Interactomics: Basics & Applications

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Lecture – 25
Basics and Applications of Reverse Phase Protein Arrays – I

Today's lecture and the next two lectures will be delivered by a guest scientist Doctor Ramesh Ummanni. Doctor Ummanni is a scientist at the CSIR, Indian Institute of Chemical Technology, Hyderabad. He focuses on identifying new potential biomarkers and understanding cell signaling mechanism driven by deregulated proteins associated with prostate cancer. Doctor Ummanni group works on proteomics platforms for differential as well as functional proteomics. He is actively pursuing investigations to understand disease molecular mechanisms and ruggable targets is specific to different solid tumors.

Doctor Ramesh Ummanni is currently also working on many comprehensive projects which are related to identifying new chemical entities with anti-cancer and anti-tubercular potential uses cell based and target based screening of a small molecule libraries. In today's lecture, Doctor Ummanni will talk to us the basics of reverse phase protein arrays which is a high throughput proteomics platform for discovery, validation and clinical applications.

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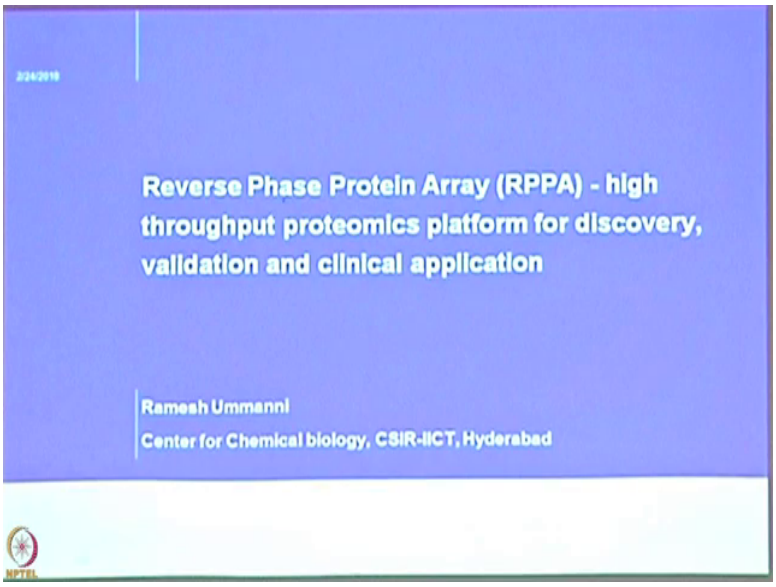

Good afternoon to everyone. I am Doctor Ramesh Ummanni from India Institute of Chemical Technology, Hyderabad. I should thank Doctor Sanjeeva for inviting me to interact with you and share the technique how this reverse phase protein arrays are useful in the array platforms. And also I would like to highlight how this RPPA platform is different from the routine protein array platforms, many people have been using for quite a bit of time.

So, my lecture I do not have any hands on training session to give you, to perform the experiments, but all the necessary steps you are doing same in this, but exactly the sample to antibody is reverse, that is it. That is why it is called as reverse phase protein arrays. So, I try to stress where it is different from the routine array routine protein arrays and what are the steps to be taken care to perform the arrays and how to do data analysis, and also I will try to show some of our studies we have performed in the past, how we use this technique and try to

address the biologically relevant question in terms of clinical samples as well as in the biomarker validation as well.

So, all in all I would like to tell you that by end of my lecture so you should feel that it is imaginary how much you can imagine to use this technique into your lab research. That is what I would like to say. So, just imagine that how I can use this technique into my science or your PhD work or maybe your postdoctoral work or maybe any biological question to be asked was to be addressed with the help of clinicians or with the help of researchers in the lab, ok.

