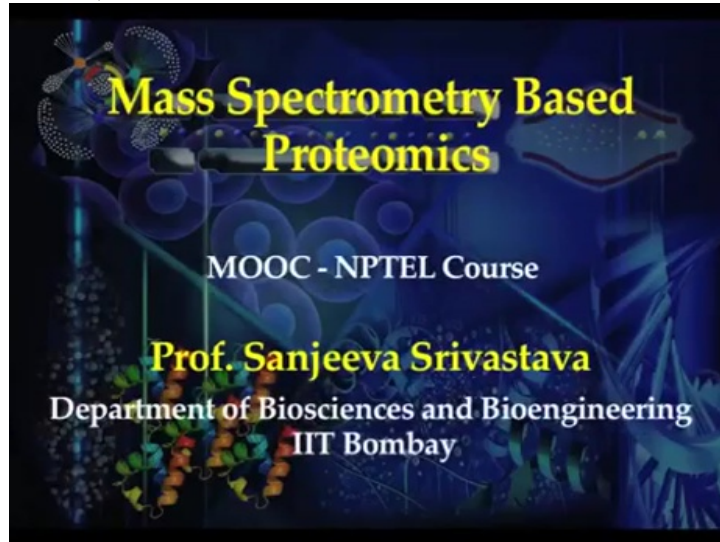
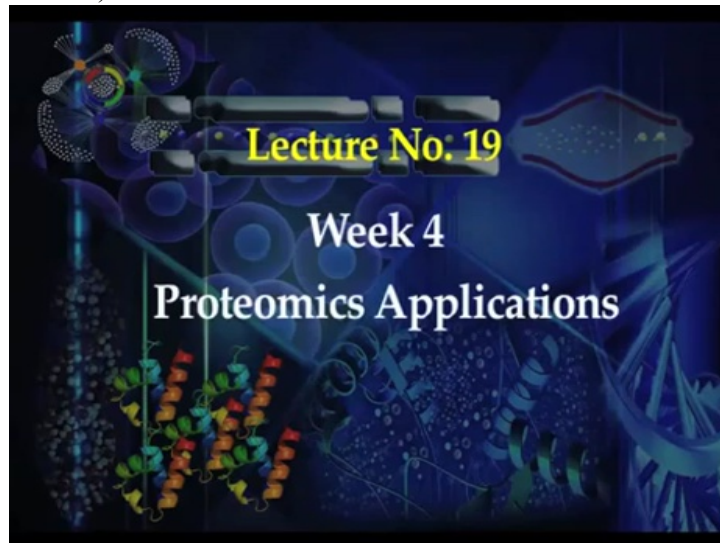


**Mass Spectrometry Based Proteomics**  
**Professor Sanjeeva Srivastava**  
**Department of Biosciences and Bioengineering**  
**Indian Institute of Technology, Bombay**  
**Mod 04 Lecture Number 19**

(Refer Slide Time 00:10)



(Refer Slide Time 00:15)



(Refer Slide Time 00:19)

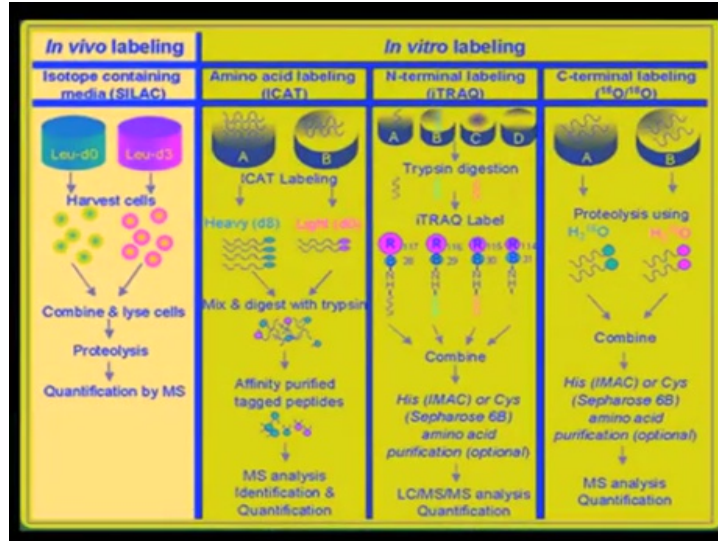
## **Topics to be discussed**

# Quantitative proteomic applications

(Refer Slide Time 00:25)

**Comparison of protein labeling  
methods**

(Refer Slide Time 00:32)



(Refer Slide Time 01:01)

### Quantitative Proteomics: Tagging Strategies

- # SILAC is in-vivo labeling method whereas ICAT, iTRAQ and  $O^{16}/O^{18}$  are in-vitro labeling methods
- # ICAT enables tagging of specific proteins
- # ICAT tags cysteine residues in proteins
- # iTRAQ tags primary amines in proteins
- # SILAC tags lysine and arginine amino acids

(Refer Slide Time 01:03)



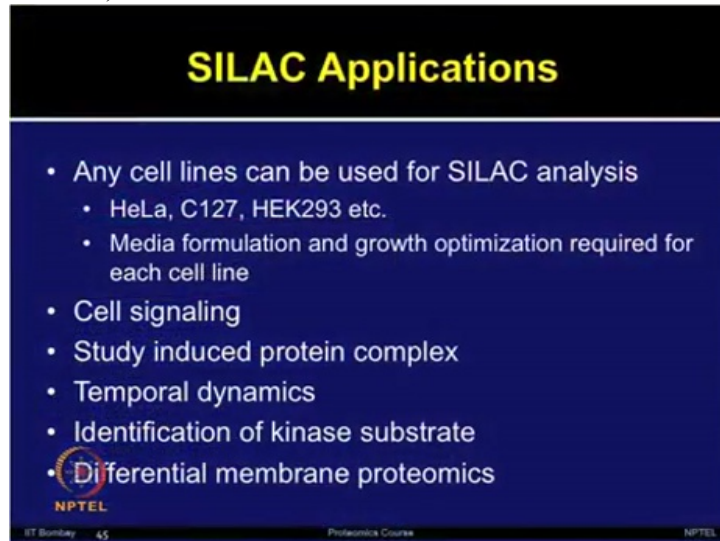
(Refer Slide Time 01:10)



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

(Refer Slide Time 01:24)



**SILAC Applications**

- Any cell lines can be used for SILAC analysis
  - HeLa, C127, HEK293 etc.
  - Media formulation and growth optimization required for each cell line
- Cell signaling
- Study induced protein complex
- Temporal dynamics
- Identification of kinase substrate
- Differential membrane proteomics

NPTEL  
IT Bombay 45 Proteomics Course NPTEL

...any cell line, whether it is HeLa cell, C127, HEK293 or different types 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.

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

(Refer Slide Time 02:20)

**SILAC: applications**

Relative quantitation of changes in protein expression during the time course of myoblast differentiation in mouse C2C12 cells

- SILAC application was first demonstrated in this study
- Ref: Ong et al. (2002) *Mol. Cell Proteomics* 1, 376–386.

NPTEL  
IT Bombay 46 Proteomics Course NPTEL

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

(Refer Slide Time 02:31)

**SILAC: unique metabolic-labeling strategies**

Tyrosine

Identification of tyrosine kinase substrates using [<sup>13</sup>C] tyrosine  
Ref: Ibarrola et al. (2004) *J. Biol. Chem.* 279, 15, 805

← Labeling →

In vivo methylation sites by heavy methyl SILAC  
Ref: Ong, et al. (2004) *Identifying and quantifying Nat. Methods* 1, 119–126

Methionine

NPTEL  
IT Bombay 47 Proteomics Course NPTEL

Researchers have reported various unique metabolic-labeling strategies.

