Lecture – 18
Basics and Applications of Reverse Phase Protein Arrays-I

Welcome to MU codes, on applications of Interactomics, using genomics and proteomics technologies. Today's lecture and the next two lectures will be delivered, by a guest scientist, Dr. Ramesh Almani. Dr. Almani is a scientist, at the CSIR, Indian Institute of chemical technology, Hyderabad. He focuses on, identifying, new potential biomarkers and understanding cell signalling mechanism, driven by, deregulated proteins, associated with Prostate cancer. Dr. Almani's group works on, proteomics platforms, for differential, as well as, functional proteomics. He is actively pursuing, investigations, to understand, disease Molecular mechanisms and druggable targets, is specific to different, solid tumors. Dr. Ramesh Almani is currently also working, on many comprehensive projects, which are related to, identifying, new chemical entities, with anti-cancer and NT tubercular potential,
uses cell based and target based, screening of, a small molecule libraries. In today's lecture, Dr. Almani will talk to us, the basics of protein arrays, which is a highly throughput, proteomics platform, for discovery, validation and clinical applications. Good afternoon, to everyone. I am Dr. Ramesh Almani, from India Institute of chemical technology, Hyderabad. I should thank, Dr. 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 RTP a platform is, different from the routine, protein array platforms. Many people have been using, for quite a bit of time.
So my lecture, I don't 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 sampled antibody is reverse. That's it. That's why, it is called as, 'Reverse Phase Protein Arrays'. So I'll try to stress, where it is different from, the routine array or 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's 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 by, to be addressed, with help of clinicians or with the help of researchers, in the lab. Okay, so essentially, here if you look at it, here is a central dogma of molecular biology. Okay? 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's why.
So if you look at the correlation, between the gene to protein, it's very poor. If you look at the gene, gene to RNA, the correlation is 04only 60%. That means, 40 percent of genome information is already missing, in the Transcriptome level. When you look at the correlation between, Transcriptome proteome level, again the correlation is very, very poor, it is only 40 percent. So ultimately if you look back, protein the genome, 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. Okay? But advance mass spectrometry, definitely tells us, tells you about the, activation status of the proteins, in terms of either Phosphorylation or the post translational modifications, such as Isolation Glycosylation and so on. So that's 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, disease state. If you look at here, any illness or disease of genes, integrated at the protein level, that what the protein functional level. So that is one of the reason, you should perform the functional proteomics, rather than the, differential proteomics. 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, up regulation 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's

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, on 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, provided valuable platform, for high-throughput analysis, of thousands of proteins, simultaneously. Okay? 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, what it will do the cell? That way they use to analyze, one protein, by one protein, that's 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 micro arrays, are also broadly classified, into, three different types; Analytical micro arrays, 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, nomenclatured, as on today.

So particularly, I don't 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 these analytical micro arrays and functional micro arrays. That's the reason; I would like to concentrate, only on the reverse phase protein array. 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 micrograms. 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 of evolved, into such a 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. So, microarray 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's a DNA array. I didn't 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's what, I always claimed, in any of the open platforms, in indeed. So, nevertheless, if you look at this picture, this nice cartoon here, there are different types of arrays.

In the first cartoon, can see the same slide, with a barcode. And one glass slide, printed with protein, express libraries. And if you can add, the protein which is already, pre-labelled, to the glass slide. If those proteins, printed on the glass side, have the interact and 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 labelled, bound to the protein Y, on 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 disease state or non disease State or not. The second way of approach is that, again the glass slide. So we all talks about, glass slides today. So glass slides, printed with the antibody or of course antigen also, 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 antigen, antigens, which are specific to antibody, captured by the, capture antibody, of course, this protein is not labelled here, that's why, we need a detection antibody. So the detection antibody, is can be antibody related any bi-accumulated antibody or any, choice of your interest. So then., you can detect, the bi, this protein, which is immobilized, which is captured, by the captured ,antibody, by looking at the signal here. So this signalling methods, I will explain you, what are the different methods can be used. And here, this is a real approach, what we are going to talk, next half an hour or 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 supernatant contains a pool of proteins. That sample, directly 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 can call it as a, 'Dot Blot or Micro Western blotting approach', here. Okay? So then the last sample. Here, we can make the peptides, can small peptides or engineered protein, like morning, Sanjeeva explained. Can spot them here. And we can also take a complex mixture of proteins, already pre labelled, add that mixture into the slide, so those proteins which have affinity, towards these peptides, are 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 true printed protein, the full-length protein is spotted here, so 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's a hundred amino acids, you chopped down into, four 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. 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, A2 G proteins. So in a single spot, it contains a protein of interest, along with other proteins. Then you had 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's the 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, labelled 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. So this is basically the, reverse phase protein array. This concept, has been developed by, these two great Scientists, Petra Cohen 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, which are technical challenges. So, this cartoon Explains, the spotting of the samples, it's a single lysate spot. That means, if you have thousand samples, so thousand samples, will have thousand spots here. Individual spot belongs to, one sample, remember that. Not one protein, that's where always we think that, one spot will contain, one protein that is a forward approach.

In Reverse approach, one spot will you contain, thousands 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, one Nano litre. Think about, from one nano litre, you can quantify one protein. In Western blot, you need 20 microgram. That means is 20,000 nano grams. So, now, sorry, a, 1 to 8 nano gram protein. Let's Say, 10 nano gram is required. So if you want 20,000 divided by, so you can quantify 2,000 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 nano gram of total protein. Once you spot, the array slide, looks

like, here. 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 2,000 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 one nano gram, a 8 nano gram, the last slide also, should have, 8 nano gram 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 don't know. Those antibodies are, very, very limited and we cannot use them for reverse phase protein array approach. That's where, this reverse phase protein Array, is difficult to perform in protein laboratories. 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 labelling and all. So, in our approach, in our lab, previously, we used, infrared labelled secondary Antibodies, to visualize the primary antibody signals. Why? So IR signals always have a, high dynamic range. Okay 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's the reason, to avoid that to have a broad range of detection range, so we used, infrared imaging labels, infrared even infrared label secondary dyes. 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, there is a detection antibody and near-infrared dye labelled, secondary antibody. It's the same, simple, western blotting approach, explaining to, +2 standard student. Okay, advantage, single spot diameter is, 300 micrometer, that's why you can accommodate, more number of Samples, on slide.

Then you can perform the array and then you get the signals like this. Here is a complete workflow; I will walk through, this complete workflow, in next 1 hour, each, step-by-step. First is a, tissues are 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? So as you are aware, that protease 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 be 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 empty bodies, 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 Dr. Ramesh Almani. So I hope today you have learned, about reverse phase protein arrays, the basic workflows and how it looks, once it printed, on the chip and the advantages of using, RPPA. This talk, will be continued in the next lecture. Well Dr. Almani will talk to us, the entire workflow, of reverse phase protein arrays, in some more detail. Thank you