Interactomics: Protein Arrays & Label Free Biosensors
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MOOC NPTEL Course
Indian Institute of Technology Bombay
Module 8
Lecture No 37
Application of protein
microarray in biomarker discovery-I

Welcome to mooc interactomics course. In today's lecture we will talk about applications of protein microarrays. The protein microarray technology has potential to one of the integral tools in proteomics field. Due to the enormous potential in diagnostic and drug discovery these high density protein arrays which are printed on glass the arrays have been used for proteome wide screening. Whether it is human, yeast or different type of bacterial species, these wide variety of microarrays have shown different applications and its potential for high throughput studies.

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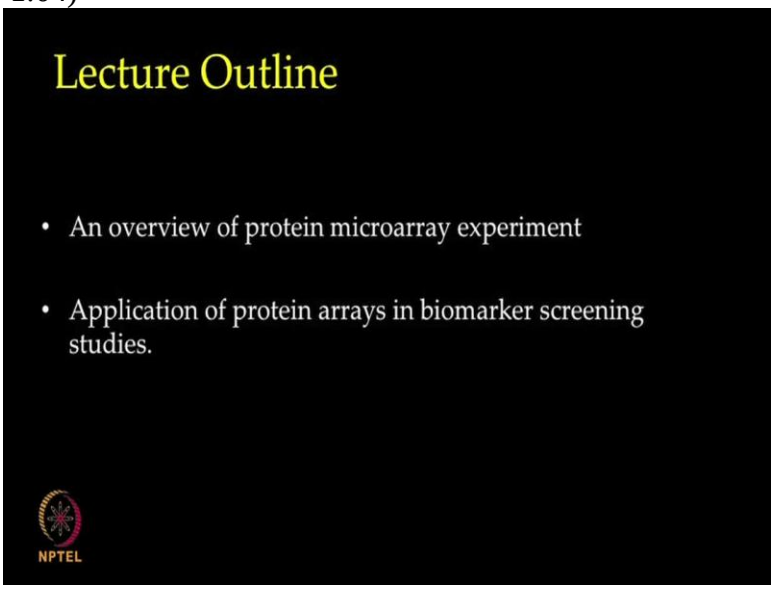

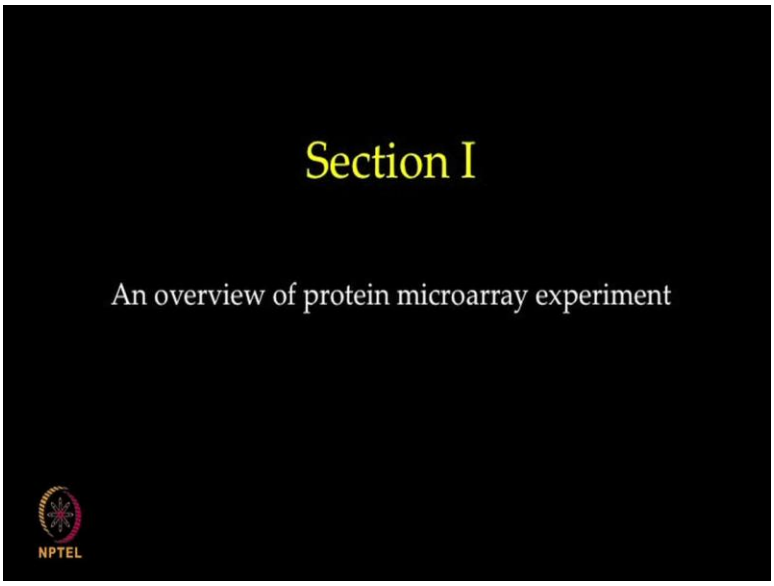

In today's lecture, first I will give you an overview of protein microarray technology which we have already discussed in great detail in the previous lecture. I will then provide you an overview of how to perform protein microarray based experiment and how can it be applied for biomarker screening studies. After discussing the overview and general strategies for protein microarray experiments. We will then talk about different applications in the form of case studies.

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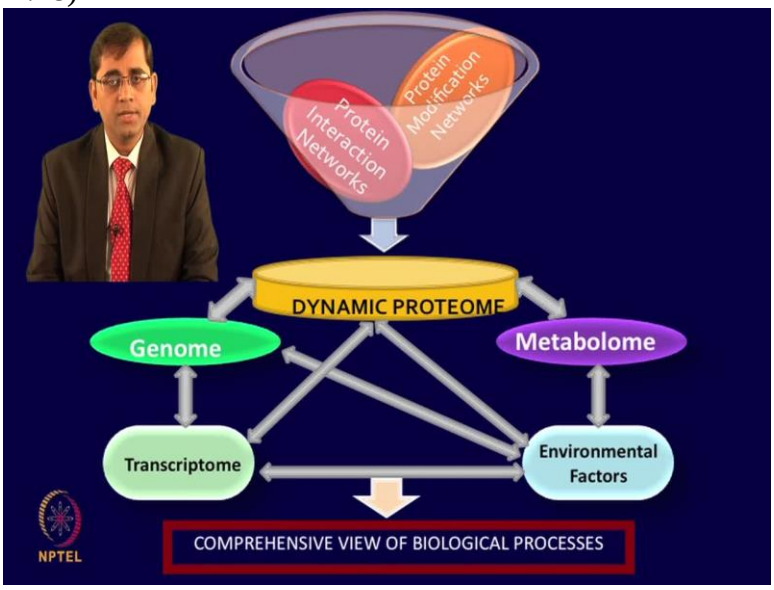

As you know the proteome is very dynamic and it represents very high complexity whether we are studying it at the gnome level, transcriptome or proteome or metabolome level. That is why proteomics on one hand aims to study the various types of biological problems, simultaneously on other hand researchers are trying to integrate various type of homic technologies so that a robust high throughput platform can be generated to study the dynamic proteome.

As you can see in the slide till dynamic proteome are very much linked with physiological actions happening in the biological systems and that is why we need to integrate many of these to obtain the compressive image of what is happening inside eleven system.

We want to study the proteins but we cannot do that in isolation. We need to study them in high throughput manner by identifying to whom they interact and work as a part of team player of a complex signal transaction cascade. To answer many of the questions, protein microarray platform as well as some of the other techniques have been generated and shown potential for high throughput applications.

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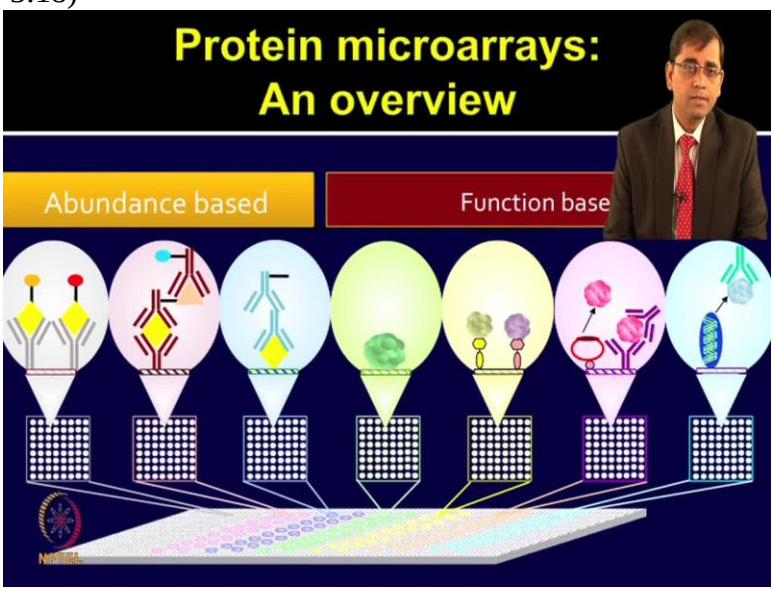

In previous lecture I provided you an overview of different type of protein microarray technology, we discussed both abundance based and function based protein microarray platforms. We have talked about different type of labeling which includes direct labeling, sandwich assay type of technology, we also talked about reverse phase protein arrays and then discussed different ways of making functional based protein arrays.

It includes immobilizing the proteins which is purified or peptide fusen with the tag as well as different type of cell free expression based approaches. With this now I hope you recall all the topics covered this far and that there are many kind of protein microarray platform which could be used to address different type of biological questions.

Let me show you one video to provide an overview of this process of using protein microarrays.