For example, by using tyrosine; identification of tyrosine kinase substrates using <sup>13</sup>C tyrosine Labeling is also performed by using methionine, the in vivo methylation sites by heavy methyl SILAC.

(Refer Slide Time 02:57)

**SILAC: global protein profiling**

Global protein expression profiling

- Analyzed expression levels of > 440 proteins in microsomal fractions of prostate cancer cells with varying metastatic potential  
Ref: Everley et al. (2004) *Mol. Cell Proteomics* 3, 729–735.
- Investigated early stage of apoptosis by inducing the p53 up-regulated modulator of apoptosis  
Ref: Gu et al. (2004) *J. Proteome Res.* 3, 1191–1200.

NPTEL  
IT Bombay 48 Proteomics Course NPTEL

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 the study by Everley et al. in 2004 analyzed the expression levels of more than 440 proteins in the microsomal fractions of prostate cancer cells with varying metastatic potential.

Another study by Gu et al. investigated the early stage of apoptosis by inducing the p53 up-regulated modulator of apoptosis.

SILAC has also been used for functional assays to study the protein-protein interactions.

Study by Blagoev et al. used



(Refer Slide Time 04:00)

**SILAC: protein-protein interactions**

Functional assays to study protein–protein interaction

- Differential labeling of proteins in EGF-stimulated versus unstimulated cells  
- Ref: Blagoev et al. (2003). *Nat. Biotechnol.* 21, 315–318
- Quantification of proteins interacting in an attachment-dependent manner with focal adhesion proteins  
- Ref: de Hoog, C. L., Foster, L. J., and Mann, M. (2004) *Cell* 117, 649–662

NPTEL  
IT Bombay 49 Proteomics Course NPTEL

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

Study by de Hoog et al. did quantification of proteins interacting in an attachment-dependent manner with focal adhesion proteins.

These are 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; a study by Foster et al ...

(Refer Slide Time 04:42)

**SILAC: functional analysis**

Identification of proteins enriched in specific cellular structures

- First functional proteomic analysis of rafts
- Specific detection of proteins depleted from rafts by cholesterol-disrupting drugs  
- Ref: Foster, et al. (2003). *Proc. Natl. Acad. Sci. USA* 100, 5813–5818.

NPTEL  
IT Bombay 50 Proteomics Course NPTEL



...used the first functional proteomic analysis of rafts and they showed that 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.

(Refer Slide Time 05:04)

**SILAC: comparison of cellular state**

Multiplexed analysis to compare cellular states

- Quantitative analysis of the proteome of human nucleoli  
- Ref: Andersen et al. (2005). *Nature* 433, 77–83
- Temporal analysis of phosphotyrosine-dependent signaling networks to compare proteome of three cell populations  
- Ref: Blagoev et al. (2004). *Nat. Biotechnol.* 22, 1139–1145
- Analysis of divergent growth factors in mesenchymal stem cell differentiation  
- Ref: Kratchmarova et al. (2005). *Science* 308, 1472–1477

NPTEL  
IT Bombay 51 Proteomics Course NPTEL

Andersen et al. showed the quantitative analysis of the proteome of human nucleoli.

Blagoev et al. performed a temporal analysis of phosphotyrosine-dependent signaling networks to compare the proteome of three cell populations.

Kratchmarova et al. analyzed the divergent growth factors in mesenchymal stem cell differentiation. These are just few examples of multiplex analysis.

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

SILAC method has also been used to study the protein turnover.

Study by Pratt et al. used the rate of breakdown of individual proteins by analysis of mass shifts

(Refer Slide Time 06:02)

**SILAC: protein turnover study**

Studying protein turnover

- Rate of breakdown of individual proteins by analysis of mass shifts in tryptic fragments
- Analysis of abundant proteins in glucose-limited yeast cells grown in aerobic chemostat culture at steady state
- Ref: Pratt et al. (2002). *Mol. Cell Proteomics* 1, 579–591

NPTEL  
IT Bombay 52 Proteomics Course NPTEL

...in tryptic peptide fragments

The analysis of abundant protein 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 quantification of protein post-translational modifications.

(Refer Slide Time 06:30)

**SILAC: Posttranslational modifications**

Identification and quantitation of protein posttranslational modifications

- Identification and quantitation of phosphorylation sites
- Ref: Ibarrola et al. (2003) *Anal. Chem.* 75, 6043–6049
- Phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling
- Ref: Ballif, et al. (2005) *Proc. Natl. Acad. Sci. USA* 102, 667–672

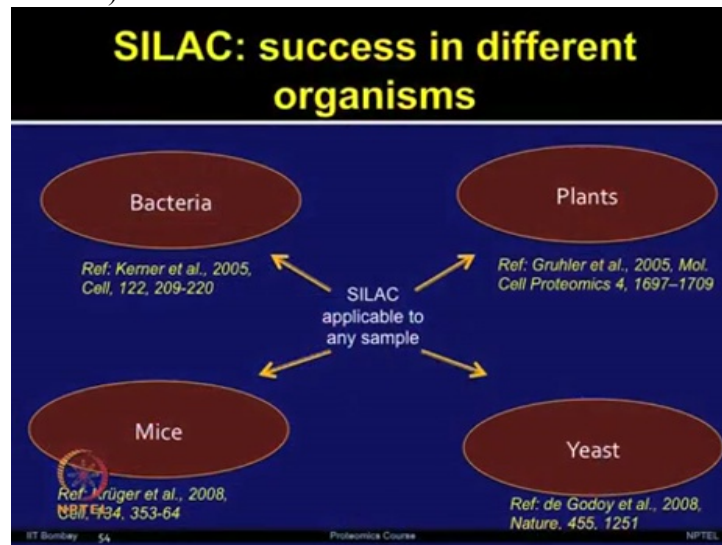
NPTEL  
IT Bombay 53 Proteomics Course NPTEL

Study by Ibarrola et al. identified and quantitated phosphorylation sites.

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

Interestingly, now SILAC method has been used in different organisms; in bacteria...

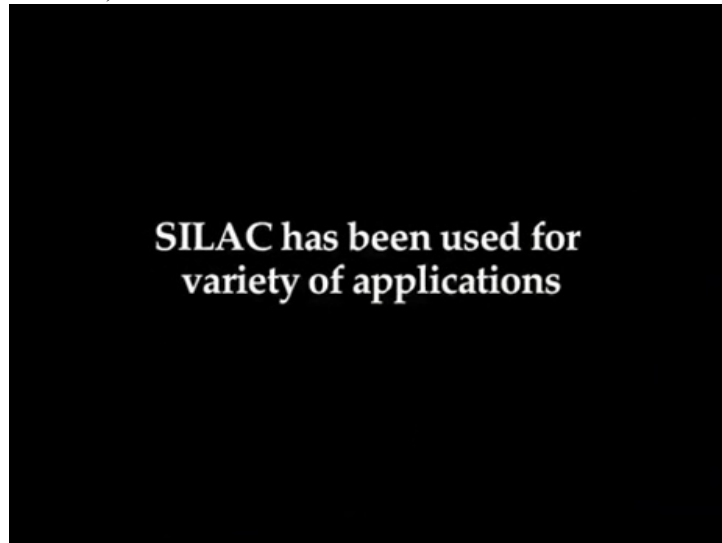
(Refer Slide Time 07:06)



...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 organisms.