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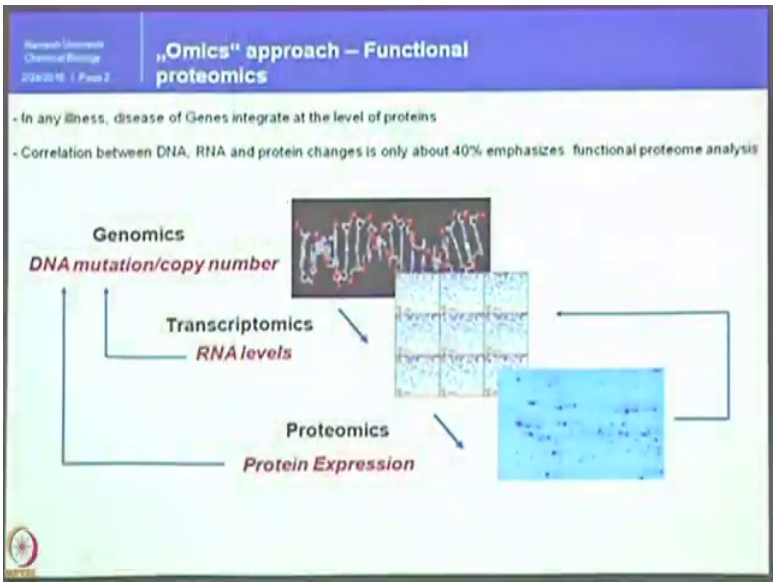

So, essentially here if you look at it here is a central dogma of molecular biology, ok, so DNA to RNA, RNA to protein. We all know that, so all the essential functions to be done to be carried out by a cell stored its information in the form of DNA in the form of genes, but those

genes alone cannot do anything, but it is the functions or the molecular functions of a cell are to be done by the products of the genes that is why.

So, if you look at the correlation between the gene to protein it is very poor. If you look at the gene to RNA the correlation is only 60 percent; that means, 40 percent of genome information is already missing in the transcriptome level. When you look at the correlation between transcriptome to proteome level again the correlation is very very poor, it is only 40 percent. So, ultimately if you look back protein the genome the correlation is not more than 30 percent on average.

And also I should emphasize here the proteomics has been evolved and advanced much more in this recent past to address many biologically relevant questions which are unanswerable by any of the genomics approach or transcriptomics approaches. Nevertheless and also it is important to mention here that mass spectrometer will tell about the abundance of the proteins, ok. But advance mass spectrometry definitely tells you about the activation status of the proteins in terms of whether phosphorylation or the post translational modifications such as oscillation, glycosylation and so on.

So, that is where it is very very important to look at the protein function really. Sometimes protein may express, but it may keep quiet. It says like a silent mutation in the gene, it may keep quiet nothing to do. So, ultimately if it is a functional protein then only it reflects in the DC state. If you look at here any illness or disease of genes integrated at the protein level that too at the protein functional level. So, that is one of the reason you should perform the functional proteomics rather than the differential proteomics.

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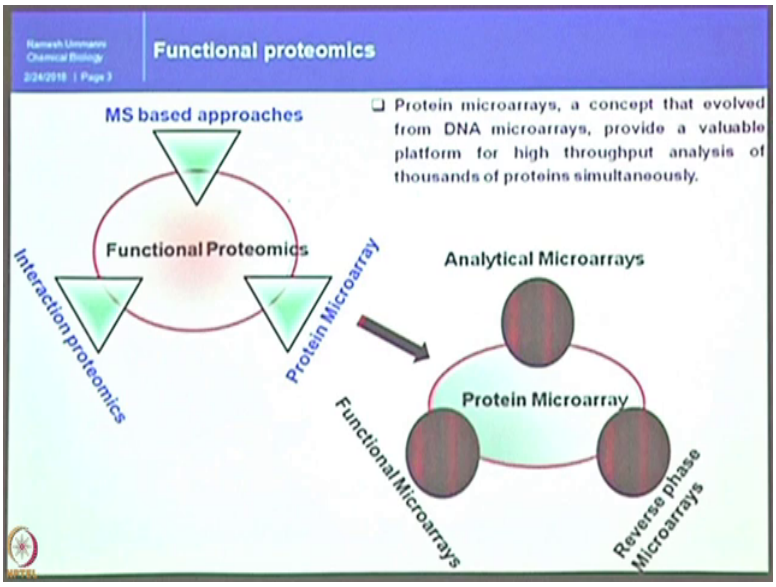

If you look at any proteomics books available in the library or maybe in the internet see that proteomics is broadly divided into different areas like a differential proteomics, and the structural proteomics and also the functional proteomics.