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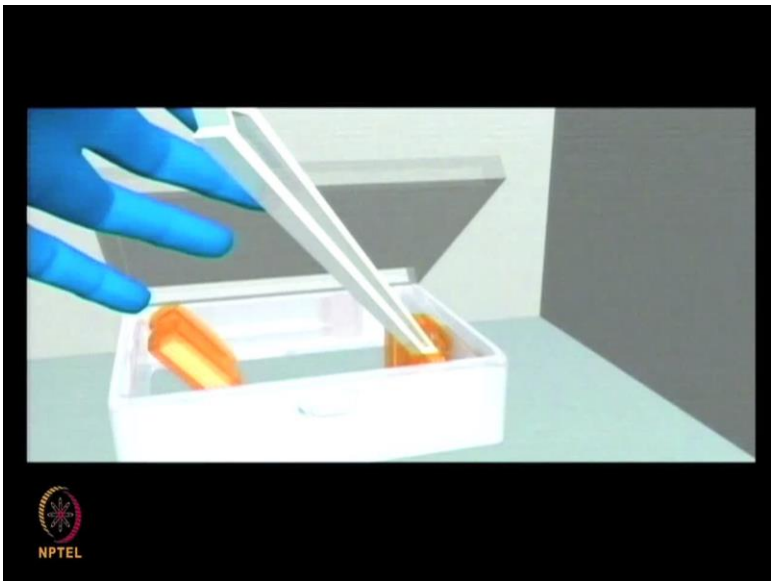
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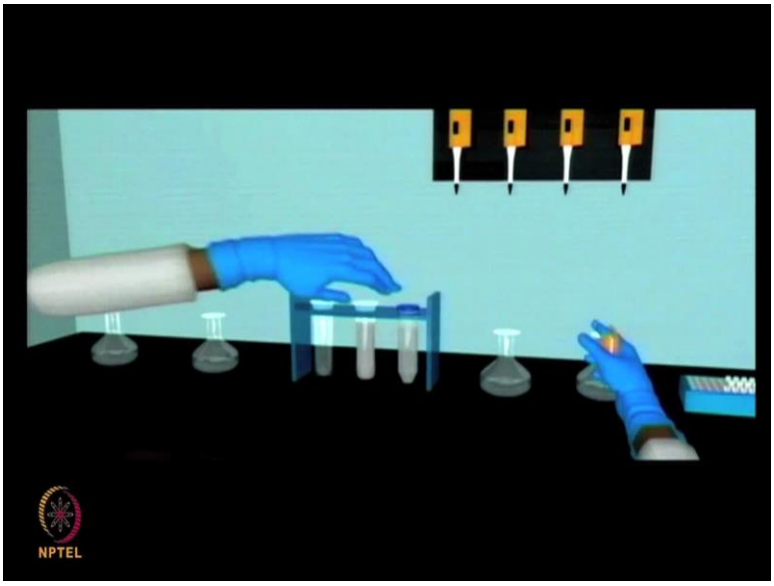
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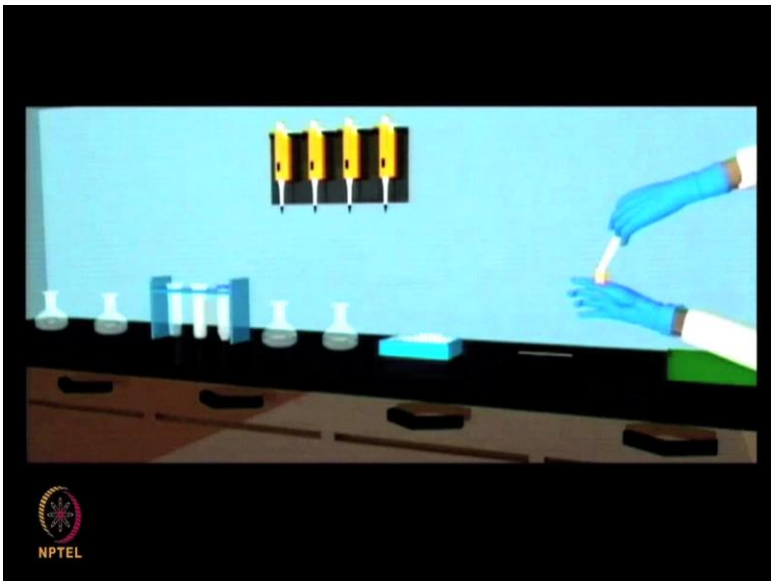
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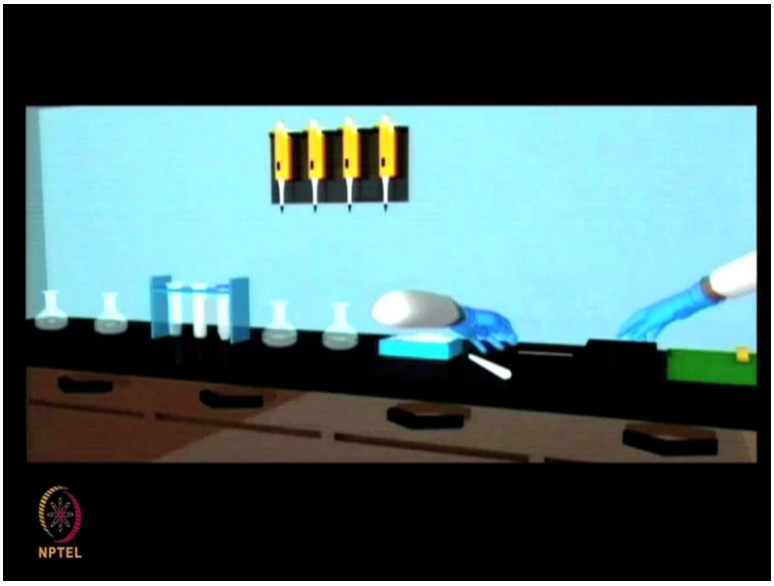
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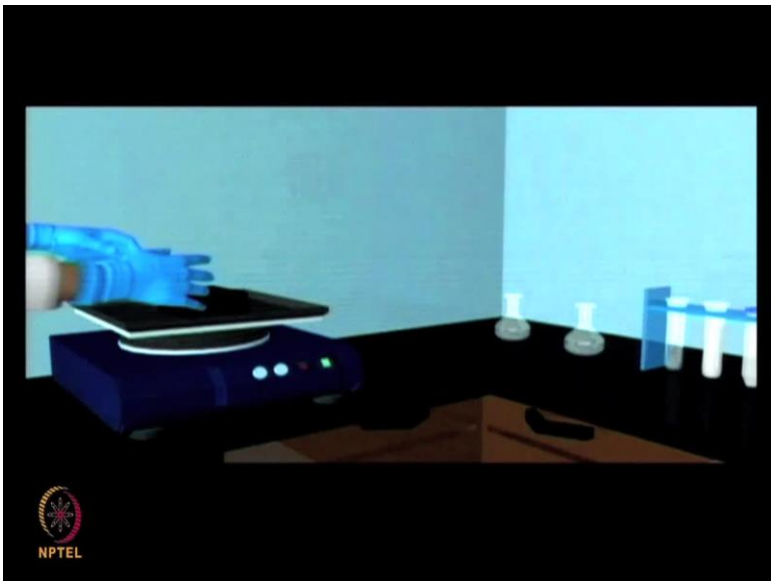
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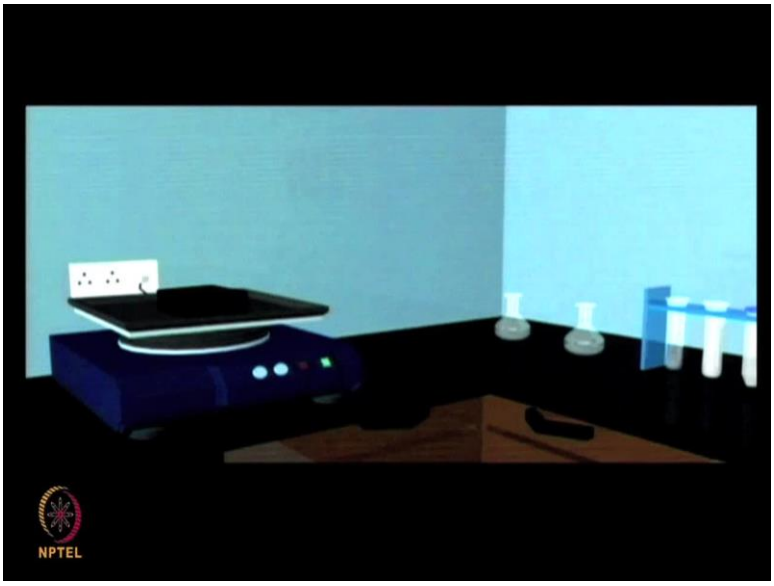
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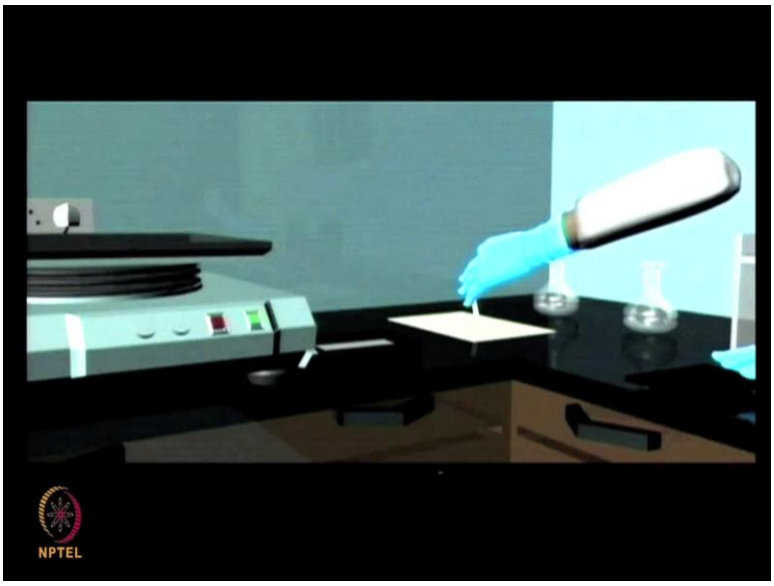
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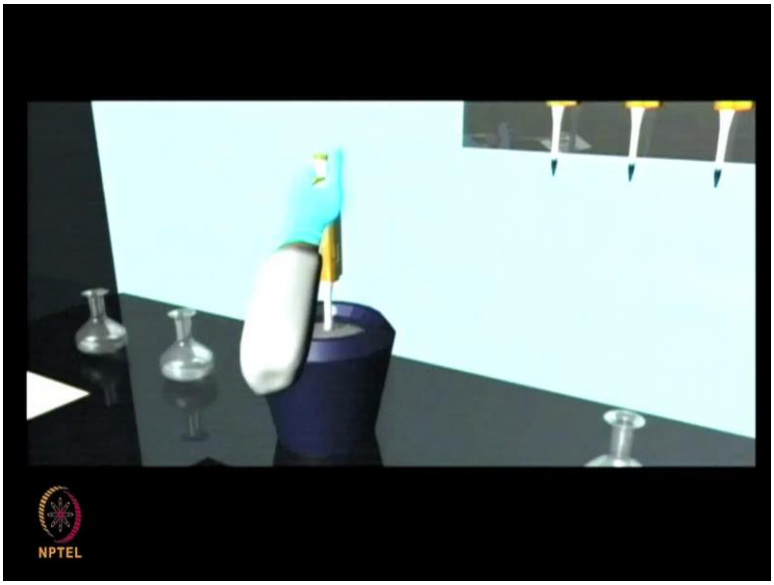
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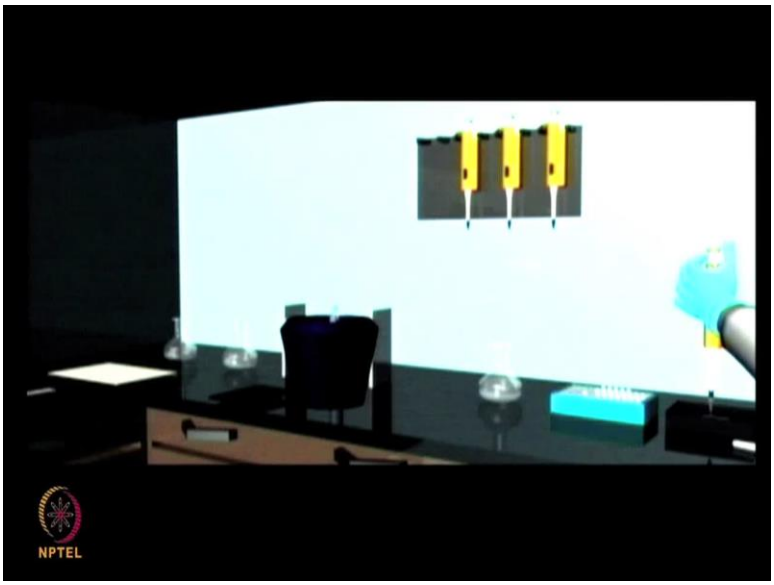
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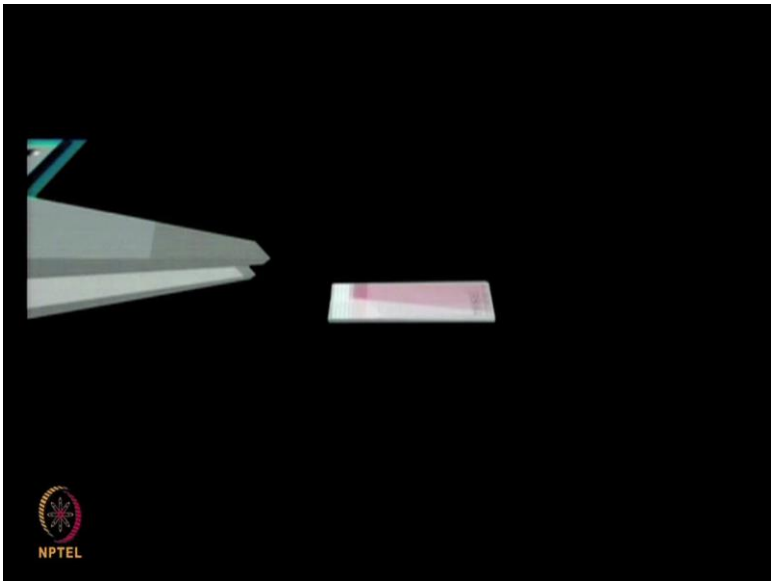
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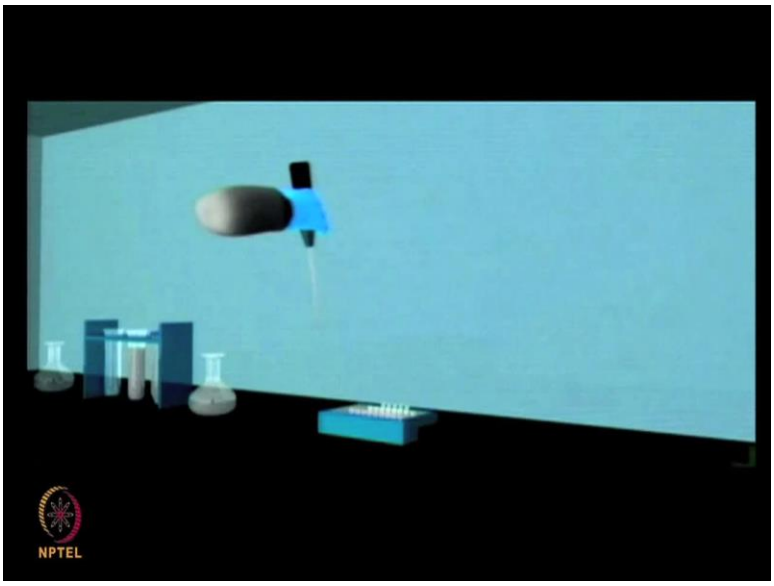
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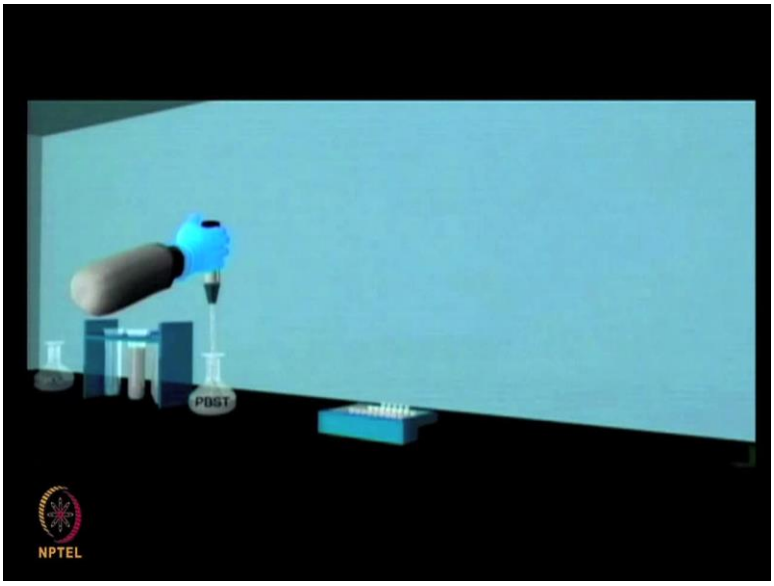
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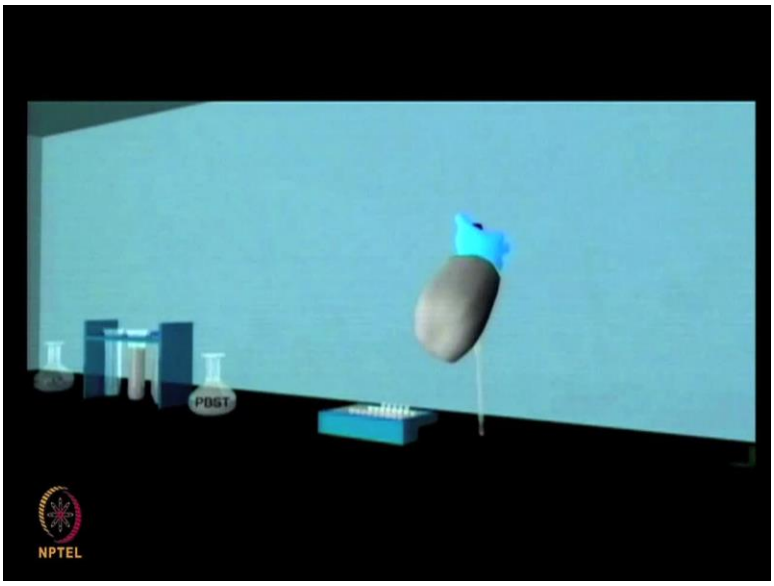
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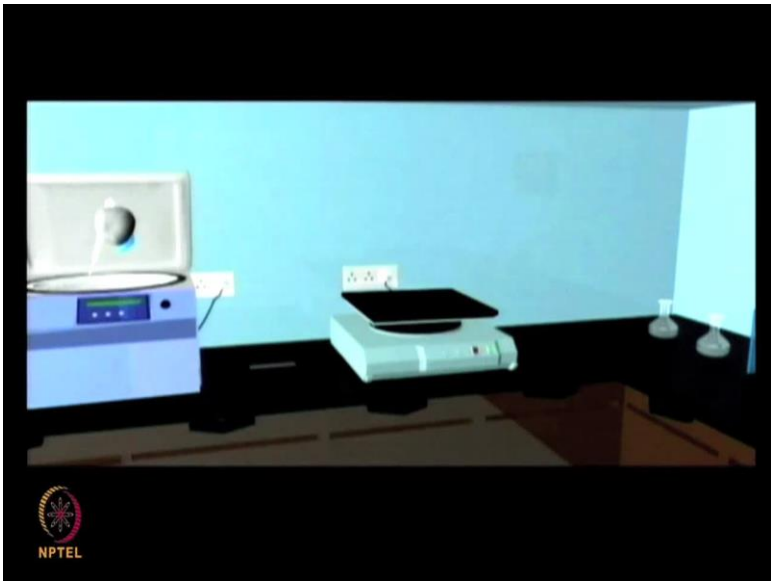
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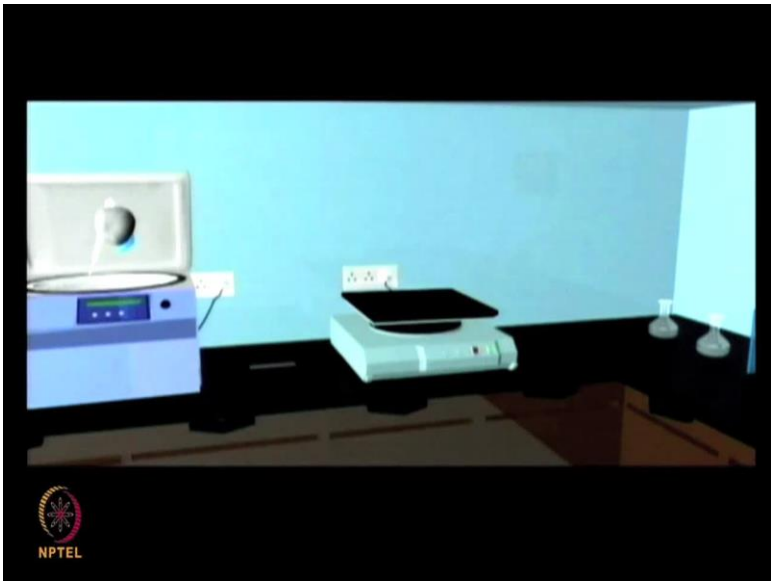
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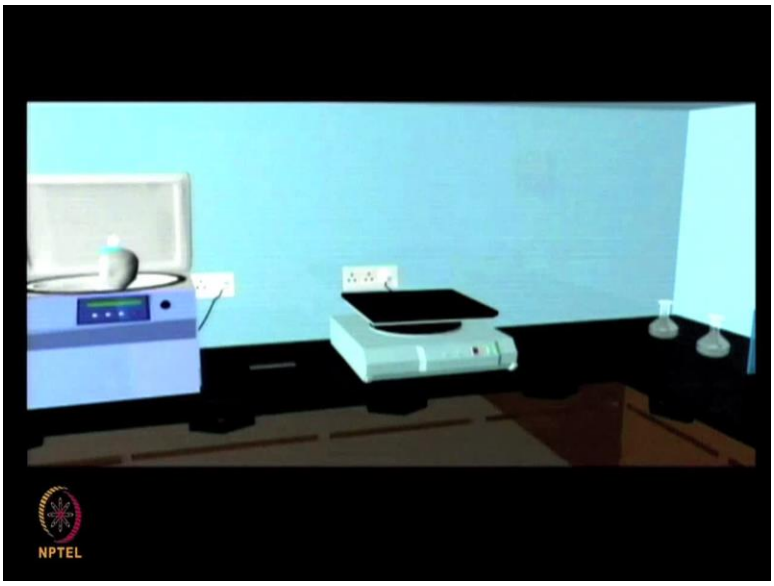
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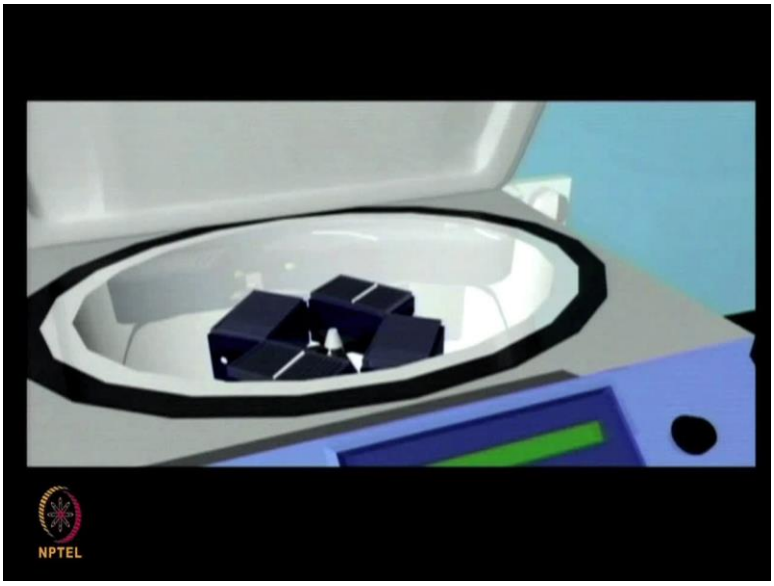
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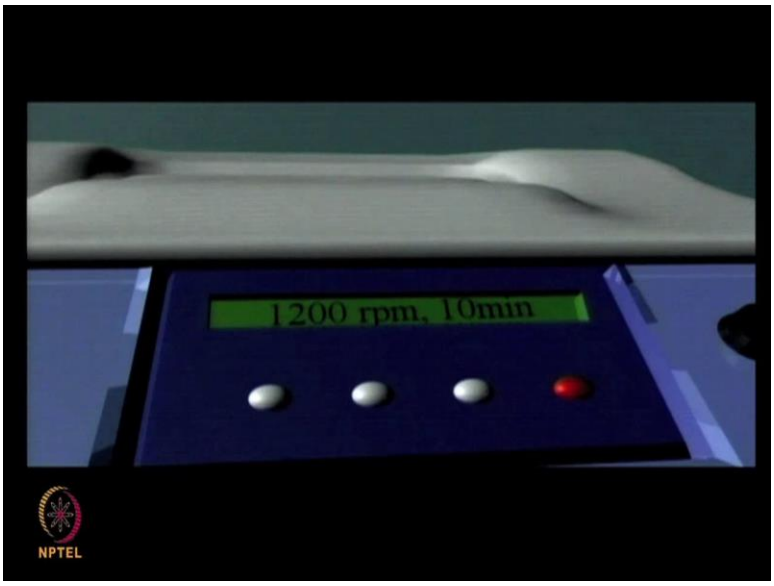
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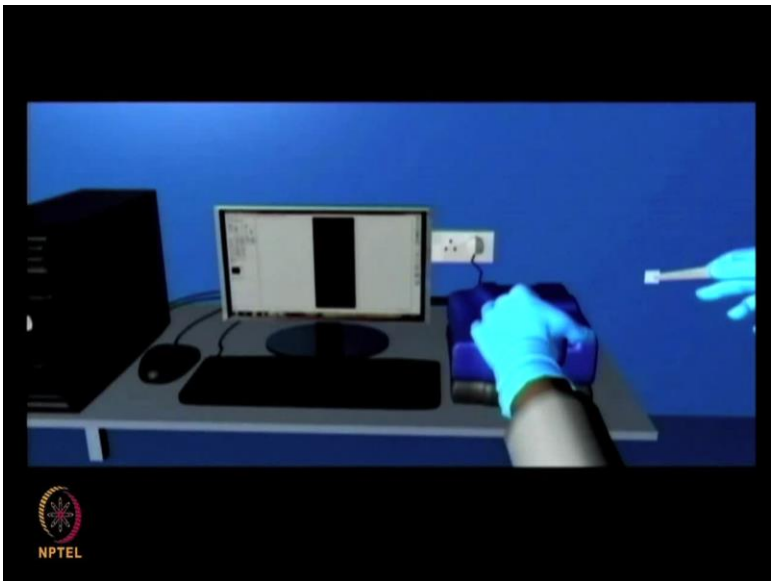
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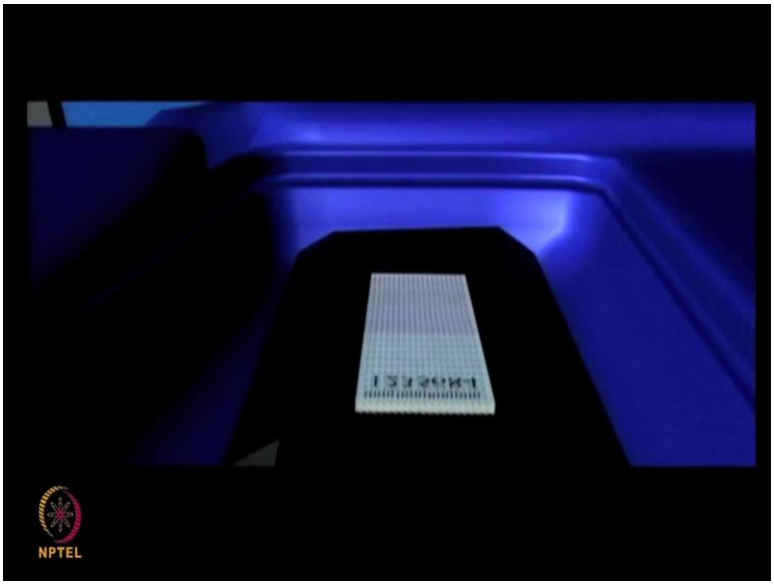
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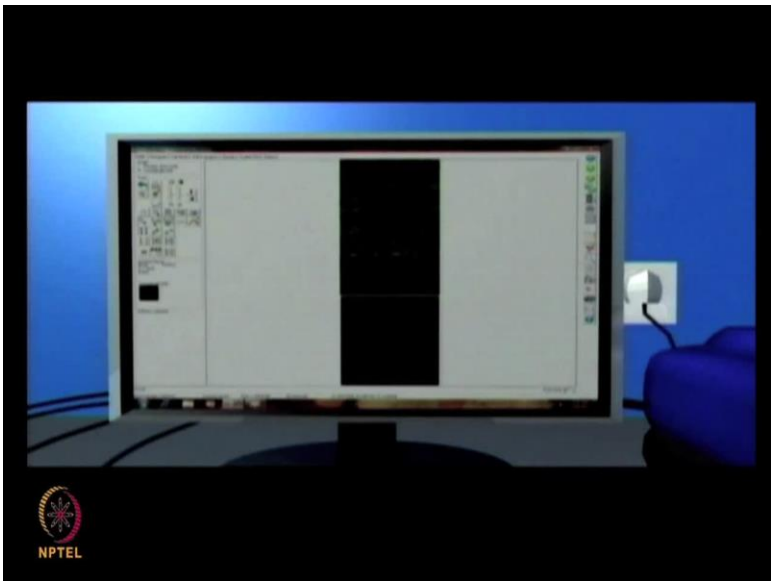
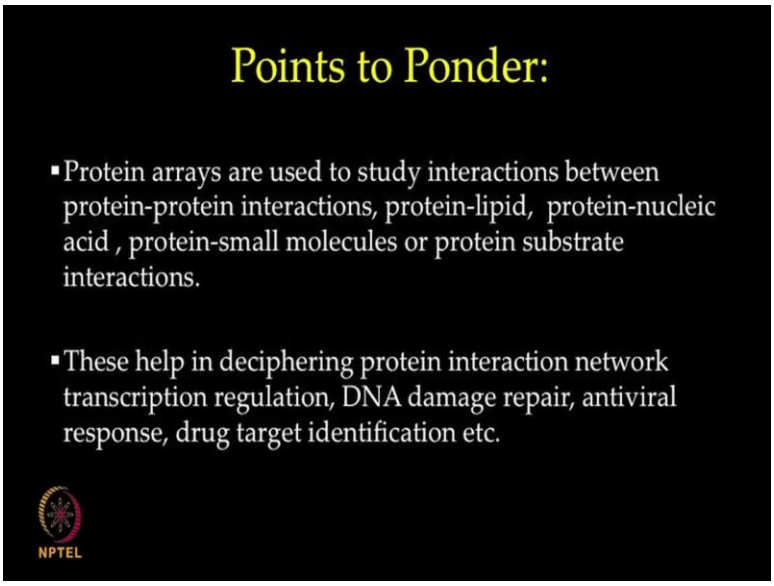


