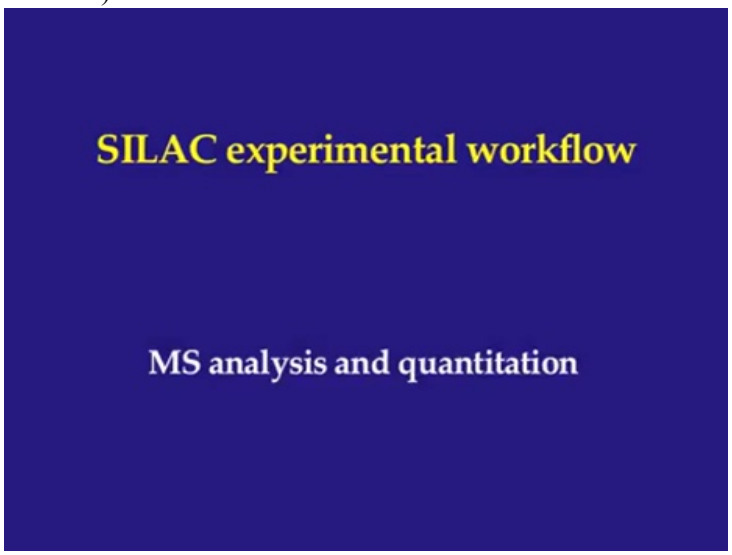

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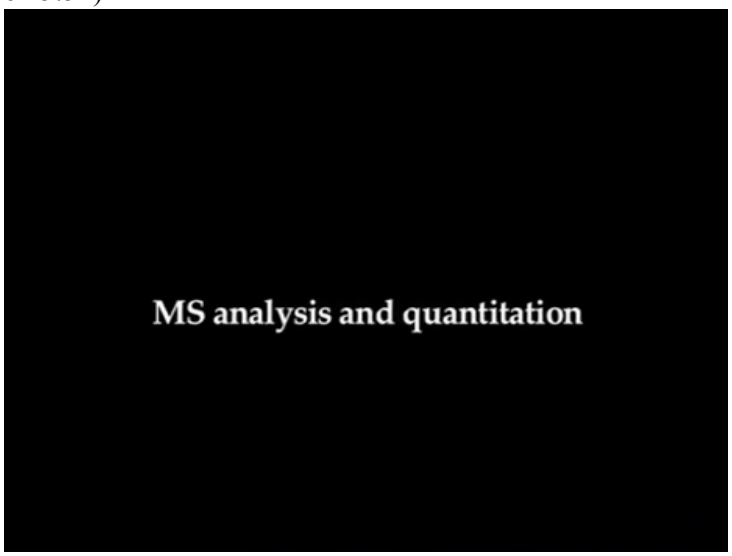

In the SILAC experimental workflow, the last and most important point is MS analysis and protein identification and quantification.

So, as we discussed earlier, for prior to MS analysis one need to do …

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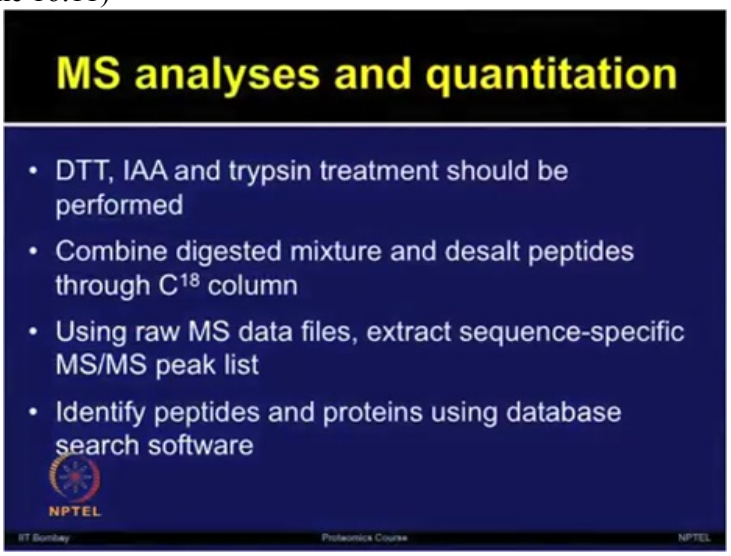

…. dithiothreitol treatment to cleave the disulphide bonds, iodoacetamide treatment for alkylation and trypsinization for protein digestion

These treatments should be performed and then the combined digested mixture and desalted peptides can be further used. The desalting can be 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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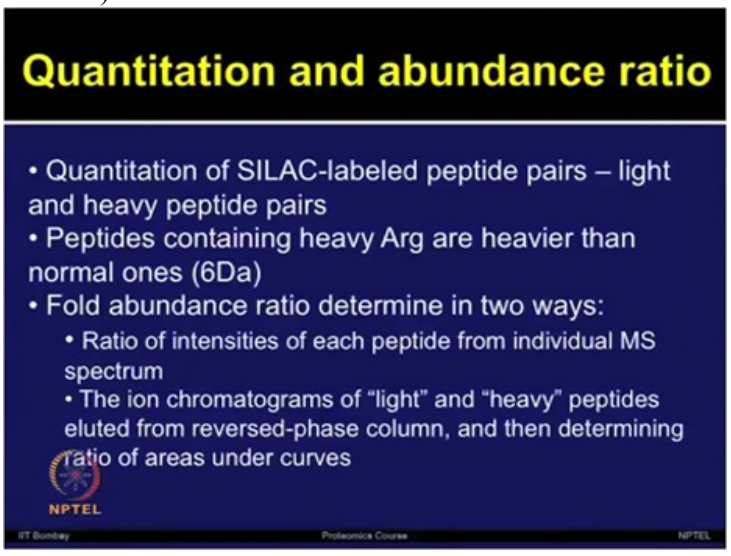

The quantification of SILAC-labeled peptide pairs which are light and the heavy peptide pairs; so, these peptides containing for example, heavy arginine are heavier than 6 Dalton of the normal ones. So, these 4 abundance ratios can be determine in different methods.

Here the two ways 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 the reversed phase columns and then it can be used to determine the ratio of areas under curves.

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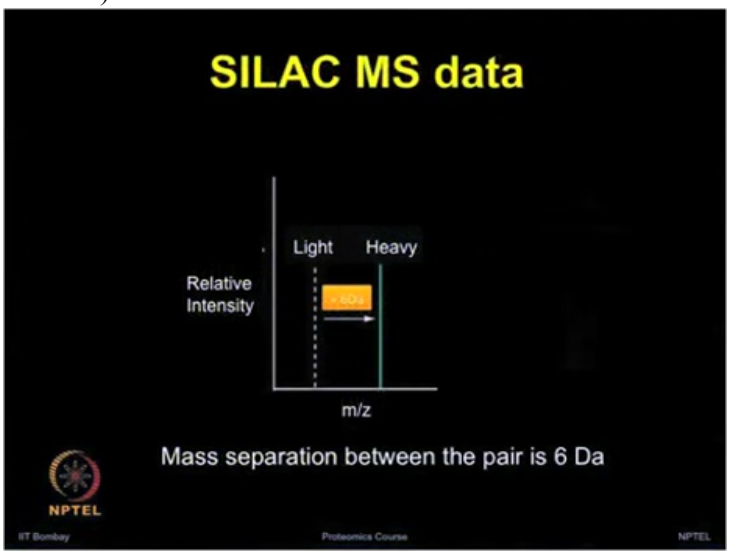

So in this MS spectra, the pairs of chemically identical peptides of different stable isotopes can be differentiated due to their mass difference. The ratio of peak intensities for such peptide pairs demonstrate the population ratio for 2 proteins. So, here I have shown the light and heavy form separation with the 6 Dalton difference.

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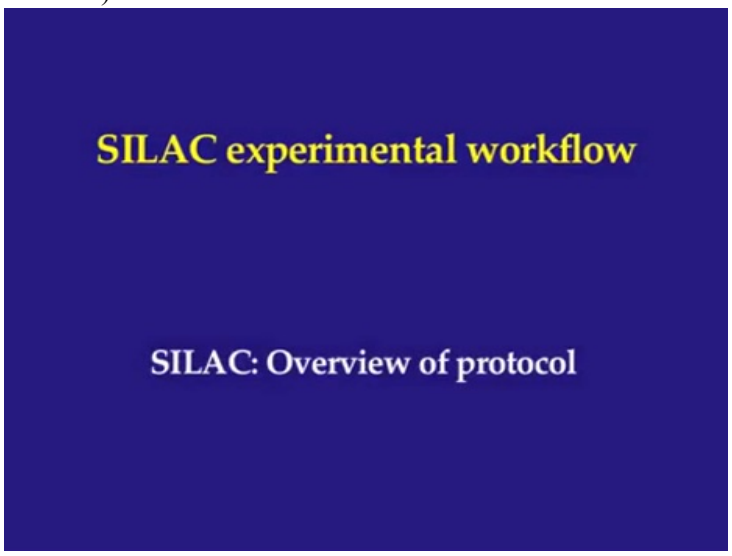

So, having discussed the work flow for performing a SILAC experiments let us have an overview of the protocol.

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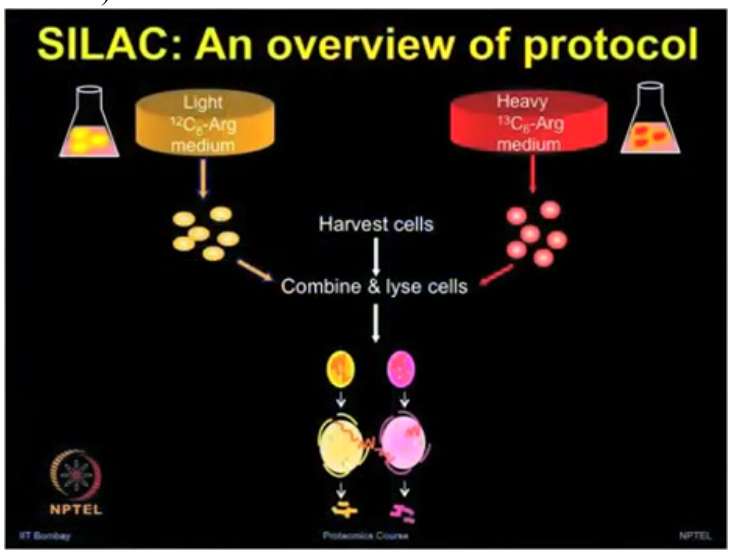

In SILAC, two different cell populations can be grown in DMEM media containing 13 C 6 stable labeled form of arginine in place of normal or light arginine.

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.

So, continuing into the protocol once we have combined these heavy and light populations then this can be separated on the SDS page gel…

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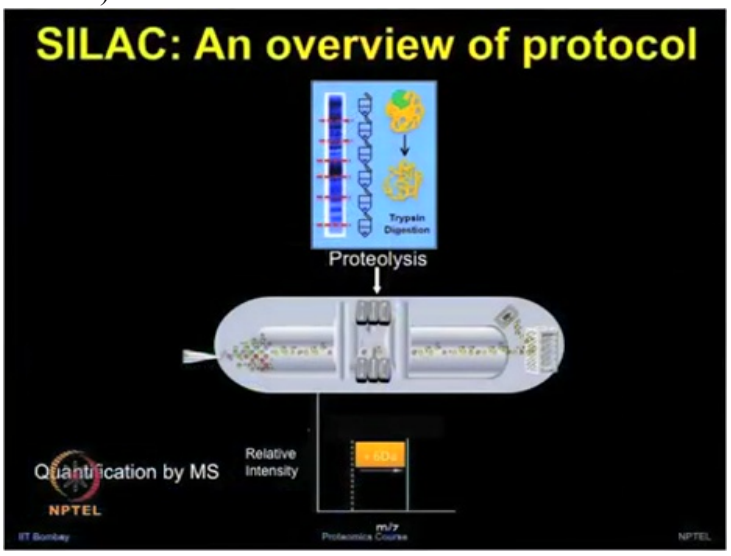

…and each of these bands can further be digested by adding trypsin.

So after digestion of isolated proteins to peptides by trypsin, the arginine containing peptides will be 6 Dalton heavier than their light counterparts which can be analyzed by using mass spec.

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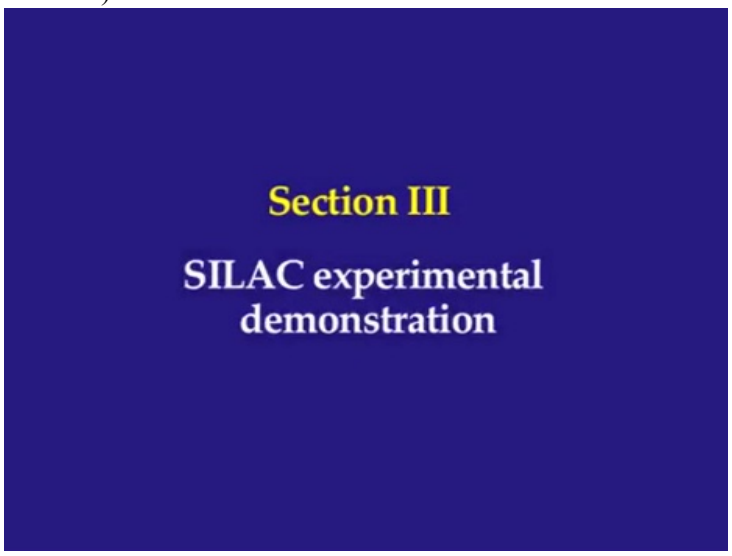

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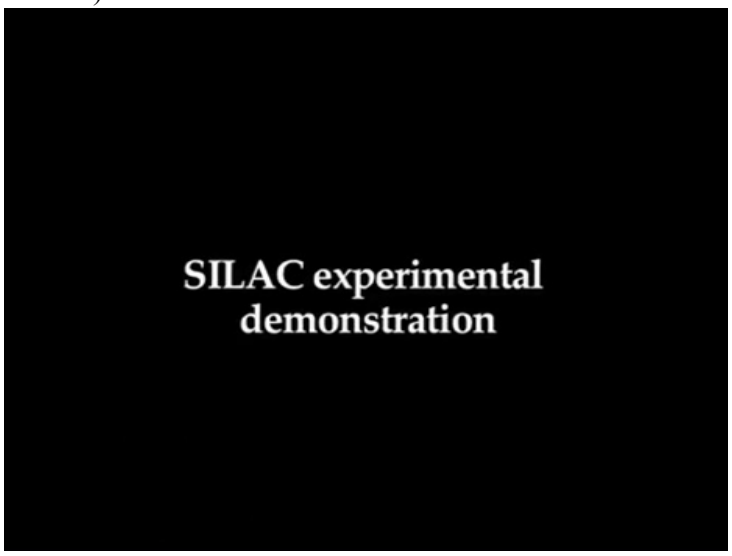

So, now I will show you an animation of a Stable Isotope Labeling by Amino acids in Cell culture or SILAC.