So, differential proteomics always tells about the upregulation down regulation. So, what? Gene is also up regulated, down regulated. We have to see whether it is up regulation mean it is really influencing the disease or not. So, that is where the functional proteomics came into picture and it gained the importance to work on this area further.

There are many different approaches, one can do functional proteomics. So, particularly MS based approaches, and interaction proteomics, and protein micro arrays. So, here already you can see that we are moving towards the theme of our course work here. So, in this case look at as Sanjeeva highlighted in the morning, so the basically how the concept has been evolved,

how the protein micro arrays are evolved in the research field. So, from the least concept has been evolved from DNA microarrays provide a valuable platform for high throughput analysis of thousands of proteins simultaneously, ok.

We all know that the traditional biochemistry labs, I did not have the nice cartoon to show here every time people used to clone one gene, make a vector, express it and wait what it happens, what it will do the cell. That way they used to analyze one protein by one protein that is traditional biochemistry labs. Now, the ones omics has been evolved the thousands of proteins functions can be analyzed in a simultaneous manner, so that definitely or certainly can be done using this array platforms.

So, particularly I wanted to emphasize on the protein microarray this protein microarrays are also broadly classified into three different types, analytical microarrays, the functional micro arrays and reverse phase micro arrays. So, based on the principle involved in this protein array technology. So, this classification has been nomenclature as a 2D. So, particularly I do not want to emphasize on all of them today, I will maybe in the next lectures I see in the program see they will cover all this analytical micro arrays and functional micro arrays that is the reason I would like to concentrate only on the reverse phase protein array here. And also all of you have done western blots in the lab.

So, to do one western blot at least you need a minimum 20 micro gram protein, and of course, you have to measure about the housekeeping gene you need another 40 micro gram protein another 20 micro gram. So, total all in all 40 micro gram protein is required to measure whether the protein is up or down compared to one of the other samples. This case 40 micro gram protein is a lot on today when the all proteomics platforms are evolved into such an advanced stage. So, to avoid that definitely the technique what we are going to speak about today reverse phase protein arrays are the alternative to western blotting technique.

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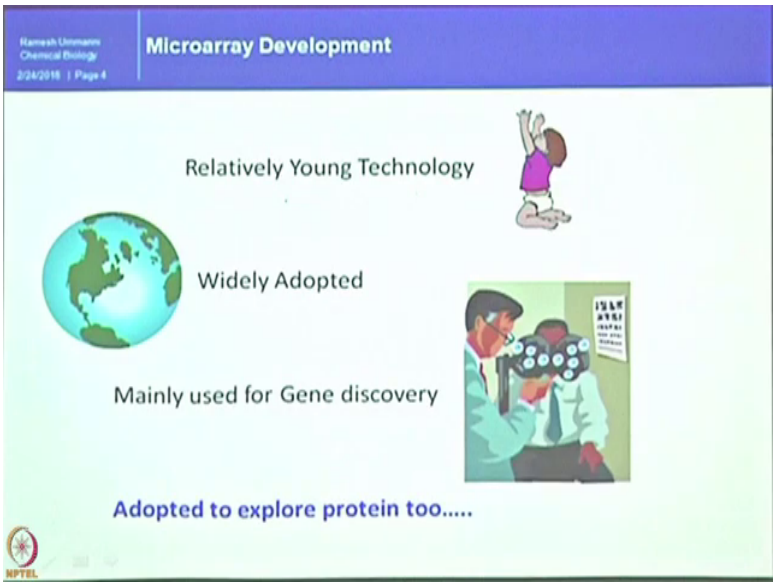

So, micro array development if you look at it relatively very young technology, widely adopted by many researchers for many different applications and mainly this is used for gene discoveries. But our clever proteomic community has been adopted to protein analysis as well.

So, when I started protein microarray projects I thought only array means it is a DNA array, I did not know that protein array was something like that was there at all. So, then I understood that in fact, this array name is more exploited by the proteomics people than the genomics people. That is what I always claim in any of the open platforms in indeed.