Points to Ponder:

- The potential of proteomics in biological sciences is tremendous in consideration of its downstream functional role in a cell.
- To monitor the dynamic changes in the proteome, high-throughput approaches like protein microarrays are extremely useful.
- The over-all workflow of protein microarray experiment involves blocking of the microarray slide, interaction of protein of interest/ test sample onto the chip followed by its detection using Cy-dye labelled antibodies along with interim washing steps.



Here I am giving you an overview of a protein microarray experiment. Regardless of what application one want to study, the overall workflow of the protein microarray experiments remain the same. Here I am showing you the experiment of protein interactions performed on the E. coli proteome chip. The first of all these chips are stored at the - 80 degree if they are purified proteins you want to store them at the very freezing condition so that protein can remain functional.

Now these chips should be carefully removed from frigid and that allow to thought briefly followed by the washing instance. First of all transfer these chips from the - 80 freezer to a fresh slide holder. After these chips are thawed briefly at the room temperature, then one can either directly block it in the blocking solution or use the PBS twin for brief rinsing. Blocking is usually performed at room temperature for an hour or at four degrees overnight depending on different type of applications.

PBS and milk or super block or BSA are commonly used blocking solutions. One can typically use even a pipette box or a small box at approximately 30ml or 50ml of the blocking solution, dip the microarray slides inside the solution. And once the blocking is completed, you can remove the slide and tap again the paper towel to remove the excess milk.

By performing the blocking experiment or blocking step make sure continuously makes the slide even if you are preparing the entire set. If slides are left sitting on their side without mixing, then

they will dry and then the slide will appear dark background after the scanning. After thawing steps are completed then depending on your application you can either apply primary antibody if you want to do a quality control chip for example or one can use a query protein for example if you want to study the protein-protein interaction.

So in this study we are talking about protein interactions. So let us say a query protein of interest which for which you want to study the protein interactions you can take that protein query protein and then apply that on the protein microarray slide. After addition of this query protein you need to cover it with the cover slip and incubate it at the room temperature for an hour or one can optimize this incubation condition depending upon their experimental requirement.

Once this step is done, then you need to wash the slide with PBS twin for three times, usually five minutes wash for three times at the room temperature with a gentle shaking on a rocker shaker is most commonly used. In the microarray experiment one need to ensure the proper washing and gentle shaking throughout various steps to ensure that the slides are washed very neatly, otherwise you will see very high background on the slides.

After this step, one can add the secondary antibody labeled with the either cy 3 or cy 5 conjugate labels or one can use the HRP based detection system for detecting the signals but prior to the this step one need to dry the chip by centrifugation. So rinse the slides quickly and then centrifuge it to dry it or one can also use the compressed air for drying these slides.

Once the slides are dried, then they can be scanned at the appropriate wavelength. With this gives you a glimpse of, an overview of a microarray experiment which is shown in the 3d animation here, but depending upon your biological question various type of modifications can be made.

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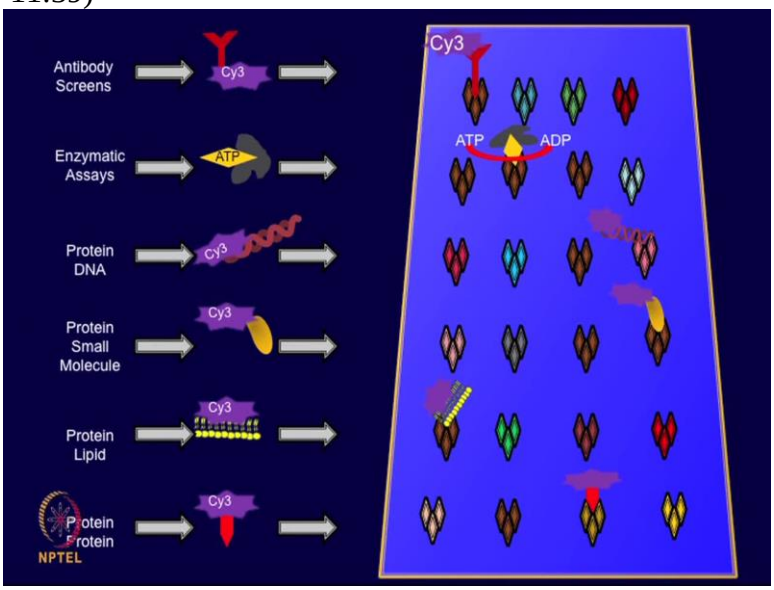

After giving you an overview of protein microarray experiment, let us now talk about protein microarray based applications. Protein microarray chips have been used to assay for wide range of applications such as protein-protein interactions, protein lipid, protein nucleic acid which includes protein DNA and protein small molecules or protein substrate identification.

Protein chips have also been used for drug and drug target identification and kinase substrate identification. I will try to summarize few more studies in detail to familiarize you with different type of applications which can be performed using protein microarrays.

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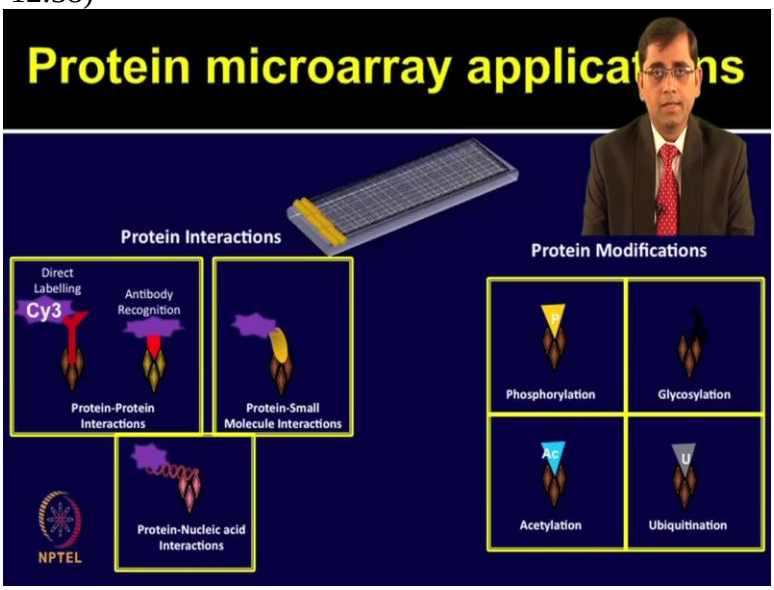

Protein microarrays have been widely used for biomarker identification and protein-protein interactions as well as different type of protein modifications. As you can see in the slide the protein interaction network, transcription regulation, DNA damage repair, antiviral response, drug target identification are various type of applications which researchers have tried using protein chips.

Similarly, studying the kinase networks, the dynamic function regulation as well as studying protein turnover and protein modification have been used on protein chips.

Let us further discuss biomarker detection, this is one of the most commonly used applications which researchers have tried using protein arrays. Because the clinical samples are very precious very limited and usually do not have access to the large volume of clinical samples. So on one hand you would like to study as many as protein possible, but at the same time we would like to use very small volume.

So protein chips provide this high throughput platform to screen patient samples in a very volume. Therefore protein microarrays offer a very appreciable platform because you can use few microliters of sample and that can be used to prove thousands of proteins. On one hand, we are using very small amount of sample that you can actually probe for large number of proteins.

Often some of these samples are very challenging, but by using protein microarray applications one could get some very useful information for biomarker discovery.

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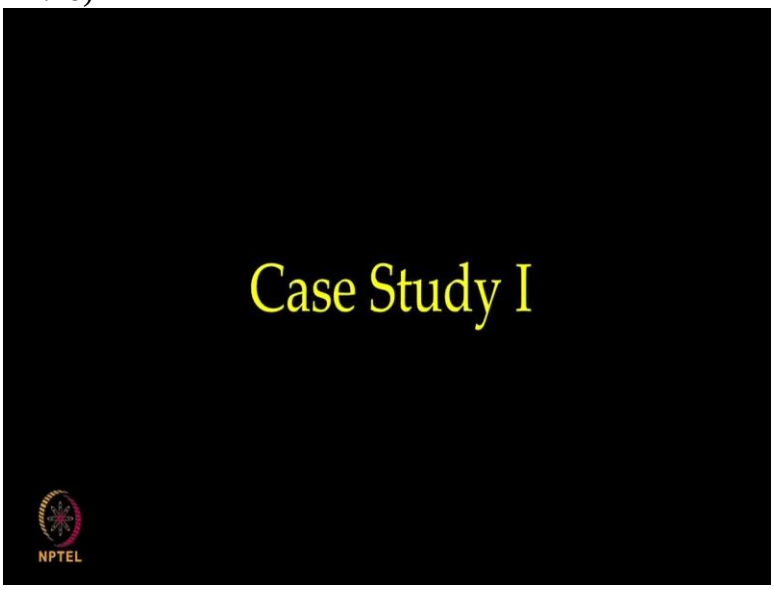

Let us start with case study first, identification of differentially expressed proteins (())(14:51) in cancer using high density protein microarrays, a study performed by Hudson Nitol.

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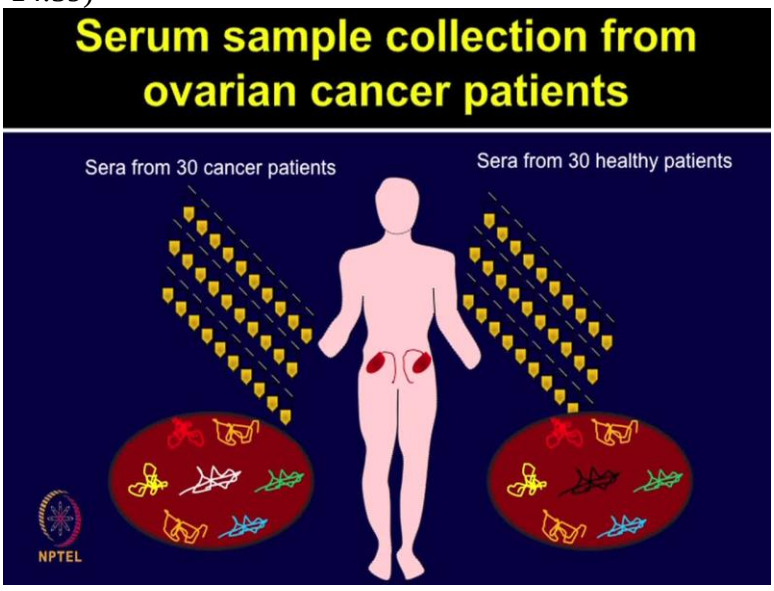

In this study authors took serum from 30 individuals suffering from ovarian cancer and 30 healthy individuals. For each of this type of samples they used 300 microliter of serum sample, diluted in correct buffer and then applied that on human proteome arrays.

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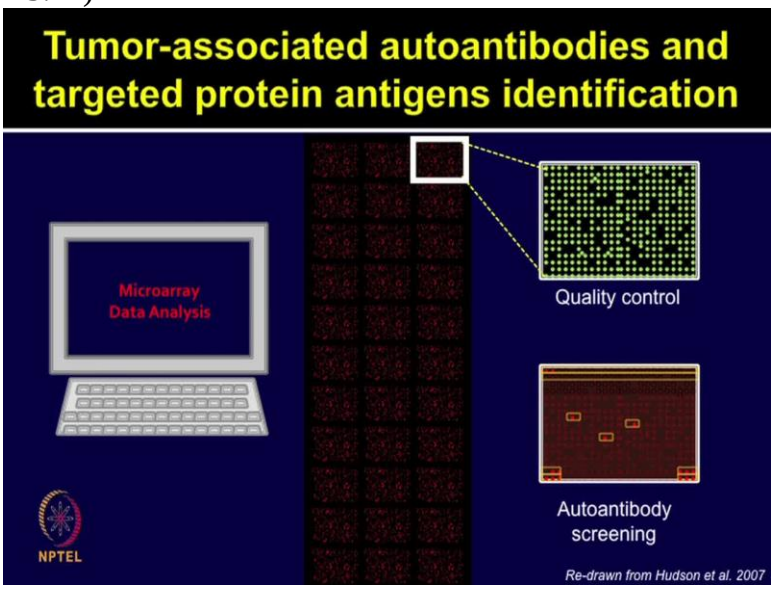

These arrays are obtained commercially, so they wanted to compare antibody response of various type of proteins in ovarian cancer serum as compared to the healthy controls.Identification of tumor associated antibodies and targeted protein antigens were performed on protein microarrays. These protein microarrays contain more than 5000 GST fusion proteins which were first probed with antigens tmt body.

As you can see in the slide, first of all quality control experiments were performed by using entire GST antibody because all the clones contain GST tag. So first of all there was a need to ensure that all the clones express protein uniformly and whole of the protein array can be used for uniform screening.