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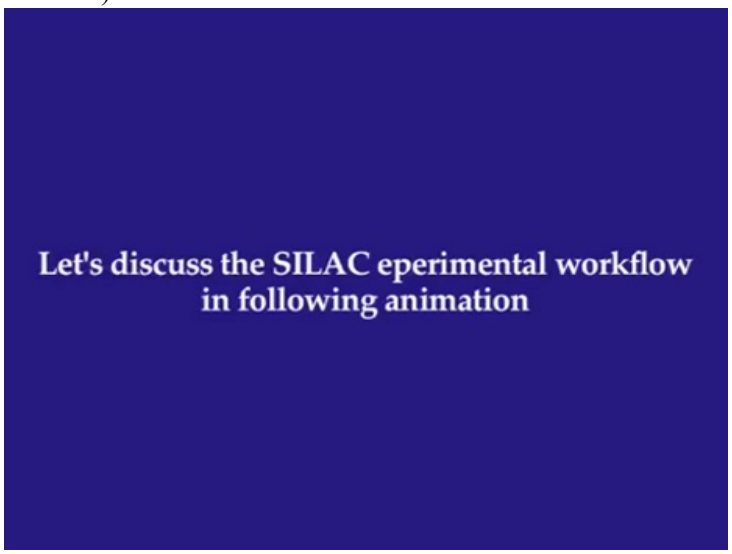

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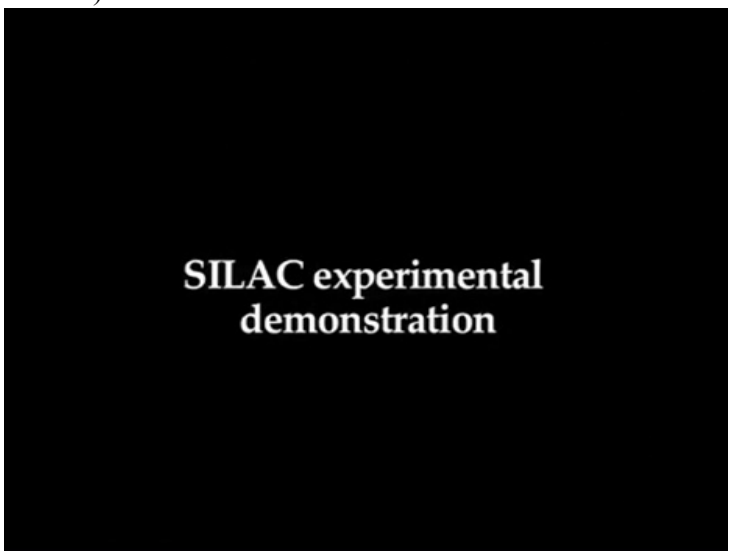

So, let us discuss about SILAC method. SILAC is a simple method for in vivo incorporation of a label into proteins for the quantitative proteomics purposes.

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In SILAC method, two group of cells are cultured in media that are identical in all aspects except that one contains a heavy medium…

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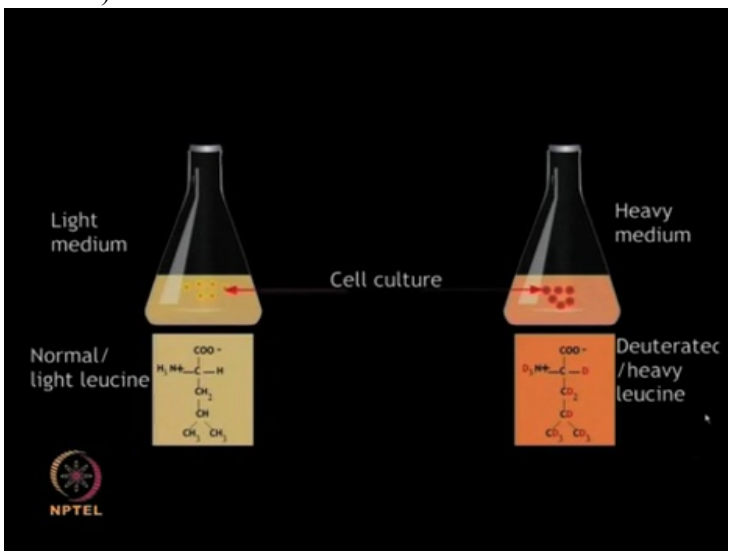

..a heavy isotopic analog of an essential amino acid …

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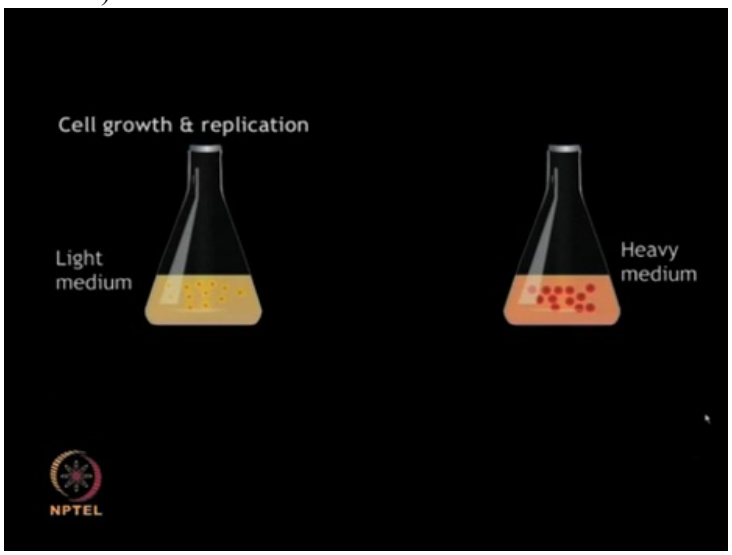

…while the other contains the normal or light amino acid

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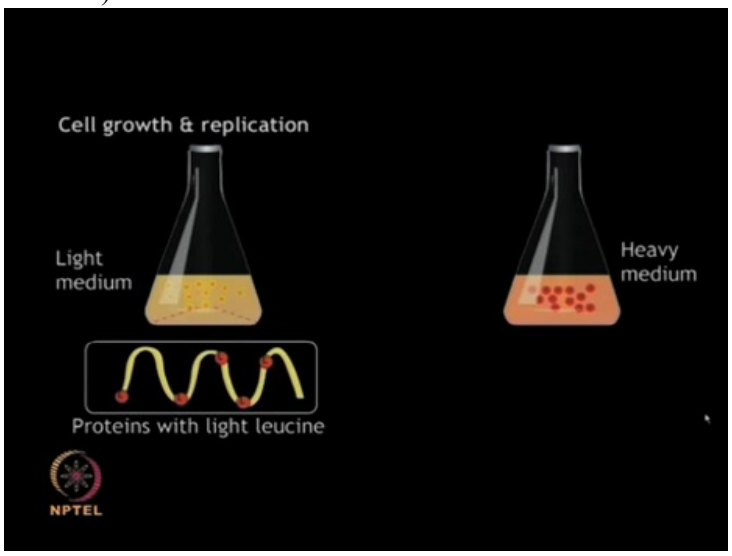
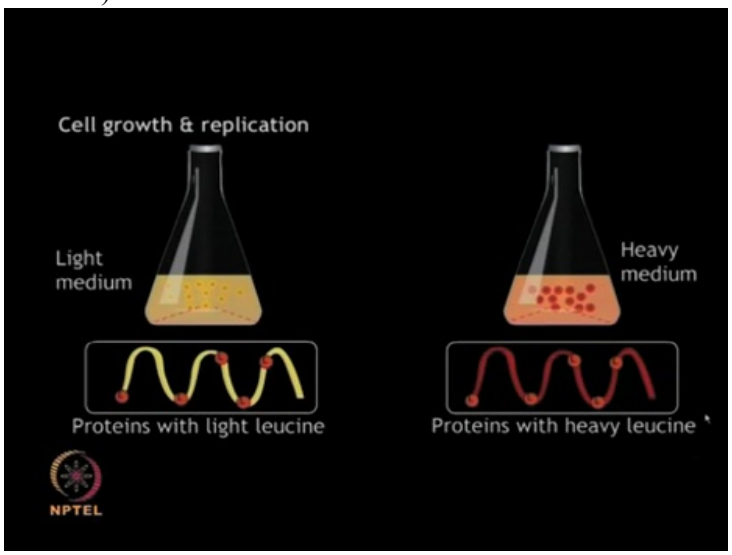


The essential amino acids …

(Refer Slide Time 20:57)

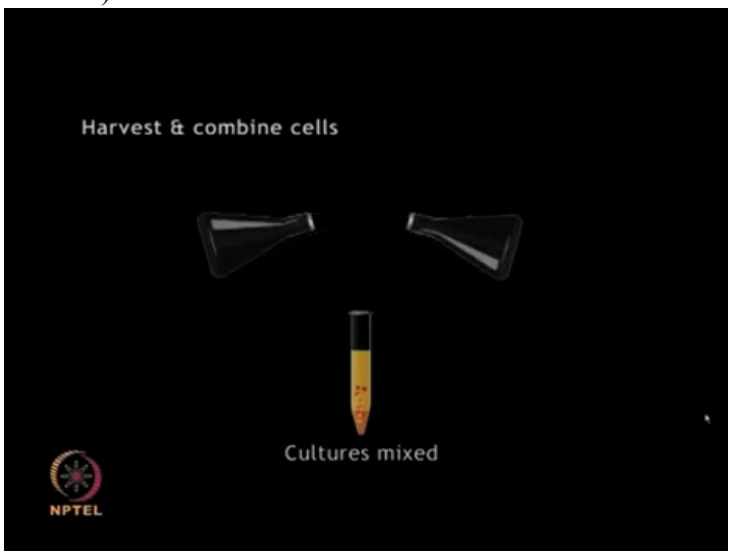


…which are obtain from the cell culture medium are incorporated into the corresponding newly synthesize proteins during growth

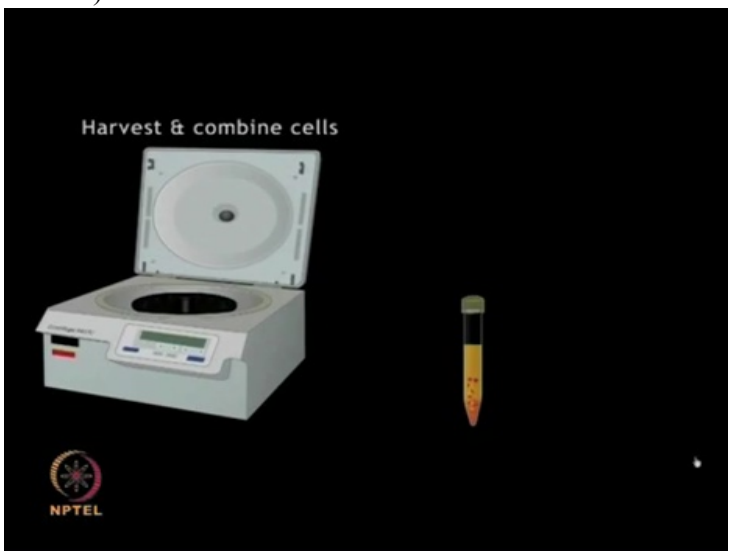
(Refer Slide Time 21:08)

… and replication

(Refer Slide Time 21:10)

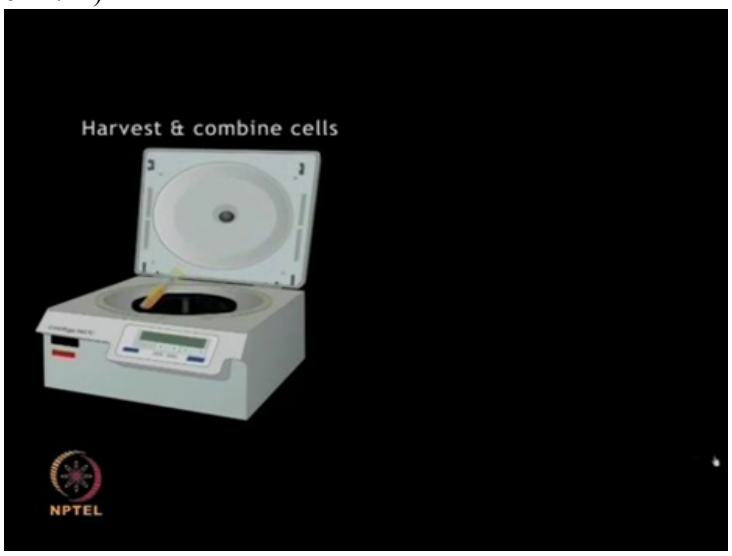


(Refer Slide Time 21:14)



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.