(Refer Slide Time 07:28)

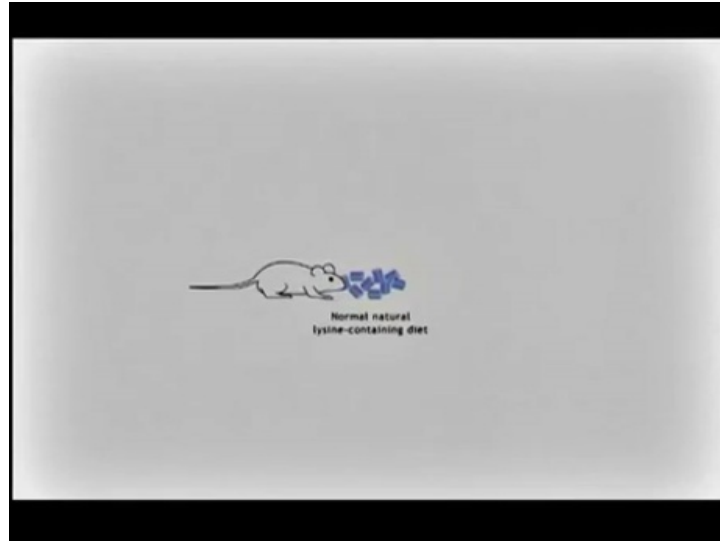


(Refer Slide Time 07:36)



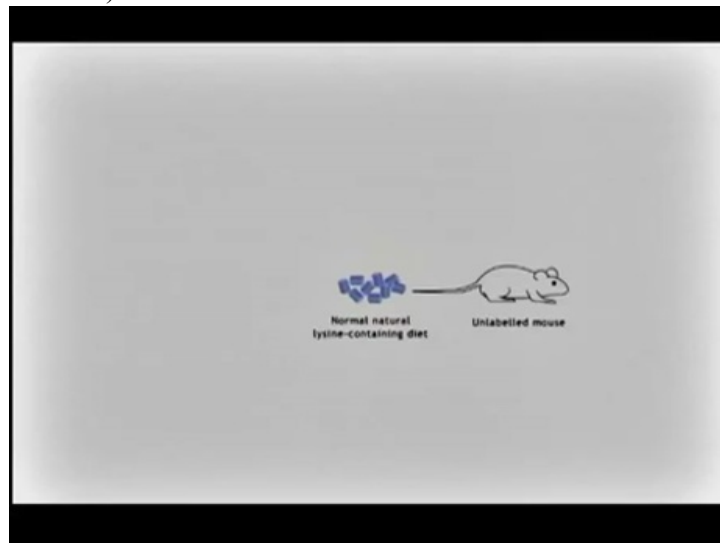
In an experiment conducted by Kruger and colleagues

(Refer Slide Time 07:38)



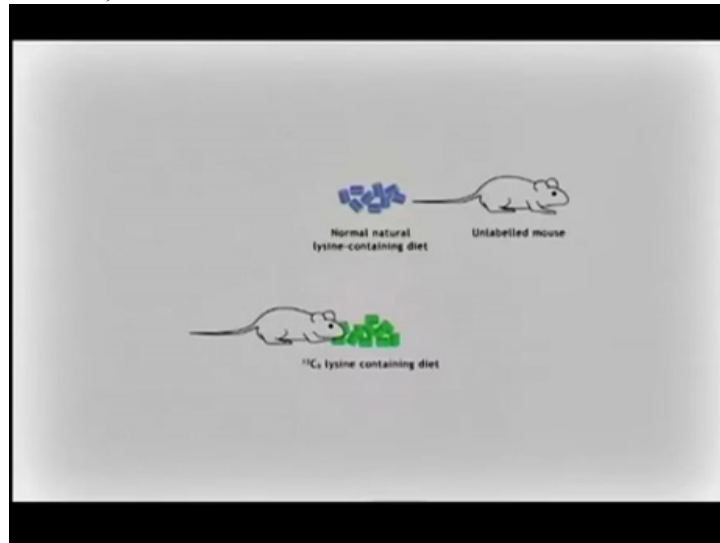
, a set of mice were fed

(Refer Slide Time 07:41)



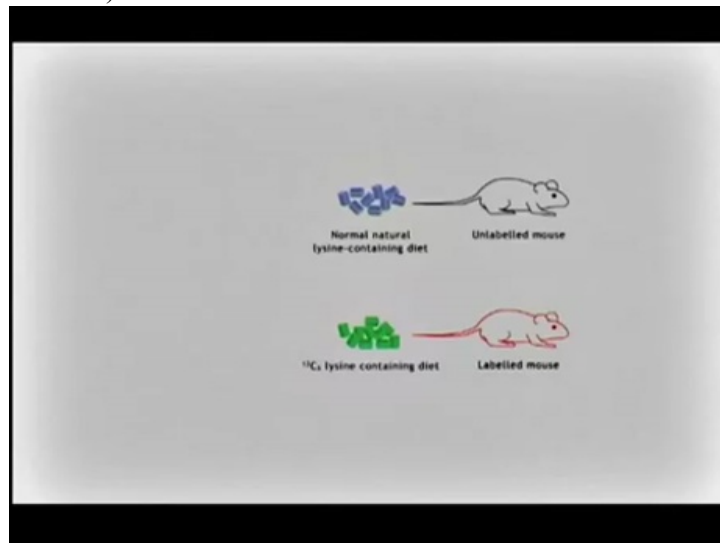
with diet containing natural lysine Another set of mice were fed

(Refer Slide Time 07:50)

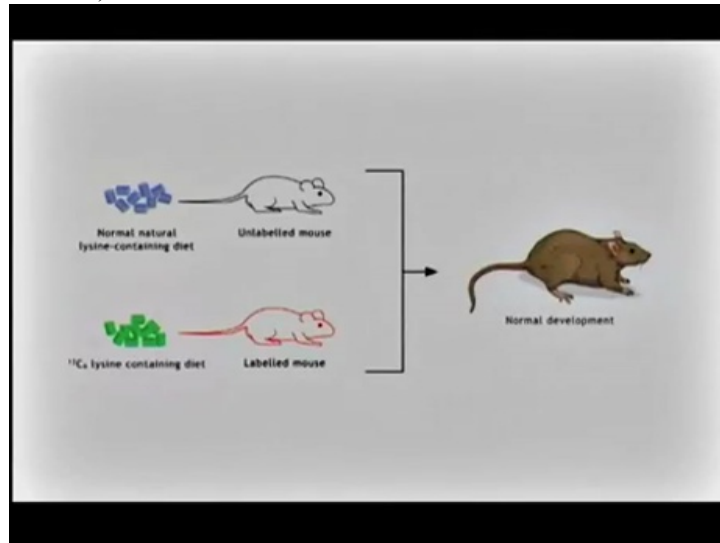


...with diet containing C13 labeled lysine.

(Refer Slide Time 07:58)



(Refer Slide Time 08:03)



The researchers tracked the incorporation of Lysine 6 into the mouse proteome over 4 weeks by providing the C13 containing lysine dye.