Now once the quality control experiment is done, then you can start actual screening of large number of samples on these arrays for discovering biomarkers. In this study, the authors used sample from ovarian cancer and healthy individuals and then applied it using human proteome chips. The different boxes which are shown on the bottom panel on the right hand side indicates there are several positive and negative controls which were printed on the chips to unsure that

assay is working fine and there are certain proteins shown in the centers which are potential biomarkers.

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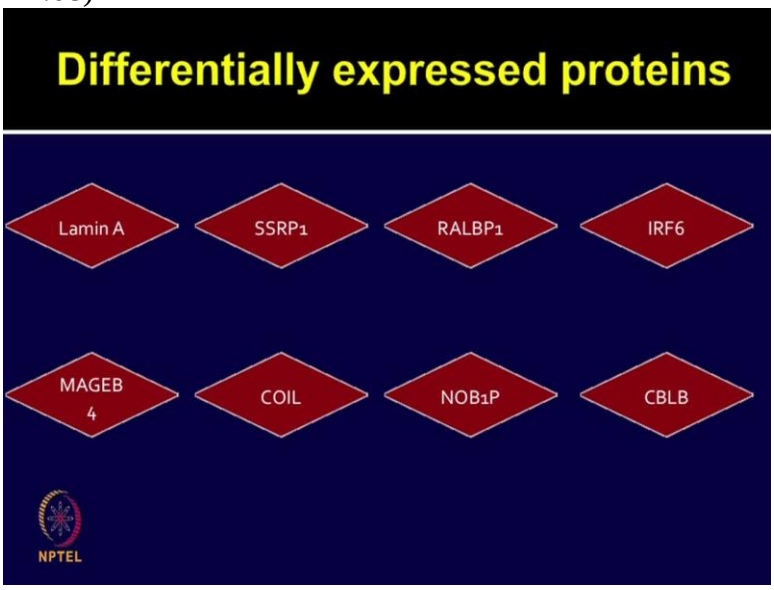

Based on this study, authors werel able to identify several differentially expressed proteins which included lamin A which is one of the nuclear membrane organization protein. Structure specific recognition protein SSRP1 which is involved in the regulation of transcription, RAL binding protein which is important in the transportation, IRF6 which is crucial for transcription regulation, MAGEB4 is reported as cancer biomarker, COIL which is found in the nuclear body although its function is unknown, NOB1P which is adenocarcinoma antigen the function of this protein is unknown, CBLB which is involved in signal transaction.

By using high throughput approach, high throughput protein microarrays and screening for more than 5000 proteins authors will able to identify several differentially expressed proteins. These are some of the proteins which they conceded quite interesting and selected them for further validation experiments.

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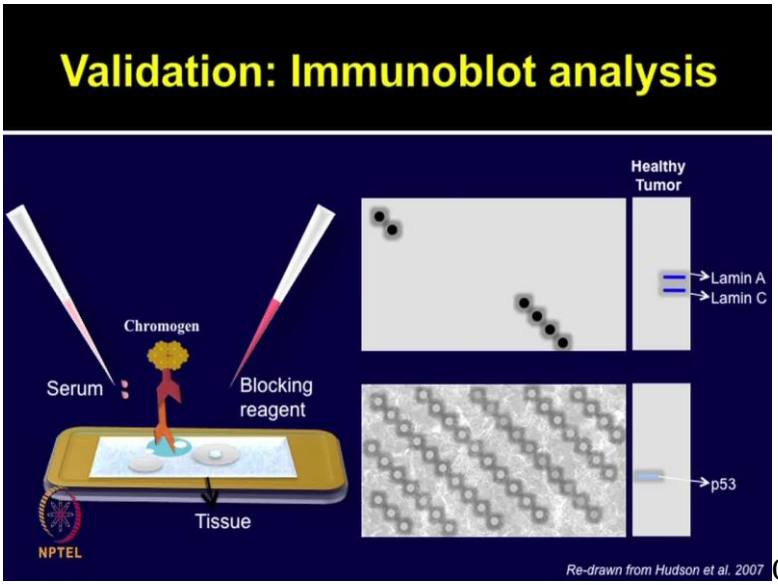

Once protein targets were identified, authors used immunoblot and western blots to validate their findings. The protein sample of cancer and normal cells were analyzed by using immunoblot assays and they used antibody the specific for Lamin A or C and SSRP1 proteins. These proteins were considered interesting based on their proteomic finding.. The Lamin A and C were greatly elevated in cancer samples as compared to the healthy individuals and appeared quite interesting although the western blot analysis was performed by using healthy and ovarian patients samples and then they used antibodies specific for Lamin proteins as well as p53 protein.

As you can see on the right hand side of this slide the Lamin A and C were probed by using western blot. Similarly, p53 protein was probed by using anti p53 antibody so these immunoblot assays they have performed. The western blot analysis confirmed and validated that the proteomic findings based on protein microarrays were quite relevant and elevation of this proteins were validated by independent techniques.

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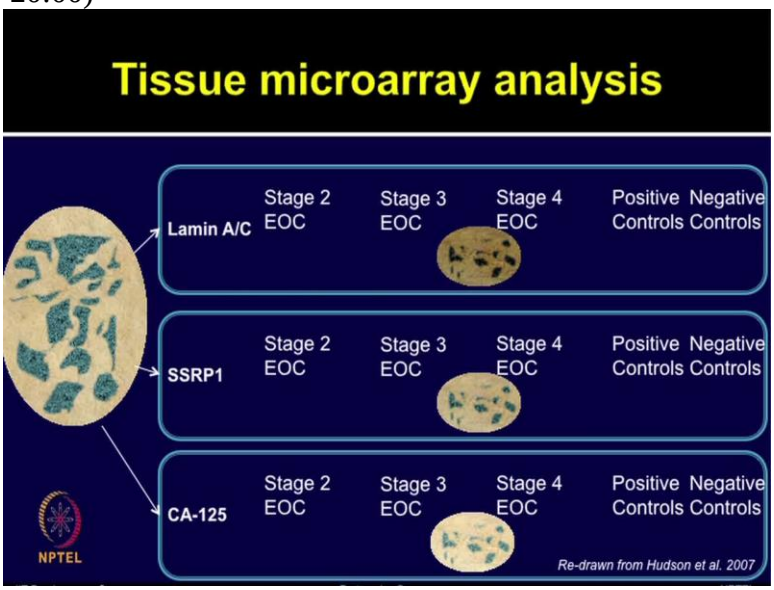

Now further to the immunoblot assays, authors also used tissue microarray based analysis. So the tissue microarray analysis of different stage of ovarian cancer was performed. These microarrays containing the representative tissues from various stages of ovarian cancer such as stage 2, 3 and 4 tumors were probed for Lamin A or C, SSRP1, and cancer antigen CA-125. These results are shown in the slide which is redrawn from the results presented in the manuscript.

Lamin A and C shown in the top panel, SSRP1 shown in the middle panel, and cancer antigen 125 shown in the bottom panel. Overall this was very interesting study which revealed that these three can add a tissue markers which were immunostained, can produce a very robust signature of ovarian cancer in tissue specimen.

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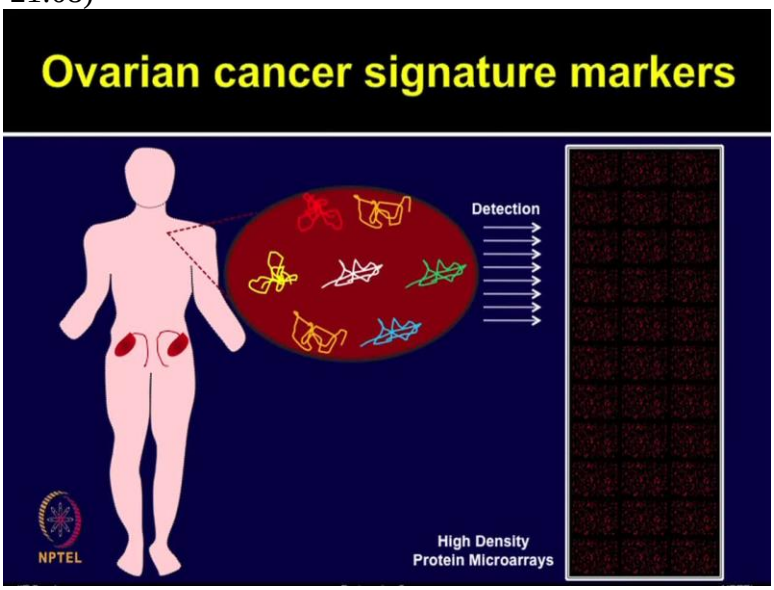

Although authors have used serum and their motivation was to develop the serum or blood based assays, they thought that at least the robust signature in the tissue indicates that these proteins are overly presented in serum sample and probably this marker could be used for blood based or serum based assays.

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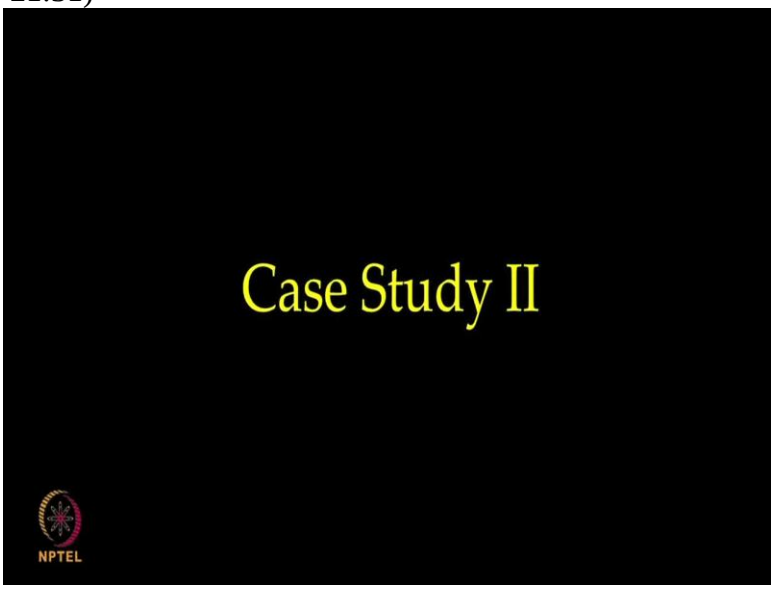

Let us now move on to case study 2-identification of novel serological biomarkers for inflammatory bowel disease using E. coli proteome chip, crone disease and ulcerative colitis.

These are chronic, idiopathic and clinically heteropathic intestinal disorders which are collectively known as inflammatory bowel disease also known as IBD.

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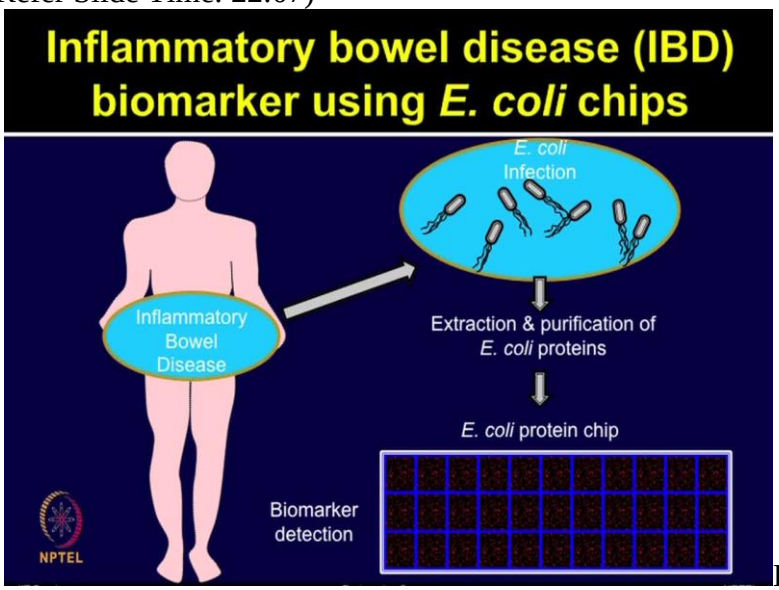

In this study, authors have used E. coli proteome chip to screen and identify novel biomarkers associated with inflammatory bowel disease, study was performed by Chan Etal in 2009. In this study first of all authors have used E. coli proteome chip, they used more than 4200 proteins obtained from E. coli and then they collected serum from healthy controls and clinically well characterized patients with inflammatory bowel disease. (())(22:42) examples from chrome disease and 29 from ulcerative colitis were collected and compared with 39 healthy individuals.

The protein spots were recognized by serum antibodies and visualized by using cy 3 labeled goat antihuman antibody. After looking at the overview of the experiment, let us now discuss some of the findings.

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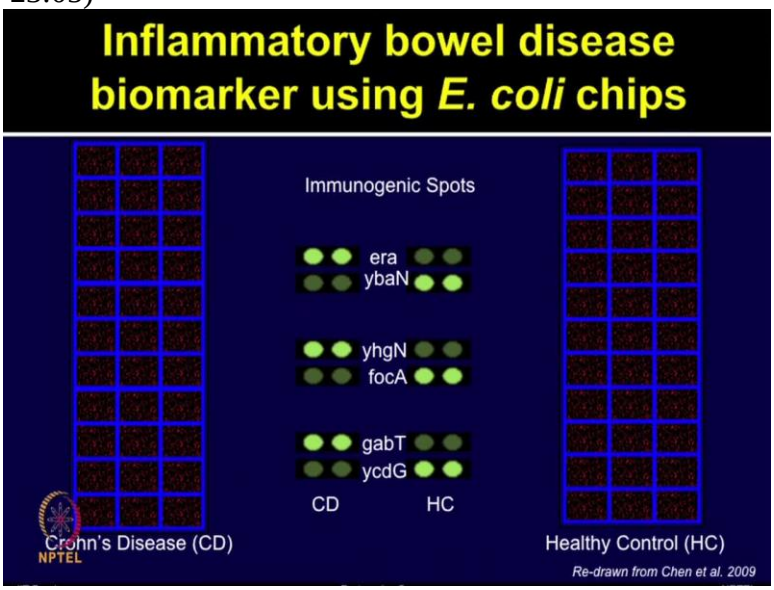
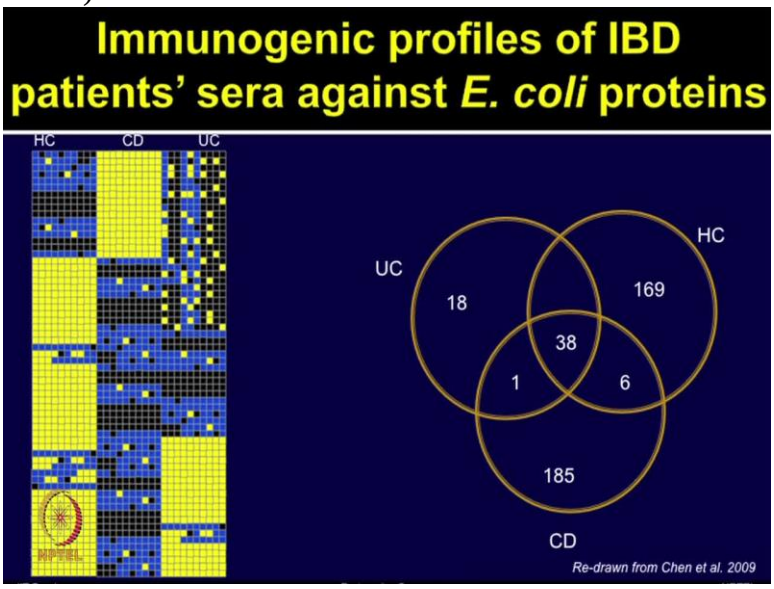


This E. coli proteome chips were probed with serum from CD patients which is shown on the left side which is crohn's disease and healthy control is shown on the right panel in the slide. The cy 3 labeled antihuman immunoglobulin antibodies were probed on the chips which allowed the visualization of immuno reactive protein spots.

Some of this spots are shown in the center and it shows the comparison of crohn's disease versus healthy individuals.

(Refer Slide Time: 23:47)



After performing this screening experiment on chip, author generated a heat map of 273 differentially expressed proteins which were identified using healthy controls and crohn's disease samples. They also performed a comparison of UC, HC and CD which are shown on the slide in Venn diagram on right side. It shows that differentially expressed proteins should very limited overlap even the HC versus CD and CD versus UC.