(Refer Slide Time 21:41)

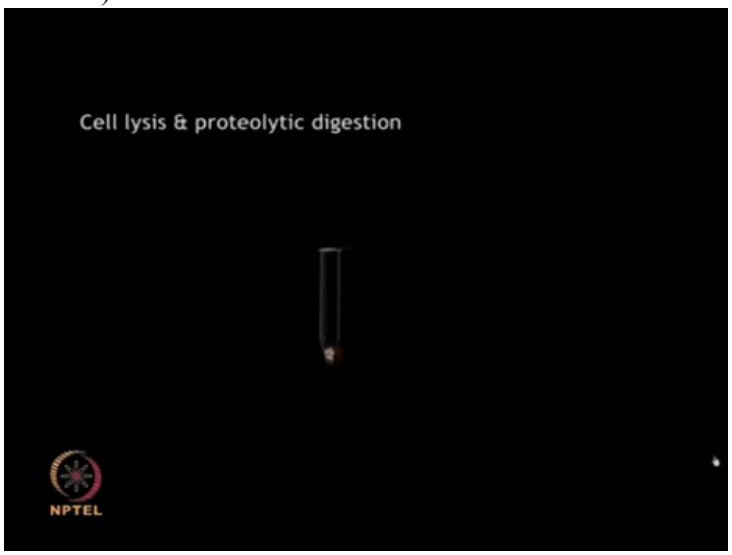


Centrifugation of the mixture will result in pelleting of cells which can then be use for …

(Refer Slide Time 21:48)

…. further analysis

(Refer Slide Time 21:55)



The grown cells

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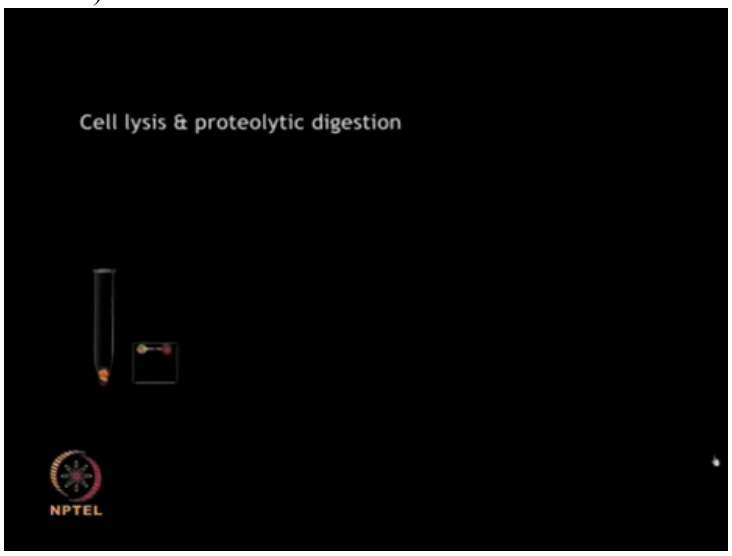

… are then lysed ….

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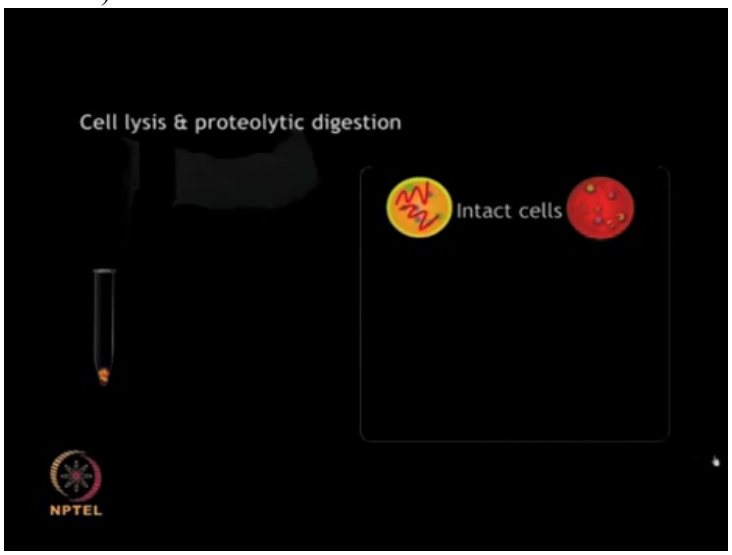
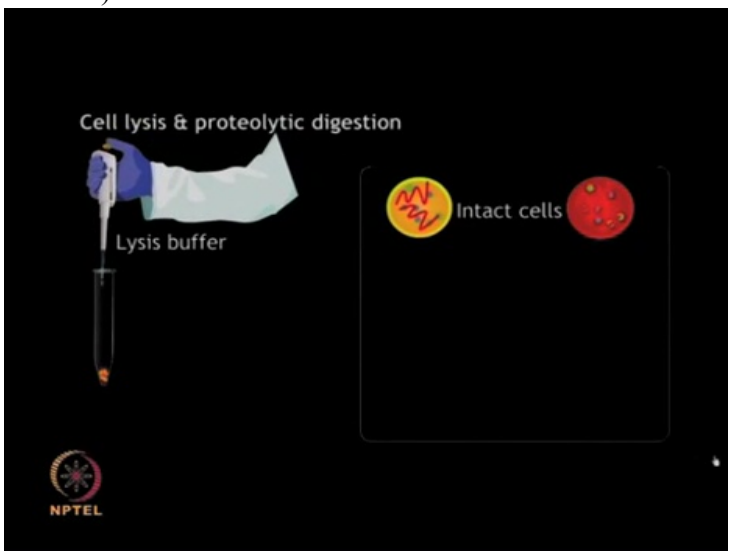


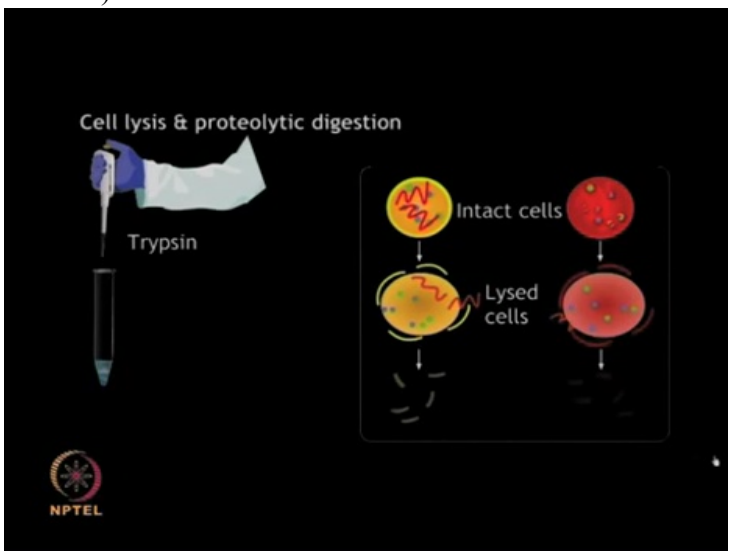
using a suitable

(Refer Slide Time 22:01)



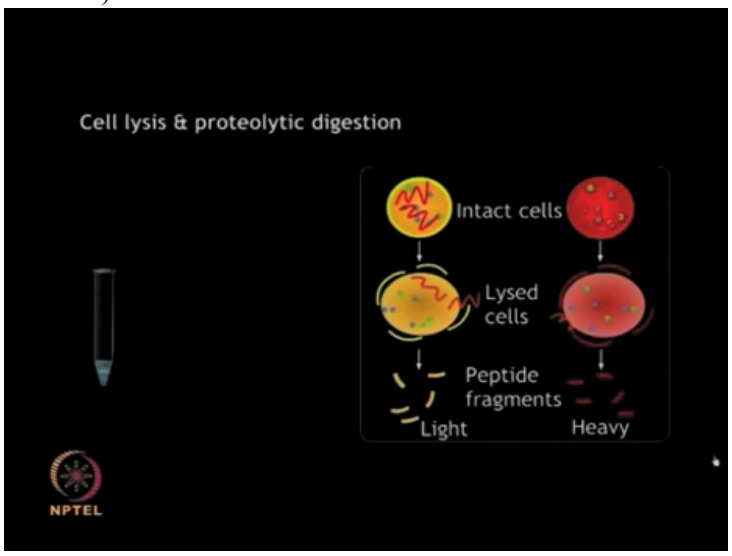
lysis buffer and proteins are cleaved by using a proteolytic enzyme

(Refer Slide Time 22:09)



..such as trypsin This results in a mixture of

(Refer Slide Time 22:15)



light and heavy peptide fragments which can be

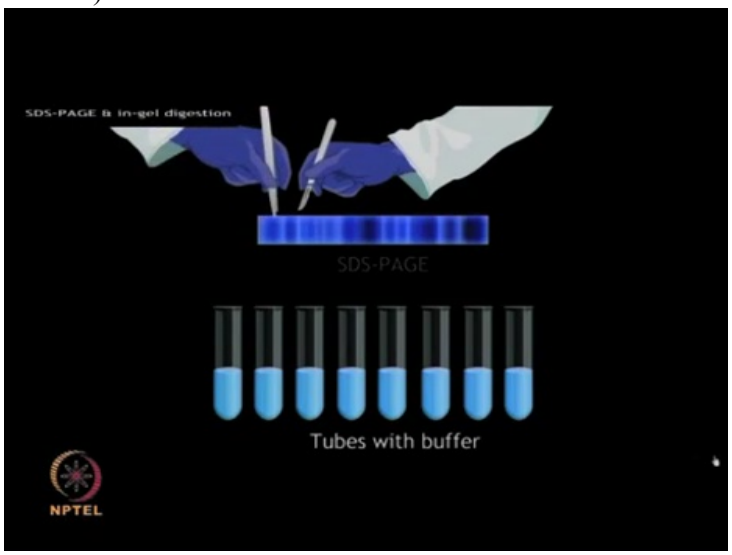
(Refer Slide Time 22:20)

quantified further by using

(Refer Slide Time 22:23)

…mass spec The complex mixture of peptide fragments is further separated

(Refer Slide Time 22:30)



...by SDS PAGE to simplify this analysis

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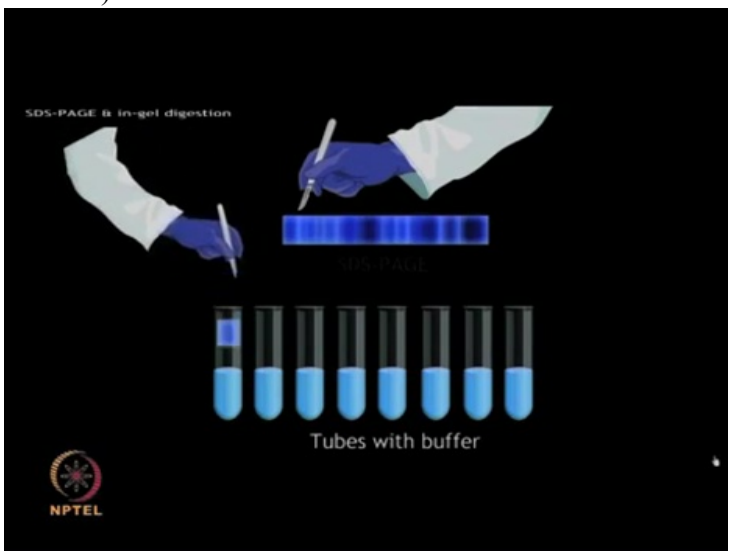

Each band of the SDS PAGE gel is cut out

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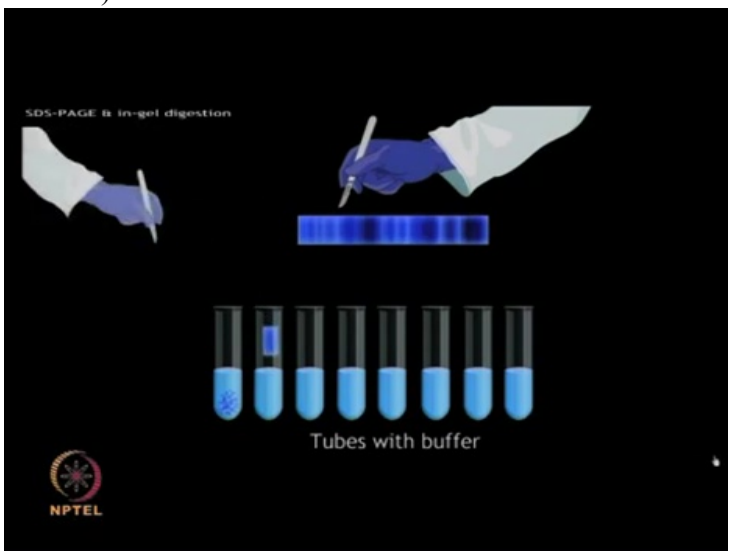
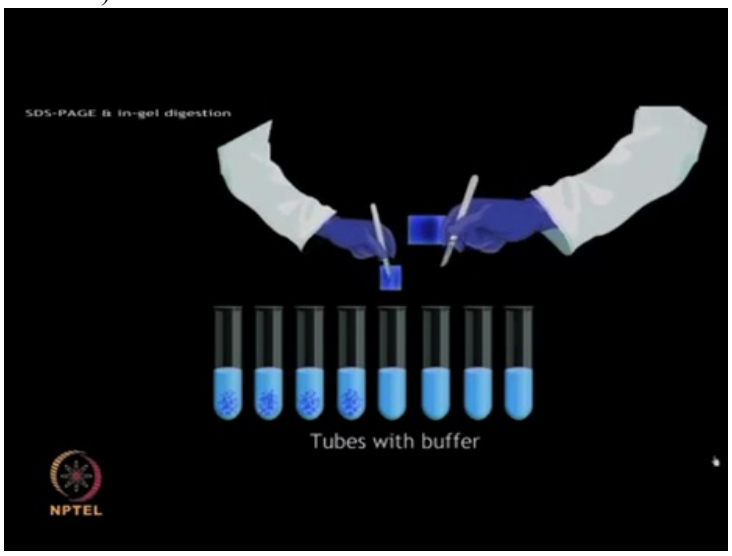


…and redissolved in a suitable buffer solution These simplified peptide fragments can then further be used for

(Refer Slide Time 22:54)



mass spec analysis

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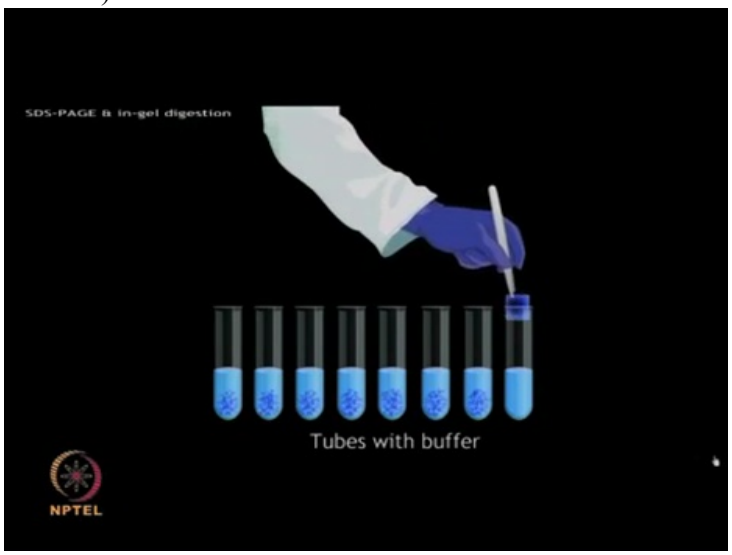

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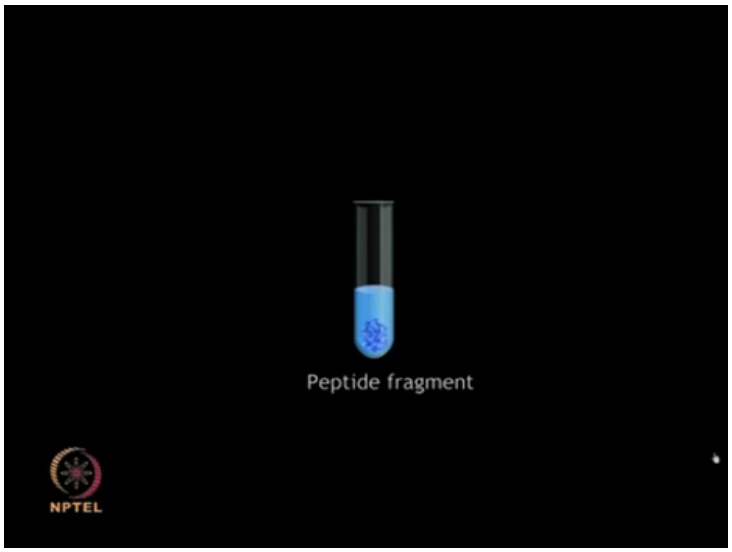

Further purification can be carried out by liquid chromatography.