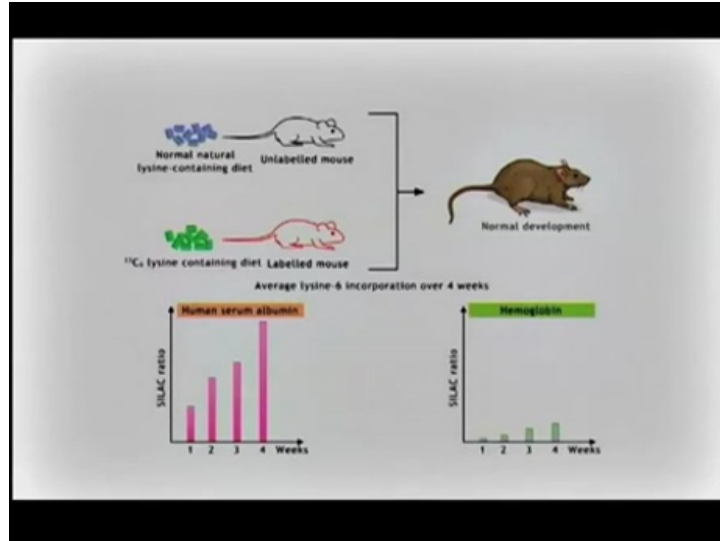
(Refer Slide Time 08:18)



Their developmental growth and behavior were observed in addition to sampling various blood proteins. The labeled mice were found to develop normally



(Refer Slide Time 08:31)



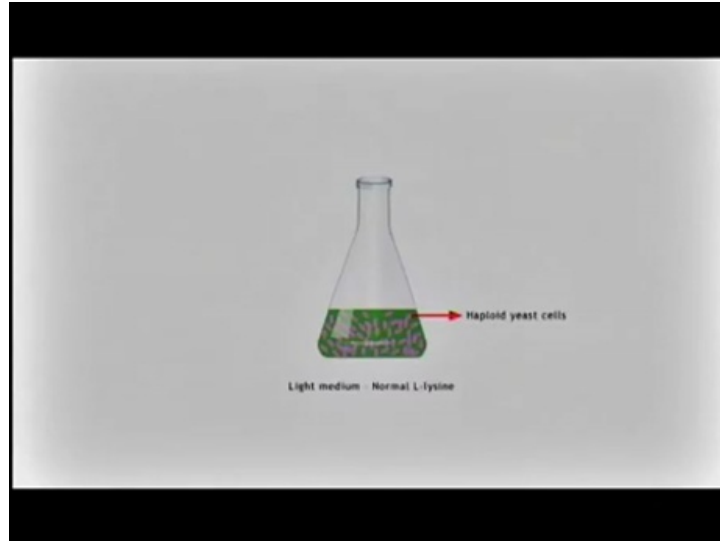
... average Lysine 6 incorporation over 4 weeks in human serum albumin and hemoglobin is depicted in the graph

(Refer Slide Time 08:42)



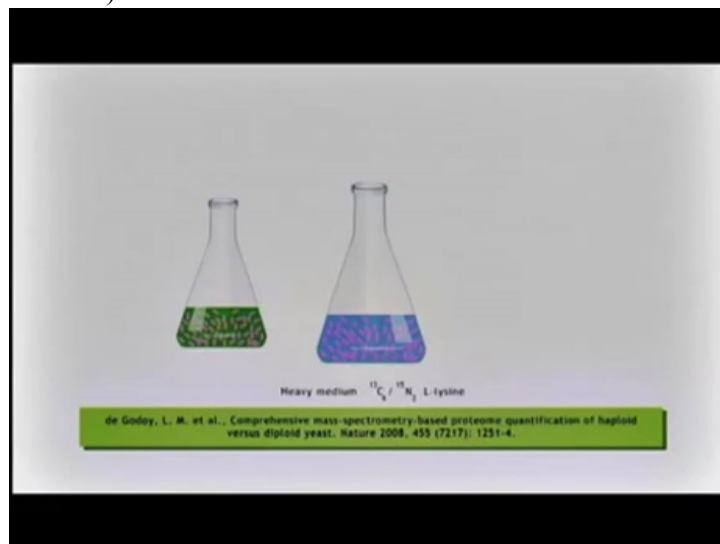
de Godoy and colleagues determined the fold change

(Refer Slide Time 08:46)



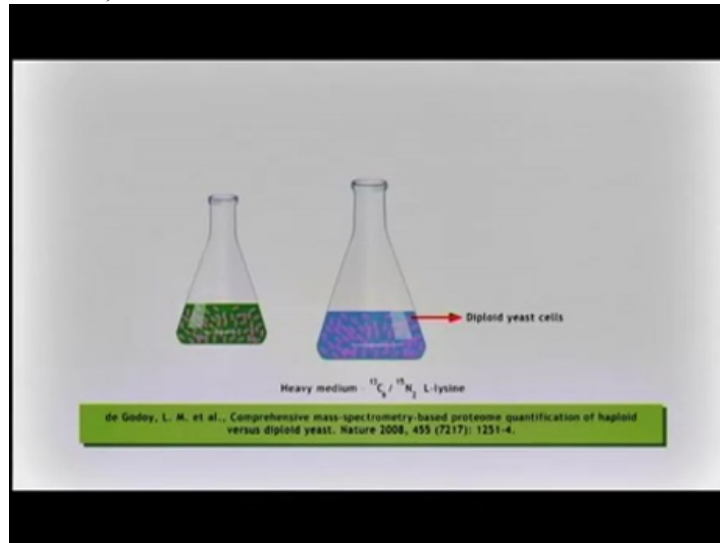
of peptide pairs between haploid and diploid yeast cells using SILAC. Labeled lysine residues were used to grow the diploid yeast cells

(Refer Slide Time 08:57)

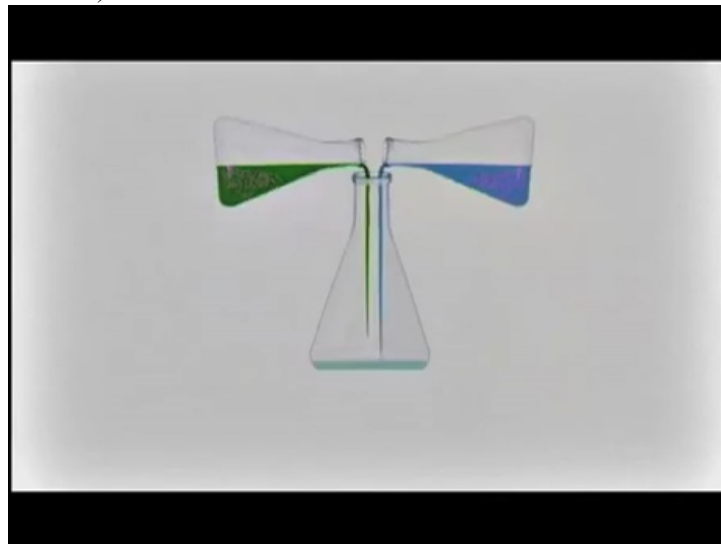


...while haploid cells were grown in normal lysine medium

(Refer Slide Time 09:03)

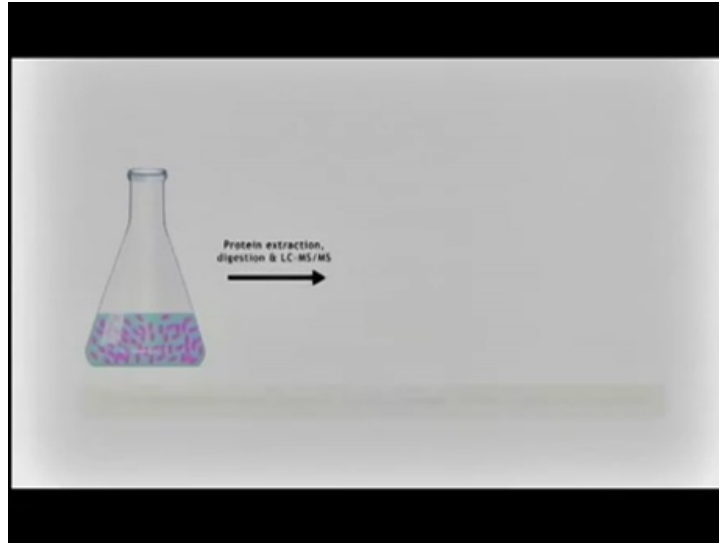


(Refer Slide Time 09:17)