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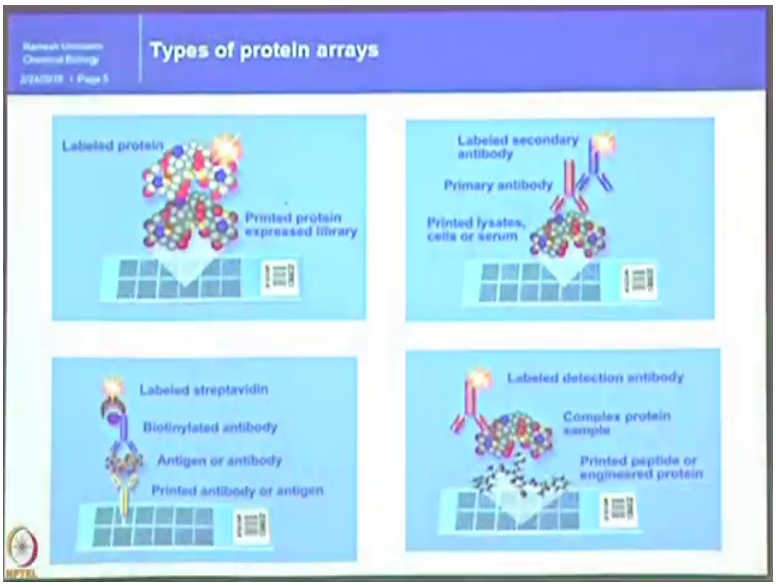

So, nevertheless, if you look at this picture this nice cartoon here, there are different types of arrays. In the first cartoon you can see the same slide with a barcode and one glass slide printed with protein expressed libraries, and if you can add the protein which is already pre-labeled to the glass light, if those proteins printed on the glass slide have the interactant partners in the lysate they will attract and immobilize them. When you wash out, so other proteins will get washed away and those bound to that remain on the glass slide and we can visualize them.

Then you can see that, so particular protein x which is labeled bound to the protein y and the glass slide; that means, they are interacting with each other. Then you can proceed to see that whether this interaction has any physiologically relevance in a DC state or non-DC state or not.

The second way of approach is that again the glass slide. So, we all talks about glass slides today. So, glass slide printed with the antibody or of course, antigen also you can quote it in this case antibody, and then add the lysate which is a pool of all the proteins which are expressed by the Orpheum then those antigens which are specific to antibody captured by the captured antibody. Of course, this protein is not labeled here that is why we need a detection antibody.

So, the detection antibody is can be any biotinylated antibody or any choice of your interest. So, then you can detect the this protein which is immobilized, which is captured by the captured antibody by looking at the signal here. So, this signaling methods I will explain you what are the different methods can be used.

And here that this is a real approach what we are going to talk next of an hour of 45 minutes. So, here the lysates can be printed. So, today morning Sanjeeva explained that he is also printing a protein, but it is a externally expressed pure protein in a each spot. But in this case we are going to print as such protein lysate. Take a cell lysate, when you scrape them in a cell culture lab and lyse them and spin down the supernat contains a pool of proteins, that sample directly you are going to spot here.

After spotting then you add a primary antibody. Then primary antibody binds to the specific protein of interest, protein in the spot and then the secondary antibody will detect the signal. So, it is same essentially you can call it as a dot blot or micro western blotting approach here, ok.

So, then the last sample. Here you can make the peptides, you can small peptides or engineered protein like morning Sanjeeva explained you can spot them here and you can also take a complex mixture of proteins already pre-labeled add that mixture into the slide. So, those proteins which have affinity towards these peptides or engineered proteins will bind.

So, more or less these two slides, these two cartoons explain a similar approach, but there is a little different here. Here you see that printed protein, the full length protein is spotted here.

This only peptide is spotted here. The difference is here. You can also use this approach for epitope mapping.

So, you can make a fragmentation of the peptides. So, those peptides can be printed separately and you can map on the which domain of the complete protein is binding to the binding partner. See maybe it is a 100 amino acids, you chop down into 4 different parts 25, 25, 25. So, the interacting partner the bait prey protein may bind to the peptide between 51 to 75. So, how can we map that? So, this is approach can be used to map the really binding domain or the interacting domains between the two proteins.