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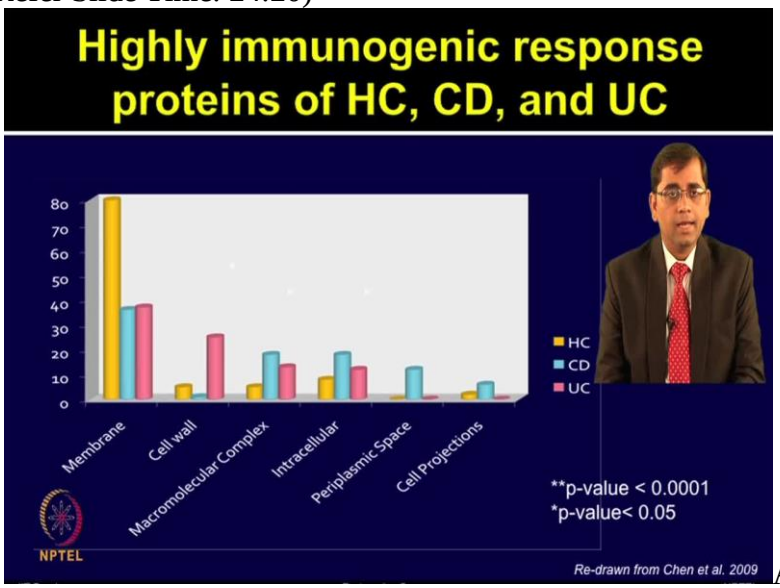
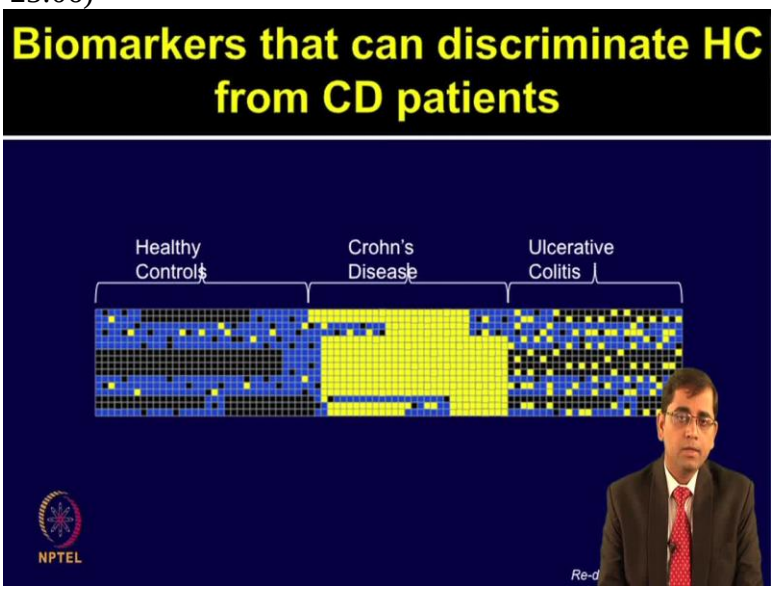


After studying these differentially expressed proteins, authors used these proteins to understand there functional role and how they are distributed in the silver compartments. HC, CD and UC were then further used to define the silver components and functional role which is show in this slide. Authors tried to categorize the proteins in all the three groups as per that silver location as membrane, cell wall, macromolecular complex, intracellular and periplasmic space and the cell projections and looked at the differential response.

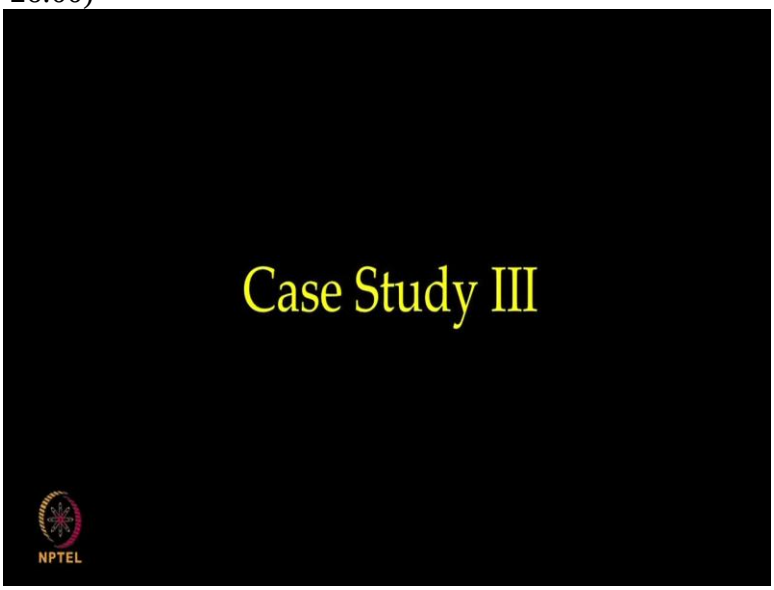
(Refer Slide Time: 25:06)

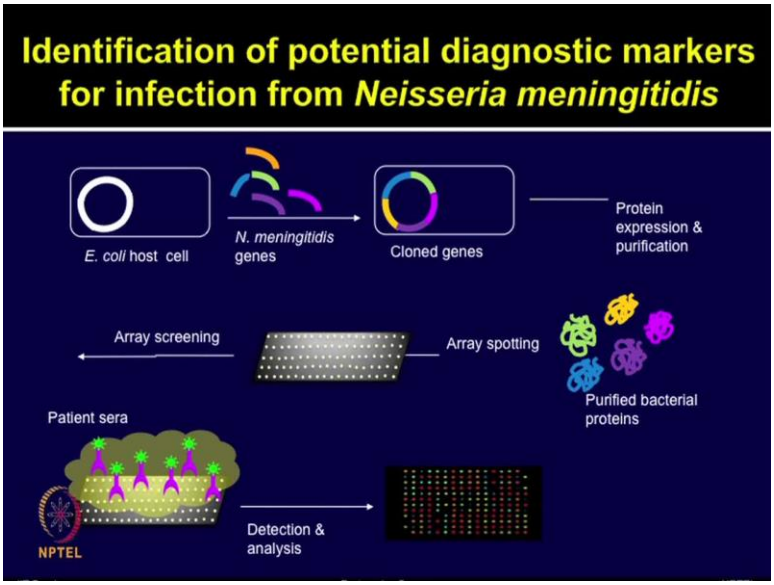


By using the supervised learning algorithms ktop scoring peers, authors identified two sets of serum antibodies that were novel biomarkers for specifically distinguishing crohn's disease from healthy controls. As you can see in the slide that healthy controls, crohn's disease and ulcerative colitis all these samples can be distinguished and especially the healthy controls versus crohn's disease cross by using supervised learning algorithms.

After studying the application one based on the biomarkers and another based on E. coli proteome chips let us now look at few more examples of biomarker discovery in brief to give you an overview of different type of applications and data analysis which can be performed using protein microarrays.

(Refer Slide Time: 26:00)



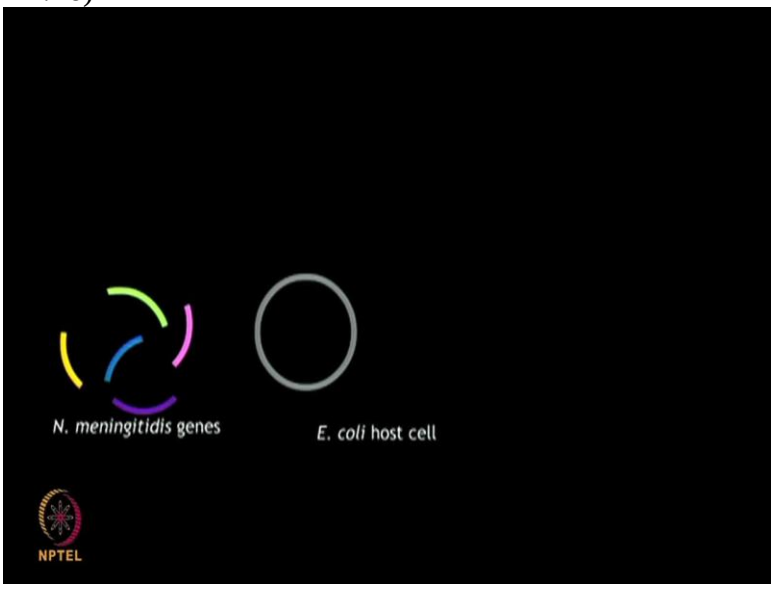


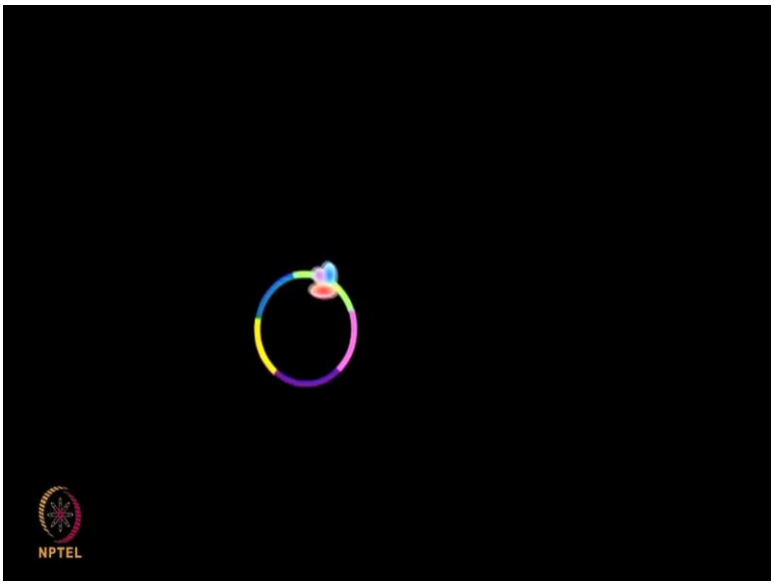
In this study, we will talk about identification of potential diagnostic markers for infection from neisseria meningitides. Neisseria is most common cause of meningital disease and also causes epidemic outbreaks. To investigate the immune responses to the phase variable express proteins (())(26:28) apply protein microarrays to screen the meningitidis patient serum.

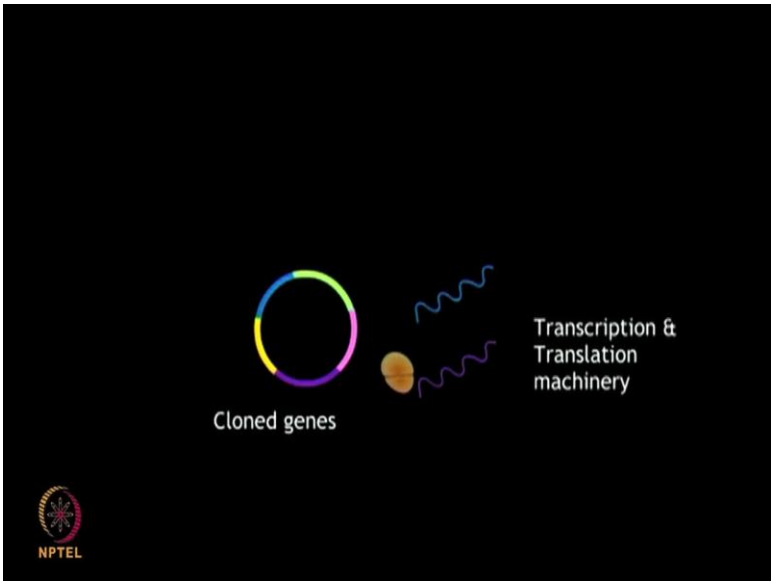
This is the first study which aims to investigate the genetic phase variations in the pathogens. Authors first amplified all the 102 known phase variable genes obtained from the neisseria meningitides and expressed and purified these proteins in hydrologal system using E. coli. They were able to purify 67 recombinant proteins because not all the proteins are able to produce and therefore they were limited with 67 purified proteins for further investigation using protein microarrays.

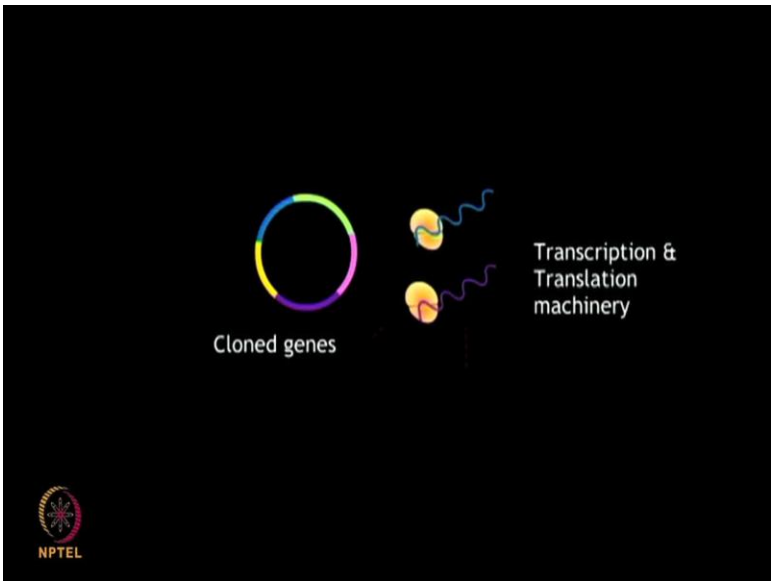
This protein microarray platform was used to screen 20 patients serum and healthy individuals. After screening, authors identified 47 immunogenic proteins out of which 9 proteins were quite reproducible including its phase variable opacity protein OPAV which was very reproducible in many patients. Let me provide you the overview of this study in following animation.

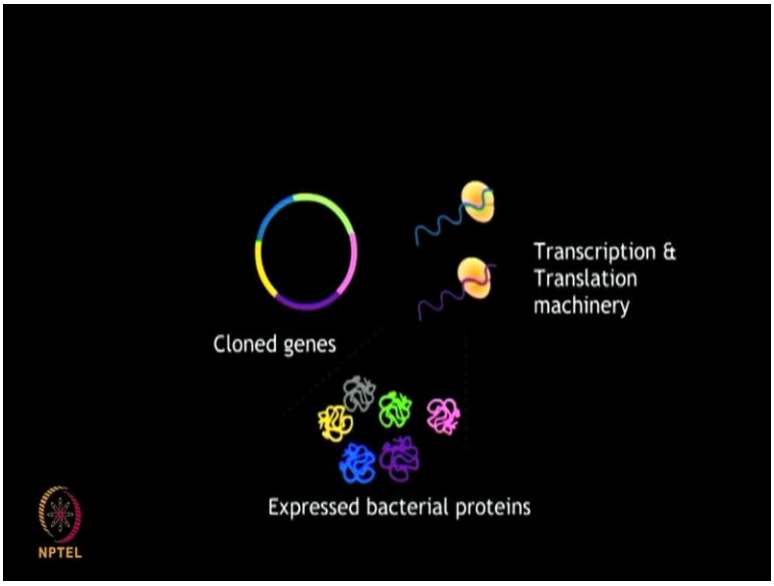
(Refer Slide Time: 27:46)