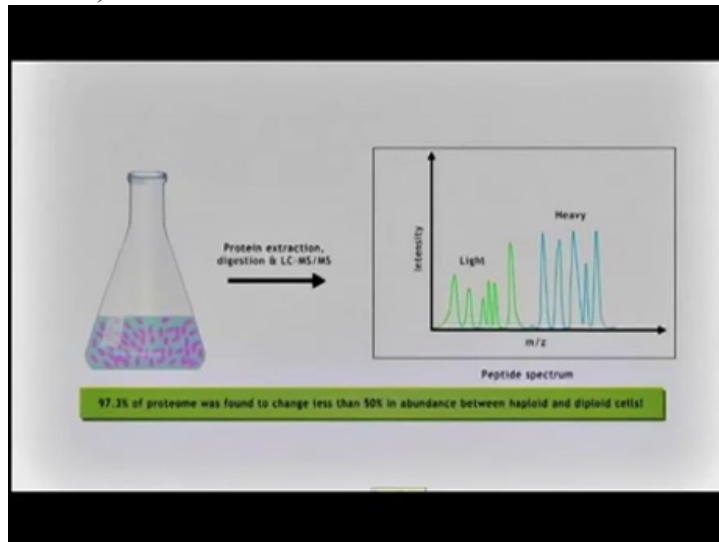


The cultures were mixed, proteins extracted and analyzed by LC-MS/MS.

(Refer Slide Time 09:27)



(Refer Slide Time 09:30)



Protein ratios between haploid and diploid cells were determined with high accuracy. Comparison revealed 97.3% of the proteome changes less than 50% in abundance between haploid and diploid cells.

(Refer Slide Time 10:03)

## **Tumor Progression**

- # Tumor metastasis is one of the interesting field of research
- # Tumor progress in different stages and difficult to find the changes in early stage
- # SILAC based studies have helped to study the expression of proteins at different stages to understand tumor progression
- # Gieger et al., had quantified 7000 proteins in breast cancer patients with SILAC using MS based approach
- # Validation of the biomarkers were performed using immunohistochemistry

(Refer Slide Time 10:10)

## **Quantitative proteomic applications**

iTRAQ applications

(Refer Slide Time 10:16)



Let us briefly look at the iTRAQ applications and I will show you this in this animation. In this animation we will look at one application of iTRAQ method.

A study performed by Boylan et al. in 2010 used iTRAQ for the identification of candidate biomarkers in ovarian cancer serum.

(Refer Slide Time 10:50)



Serum samples from 1 control and

(Refer Slide Time 10:55)



... cancer patients were first of all, depleted by using ...

(Refer Slide Time 11:00)



...a multiple affinity removal system to carry out immune-depletion of serum samples from normal controls and ovarian cancer samples



(Refer Slide Time 11:14)



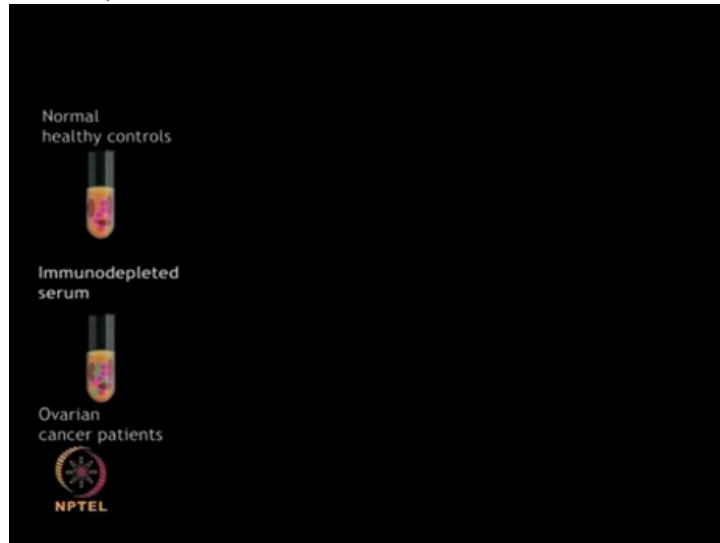
This step helped in removing the high abundance proteins, leaving behind only the medium and

(Refer Slide Time 11:22)



low abundance proteins

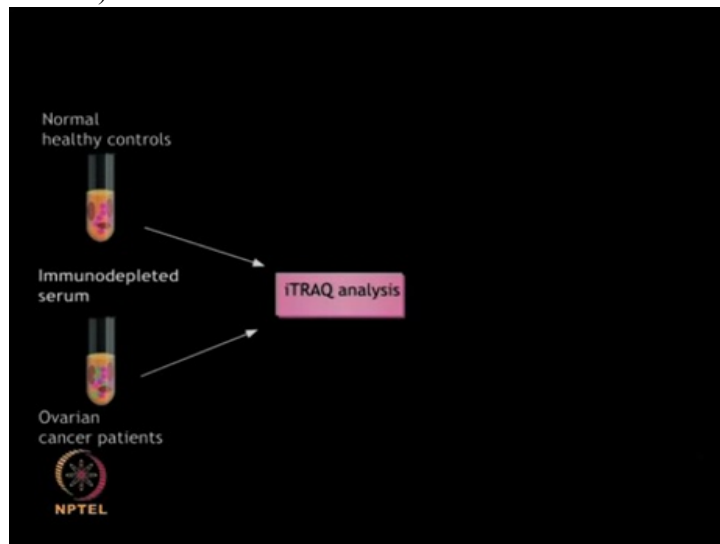
(Refer Slide Time 11:24)



... for iTRAQ analysis

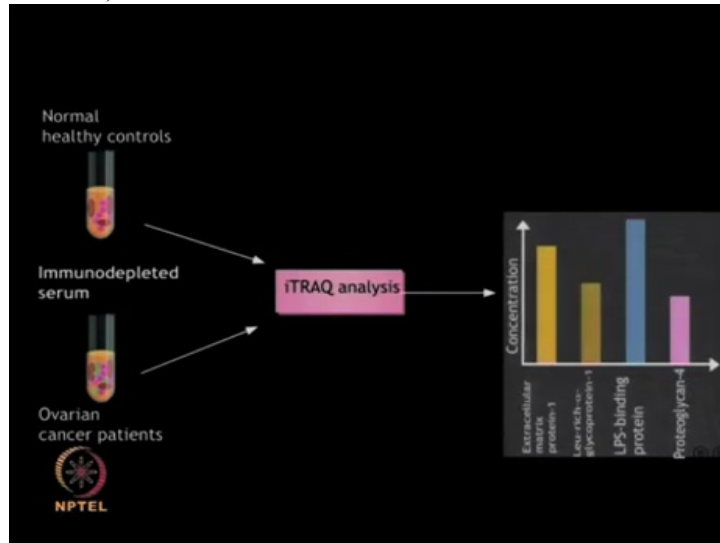
The immuno-depleted serum samples were then labeled with the iTRAQ reagents ...