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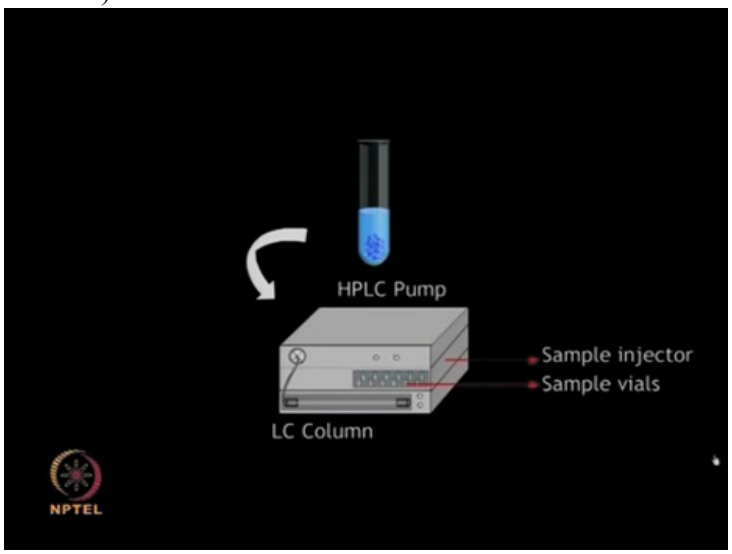
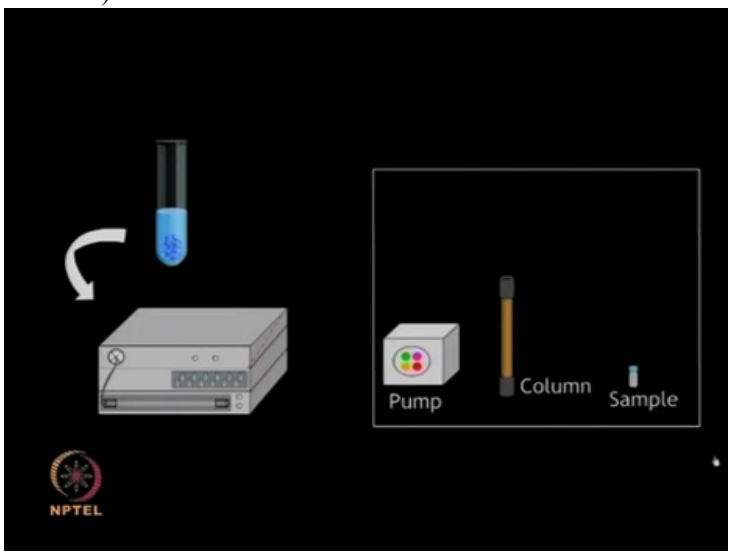


wherein the sample is passed through the column containing

(Refer Slide Time 23:27)



…a packed stationary phase matrix that selectively adsorbs only

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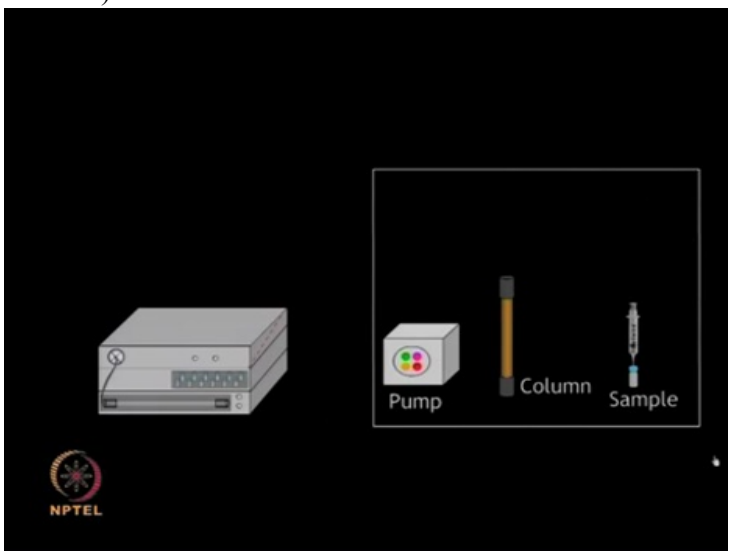
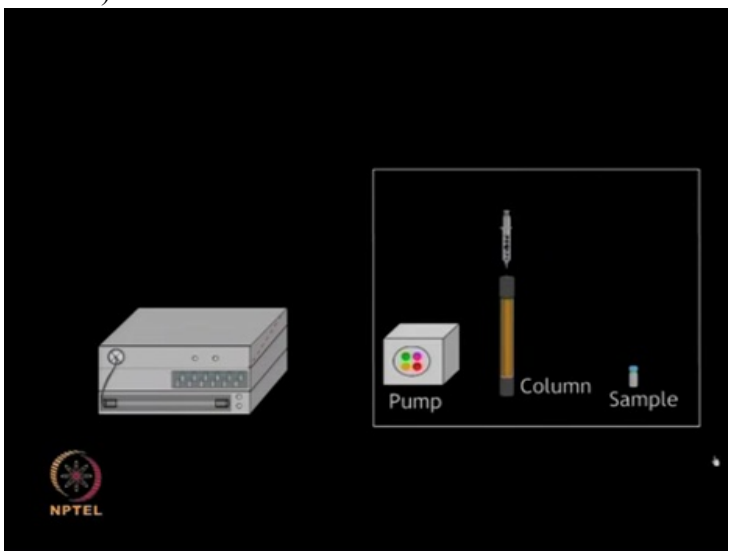


…certain analyte molecules The reverse phase and

(Refer Slide Time 23:39)



strong cation exchange chromatography are the most commonly used methods.

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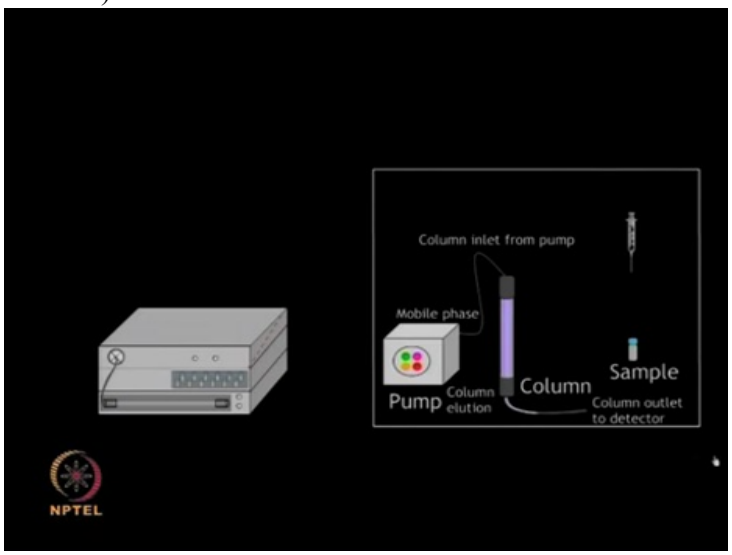

These eluted fractions are further

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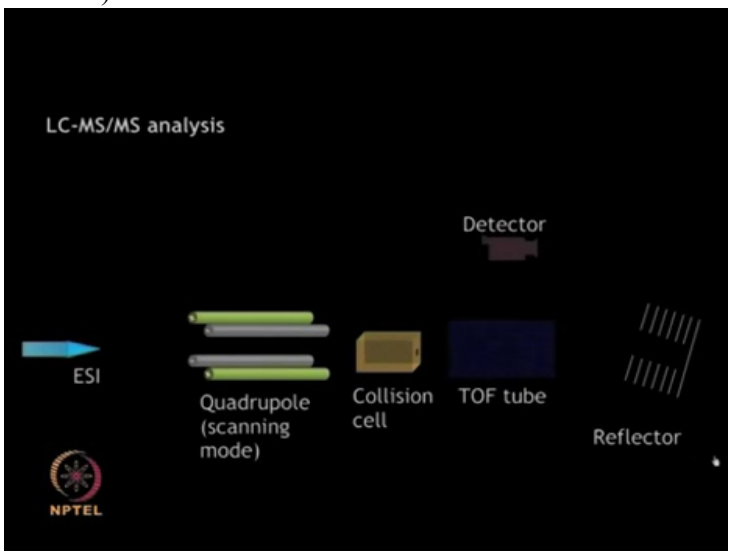

…characterized by MS

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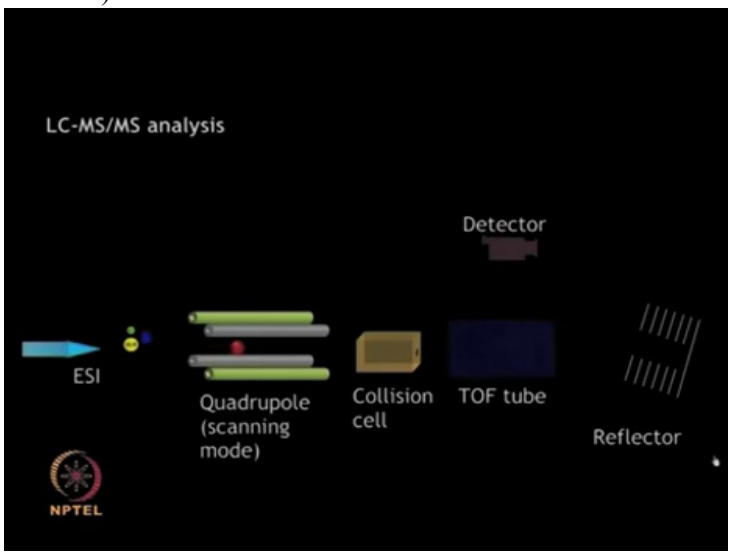

The purified peptide fragments are analyzed by MS/MS.

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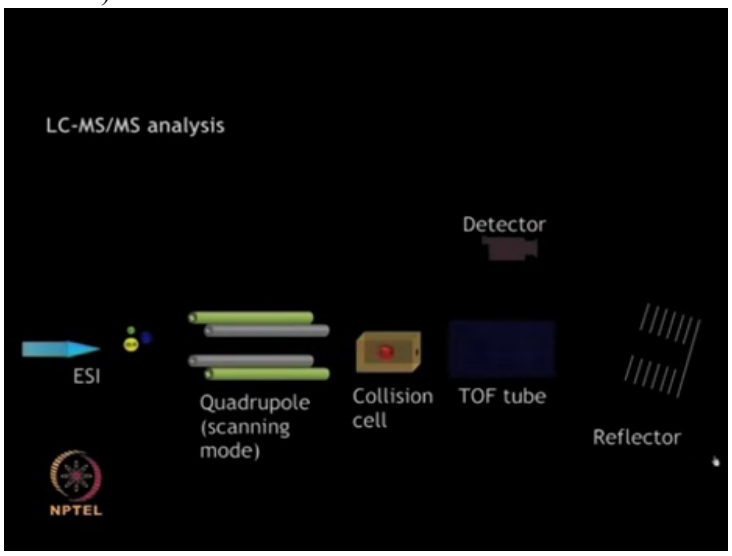
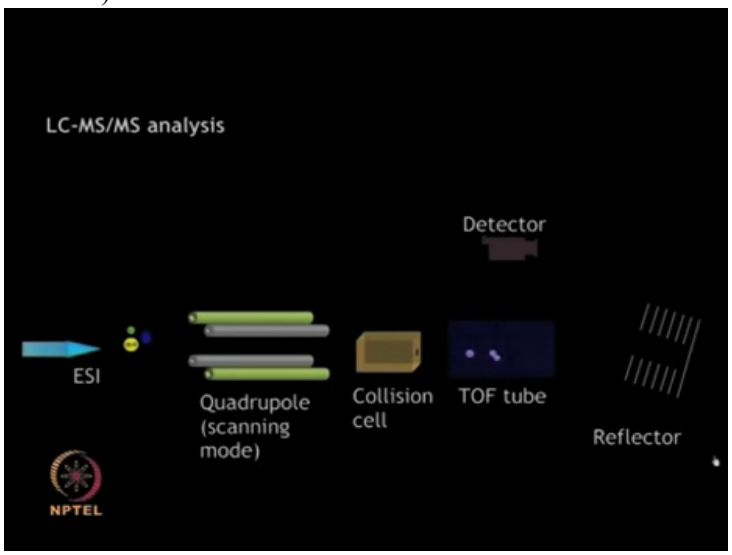