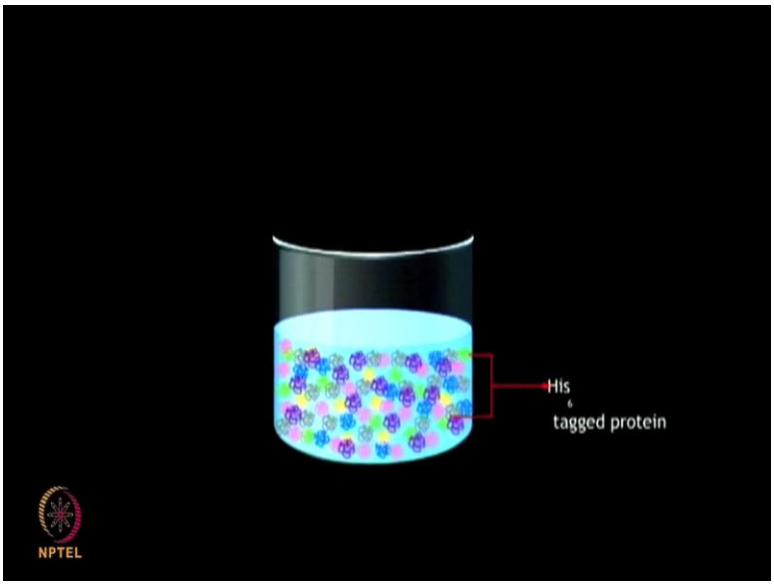


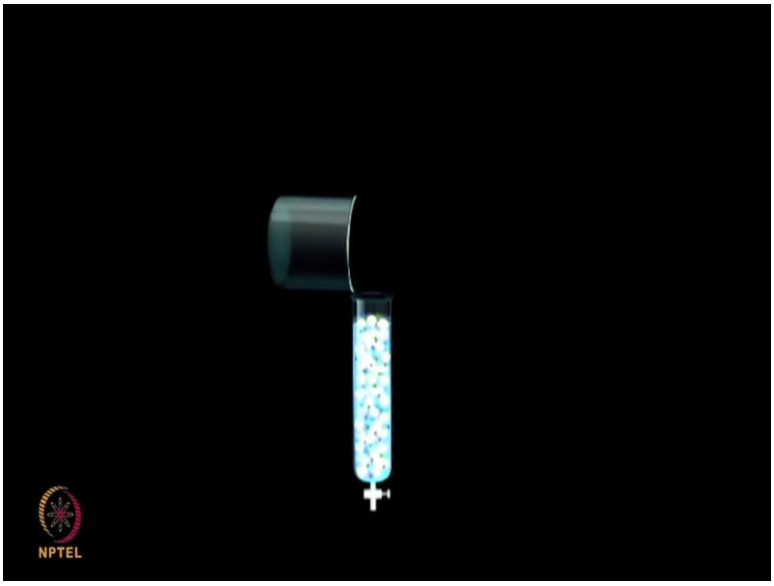

NPTEL

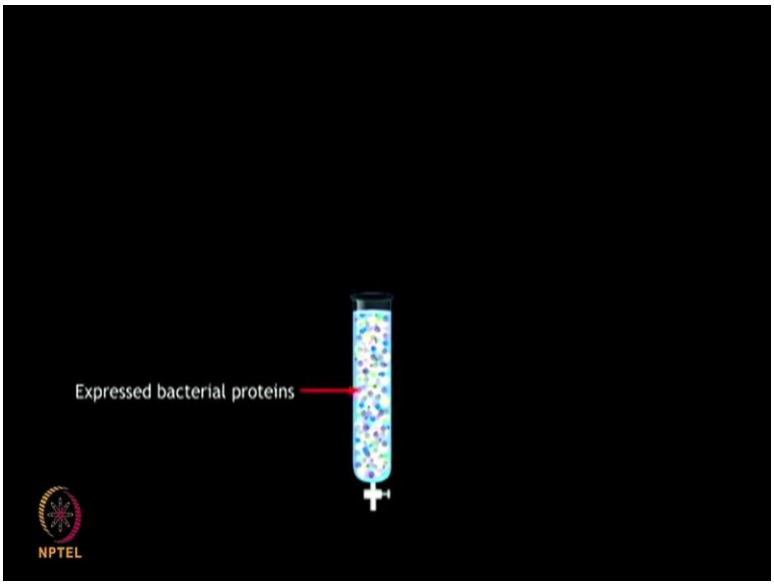

Transcription &
Translation
machinery
Cloned genes
NPTEL

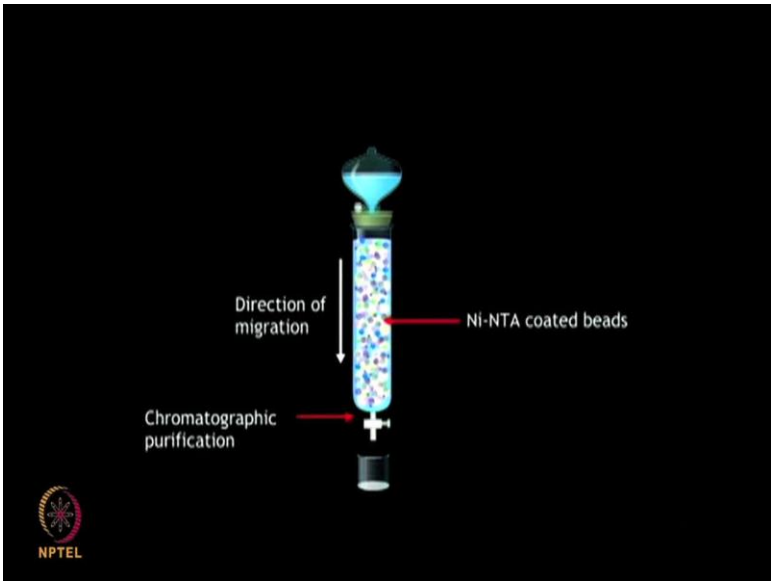

Transcription &
Translation
machinery
Cloned genes
NPTEL

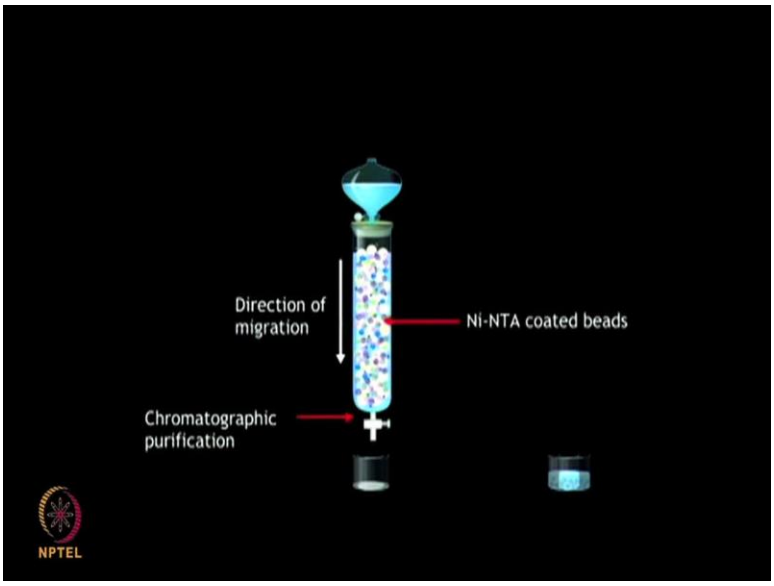

Transcription &
Translation
machinery
Cloned genes
Expressed bacterial proteins
NPTEL

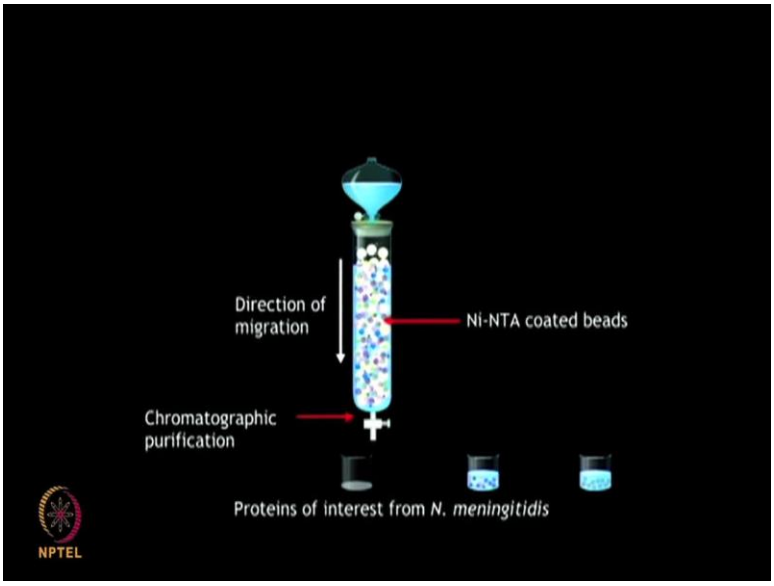

His
6
tagged protein
NPTEL

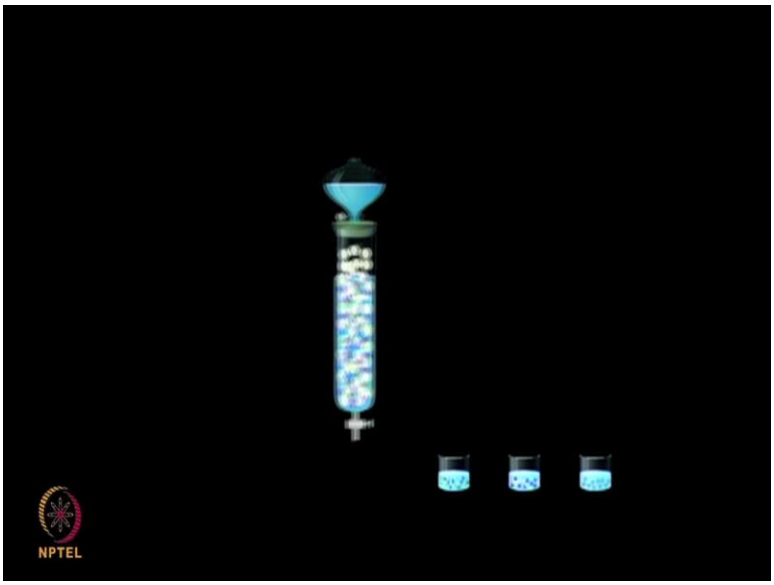

NPTEL

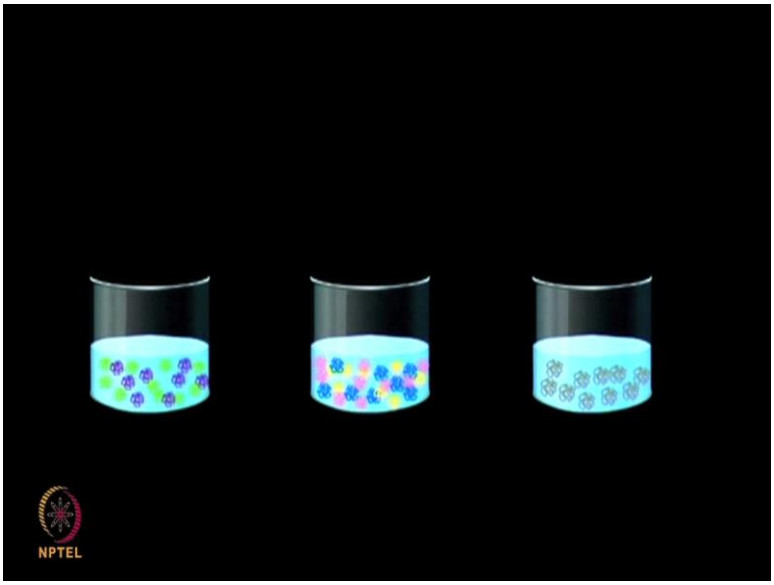

Expressed bacterial proteins
NPTEL

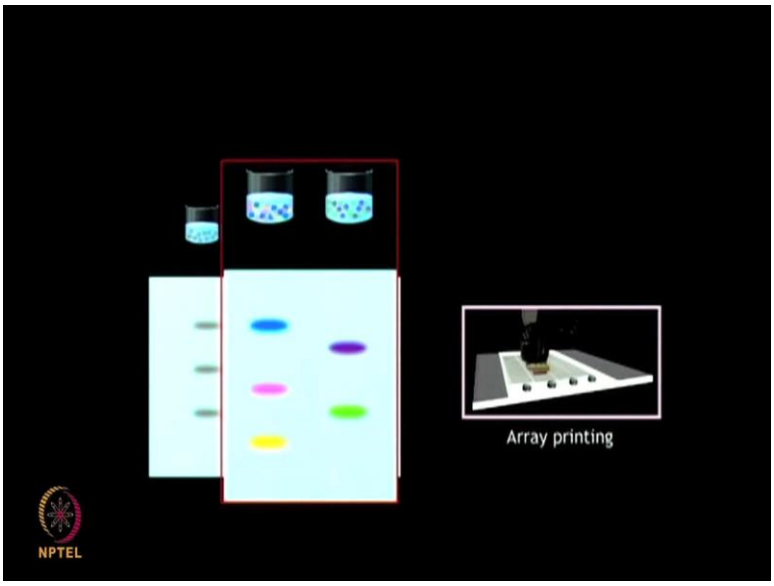

Direction of migration
Ni-NTA coated beads
Chromatographic purification
NPTEL


Direction of migration
Ni-NTA coated beads
Chromatographic purification
NPTEL


Direction of migration
Ni-NTA coated beads
Chromatographic purification
Proteins of interest from N. meningitidis
NPTEL


NPTEL


NPTEL

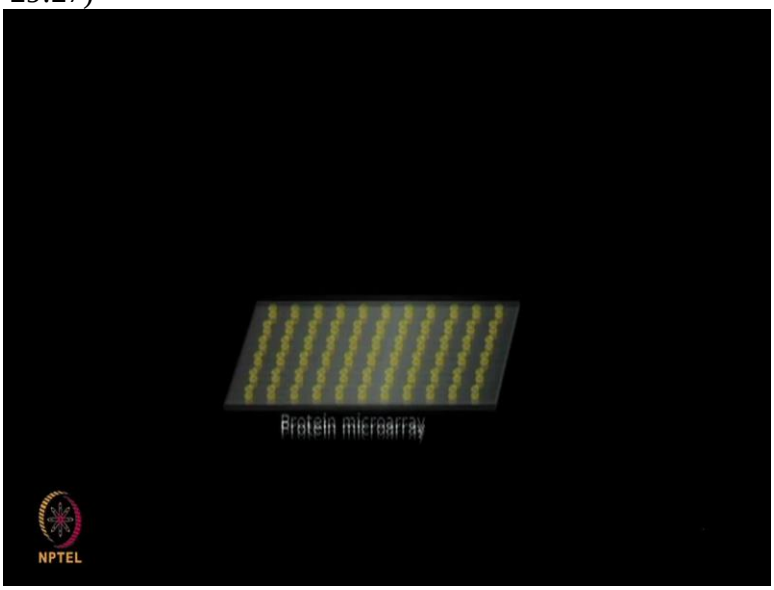


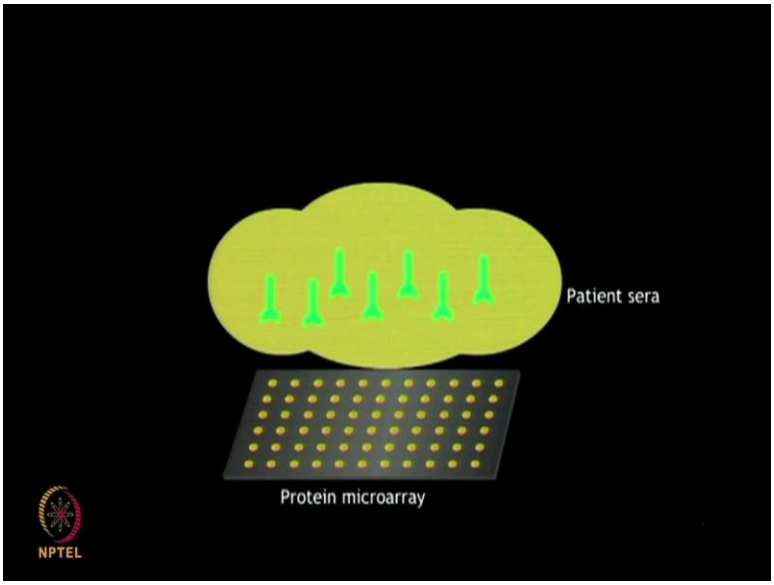
This study was performed by Stellar et al. Bacterial protein microarrays for the identification of new potential diagnostic markers for neisseria meningitides infections. Authors simplified and sub cloned 102 genes from the neisseria species for expression in the E. coli. These clones were grown for overnight at 37 degrees centigrade in the antibiotic containing medium after which the protein expression was induced by addition of IP2G. The cells were harvested 4 hours after induction and then protein was purified.

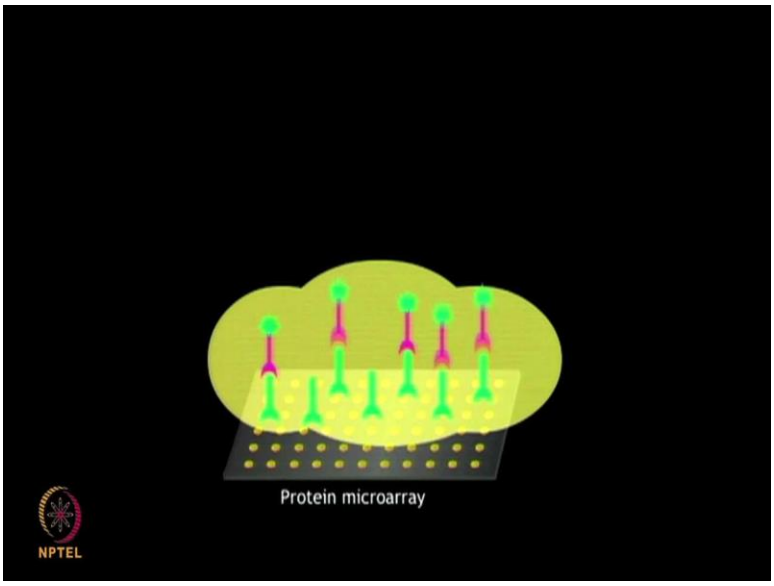
The proteins were purified based on the specific nickel NTA binding, after various elutions were collected these fractions were further separated on the SDS page gel to check the purity of these probes. This shows the SDS page separation of these proteins based on the molecular weight.

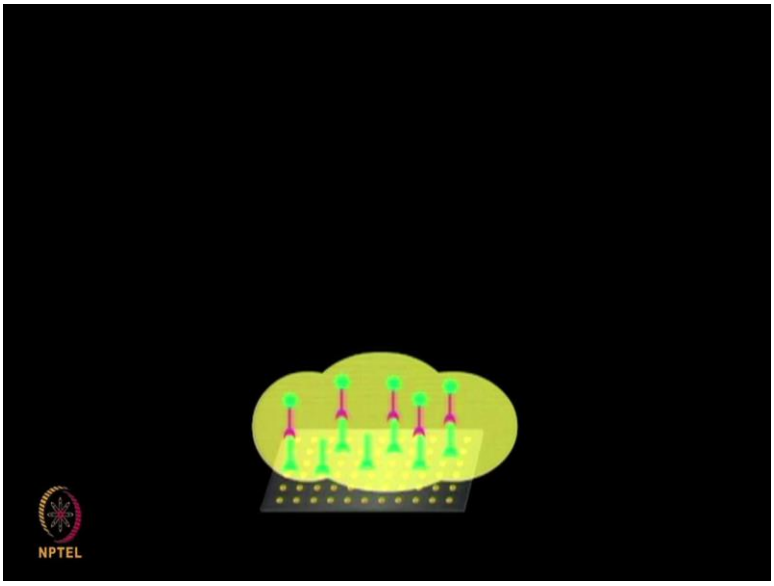
Now authors were successful in purifying 67 proteins and then these purified proteins were further printed on the nitrocellulose coated glass slide by using the robotic printer.