(Refer Slide Time 11:35)



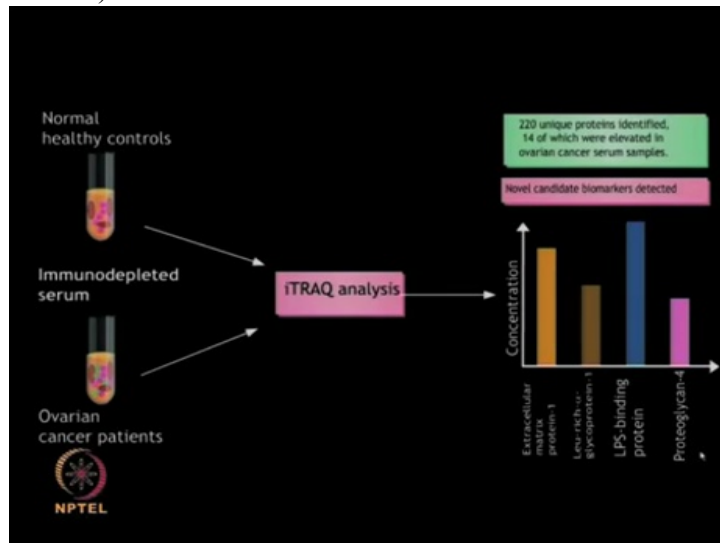
...and further analyzed...

(Refer Slide Time 11:40)



... in MS/MS

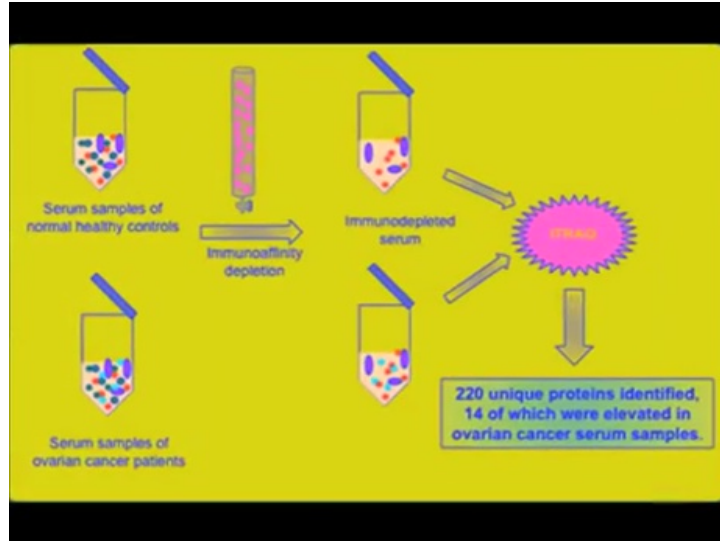
(Refer Slide Time 11:45)



The authors detected a total of 220 unique proteins of which 14 were found to be elevated in the ovarian cancer serum samples as compared to the healthy controls and 4 novel candidate biomarkers were first time reported.

These results were further validated by Western immunoblotting. This just gives you an overview of how iTRAQ reagents can be used for various types of applications including biomarker discovery.

(Refer Slide Time 12:30)

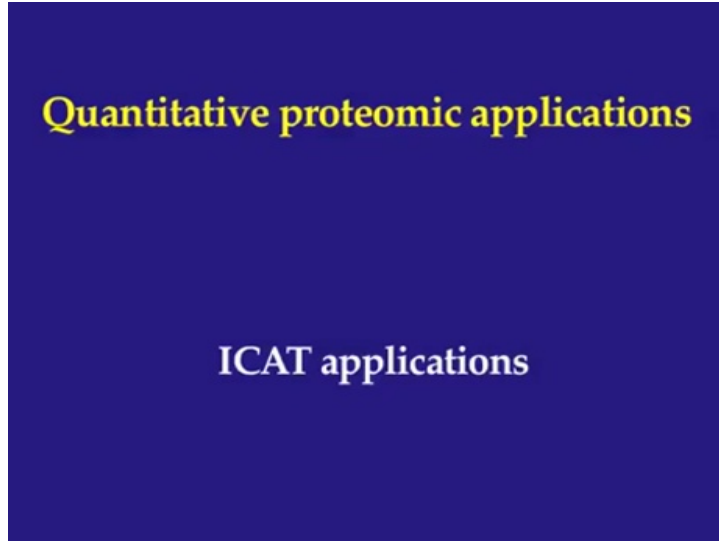


(Refer Slide Time 12:49)

## Biomarker Discovery

- # Biomarker detection is very essential for early disease detection
- # As discussed in this example, biomarker discovery in colorectal cancer was performed using iTRAQ labeling followed by MS analysis
- # Orosomucoid 2, 24 kDa is an inflammatory protein and was found to be up-regulated
- # The validation of the potential target was performed using ELISA

(Refer Slide Time 12:55)

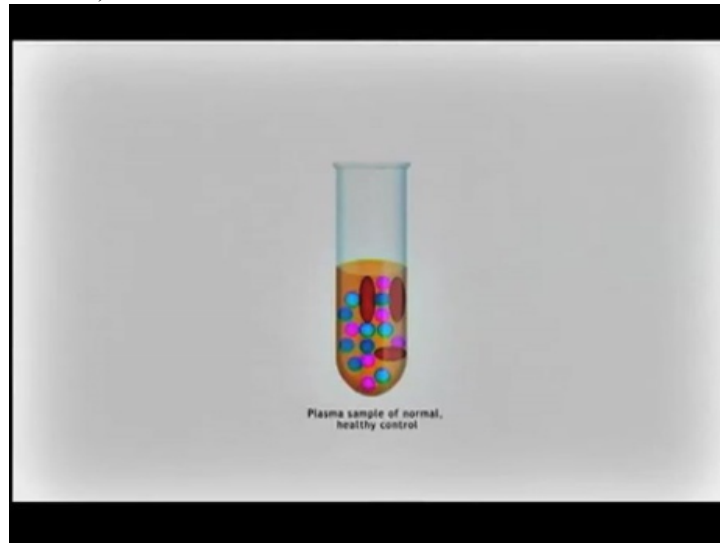


(Refer Slide Time 13:01)



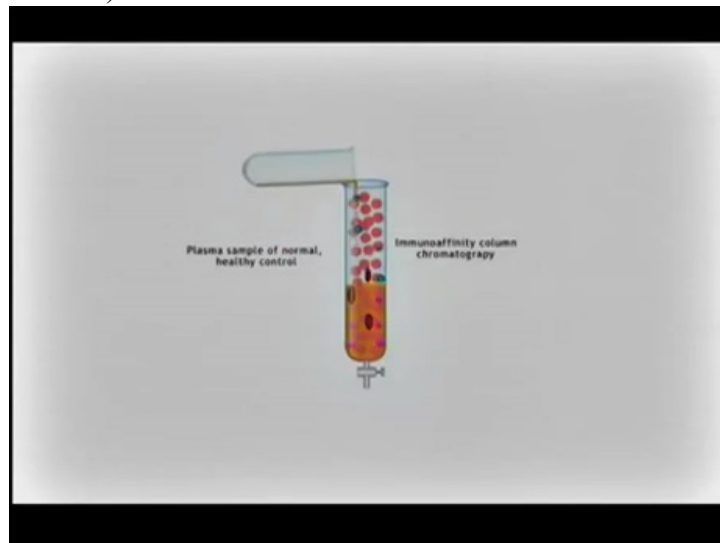
ICAT has found many applications for proteomic studies. In one such clinical application, Kang and coworkers obtained ...

(Refer Slide Time 13:15)



...plasma proteome from 6 breast cancer patients and 6 healthy controls.