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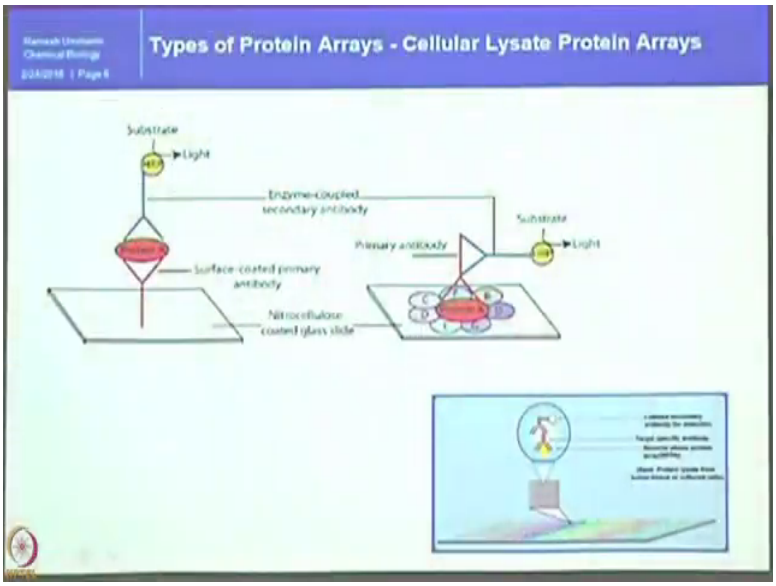

So, you can see that this is a forward array where antibody is spotted and the protein A is captured by the antibody from the pool of lysate, and the secondary antibody specific to protein A is used for the detection. So, this is coupled to enzyme linked secondary antibody.

If you look at here have a glass slide which is coated with a nitrocellulose membrane and is a pool of lysate along with your protein of interest. Here we have A to G proteins. So, in a single spot it contains a protein of interest along with other proteins, then you add a primary antibody against protein A. So, it will bind to the protein A in the mixture. Then use a secondary antibody directly against to the primary antibody you have used. So, you can see that this red Y is now detecting the protein A. Here in this case red Y is capturing the protein A that is a difference between forward array and reverse array.

So, once you have the primary antibody bound to the protein A after several washings, you can use the detection antibody which is nothing but a secondary antibody labeled with specific visualization method then you can detect it. So, this can be miniaturized into high throughput array by spotting many different number of samples.

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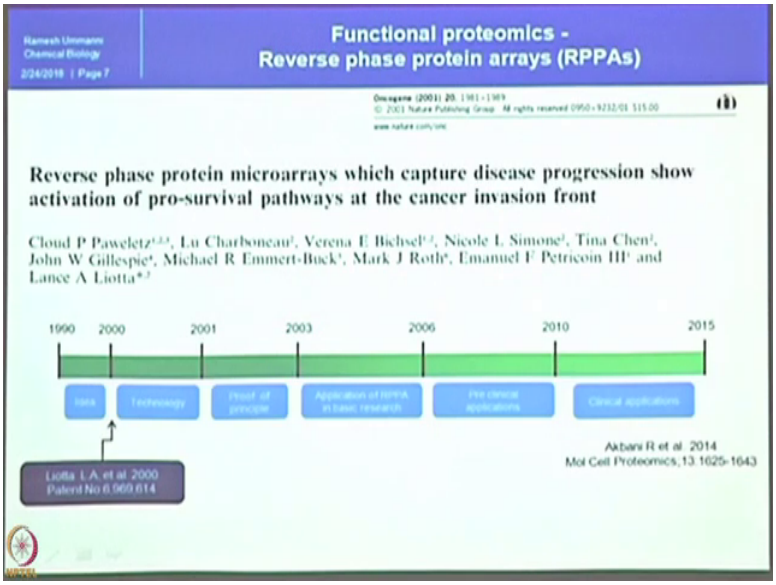

So, this is basically the reverse phase protein array. This concept has been developed by this two great scientists Petricoin and Lance Liotta from US MD Anderson. And they started to optimize this technique or they started to establish this technique at 1990 finally, it came into the light in 2001 after proof of concept and so on. Further it is progressing, now in 2015 people announced that this can go really into the clinic towards the personalized medicine. Now, how? I will try to convince you that how it can be used in the personalized medicine. And then obviously, it requires lot of technically technical challenges.