(Refer Slide Time: 29:27)






Protein microarray
NPTEL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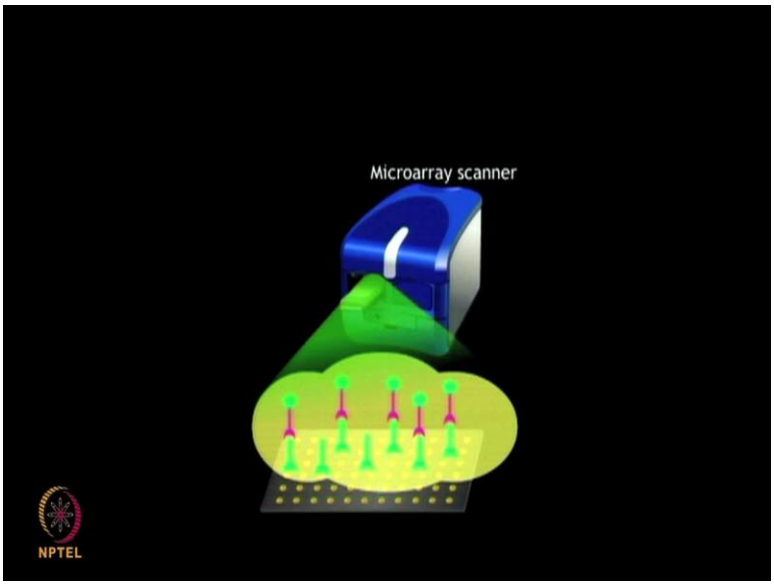
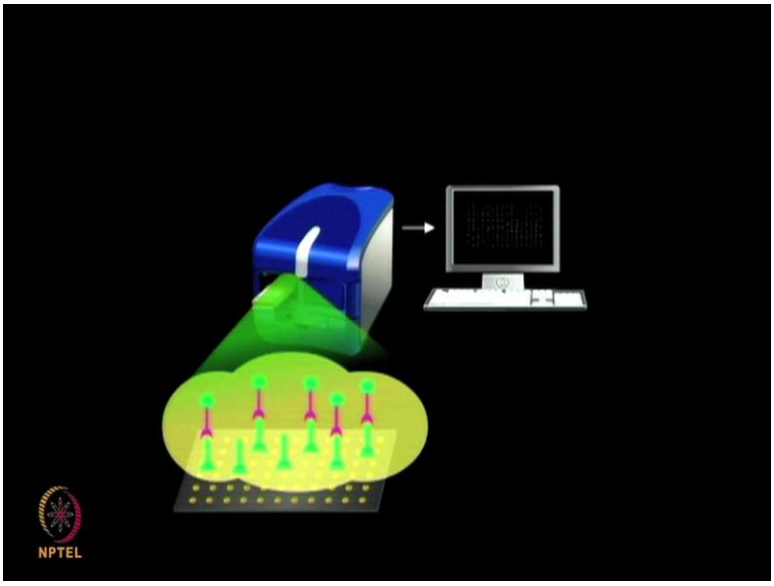
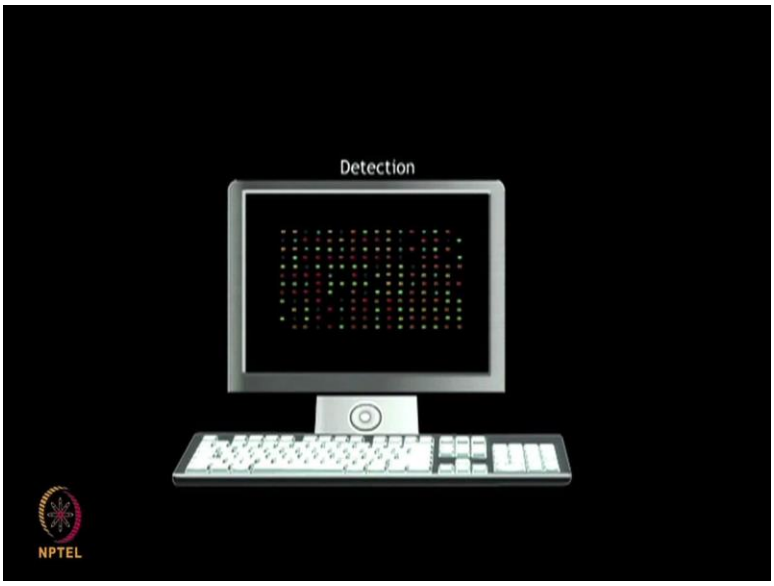
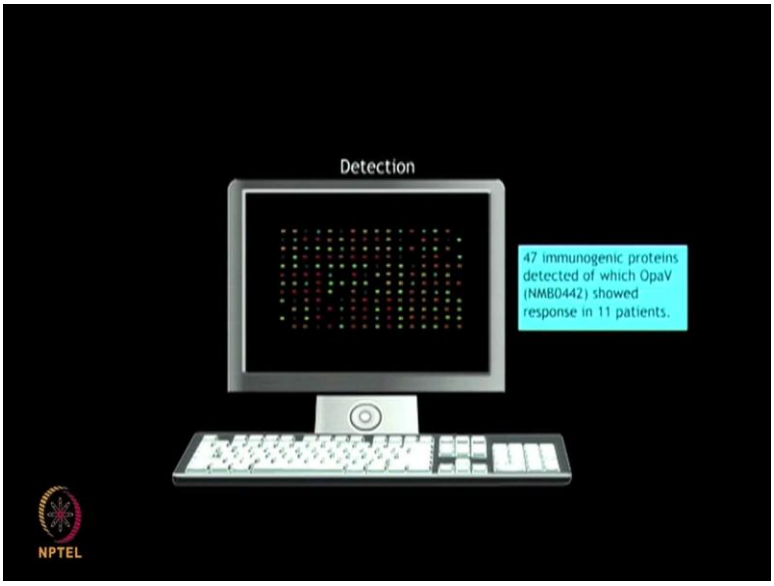

Microarray scanner
NPTEL


NPTEL

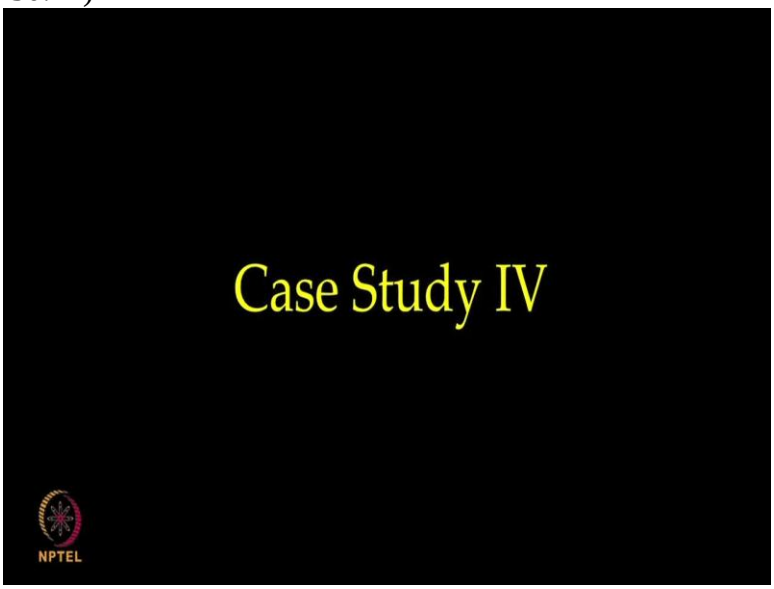

Detection
NPTEL

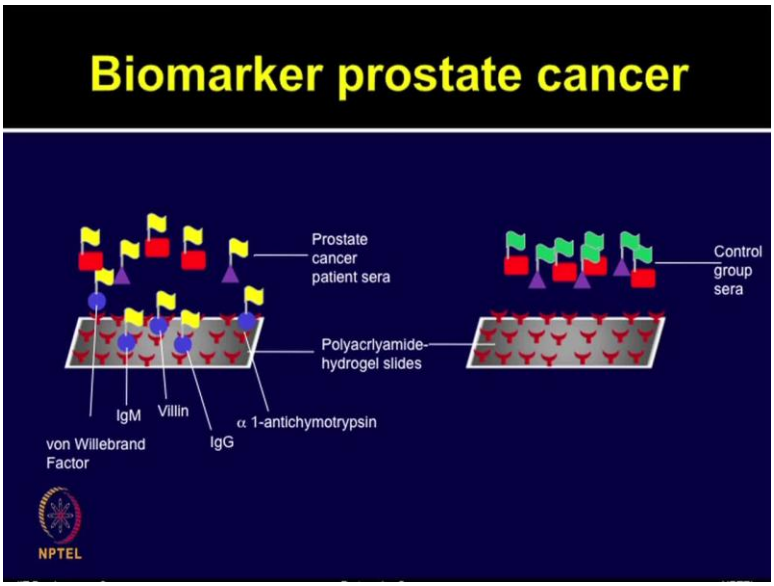

Detection
47 immunogenic proteins detected of which OpaV (NMB0442) showed response in 11 patients.
NPTEL

Once these protein arrays were generated, it was used to probe the serum from 20 convalescent patients by incubating it overnight at 4 degree centigrade. After overnight step array was washed with PBS and detection was carried out by using cy 5 labeled secondary antibody.

The excess detection antibody was washed off, array was dried and then scanned by using a microarray scanner. Authors detected 47 immunogenic proteins, one of which showed response in 11 of patients. This protein microarray platform was successfully used for detection of several other disease biomarkers and this is one of the application which is shown in this animation.

(Refer Slide Time: 30:42)





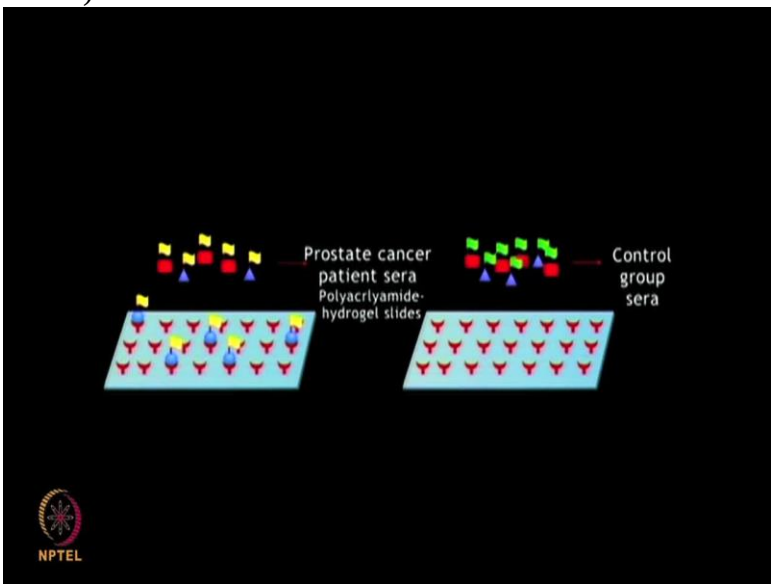
Let us now move on to case study 4, the human prostate cancer screening for identification of potential biomarkers study by Milet et al. In this study researchers used antibody microarrays containing 184 unique antibodies which were printed on microarray surface and they used two different type of substrates containing polyacrylamide as well as polylysine coated glass slides and further used to screen the prostate cancer patients for potential biomarker identification.

In this study, authors used 33 cancer patients and 20 controls and obtained serum samples from these subjects and employed to study the abundance of various proteins present on microarray slide. They optimized different parameters for the measurement and once these conditions were optimized, they used the microarray system for identification of various potential biomarkers.

Investigators used robotically spotted 184 unique antibodies on polyacrylamide based hydrogels and polylysine coated glasses slides which they probed with sera from prostate cancer patients and healthy individuals. Let me use this interactive animation and show you the results obtain for the hydrogel slides.

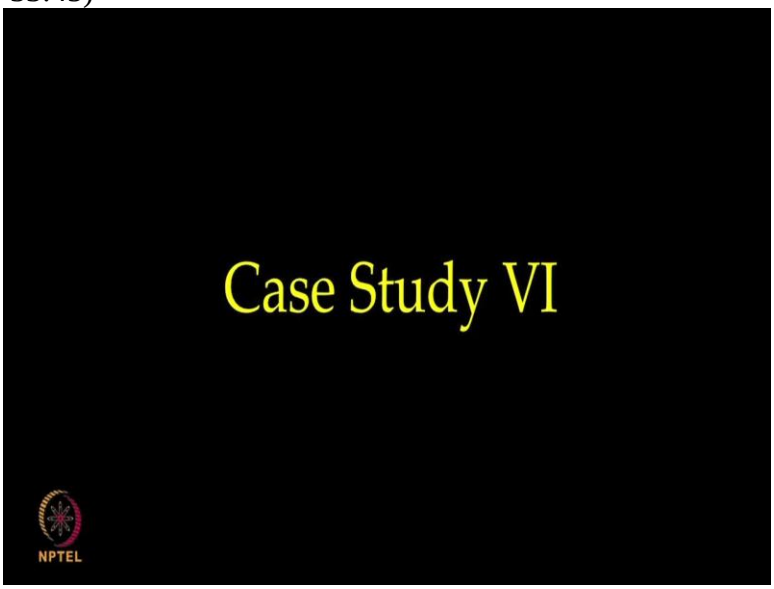
Authors used robotically spotted 184 unique antibodies on polyacrylamide based hydrogels and polylysine coated glass slides. These slides were probed with the sera obtained from the prostate cancer patients and healthy controls. Now let me use this interactive animation and show you the results obtained for the hydrogel slides.

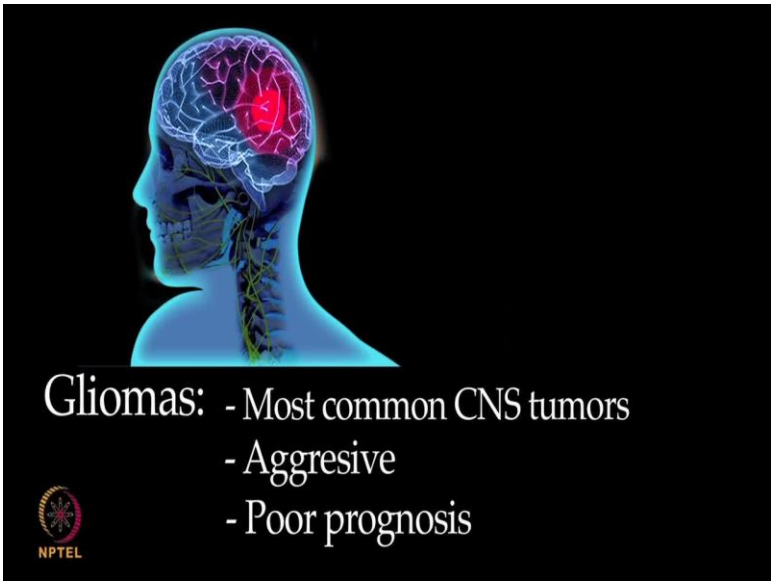
(Refer Slide Time: 32:59)



So left slide is probed with the prostate cancer patient sera and the right one is used with the control group sera. From this study five proteins were shown to have significantly differential expression in the prostate cancer patients as compared to the control group. (())(33:30) PWF protein was found to be elevated and the remaining four proteins were down regulated as compared to the control group.

(Refer Slide Time: 33:45)





We will now move on to the final case study for today which focuses on autoantibody screening in glyomous serum samples which is published by Sayed et al. Glioma is a very aggressive brain cancer with great heterogeneity. It has been classified by WHO into four grades. Grade 1 being the least benignant to grade 4 being highly aggressive and associated with poor prognosis.

The focus of this work was to devise some minimally invasive biomarkers which could help differentiate between each of the grades as well as identification of early diagnostic markers so that clinicians could identify the disease using blood at early stage as well as predict the prognosis and identify abrasions which could help them target therapeutics.

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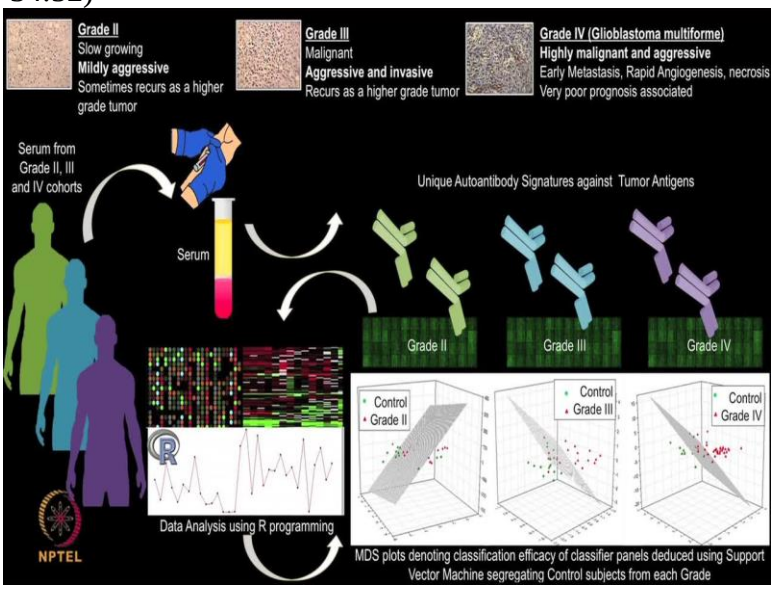
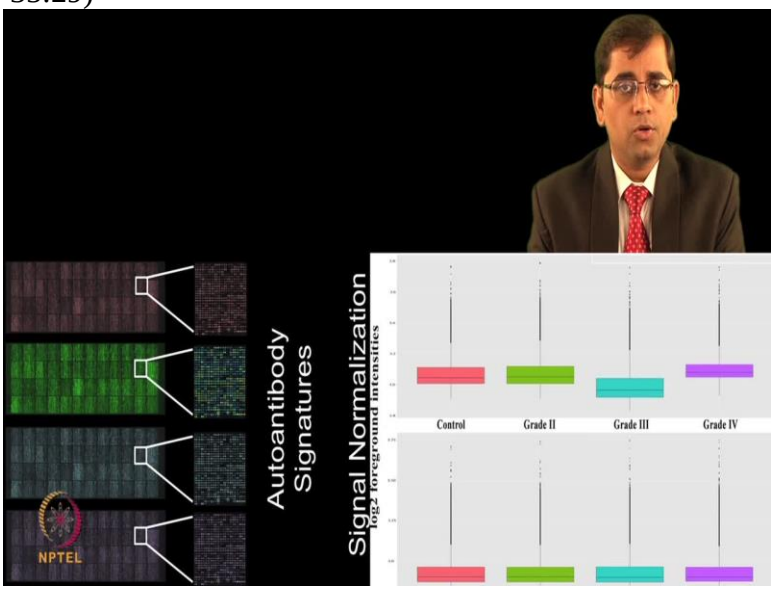


In this work investigators used serum from 17 subjects with grade 2, 18 with grade 3 and 34 with grade 4 of glioma and they screened using human proteome arrays or uprot arrays harboring over 17000 unique proteins.