(Refer Slide Time 13:22)



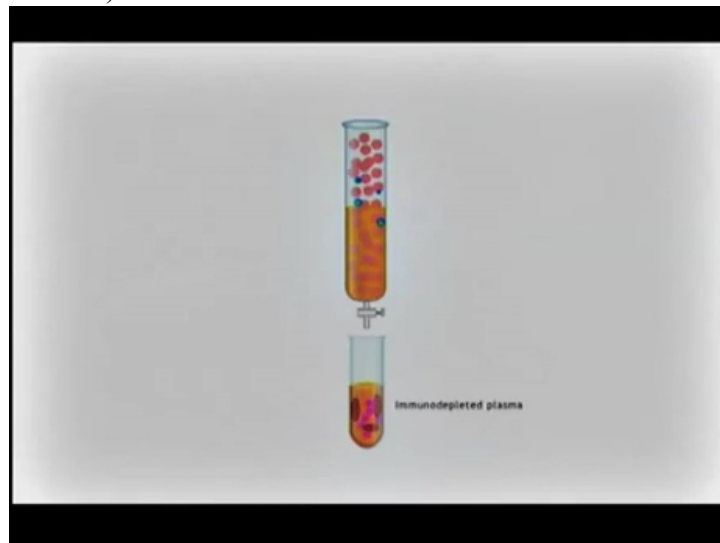
These plasma samples were first treated

(Refer Slide Time 13:27)



... on an immune affinity column ...

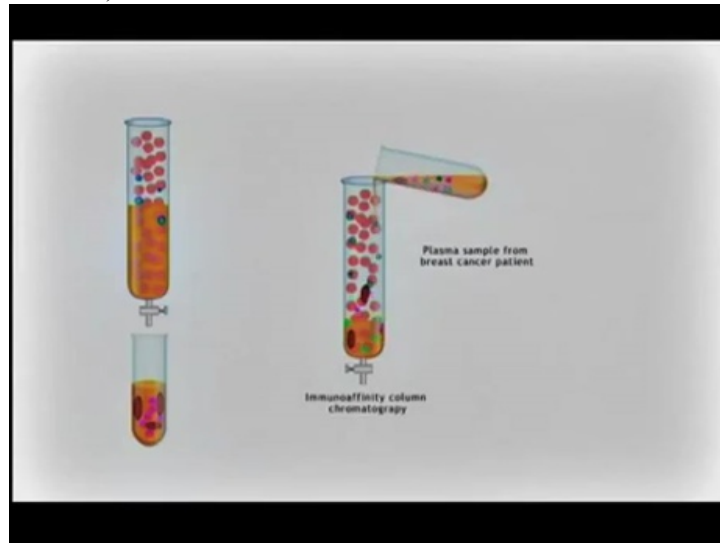
(Refer Slide Time 13:30)



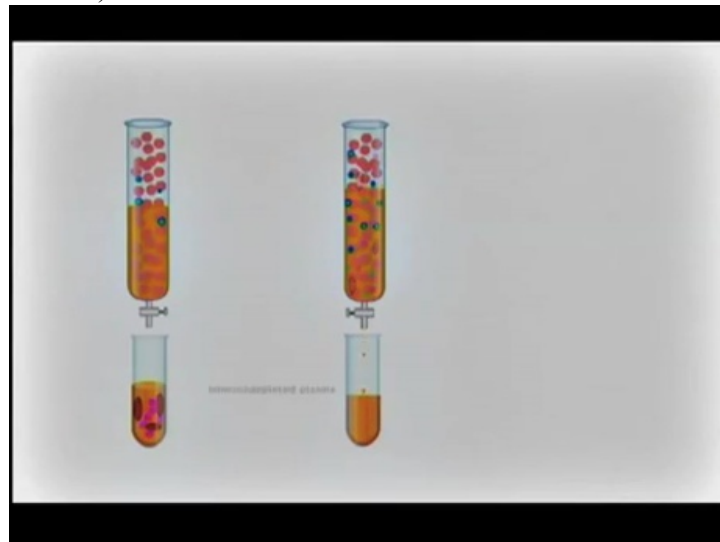
...in order to deplete them of their high abundance proteins



(Refer Slide Time 13:40)

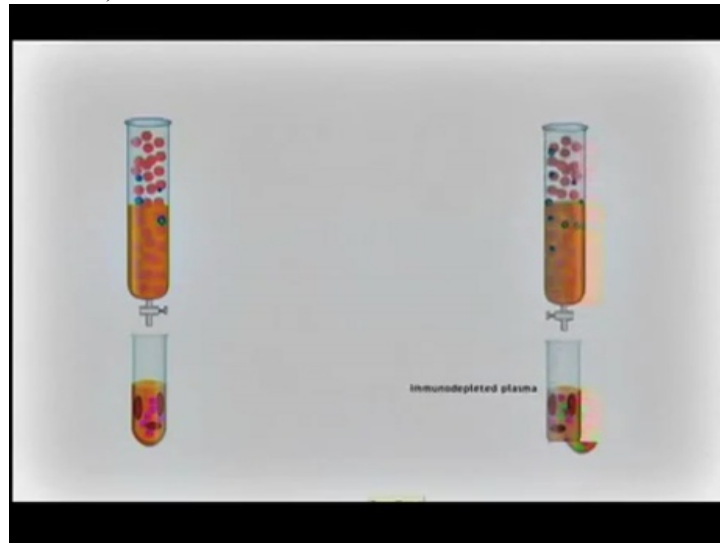


(Refer Slide Time 13:46)



The immuno-depleted serum samples were then used for

(Refer Slide Time 13:54)



further analysis by ICAT.

(Refer Slide Time 14:03)



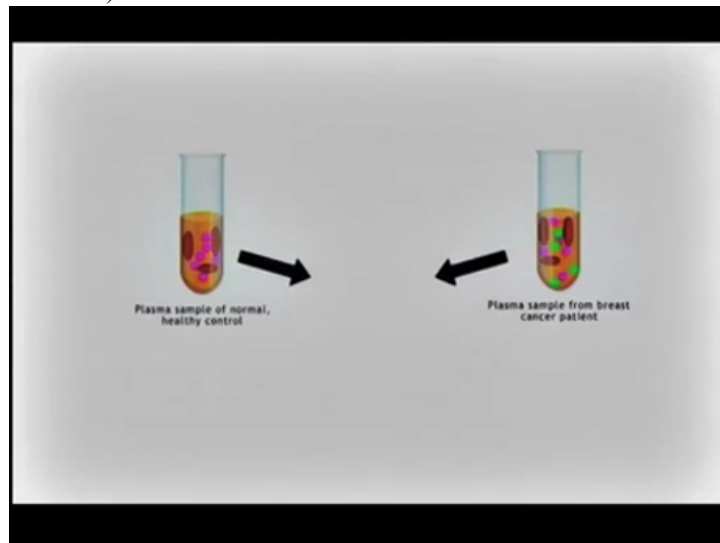
These samples were first treated on immune-affinity column

(Refer Slide Time 14:07)



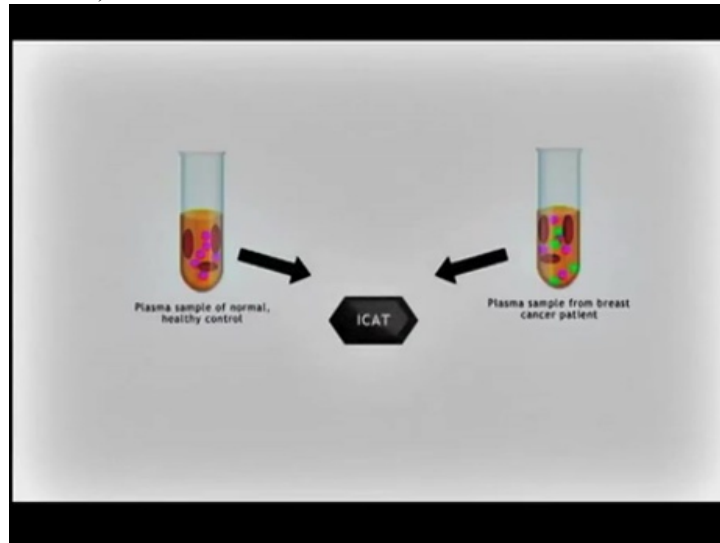
...in order to deplete the of their high abundance proteins

(Refer Slide Time 14:14)



The immuno-depleted serum samples were then used ...

