

NPTTEL VIDEO COURSE – PROTEOMICS

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LECTURE-34

APPLICATIONS OF CELL-FREE PROTEIN MICROARRAYS

TRANSCRIPT

Welcome to the proteomics course. In today's lecture we will talk about applications of cell-free protein microarrays. In previous lecture we discussed how different type of cell-free systems could be employed to generate protein microarrays. We discussed different type of approaches including protein in situ array (PISA), nucleic acid programmable protein arrays (NAPPA), multiple spotting techniques (MIST), HaloTag arrays as well as DNA arrays to protein arrays.

So in general protein microarray technology allows the identification and quantification of proteins in high throughput manner. The recent advances in the protein microarray technologies, they found unique opportunities to find novel biomarkers as well as apply this platform for several applications. The combination of proteomics technologies especially the protein microarray have potential to apply for wide variety of biological applications. The application of cell-free application based protein microarrays have seen rampant increase because of ease of synthesizing the proteins by using cell-free expression based system as compared to the cell-based traditional way of purifying the protein and then printing on the array surface.

So in today's lecture we will talk about an overview of protein microarray experiment. So first of all I will give you a broad overview how any type of protein microarray platform can be used for different applications. I will then describe few applications very briefly by taking some case studies. This will just give you an idea that how these platforms are used for wide variety of applications including biomarker screening, immunological studies, protein-protein interactions as well as protein-protein interactions. Finally we will touch upon the challenges of analyzing the microarray data regardless of you are performing an experiment to identify the protein interactors or looking for new biomarkers. The microarray experimental setup provide very high throughput platform

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and generate data for the thousands of features simultaneously therefore analyzing such data becomes very challenging. So these three points will be discussed today.

So let's first start with an overview of protein microarray experiment. So regardless of application what one wants to probe on the protein microarray there are certain steps, a work-flow is involved which need to be followed to perform such experiments. Now protein microarrays whether you have a cell-based or a cell-free platform, you need to perform certain steps. But if you are using the cell-free based protein microarray setup then you have to perform certain additional steps. Additional steps involve synthesis of protein on chip first of all and then all the steps regardless of it is cell-based or cell-free it remains the same. It means in general I will show you an overview of various steps involved in performing a protein microarray based experiment.

So let me give you an overview of various steps involved in performing a protein microarray experiment. I will show you how human proteome chips can be used for screening the biomarkers by using human serum. Now as I mentioned same overview same steps can be performed for various types of applications so these chips have to be stored so these chips have to be stored at precisely -80°C , you do not want to lose the protein activity if it is a purified protein array if you are doing cell-free expression based protein microarray then you do not have to worry. You can even store the chip at room temperature. So there is only one difference between cell-free expression based protein microarrays and cell based protein expression microarrays, in the cell-free expression one you have to add the in vitro transcription and translation machinery to synthesize the protein and then whole assay can be performed in the chip, in the protein microarray cell based system purified proteins are printed on the chip and those are stored at the -80°C . As you can see in the 3D animation very carefully the slides were removed from the -80°C freezer and now one need to thaw those slides very gently to avoid any diffusion type of effect. The typical laboratory setup where you do not require very fancy setup here because similar to the western blot all the steps can be performed. After removing chips from the -80°C freezer or synthesizing the proteins by using cell-free expression based system, first of all you would like to block those areas

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which do not have the spot features so to avoid non-specific binding. First of all, one needs to add a blocking solution. Blocking can be performed by milk, it can be performed by BSA, superbloc as well as scientist prefer a cocktail of different reagents which could be used for the blocking solution.

Typically blocking can be performed at the room temperature for an hour on a rocking shaker or it can be performed 4°C overnight in the cold condition. A small pipette tip box even can be used for this purpose where you can add the superbloc or the blocking solution and then emerge the slide. One need to ensure the proper shaking while performing the blocking experiment, we don't want the milk or the blocking reagents should be dried. It should be submerged on the chip surface so it has to be very uniform and gentle shaking. After blocking step is completed remove the slide from the blocking solution and tap against a paper towel so that one can remove the excess milk. So as I mentioned one need to ensure that this continuous mixing of the slides because if it is left sitting on the rocker without mixing then slide will dry and the slide will appear dark during scanning.