The work flow of this study is shown in the slide which is very similar to the one which we have discussed in case study 1. However investigators here used different type of data analysis scheme.

(Refer Slide Time: 35:29)



In this study stringent quality control checks were performed, after data acquisition, quintile normalization was performed to reduce the technical variability. This normalized data was further subjected to dimensionality reduction techniques like correspondence analysis or CA. CA provides a shorter list of differentially expressed proteins that are statistically more significant.

This data was then subjected to recursive feature elimination models such as support vector machine, SVM to deduce a list of significant classified proteins from this list.

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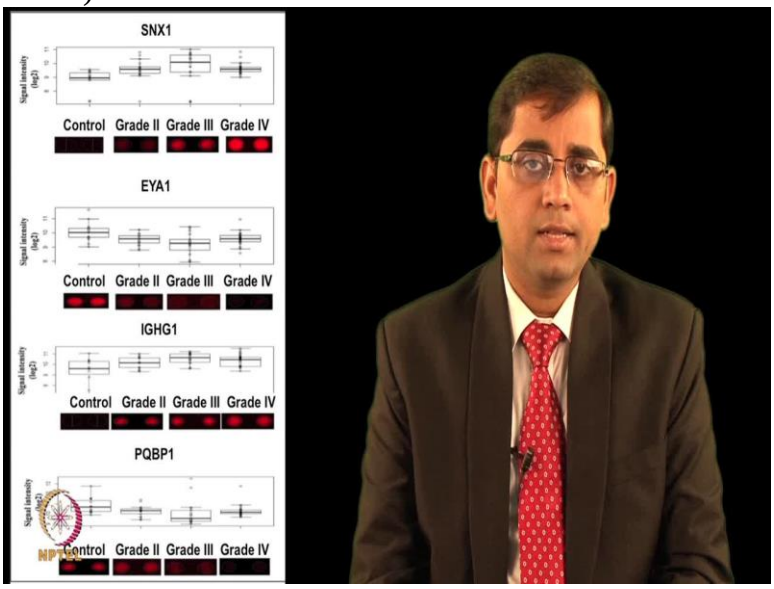
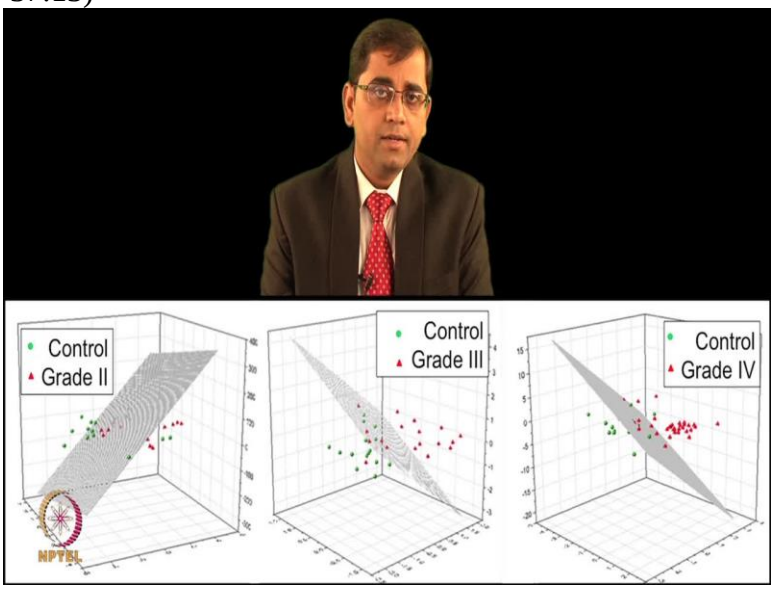
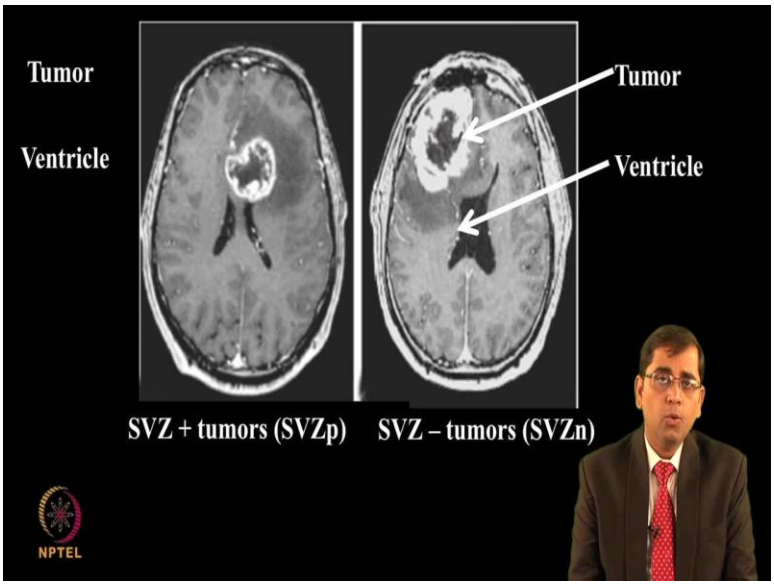


The study revealed panel of interesting proteins like IJG1, SNX1. They were up and PQBP1 I1 proteins as down regulated. The sorting nexin 1 or SNX1 is known to interact with EGA and is highly relevant in gliomas. EYA1 protein has putative role in in net immunity, DNA damage repair, angiogenesis and cancer metastasis.

IJG1 or immunoglobulin heavy constant gamma 1 protein may play a role in immune system evasion mechanisms. The polyglutamine binding protein 1 or PQBP1 binds to be add into an POU3 class of neural transcription factors which inhabits the transcription activation of BRN2. BRN2 is known to be expressed in gbms and associated with development of neural and clear cells.

(Refer Slide Time: 37:13)

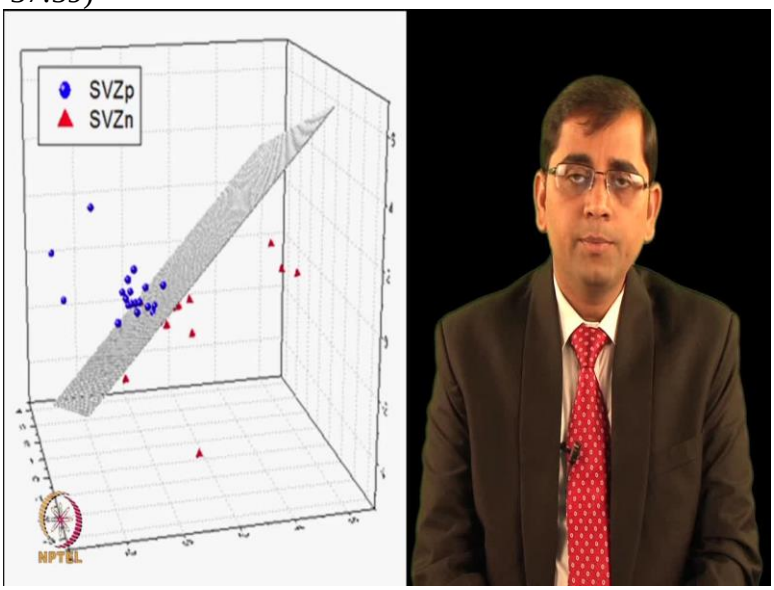




As you can see in these MDS plots that the disease cohorts show a significant segregation from healthy and disease cohorts when this specifier proteins are applied. Apart from differentiation between grade and healthy controls the authors have also used a same platform to identify the productive markers for better prognosis.

It has been observed by the clinicians that if glioblastoma multiforme tumors occur in (())(37:44) zone of the brain known as SVZ positive, the prognosis of such of patients are poor as compared to the grade 4 tumor occurring outside the SVZ region or SVZ negative.

(Refer Slide Time: 37:59)



Using similar data analysis strategies, they investigators here identified protein net nine, a protein which is involved in cell migration as one of the differentiating protein for SVZ positive with SVZ negative.

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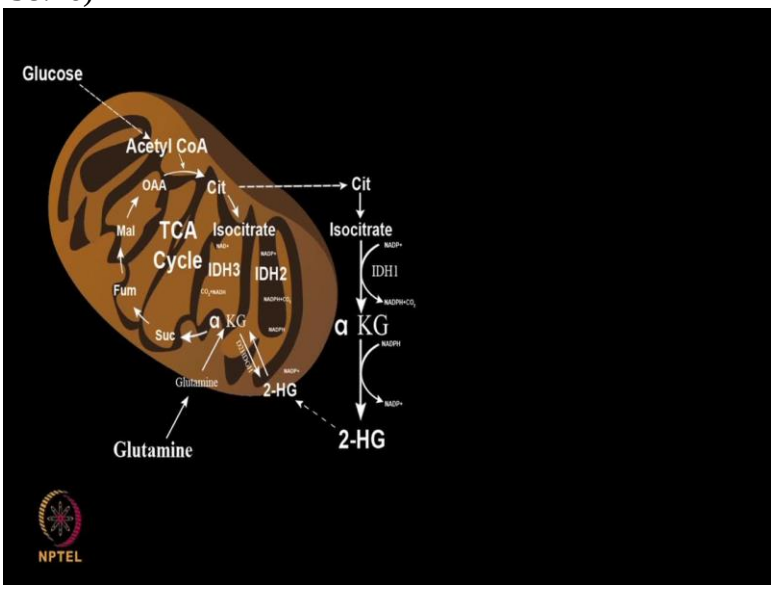

Mutation in a gene called isocitrate dehydrogenase IDH is also associated with better prognosis in glioma patients. So patients with IDH1 mutation or IDH positive show better prognosis than those with IDH1 wild type gene.

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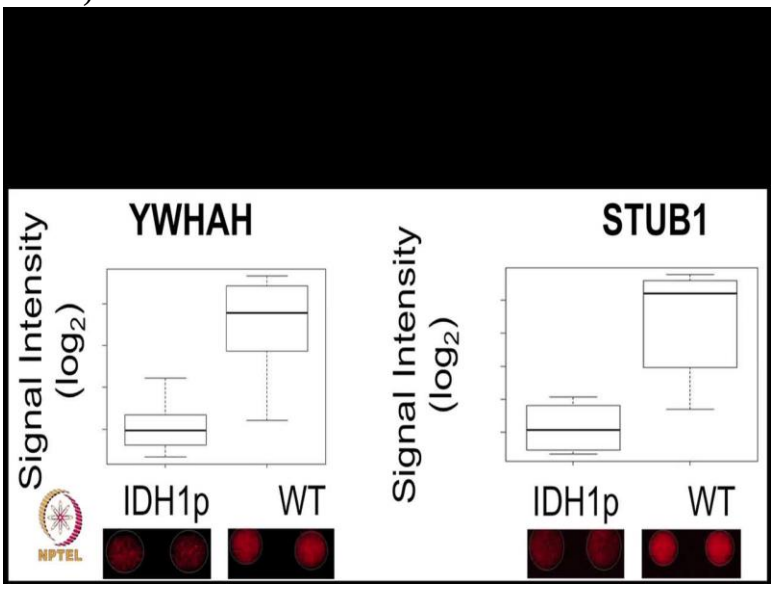

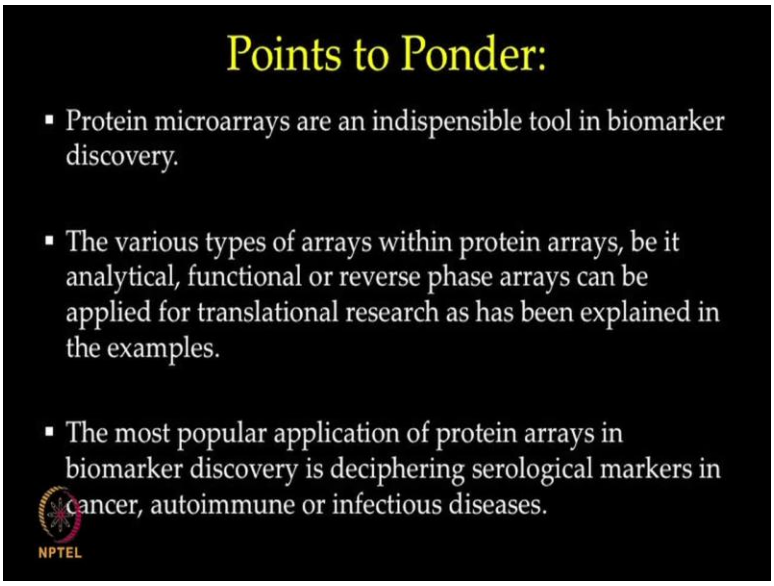

Similar comparisons using protein array data led to the identification of a protein YWHAH and STUB1 which were two of the twenty two distrivet proteins investigators have identified which are known to be involved in proliferation of glioma cells. They were down regulated in IDH1 patients which allowed the authors to correlate the pathological prognosis with their findings.

So today we have discussed several examples how protein arrays can be used for verity of clinical research questions, especially pertaining to the biomarker identification. Considering the vast diversity of protein array types each array can be used for multiple set of applications. In the next lecture we will talk about some of the example as to how to understand the scope of this powerful technology especially to understand protein-protein interactions studies using protein microarrays. Thank you.

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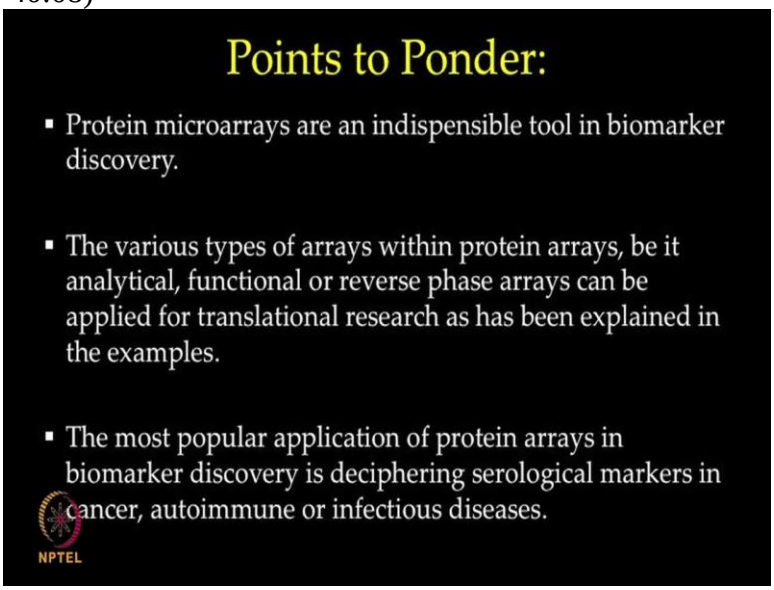

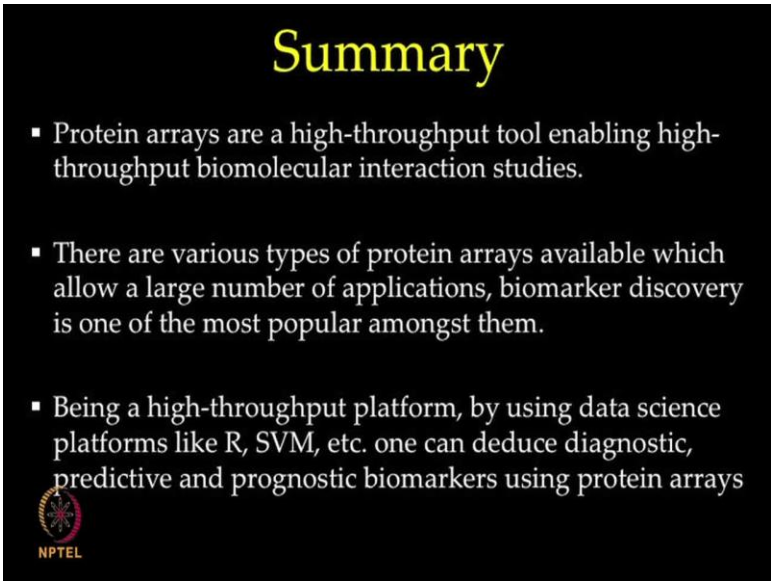

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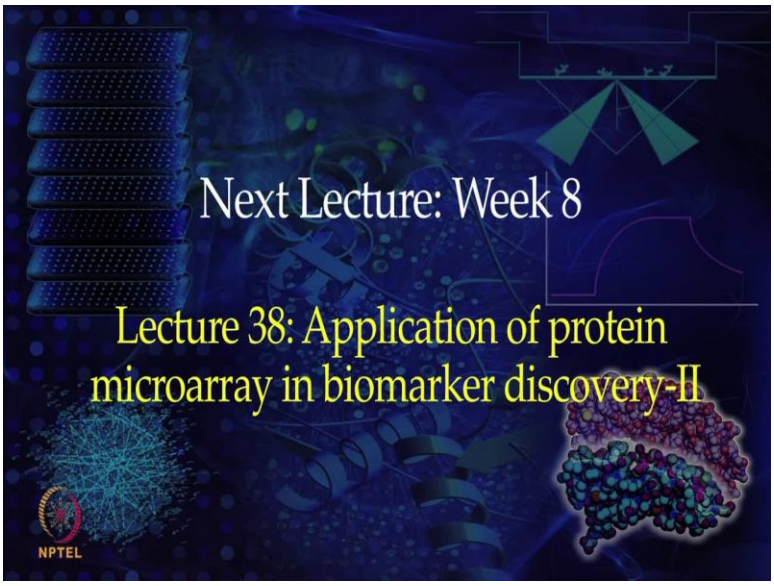
Next Lecture: Week 8
Lecture 38: Application of protein microarray in biomarker discovery-II
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