HANDOUT

LECTURE-34

APPLICATIONS OF CELL-FREE PROTEIN MICROARRAYS

Slide 1

In today's lecture we will talk about applications of cell-free protein microarrays. In the previous lecture, we discussed how different type of cell-free systems can 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.

Slide 2

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 found unique opportunities to identify novel biomarkers as well as apply this platform for several applications. The combination of proteomics technologies especially the protein microarray have potential to be useful in a 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 systems as compared to the cell-based traditional way of purifying the protein and then printing on the array surface.

Slide 3

So in today's lecture we will talk about an overview of protein microarray experiment, including:

- A broad overview how any type of protein microarray platform can be used for different applications.

- I will then describe few applications very briefly using 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 and generate data for the thousands of features simultaneously therefore analyzing such data becomes very challenging. So these three points will be discussed today.

Slide 4

Animation 1

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. Note, protein microarray chips have to be stored at -80 C, while cell free-expression based microarray slides can be stored at RT.

- 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.
- First of all you would like to block those areas 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, superblock 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 superblock or the blocking solution and then emerge the slide.

- One need to ensure the proper shaking while performing the blocking step, to avoid drying of milk or the blocking reagents It can be emerged 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.
- 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 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.
- 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 scientists try different approaches. Few people prefer using overnight incubation condition at 4°C and some groups prefer using one hour at room temperature. A different school of thought here one is that if you are incubating with serum for a 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 tween. This is to ensure 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 TSA 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 continue to accumulate and therefore one can see the good signal by the TSA based detection system. Now when you are incubating the secondary antibody one can also use cy3 and cy5 conjugated antibody and those could be directly detected. After secondary antibody incubation, then one needs to wash again with PBS tween three times similar to what we performed in the last step.
- After the 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 air to dry the slides. You have to ensure the right type of rotors while

you are centrifuging the chips by using the centrifuge.

- Now once the drying process is completed, the chips can be scanned by using scanners at the appropriate wavelength.

Slide 5

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

Slide 6

Let's first discuss the biomarker identification.

Slide 7

Biomarker discovery for the disease detection and pre-screening

- This has been one of the major areas of interest for the proteomics field. Biomarkers have the 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.
- 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 samples are the 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 useful

because with few microliters sample once can screen thousands of proteins features simultaneously on the same platform. So biomarkers have potential for early identification of the disease state, monitoring a disease treatment response and can also be used to 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 first case study.

Slide 8

Case Study-1

Slide 9

Detection of p53 autoantibodies in human serum using cell-free expression based NAPPA microarrays

- This study was performed by Anderson et al.
- Antibodies to several tumor antigens are identified 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 antibodies using NAPPA microarray approach. So let's discuss the study in this animation-

Slide 10

Animation 2

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, BS^3, BSA and capture antibody, 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.

Slide 11

Case Study-2-

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

Slide 12

<u>A bead-based assay for multiplexed detection of antibodies to EBNA-1 and p53</u> –

- The study was performed by Wong et al. in 2009.
- Wong et al. used a Luminex suspension bead platform for the rapid detection of antibodies in the sera. A programmable multiplexed immunoassay was used for the rapid monitoring of the humoral immunity.
- 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 tumor antigens identification. So let's see the steps involved in this experiment in this animation.

Slide 13

Animation 3

- 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 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.

Slide 14

Let's now move on to other application- immunological studies.

So on one hand there are several studies which 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.

Slide 15

Case Study-3

Slide 16

Profiling humoral immune responses to P. falciparum infection with protein microarrays.

- The study was performed by Doolan et al., 2008.

- Doolan used an *E. coli* based cell-free in vitro transcription and translation based system to produce 250 Plasmodium falciparum sequences generated by the polymerase chain reaction and recombinational cloning approaches.
- After synthesizing the proteins from these 250 ORFs, the authors tried to identify antibodies that develop after natural or experimental infection or after the vaccination with the attenuated organism.
- Serum from malaria patients exposed to the *Plasmodium falciparum* either naturally or experimentally (to radiation-attenuated spoorozoites) were screened by using protein microarrays.
- In this study authors identify 72 highly reactive *Plasmodium falciparum* antigens.
- The proteins were expressed 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.

Slide 17

Animation 3

- 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 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 experimenttally 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, can serve as potential vaccine targets.

Slide 18

Case Study-4

Slide 19

Identification of immunogens of Q-fever-causing *Coxiella burnetti*

- The study was performed by *Beare et al. 2008.*

Slide 20

- Q-fever is widely spread disease caused by *Coxiella* species. Identification of immunogens of Q-fever causing this disease was carried out by using protein microarray based approach.
- In this study authors used *Coxiella burnetti* protein microarrays to identify immune-dominant antigens. Almost 2000 ORFs were generated by using the cell-free expression based approach and the *E. coli* IVVT system.
- The authors then employed this protein microarray platform for identifying the immune-dominant antigens. So some of the steps involved in this experiment will be discussed in the following animation.
- 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.

Slide 21

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.

Slide 22

Study of 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, which was printed by the students taking that course.
- The array lay out shows that 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 investigate Jun and 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.

Slide 23

Now previous array was a small test array, but this slide shows 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.

Slide 24

Case Study-5

Identification of novel protein-protein interactions using NAPPA microarray –

- study by Ramachandran et al., 2004.

Slide 25

- 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 to study the regulation of CDT1 binding to the selected replication proteins and mapped its girminin-binding domain by using NAPPA approach.

  So let me describe some of the steps involved in this experiment by showing you this animation.

Slide 26

Animation 4

- Ramachandran et al. 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 ones.

Slide 27

Case Study-6

Slide 28

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.

Slide 29

- Theprevious study was more proof of concept where a handful of proteins were taken for studying the interactions whereas this time a high density array was used where thousands of features were printed.
- So Ramachandran el 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 is 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 directions. In addition to demonstrating protein expression and protein interaction, one thing that is interesting from the study that a co-expression was performed wherein there was no need to purify the proteins which was used as 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 an 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 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.

Slide 30

Animation 5

- In this study, authors made use of high-density NAPPA 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.

Slide 31

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 experiment 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 immense.

It becomes important to develop the appropriate informatic system so that one can analyze this data uniformly in order to generate valid inferences from this whole analysis.

Slide 32

- 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.

Slide 33

- 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 protein on neighboring protein spots that is going to affect the values for the neighboring spots.

- This slide shows how protein is diffused in the neighboring spot. In such a case the neighboring spot should be removed. Similarly background corrections and various other parameters one need to perform a good microarray data analysis.

Slide 34

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

Slide 35

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.