In a typical microarray experiment as I mentioned regardless of your application one need to perform certain set of steps which are quite generic and then depending upon the requirement one can make changes and optimize the condition for those experiments. So a typical experiment includes a primary antibody where one can use anti-query proteins if you are looking for the protein-protein interactions or one can add the patient serum which we are going to show you in this one for the immune response detection and a marker-linked secondary antibody which is usually the HRP-conjugated anti-mouse IgG or Cy3 Cy5-conjugated anti-Human IgG can be used for signal detection so now once blocking is completed. One can apply the primary antibody as I mentioned it can be a primary antibody or it can be serum if you are looking for the immune response detection. One needs to ensure the right dilution because most of the time serum gives very high background on the chip surface. So, appropriate dilution can be optimized based on the requirements. Often these conditions are quite similar to the one uses for the western blots. Once you are ready with the appropriate dilution of the

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serum then you can apply those on chip surface and place the cover slip for an hour. Similarly you can add the primary antibody and then place the cover slip. As I mentioned these are generic steps which can be used for variety of applications. So once the primary antibody or the serum is placed on the chip surface then one need to incubate for at least for one hour and again different scientist try different approaches. Few people prefer using overnight incubation condition at 4°C and some groups prefers using one hour at room temperature. A different school of thoughts here one is that if you are allowing serum for long time it is possible that it is going to give enough time for identifying the right targets. On hand, if you are allowing is for very long for example overnight incubation it is possible that background will become very high so people try different type of conditions in the labs and then they apply serum or antibody and then adjust the incubation time accordingly. Once primary antibody incubation is done they you need to do the washing with the PBS tween.

The washing steps are very important in microarray experiment one need to do at least 3-4 washing with PBS and PBS tween. Just so that you are removing bound antibodies on the non-specific array surface if your washing steps are not very meticulous in the microarray experiment at the end you will see very high background and non-specific binding which will interfere with the signal detection. After washing step apply the secondary antibody for example anti-human IgG and again appropriate dilutions can be selected depending upon what dilutions work best in your experimental setup. After addition of a secondary antibody the chip can be incubated for an hour at room temperature. You need to put the coverslip on the chip to avoid any dust on the chip surface.

Different investigators use different strategies for identifying the signals for example a secondary antibody could be conjugated with the HRP based system. If that the case then one can use even a tyramide signal amplification system so TCA reagent is a tyramide molecule which is link to a label, it could be Cy3, Cy5 which is activated by horseradish peroxidase to form a free radical. As the reaction continues the label molecules continues to accumulate and therefore one can see the good signal by the

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TSA based detection system. Now when you are incubating the secondary antibody one can also use cy3 or cy5 conjugated antibody and those could be directly detected. After secondary antibody then one need to wash again with PBS tween three times similar to what we performed in the last stage. After washing step it is important to remove any liquid which is adhered on the chip surface, one can use centrifuge to remove this liquid or one can use compressed airs to dry the slides. You have to ensure the right type of rotors while you are centrifuging the chips by using centrifuge. Now once the drying process is completed the chips can be scanned by using scanners and selecting the appropriate wavelength. This just gives you an overview of different steps involved in protein microarray experiment.

Addition of primary antibody, addition of a protein for testing the interactions or addition of serum for looking the immune response, different type of samples can be applied then washing steps are involved and after that appropriate secondary antibodies can be applied and suitable detection strategies are applied for signal detection, after appropriate washing step and drying slides can be scanned and then this data can be further analyzed.

After watching this video and discussing about various steps which are involved in any protein microarray based experiment, let's now look at some applications of cell-free based protein microarrays. Let's first discuss the biomarker identification.

Biomarker discovery for the disease detection and pre-screening has been one of the major thirsts for the proteomics field. Biomarkers have potential to allow early disease detection as well as accurate diagnosis of the grade of the diagnosis. These molecular signatures can also be used for follow up of disease response, survival of patients as well as various other parameters. As you know there is need for early detection and therapy of diseases such as cancer however the discovery of specific and sensitive markers that remains challenging so people employ different type of technologies including protein microarray based systems to identify biomarkers which could be used for early disease detection as well as accurate detection and other applications.

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So protein microarrays have greatly enhanced the biomarker discovery process because they allow a high throughput platform for simultaneous and rapid screening of thousands of proteins. Many times the clinical sample are limiting factor because we do not have a large amount of clinical sample to perform a study, in that regard also the protein microarrays are very impressive because with few micro liters sample once can screen thousands of proteins features simultaneously on the same platform. So biomarkers have potential for early identification of diseases state, monitoring a disease treatment response as well as follow up on the disease prognosis.