Peptides containing

(Refer Slide Time 24:04)



the heavy amino acid show

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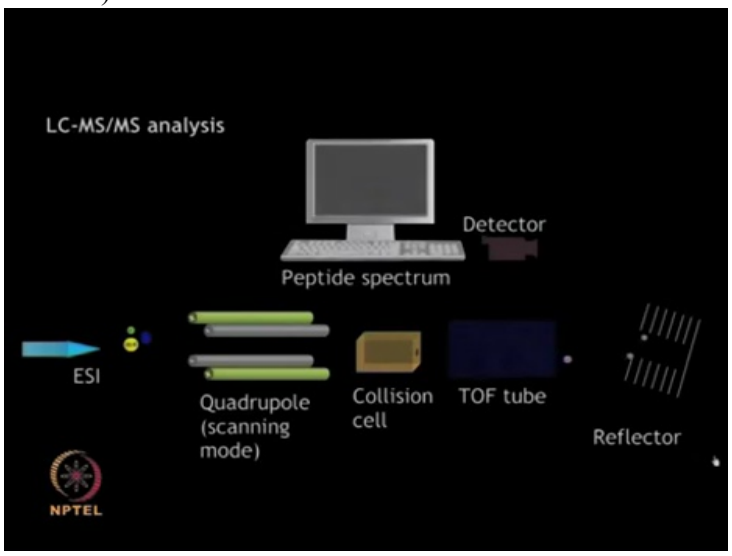

higher m by z

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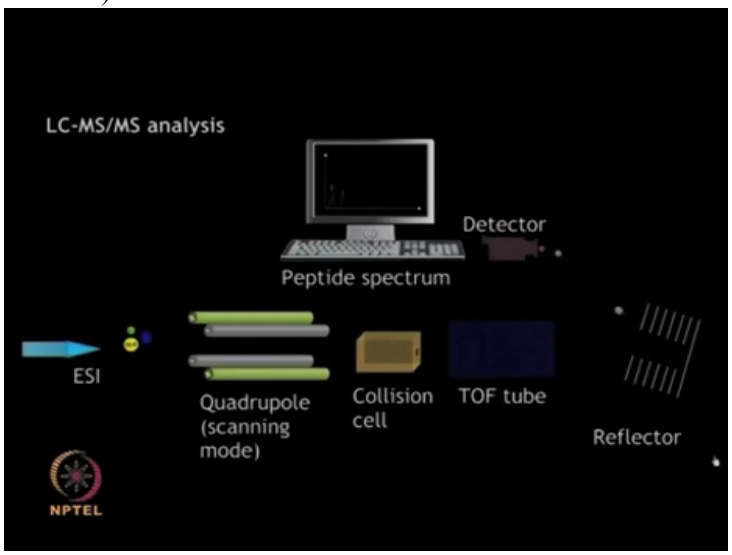

…. as compared to the corresponding peptide fragments

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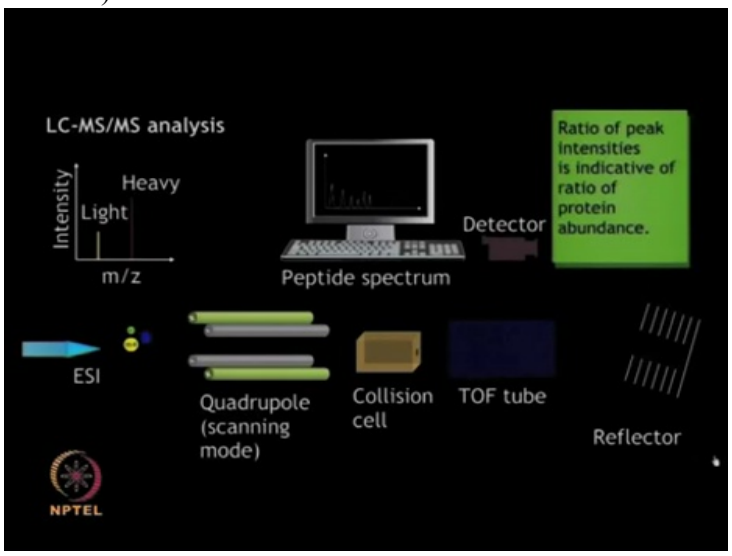

The pairs of identical peptides can be differentiated due to the mass difference and ratio of peak intensities can be correlated to the corresponding protein abundance.

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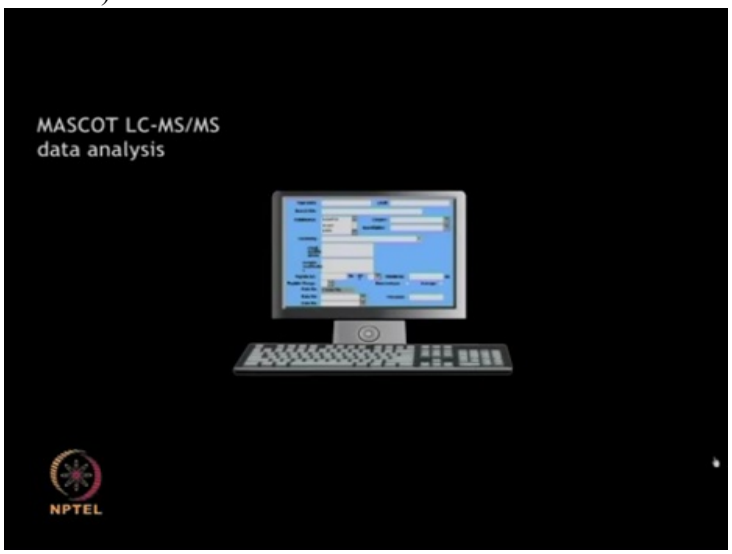

The data obtained from tandem mass spectrometry following SILAC experiment

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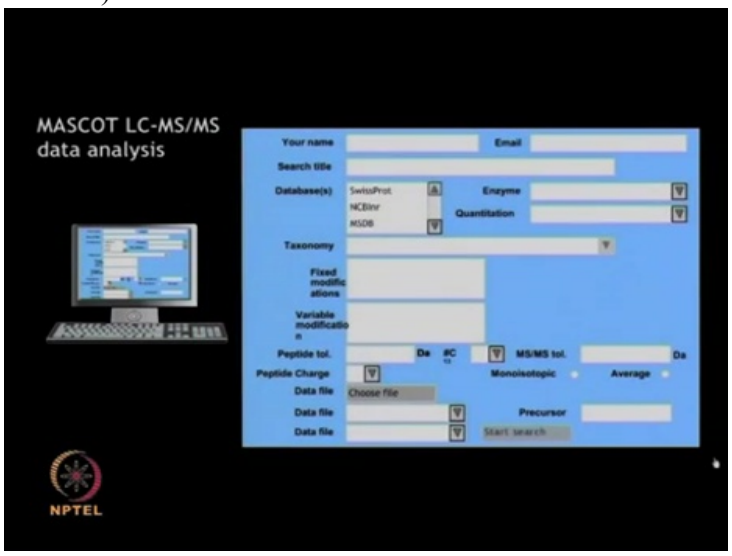

…can be analyzed by means of Mascot search engines The MS/MS data analysis shareware requires input from the user regarding the experimental parameters used such as

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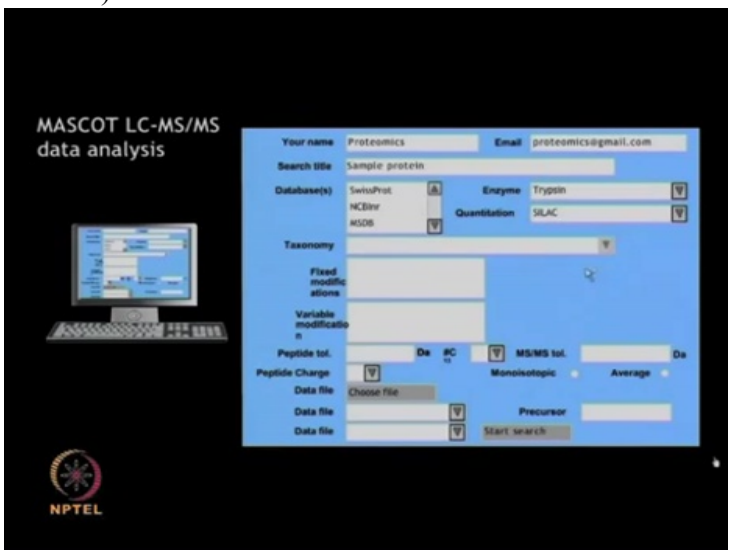
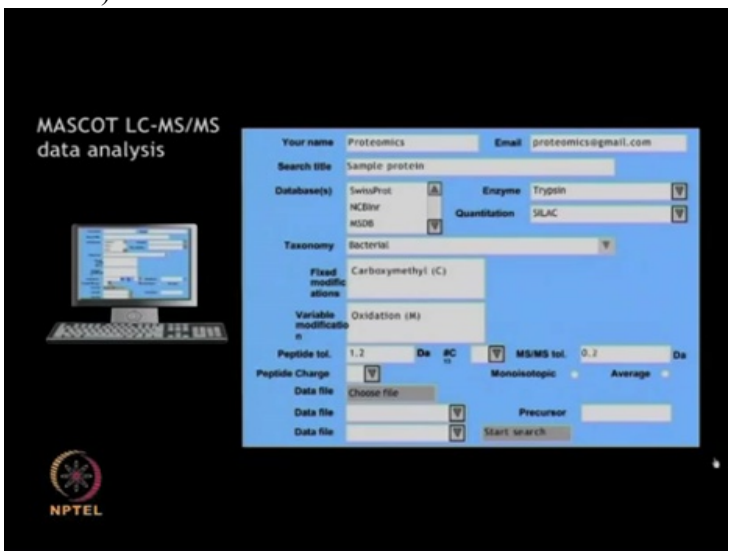


enzyme cleavage, protein name, modifications, instrument used, peptide charge etc

(Refer Slide Time 25:18)



… and the desired such criteria like taxonomy, peptide tolerance, etc.

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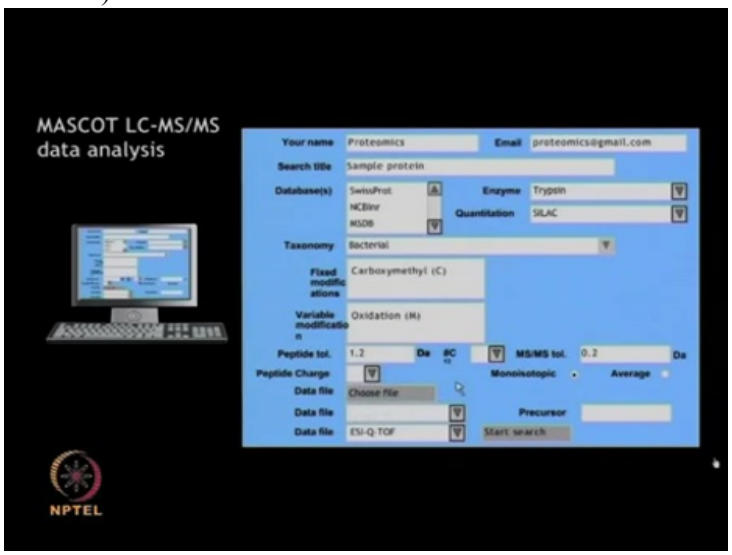
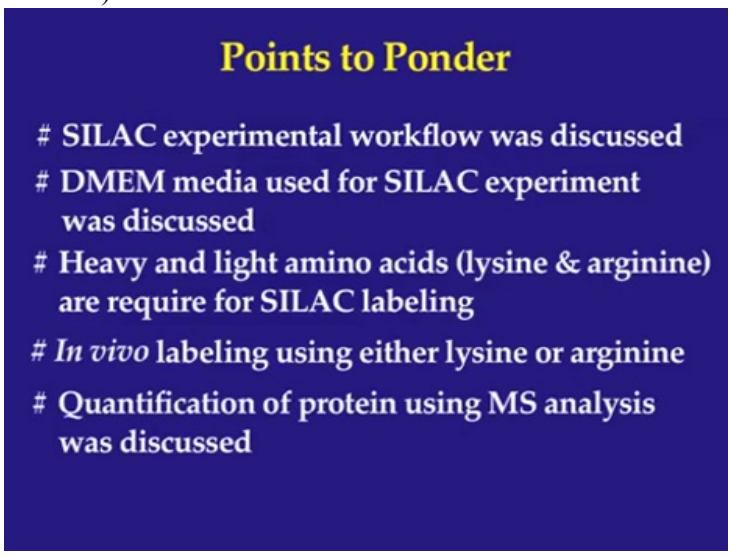


The commonly used protein databases against which the MS information is processed to retrieve sequence data include NCBI, MSTB and Swiss Prot. The data file generated from MS is uploaded and the search is carried out

(Refer Slide Time 26:01)



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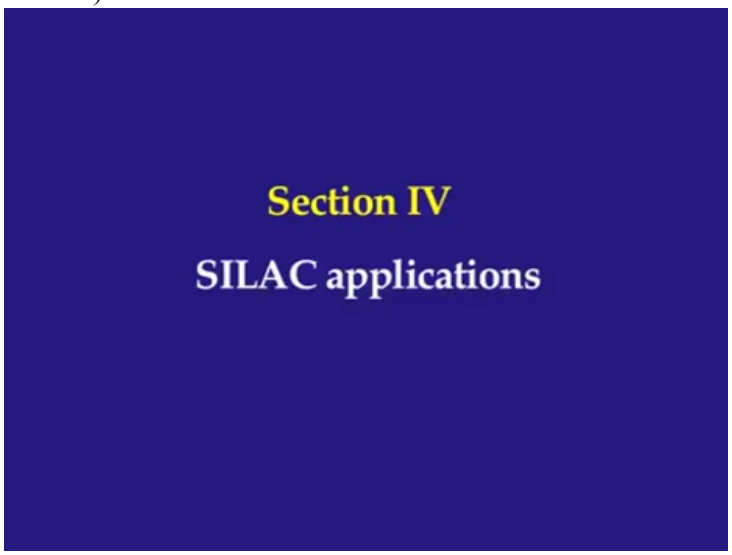

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Let us now discuss few applications of SILAC briefly. The SILAC method is very promising for any cell line. So this method can be applied for …

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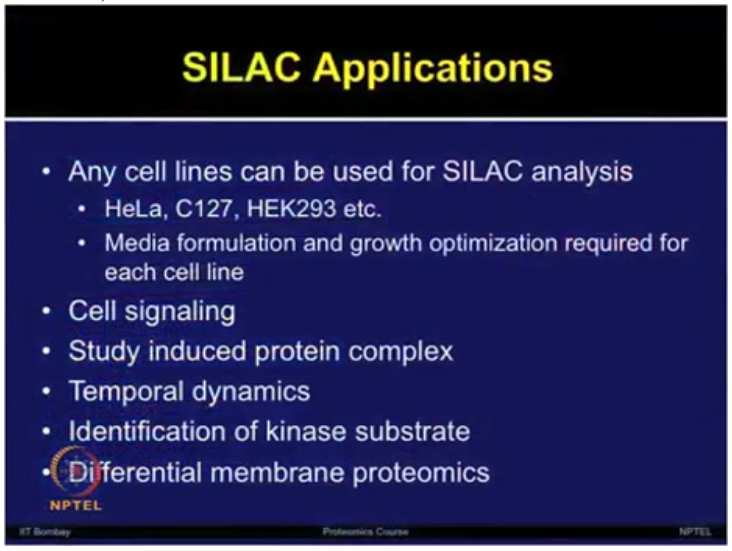

…any cell lines whether it is heLa cell C127, HEK293 or different type of cell lines people have shown. However, the media formulation and the growth optimization is required individually for each cell line.