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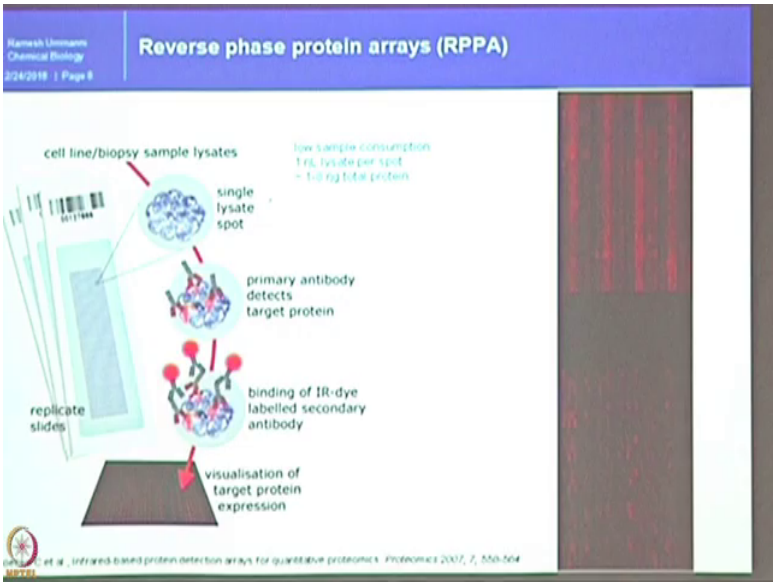

So, this cartoon explains the spotting of the samples it is a single lysate spot. That means, if you have 1000 samples, so 1000 samples will have 1000 spots here. Individual spot belongs to one sample remember that, not one protein. That is where always we think that one spot will contain one protein that is a forward approach. In reverse approach one spot will contain 1000s of proteins from one sample. So, once we spot it then we have a primary antibody to

detect and then visualization after that array use this kind of data then we have to proceed for data analysis.

So, advantage here is that in one spot requires only low volume of sample, we spot only 1 nano liter. Think about from 1 nano liter, you can quantify 1 protein. In western blot you need 20 micro gram; that means, this 20000 nanograms. So, now, sorry 1 to 8 nanogram protein. Let us say 10 nanogram is required, so if you want 20000 divided by; so, you can quantify 2000 proteins from 20 micro gram protein sample in this reverse phase protein array approach. So, the total protein you will spot depending on the intensity or the protein of interest between 1 to 8 nanogram of total protein once you spot the array slide looks like here.

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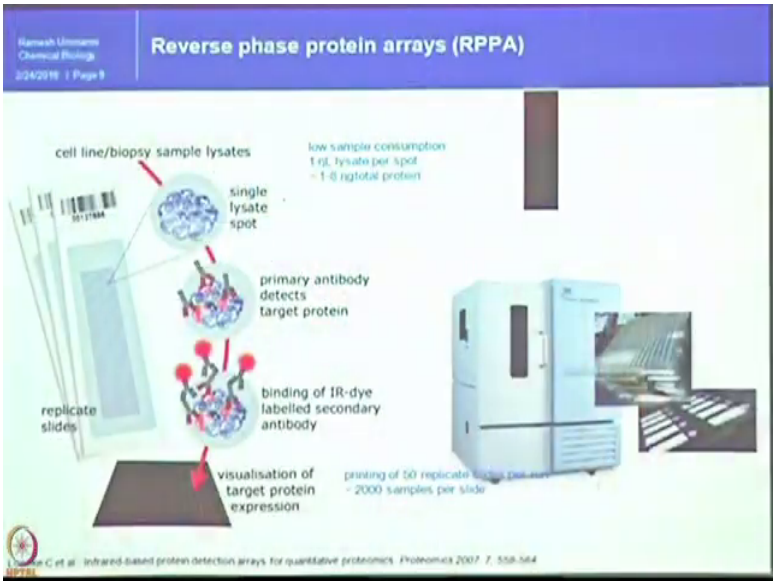

Then of course, to print such a high density array you need a sophisticated machine you cannot simply spot with pipette. And this machine is really useful nowadays meant for reverse

phase protein arrays which can print 50 replicate slides per run with the 2000 samples per slide. And also why I am highlighting about this instrument, I am not from definitely company. Thing is that when you print the first slide the same quantity should be printed on the last slide, so duration will be 3 hours. In the first slide if the one spot contains 1 nanogram, 8 nanogram, the last slide also should have 8 nanogram protein.