So let's look at how cell-free expression based microarrays have been applied for screening of the biomarkers. So I am giving you the case study-1.

Case Study-1-

Detection of p53 autoantibodies in human serum using cell-free expression based NAPPA microarrays- this study is performed by Anderson et al.

So antibodies to several tumor antigens are indentified in the breast cancer patient's sera however there is very little knowledge about the specificity and the clinical significance of antibody immune repertoire in the breast cancer patients. So Anderson et al. adapted specific detection of auto-antibodies in breast cancer patients by using nucleic acid programmable protein array. The slide gives an overview of detection of p53 auto-antibodies using NAPPA microarray approach. So let's discuss the study in this animation-

Biomarker Identification-

In this animation we will discuss about detection of p53 auto-antibodies in human serum using cell-free expression based NAPPA microarrays study by Anderson et al. 2008. In this study, authors generated protein microarrays based on NAPPA expression. As you can see in the NAPPA chemistry cDNA, BS³, BSA and capture antibody these four

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features are printed on the chip surface as a mastermix after addition of the cell-free lysate proteins are expressed which can then be further probed with the diluted sera of breast cancer patients having p53 auto-antibodies. Detection was carried out by means of HRP-linked anti-human IgG.

This study detected p53 auto-antibodies by means of NAPPA microarrays, which was further confirmed by ELISA approach. As you can see the spots are visible in the p53 positive sera which are negative in the p53 negative sera. The p53 levels were found to be directly related to tumor burden with serum antibody concentrations decreasing after neoadjuvant chemotherapy.

Case Study-2

Now let's talk about case study-2 for the biomarker screening.

A bead-based assay for multiplexed detection of antibodies to EBNA-1 and p53 – study performed by Wong et al., 2009.

So Wong et al. used a Luminex suspension bead array platform for the rapid detection of antibodies in the sera. A programmable multiplexed immunoassay was used for the rapid monitoring of the humoral immunity. As this slide show the overview of the steps performed in this experiment. The authors demonstrated that this method can be used for rapid conversion of open reading frame ORFeome-derived cDNAs to a multiplexed bead ELISA for detection of antibody immunity in infectious diseases as well as for the tumor antigens identification. So let's see the steps involved in this experiment in this animation.

In this study, authors developed a programmable multiplexed immunoassay where tagged antigens were expressed using *in vitro* transcription and translation and captured onto anti-tag coated beads. Once cell-free expression step was completed, the synthesized proteins were further immobilized on to the beads through the capturing agent. These beads were then mixed together. After mixing the beads together, the serum was added to these coupled beads and human IgG were detected by probing

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with enzyme-linked anti-human IgG. The colored reaction was observed on addition of substrate to the enzyme. The authors demonstrated that this approach for detection of antibodies to Epstein-Barr virus nuclear antigen 1 (EBNA1) and p53.

Let's now move on to other application-immunological studies. So on one hand there are several studies have shown application of protein microarrays including cell-free based protein microarrays for biomarker screening. There are several studies have also focused on the immunological studies.

Case Study-3

Detection of potential immunogenic proteins of *Plasmodium falciparum* – study performed by Doolan et al., 2008.

So Doolan et al. used *E. coli* based cell-free *in vitro* transcription and translation based system to produce 250 *Plasmodium falciparum* generated by the polymerase chain reaction and recombinational cloning approaches. After synthesizing the proteins from these 250 ORFs authors profiled antibodies that develop after natural or experimental infection or after the vaccination with the attenuated organism. Serum from malaria patient which have been exposed to the *Plasmodium falciparum* either naturally or experimentally were screen by using protein microarrays. In this study authors identify 72 highly reactive *Plasmodium falciparum* antigens. The proteins express specifically in the pre-erythrocytic stage of plasmodium which was CSP as well as some liver stage specific antigens such as LSA1, they identified successfully by applying cell-free expression based protein microarrays. So let's discuss this experiment by looking at this animation.

The use of cell-free expression based protein microarrays for detection of potential immunogenic proteins of *Plasmodium falciparum* was studied by Doolan et al. in 2008. In this study, authors carried out cell-free expression of the PCR amplified vectors using an *E. coli in vitro* transcription and translation system. They expressed 250 putative

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proteins that were printed directly onto microscopic array slides without any protein purification.