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.

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

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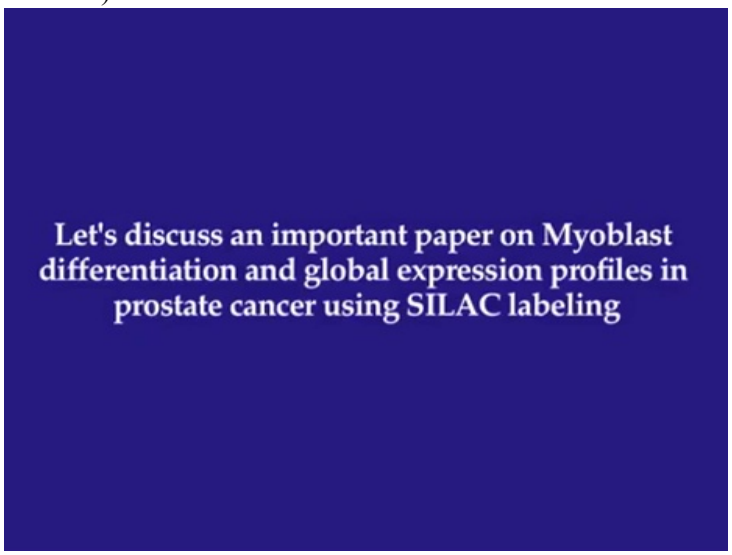

Ong et al in 2002 published a paper in molecular cell proteomics which was the first SILAC application demonstrated

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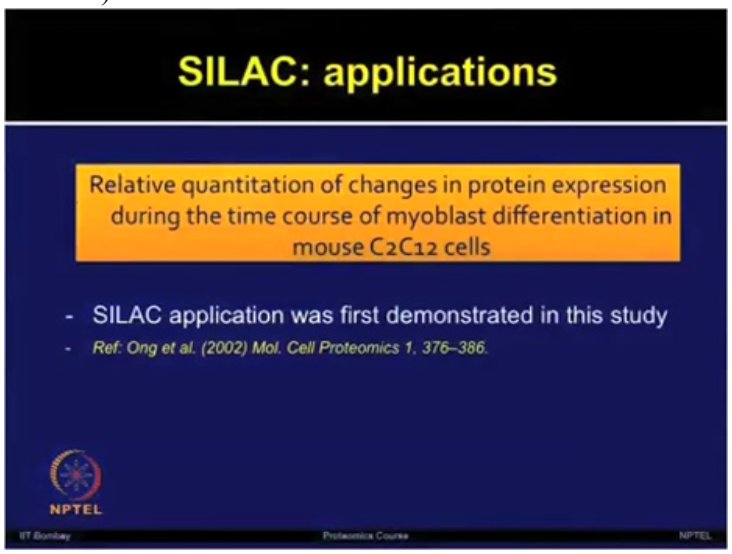

…where they used the relative quantitation of changes in protein expression during the time course of myoblast differentiation in mouse cells

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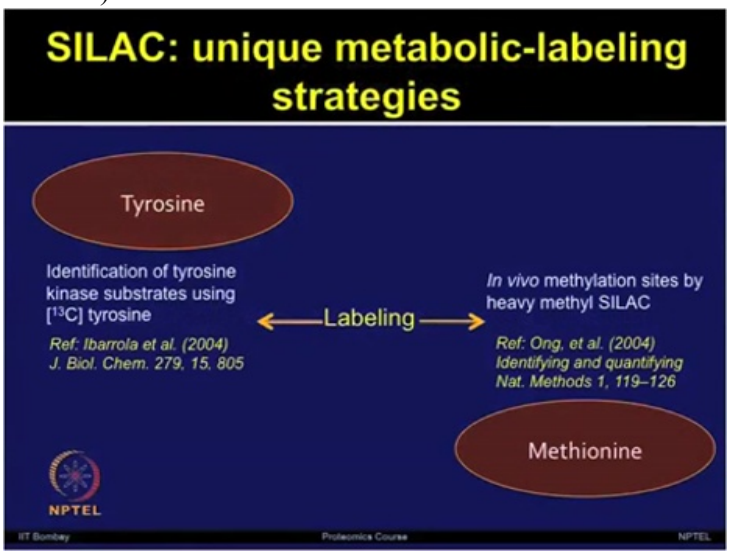

Researchers have reported various unique metabolic labeling strategies, for example, by using tyrosine, the identification of tyrosine kinase substrate using 13C tyrosine. Labeling is also performed by using methionine, the in vivo methylation sites by heavy methyl SILAC.

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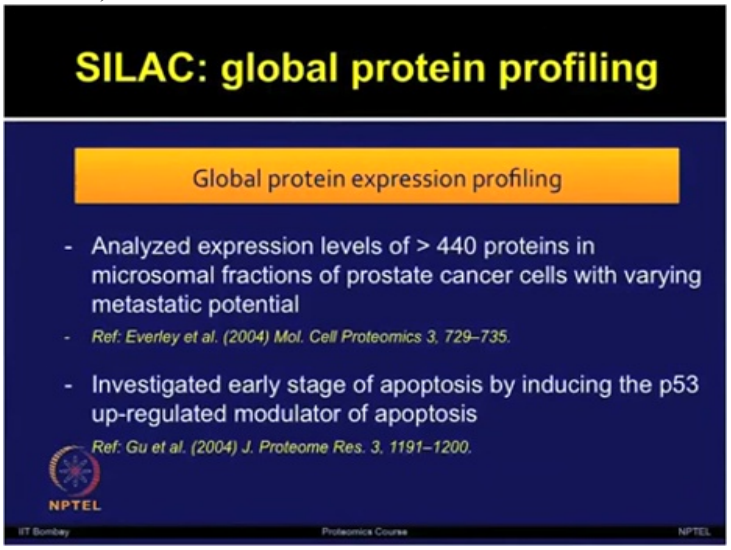

There are numerous studies based on the global protein expression profiling using SILAC method. I am just highlighting some of the very earlier studies which set up the path for performing these protein expression profiling.

So, study by Everley et al in 2004 analyzed the expression level of more than 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 p 53 up-regulated modulator of apoptosis.

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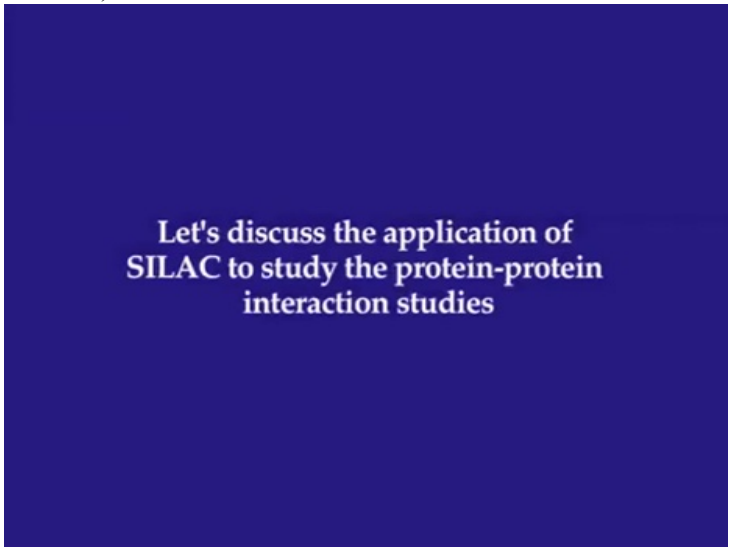

SILAC has also been used for functional assays to study the protein-protein interactions. Study by Blagoev et al used

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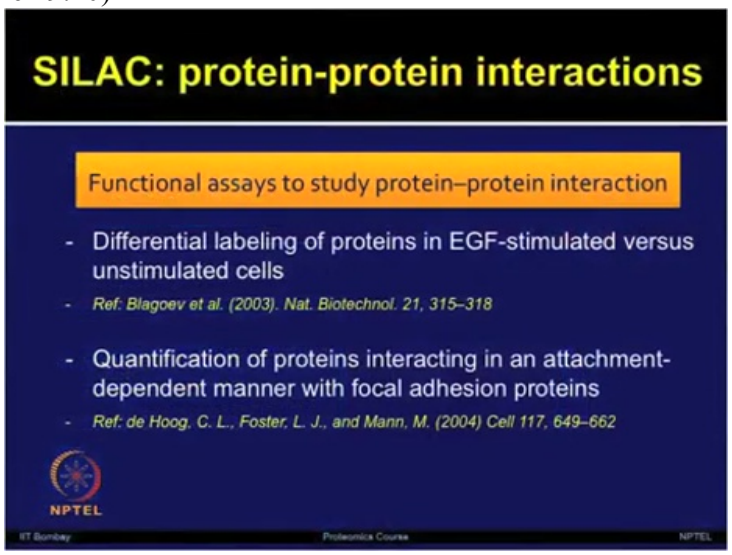
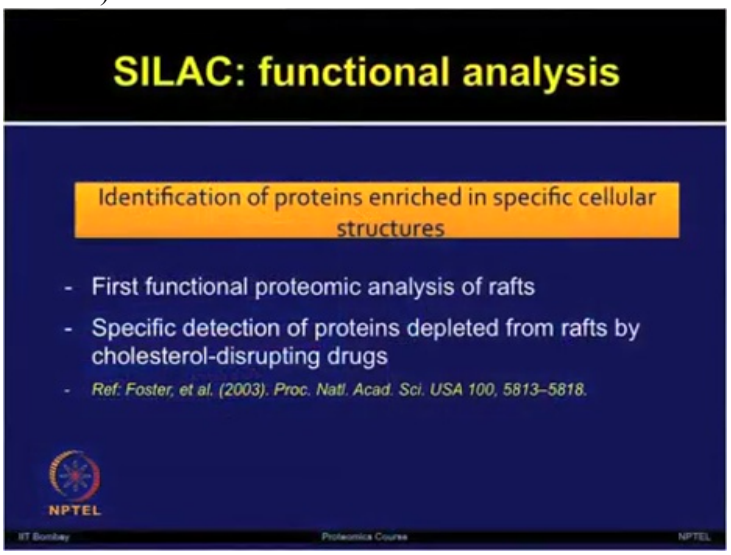


the differential labeling of proteins in EGF-stimulated versus un-stimulated cells

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, the Study by Foster et al

(Refer Slide Time 30:01)



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

SILAC has been widely used for multiplex analysis to compare the cellular states. Andersen et al

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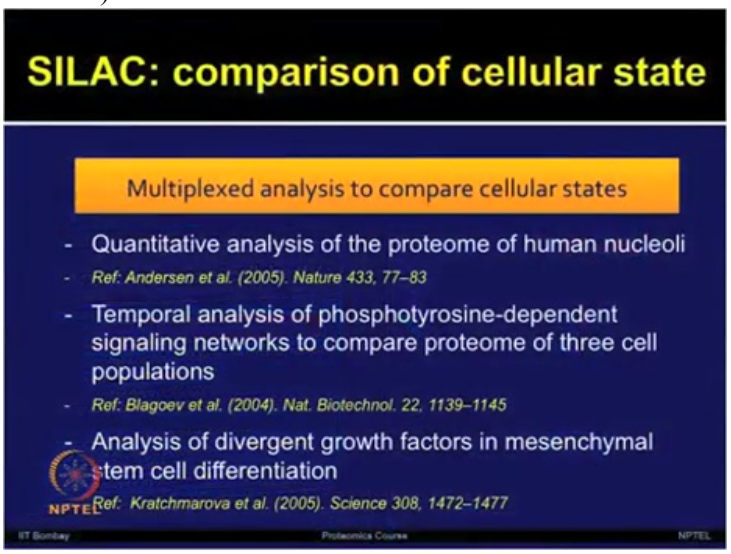

…showed the quantitative analysis of proteome of human nucleoli

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.

Now, if you look into literature, there are many studies which have used the SILAC method of comparison of cellular states.