(Refer Slide Time 14:16)



... for further analysis...

(Refer Slide Time 14:20)



...by ICAT. 155 proteins were identified...

(Refer Slide Time 14:30)



... of which 33 showed 1.5-fold abundance changes in plasma of breast cancer patients as compared with the healthy controls

(Refer Slide Time 14:54)

- Breast cancer plasma proteome analysis**
- # Dynamic protein expression profiles of plasma from healthy and breast cancer samples were analyzed
  - # Depletion of the high abundant proteins from plasma had been performed
  - # ICAT labeling followed by mass spectrometry analysis was used for establishing the protein expression profile
  - # Affinity chromatography was used for pre-fractionation
  - # 115 differential expressed proteins were identified

(Refer Slide Time 14:59)

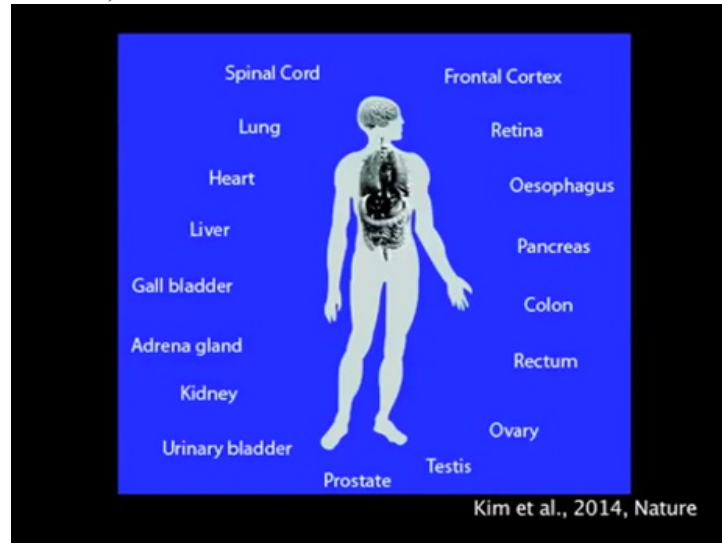


(Refer Slide Time 15:11)



In 2010, Human Proteome Organization has launched a global Human Proteome Project HPP. This project is designed to map the entire human proteins encoded by the genome.

(Refer Slide Time 15:31)



(Refer Slide Time 15:50)

### Experimental Information

- # 17 adult tissues, 7 fetal tissues and 6 primary haematopoietic cells were used for analysis
- # Protein samples were separated on SDS-PAGE and digested with trypsin
- # Liquid chromatography coupled with Orbitrap Velos was used for protein identification
- # Label-free quantification method was used for quantitation
- # Protein validation was performed using western blotting
- # Proteogenomic analysis was performed further to explore the proteomic data

(Refer Slide Time 16:11)

### Major Findings

- # The first human proteome reference map was drafted from data derived from mass spectrometry
- # 17,294 human proteins were detected in this study
- # This high resolution mass spectrometry covered 84% of the human proteome
- # This study revealed many novel protein coding genes
- # This study also provided information about pseudogenes, non-coding RNAs, Upstream ORFs
- # Proteogenomic analysis provided novel peptides and proteins

(Refer Slide Time 16:17)

The diagram shows a human silhouette with various tissues and organs labeled. The labels are arranged in two columns on either side of the body. The left column labels include: Cerebral Cortex, Hair Follicle, Creamers, Salivary gland, Saliva, Lung, Thyroid gland, Lymph node, Gall bladder, Adipocytes, Adrenal gland, Liver, Kidney, Prostate gland, Seminal vesicle, Seminal plasma, and Testis. The right column labels include: Cerebrospinal fluid, Vitreous humour, Oral epithelium, Tomils, Esophagus, Heart, Brain, Milk, Stomach, Spleen, Skin, Colon, Pancreatic Juice, Rectum, Uterus, Cervical mucosa, and Blood.

Wilhelm et al., 2014, Nature



(Refer Slide Time 16:40)

## **Experimental Information**

- # 60 tissues, 13 body fluids, 147 cell lines, 1300 affinity purifications were used for analysis
- # More than 10,000 MS raw files were analyzed from different data repositories
- # Liquid chromatography coupled with Orbitrap Velos was used for protein identification
- # Label-free quantification was used for quantitation

(Refer Slide Time 16:59)

## **Major Findings**

- # The first human proteome reference map was drafted from data derived from mass spectrometry
- # 19,629 human proteins were detected in this study
- # The high resolution mass spectrometry covered 92% of the human proteome
- # Many previously missing proteins had been detected
- # Provided information about the function and expression of human proteins
- # Identified organ specific proteins and translated LincRNAs
- # The complete data was deposited in ProteomeDB

(Refer Slide Time 17:05)



Let us now discuss some of the targeted focus initiatives.

The Human Proteome Liver Project, this is the first initiative for Human Proteome Project for human organ, tissues with an intention of generation of comprehensive protein atlas of the liver and international liver tissue network, collection and distribution of normal liver samples and validation of new discoveries.

(Refer Slide Time 18:00)

**Human Liver proteome project**

- # First initiative of human proteome project
- # This project was initiated on April 2002
- # Complete proteome of liver and its expression profile modifications, protein-protein interaction and localization map
- # It also aims to study the ORFeome, physiome and pathome of the liver
- # LC-MS/MS is the major platform used to study the liver proteome

(Refer Slide Time 18:05)



Human Plasma Proteome Project, analysis of the protein constituents of human plasma and serum.

(Refer Slide Time 18:25)

**Human Plasma Proteome Project (HPPP)**

- # This is one of the initiative of human proteome project
- # Plasma/serum is the rich source of proteins and reflects the physiological status of the human
- # Plasma/serum is widely used for biomarker detection
- # Currently, HPPP has information for more than 10000 proteins from plasma/serum
- # The data is mostly derived from mass spectrometry and MRM/SRM analysis

(Refer Slide Time 18:36)



Human Brain Proteome Project BPP focuses on the revolution of the brain related proteomics alteration, focusing on understanding neuro-degenerative diseases, aging and identification of prognostic and diagnostic biomarkers.

(Refer Slide Time 19:07)

**Brain proteome project (BPP)**

- # This is one of the initiative of human proteome project
- # Aims to decipher the brain proteome from human and mouse in both health and neuro-degenerative diseases
- # Quantitative proteome analysis using LC-MS/MS was performed for proteomic data
- # Also compared proteome data with complementary gene expression data
- # Enhances the neuroproteomics filed for better diagnosis of neuro-degenerative diseases



(Refer Slide Time 19:18)



Human Kidney and Urine Proteome Project aims to understand kidney functions, mechanism of chronic kidney diseases at a protein level and discover biomarkers and target molecules for due therapeutics of kidney diseases.

(Refer Slide Time 19:48)

**Human kidney and Urine proteome project**

- # Initiated to decipher the kidney as well as urine proteome in various kidney diseases
- # Aims to understand the kidney functions and pathogenesis of kidney diseases
- # To establish the biomarker for kidney related diseases to early diagnosis
- # Established standards for sample collection, storage, processing and analysis
- # LC-MS/MS based quantitative approaches was used for proteomic analysis used for proteomic analysis

(Refer Slide Time 19:56)