These arrays were probed with serum samples from patients who had been naturally exposed to *Plasmodium falciparum* and who were experimentally exposed by means of radiation attenuated *Plasmodium falciparum*. Authors successfully identified 72 highly immunoreactive protein antigens as well as 56 previously uncharacterized antigens that were serodominant, the study shown some of the newly identified targets, could serve as potential vaccine targets.

Case Study-4

Let's now talk about case study-4. Identification of immunogens of Q-fever-causing *Coxiella burnetti* – study performed by *Beare et al. 2008*.

So Q-fever is widely spread zoonosis disease caused by *Coxiella* species so identification of immunogens of Q-fever causing this disease were identified by using protein microarray based approach so in this study authors used *Coxiella burnetti* protein microarrays to identify immuno-dominant antigens. Almost 2000 ORFs were generated by using the cell-free expression based approach, *E. coli* IVVT system and then employed this protein microarray platform for identifying the immuno-dominant antigens. So some of the steps involved in this experiment will be discussed in the following animation. Case study-4 “Identification of immunogens of Q-fever-causing *Coxiella burnetti*” – study performed by *Beare et al. 2008*.

Beare et al., carried out *in vitro* transcription and translation of 1988 ORFs of *C. burnetti* using *E. coli* based cell-free systems. 75% of the ORFs were successfully generated as full-length proteins by using cell-free expression system and then spotted onto nitrocellulose arrays. The cell-free expression based arrays were probed with sera from patients who had been vaccinated as well as acute Q-fever patients. 50 proteins were identified that were found to react strongly with the immune sera.

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So as you have got a glimpse of applications of protein microarrays such as biomarker discovery and immunological studies. Now let's look at another widely used application protein-protein interaction by using cell-free expression based protein microarrays. So in this part of the lecture I will mainly focus of on NAPPA how they have been applied to study protein-protein interactions. In this slide I am showing you a small test array which we used to teach a course in the Cold Spring Harbor in New York. Students were making these arrays by themselves as you can see a array lay out there are only handful-5 genes printed in the duplicate on the chip along with vector control, mastermix and the water. Now If we want to Jun qnd Fos protein interactions and if we use Fos as a query protein, it will bind with the Jun spot and therefore two spots are lighting up as you can see in the slide so in all the six blocks there are duplicate of Jun proteins which are interacting with the Fos protein. So Jun-Fos interaction can be studied by using this system.

Now previous array was small test array but this slide show you a high density array again we want to test the protein-protein interaction of Jun-Fos protein pairs. The students in this study used Fos as a query protein and then identified Jun printed four times on the chip as the target. Jun-Fos was again used as a model system to demonstrate how protein-protein interactions can be studied by using cell-free expression based NAPPA microarrays. So let's look at some case studies which have used protein microarrays for studying the protein interactions.

Case Study-5

Identification of novel protein-protein interactions using NAPPA microarray – study by Ramachandran et al., 2004.

So Ramachandran el al. reported generation of self-assembling microarrays, which was one of the novel technology reported in science in 2004. In this study authors used a pair wise interaction among 29 human DNA replication initiation proteins, which re-capitulated the regulation of CDT1 binding to the selected replication proteins and

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mapped its Geminin binding domain by using NAPPA approach. So let me describe you some of the steps involved in this experiment by showing you this animation.

Ramachandran et al. 2004, tested the use of NAPPA microarrays by immobilizing 29 sequence-verified human genes involved in the replication initiation on the array surface and then expressing them in duplicate with RRL. The expressed proteins bound to the anti-GST antibodies which are the captured antibodies present on the array surface. The authors made use of each of these expressed proteins to probe another duplicate array of the same 29 proteins thereby generating a 29 x 29 protein interaction matrix. 110 interactions were detected between proteins of the replication initiation complex, of which 63 were previously undetected.

Case Study-6

Now let's discuss the case study 6. "High density NAPPA array approach for studying well characterized gene pairs" – Study by Ramachandran et al., 2008.