So, this machine has been decided designed in such a way that it will give a uniform printing of the slides. And also from one corner to other corner it should spot in a precisely the same amount of protein by maintaining the humidity and x y z parameters inside the machine.

So, this machine capacity is very good as on today, and main thing requirement as well as serious limitation in reverse phase protein arrays is the good quality antibodies. I am sure many of us might have used Santacruz antibodies, when we do western blot we see 10 different bands which belongs to our target of protein interest we do not know.

Those antibodies are very very limited and we cannot use them for reverse phase protein array approach that is why this reverse phase protein array is difficult to perform in protein laboratories.

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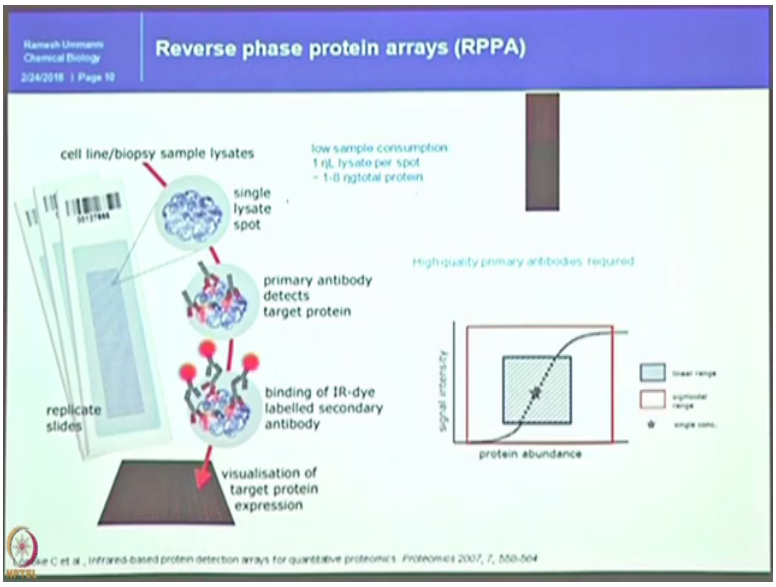

So, how to there is a way. And also there are multiple detection methods are available alkaline, phosphate detection, HRP detection, chemiluminescence, and also infrared imaging as well as site labeling and all. So, in our approach in our lab previously we used infrared labeled secondary antibodies to visualize the primary antibody signals. Why? So, IR signals always have a high dynamic range, compared to chemiluminescence method or any alkaline phosphorus method like a chromogenic substrate.

So, linearity means if the protein concentration is too high they will signal will get saturated very quickly, once the signal is saturated you cannot get a proper quantification and proper quantifiable differences between control and experimental samples. So, that is the reason to avoid that to have a broad range of detection range, so we used infrared imaging levels, infrared imaging infrared label secondary dice.