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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 …

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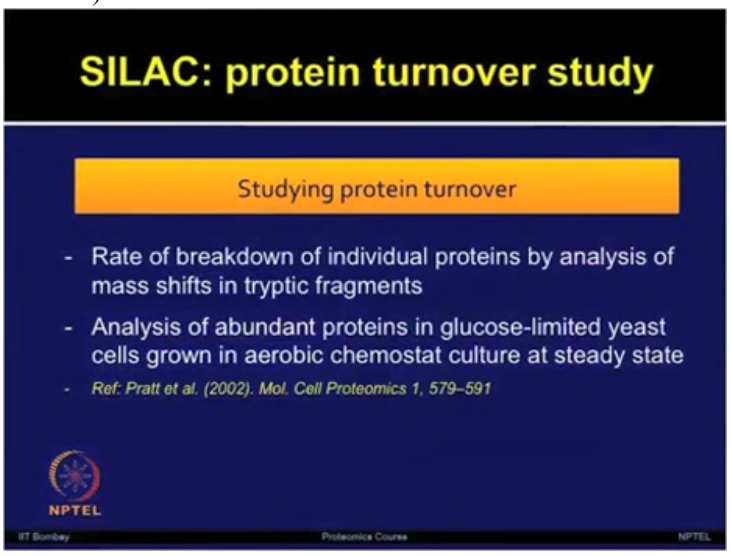

….in tryptic peptide fragments…

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.

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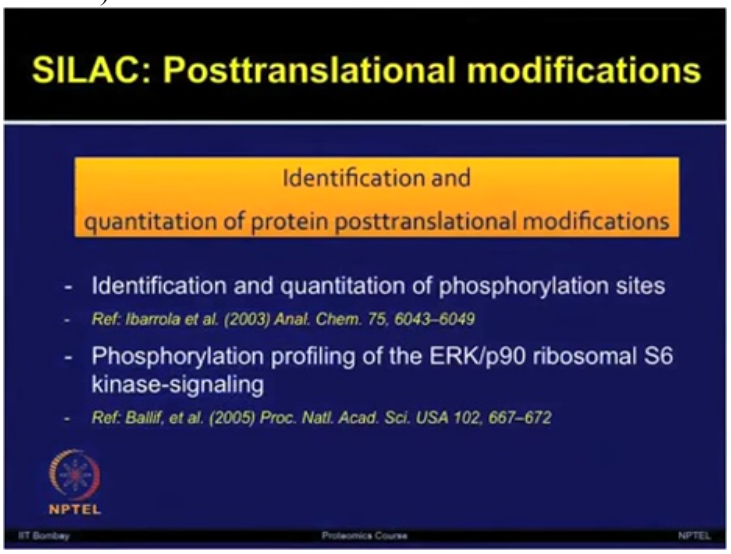

Study by Ibarrola et al identified and quantitated phosphorylation sites.

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

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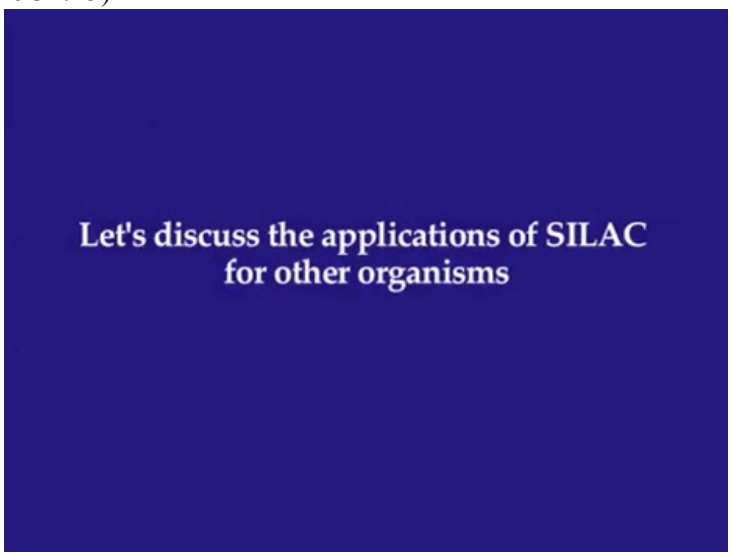

So, interestingly now SILAC method has been used in different organism; in bacteria…

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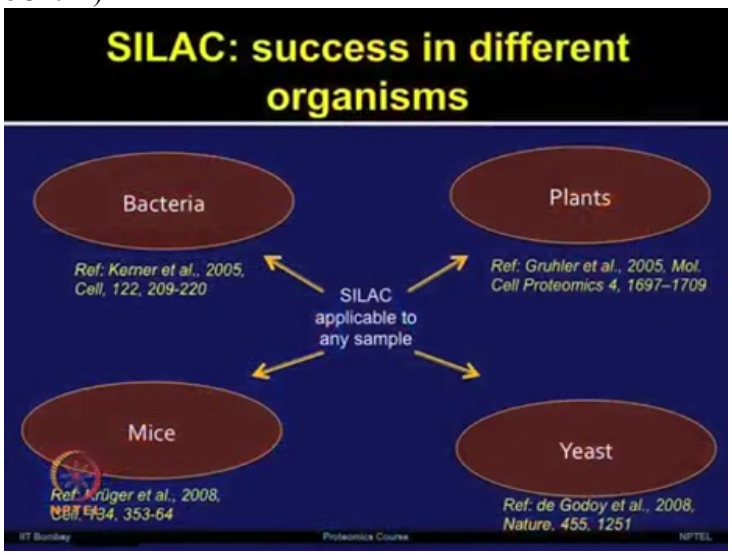

in yeast these were the more commonly used SILAC methods due to the growth in the cell culture.

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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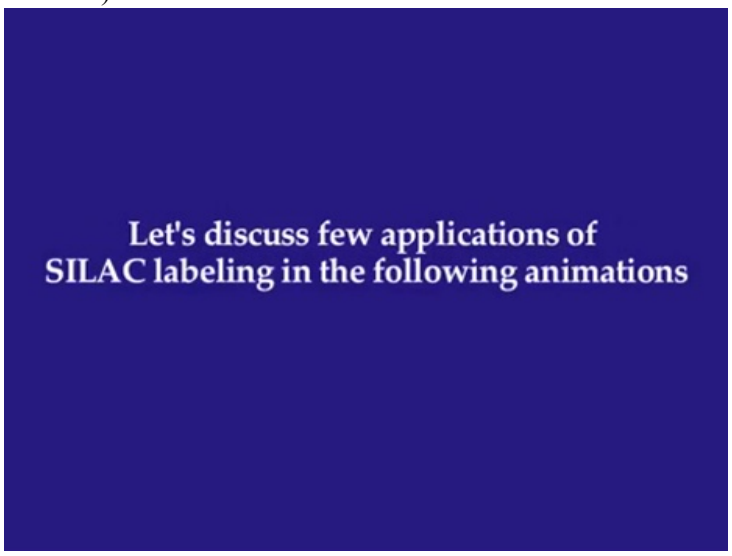

So, SILAC applications are straight forward and it only requires some initial attention to the cell culture conditions. Let us discuss the SILAC applications in an animation.

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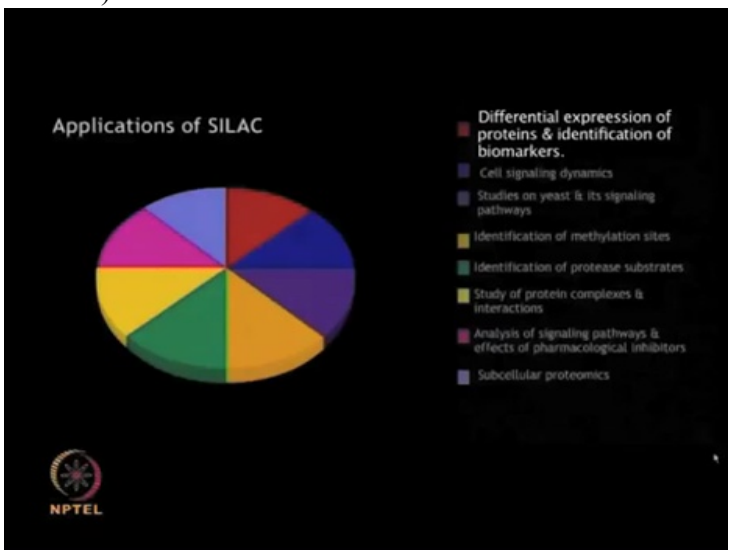

Let us now discuss the applications of SILAC. SILAC is a very useful quantitative approach which has found application for several proteomic studies.

SILAC provides an in vivo strategy to label and monitor the quantitative differences at protein level in various

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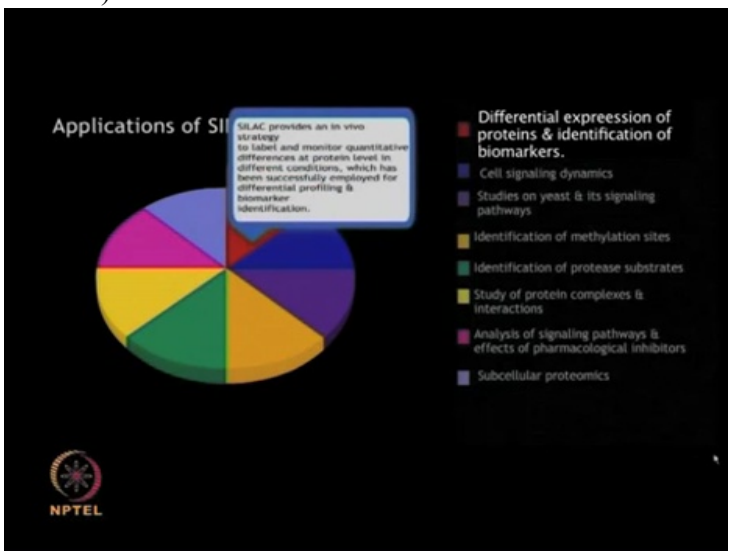

…conditions which has been successfully employed for differential profiling and biomarker identification

The temporal dynamics

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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.

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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.

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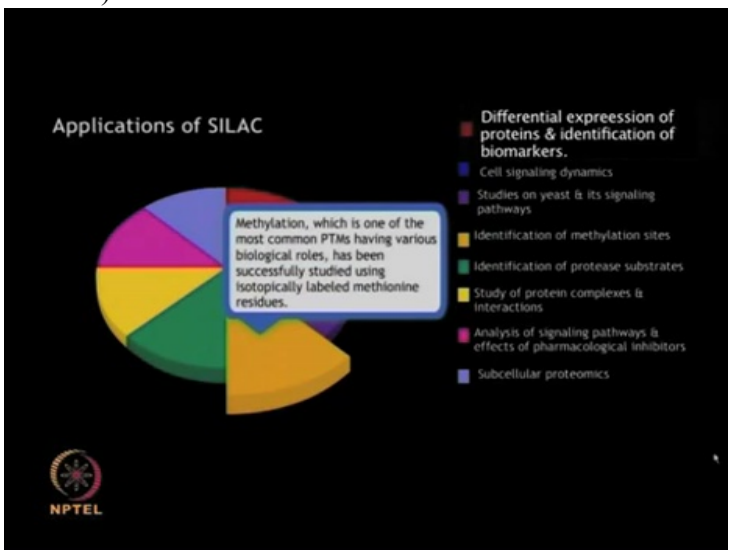

Methylation, which is one of the very common PTMs having various biological significant role, is successfully studied using isotopically labeled methionine residues.

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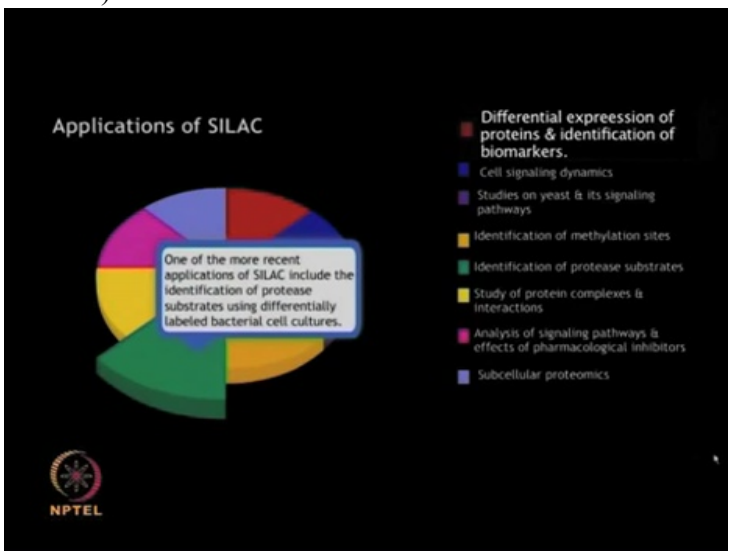

One of the recent applications of SILAC include the identification of protease substrates using differentially labeled bacterial cell cultures.

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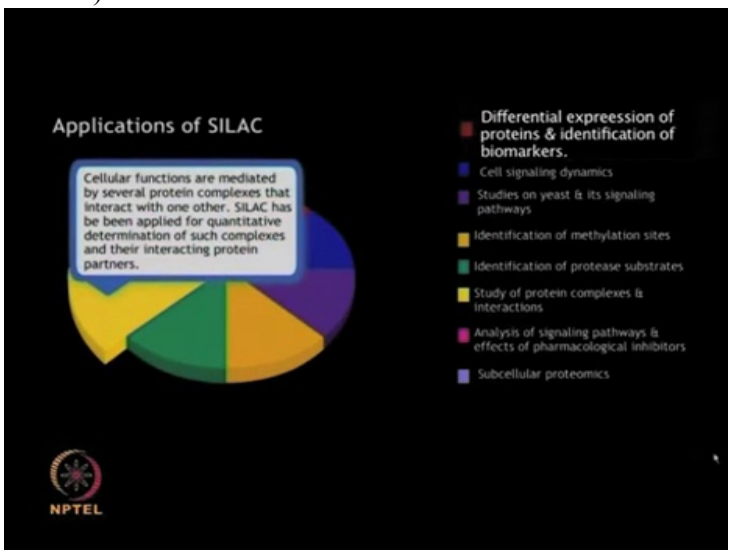

The cellular functions are mediated by several protein complexes which interact with one another. SILAC has been applied for the quantitative determination of such complexes and their interacting protein partners.

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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 studied by using SILAC.