So previous study was more proof of concept where handful proteins were taken for studying the interactions whereas this time a high density array was used where thousands of features were printed. So Ramachandran et al., used a high density NAPPA approach to study the binary interactions between several well characterized interacting protein pairs such as Jun and Fos, P53 and MDM2. Now selective binding to these interactions were identified by using a specific antibody. So in the protein interactions one thing becomes very tedious to test out protein interactions in both directions for example if one is testing the Jun and Fos interactions, it should work in either way for example if Fos is printed on the array Jun should be able to bind if used as a query protein or similarly Jun is printed on the array then Fos protein can be used as a query for showing the interaction. Many times these interactions become unidirectional; it becomes very tedious to show that interaction is working in either direction. But in this study, authors showed that protein interaction of Jun-Fos can be shown in both the directions. In addition to showing that protein expression and protein interaction works one thing that is interesting from the study that a co-expression can be

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performed for example there is not even need to purify protein which is query protein. So if you have protein microarrays, features are printed on the chip and then you have generated the contents by using cell-free expression based system now you want to study the interaction and for that you have to purify a protein and use it as a interactor. Now you can use protein specific antibody to identify the interaction or you can use tag specific antibody for detecting the interactions. But in this study, authors used co-expression it means the query proteins along with the arrayed proteins were expressed by using cell-free expression system so there was no need to purify query protein as well when the protein interaction study has to be performed you take the cDNA of Fos for example, mix it in rabbit reticulocyte lysate along with the other *in vitro* transcription and translation machinery mix the whole cell lysate on the chip surface and then after incubation when proteins are expressed at the same time query cDNA will also express the proteins and then if it finds the binding partner it is going to bind to those features which can be detected by using protein specific or tag specific antibody. So by performing this type of approach authors allowed co-expression, it means same environment for both query and the target proteins both proteins are expressed in same mammalian environment and there is good likelihood that they are going to identify right interactors. So let me show you the steps involved in the study by showing you this animation.

In this study, authors made use of high-density NAPPAs arrays to study protein interactions. 647 unique genes were printed on to the array surface and expressed by adding the cell-free expression based system. After addition of cell-free expression based system proteins containing GST-tags were synthesized and bound on to the capture antibody. cDNA of the query protein was also added to the same mixture such that the query was co-expressed but remained unbound due to the lack of a tag capturing agent. These protein microarrays were then probed with antibodies specific to the query proteins. Authors detected various protein interactions using well-known query proteins such as Jun, Fos and MDM2.

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So now we have seen the overview of how protein microarray experiments can be performed, we have looked at various applications by employing cell-free expression based protein microarrays, and we have discussed biomarker screening, immunological studies and protein-protein interactions. Now regardless of what application you want to perform on these arrays you are going to generate large amount of data so the volume of data generated from microarray experiments are prodigious. It becomes important to develop the appropriate informatic system so that one can analyze this data uniformly and make some very good output from this whole analysis.

So in the image analysis when you are talking about high density approaches the data analysis, image analysis becomes very challenging for example you can see a image here for the protein microarrays and I have shown a spot, the expression of this particular immunogenic protein is so high that it is spilling over to the neighboring proteins. Now one needs to correct for this or remove those spots which are in the periphery of this protein.

So scaling up is good approach because one wants to perform high throughput experiment so that thousands of features can be studied simultaneously however while scaling up especially when you are using cell-free expression based approaches one need to be cautious that what need to be the optimum intensity for the arrays because if there is spill over of a expressed protein on neighboring protein spots that is going to affect the values for the neighboring spots. This slide show how protein is diffused in the neighboring spot, neighboring spot should be removed. Similarly background corrections and various other parameters one need to analyze to perform a good microarray data analysis. So let's have a discussion with Dr. Sudhesh Srivastava on microarray data analysis, what are the challenges involved and in subsequent lecture we will talk in more detail about different step and details of those which one need to follow up for data analysis of microarray based systems.

So in summary, the protein microarrays offer novel technology for the simultaneous and rapid analysis of multiple biomarkers or interactors in high throughput manner.

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Microarrays have been widely used for detection of antigens as well as antibodies in blood sample and various other clinical samples. However the traditional cell-based approaches have certain limitations therefore the cell-free expression based protein microarrays have emerged and very strongly it has been shown that various applications can be performed without need to purify the proteins because you can generate the protein content in the cell-free manner. In today's lecture I gave you an overview of steps involved in performing a protein microarray experiment whether you want to do the protein interaction or any type of disease screening one has to go through all those steps then we looked at different type of applications in different case studies. Obviously the case studies discussion was very brief and you can refer to those references and read those papers for further details. Finally, we got a discussion with a leading expert Dr. Sudesh Srivastav and we will continue our discussion on applications as well as microarray data analysis challenges in the subsequent lectures. Thank you.

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