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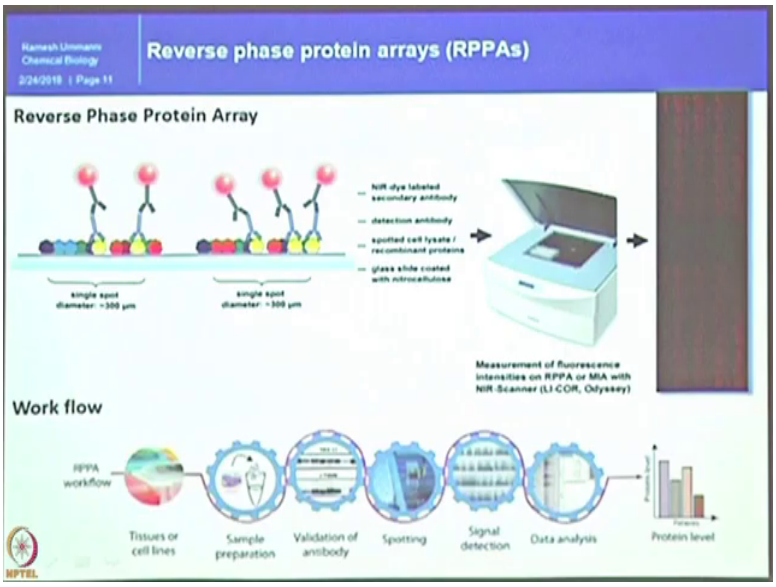

So, then here I would like to highlight how the array looks like once you print. You can see that glass slide coated with nitrocellulose, the spotted lysates which can be recombinant proteins or the total protein lysates and here is a detection antibody and near infrared dye labeled secondary antibody. It is the same simple western blotting approach explaining two plus two standard student, ok.

Advantage, single spot diameter is 300 micrometer. That is why you can accommodate more number of samples on slide. Then you can perform the array and then you will get the signals like this. Here is a complete workflow. I will walk through this complete workflow in next one hour, each step by step.

First is a tissues or cell lines, what kind of samples can be analyzed, what are the different types of methods to apply for sample preparation and then how to choose antibody, what are

the different steps to be taken care while selecting the antibodies for rpms, and how to avoid later on issues, then spotting pattern, spotting methods, signal detection methods, how different methods are advantages one on the other for detections.

And finally, data analysis, and what kind of data we see and I will show one or two examples, how we generated the data, how they are useful in literature.

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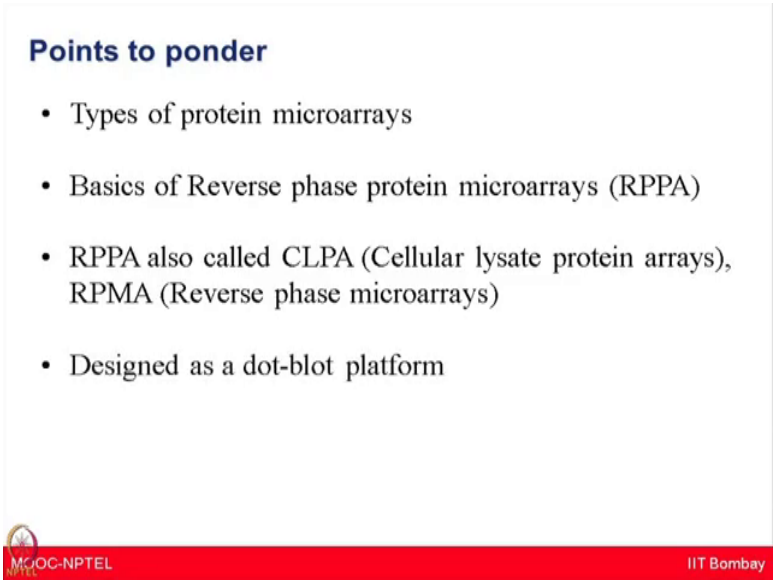

Since you are aware that proteins can be printed in a different manner on the chip and those could be used for many interesting applications. We have discussed that you can print the cDNA and do the in-vitro transcription translation to make the protein on the chip or one could do a laborious way of expressing and purifying the proteins of interest or you can have recommend antibodies to a printed or even you can have the reverse phase protein arrays

where you can take the tissue lysates or cell lysates where you can probe with the specific antibodies and proteins of interest.

In addition to just having the purified protein or antibodies printed or even having cDNA to make chips like the cell free expression based arrays, one could also try to probe directly a specific target of interest in reverse phase protein arrays as we have discussed today with Doctor Ramesh Ummanni.

So, I hope today you have learned about reverse phase protein arrays, the basic workflows and how the array looks once it printed on the chip and the advantages of using RPPA. This talk will be continued in the next lecture where Doctor Ummanni will talk to us the entire workflow of reverse phase protein arrays in some more detail.

Thank you.