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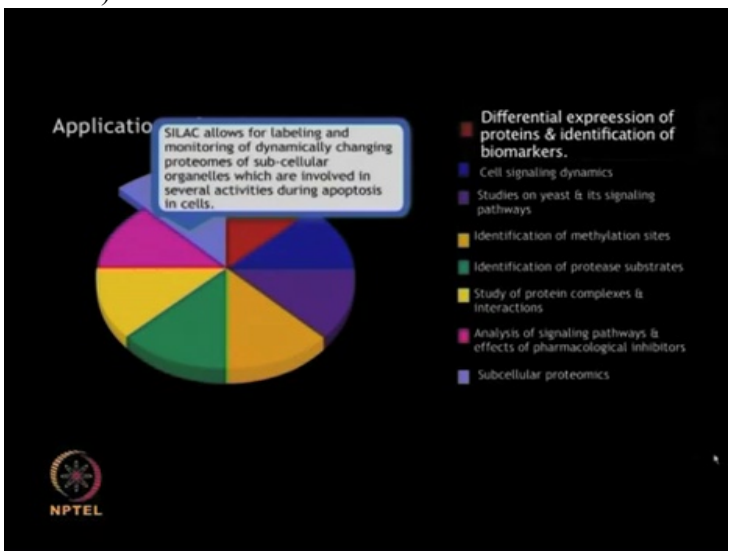

SILAC allows for the labeling and monitoring of dynamically changing proteome of sub cellular organelles; which are involved in various activities during apoptosis in cells.

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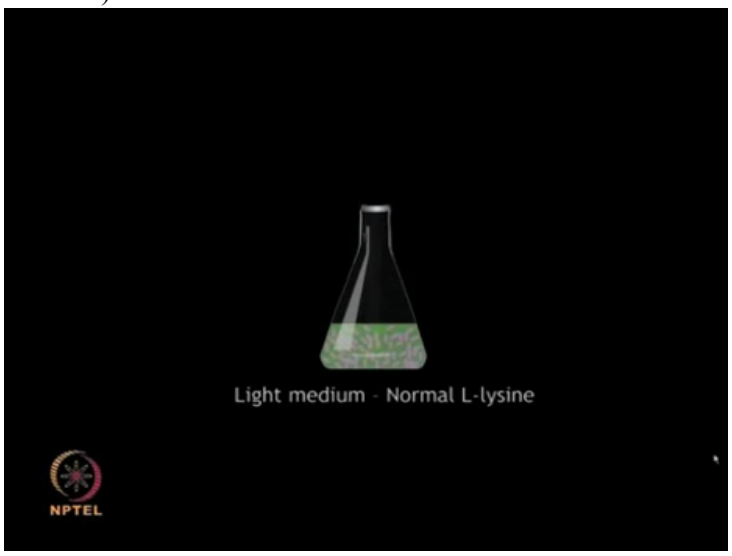

Now, let us take the case study from Godoy and colleagues …

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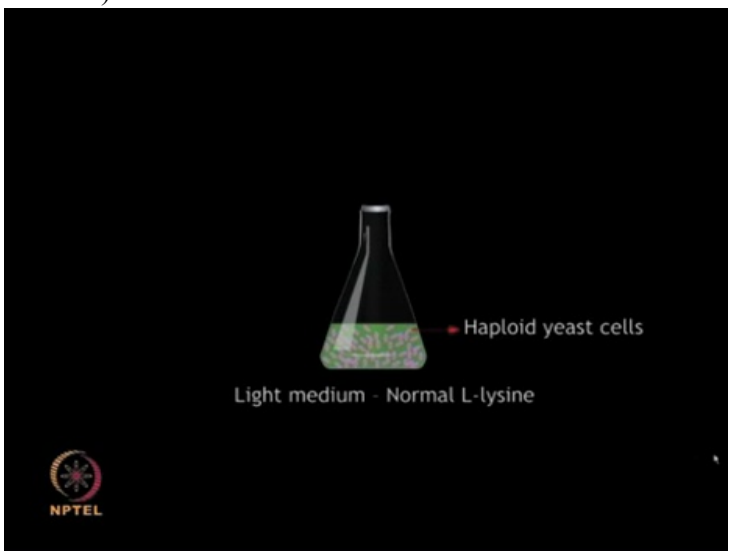

...which determine the fold change of peptide pairs between haploid and diploid yeast cells

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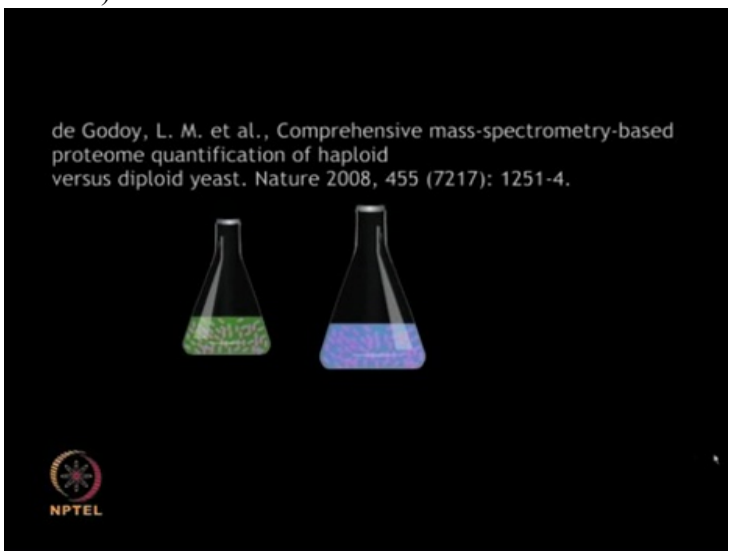

The study was published ….

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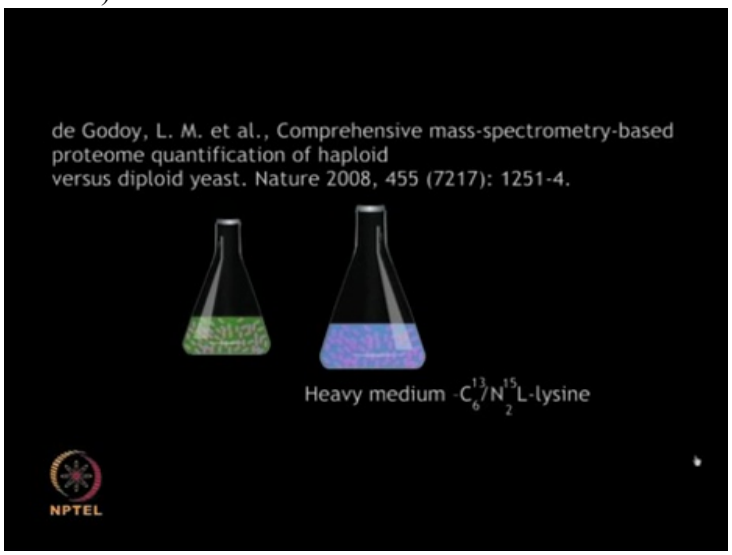

… in Nature 2008 A comprehensive mass spectrometry based proteome quantification of haploid versus diploid yeast.

The labeled lysine residues were used to grow the diploid yeast cells

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…while the haploid cells were grown in normal lysine medium

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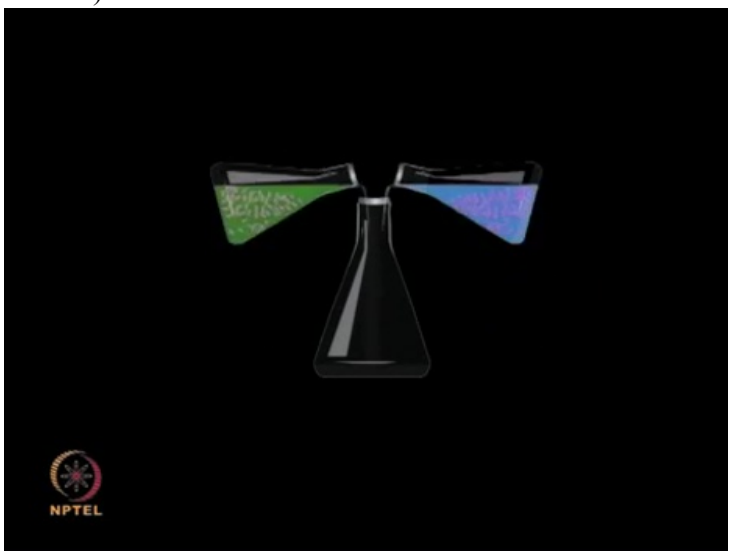

The cultures were mixed,

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proteins extracted

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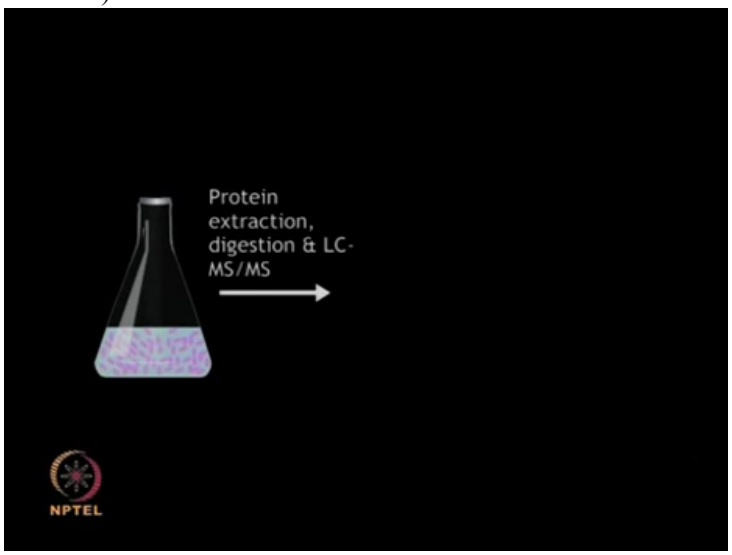

…and analyzed by LC MS/MS

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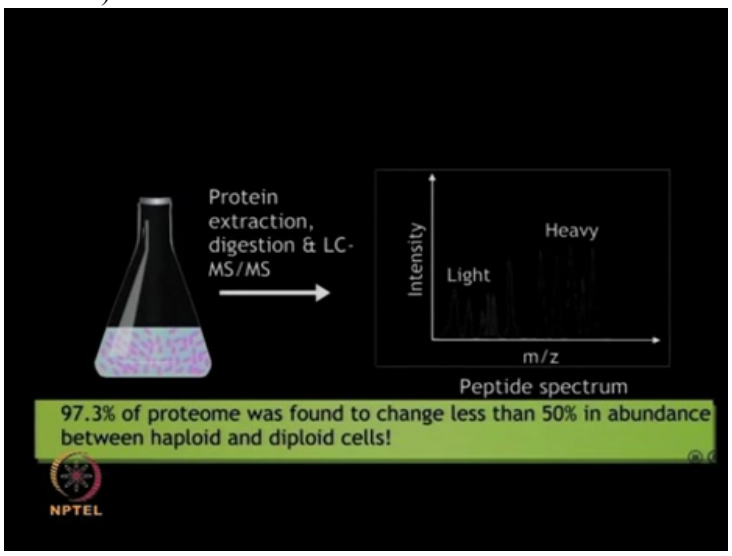

The protein ratio between the haploid and diploid cells were determined with very high accuracy. The comparison revealed that 97.3% of the proteome changes less than 50% in abundance between haploid and diploid yeast cells.

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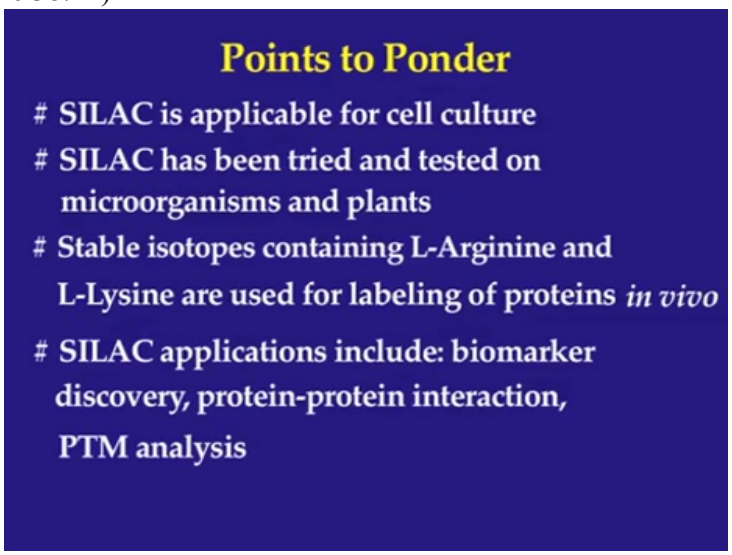

So after discussing about different type of applications of SILAC method, now let us summarize what we have studied in this lecture.

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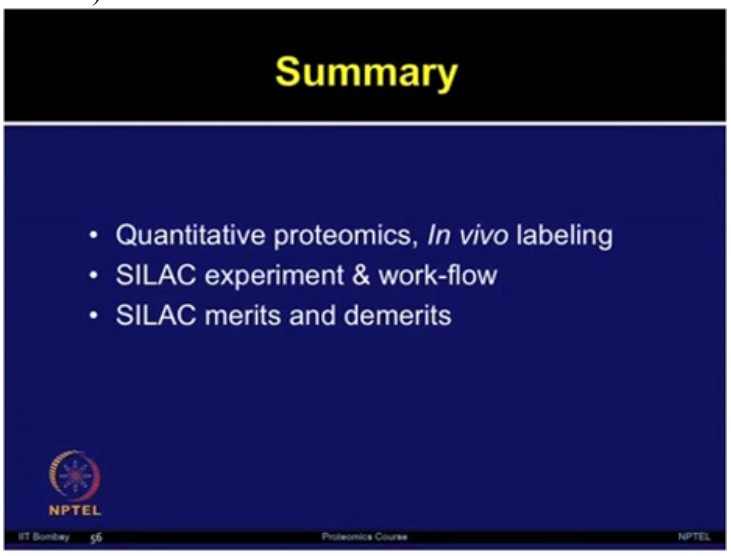

So in SILAC, in a typical experiment the control and treated cell; the cell lines are grown in different media. One was enriched with analyte form and other with the heavy isotope containing amino acids.

The peptides from each heavy and light 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 arginine 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.

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