Interactomics Protein Arrays and Label-Free Biosensors
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Module 06
Lecture 27
Cell-free Synthesis based Protein Microarrays PISSA and NAPPA

Welcome to MOOC interactomics course. In today's lecture we will talk about protein microarrays based on cell-free synthesis. The cell-free synthesis based protein microarrays provide high throughput versatile platform for large scale analysis of functional proteins. These microarrays have been used for various applications; we talk about antibody profiling, biomarker discovery, enzyme substrate identification, protein-protein interaction, etc.

The traditional cell-based methods which is used for making the protein microarrays involve protein expression in a (())(1:00) system such as E. coli however, the protein purification is very laborious process. It involves various steps and of the protein purity, integrity, stability and its functionality. If one has to generate high throughput, large number of proteins which is required for performing protein microarray studies, it is very tedious because one need to purify large number of proteins at the scale of thousands and then maintain the functionality and keep them properly folded, it becomes very tedious. Therefore, these limitations of traditional protein purification and protein microarrays generated by the purified proteins have been the major motivation of cell-free expression based microarray field.

The cell-free expression based system overcomes various limitations of protein purification and they perform in-situ transcription and translation. During the last decade various methods have immerged as a strong platform for protein microarray generation by applying cell-free expression system. Today, I will talk about cell-free expression based protein microarrays and provide you an overview and the basic principle involving these cell-free expression based protein microarrays.

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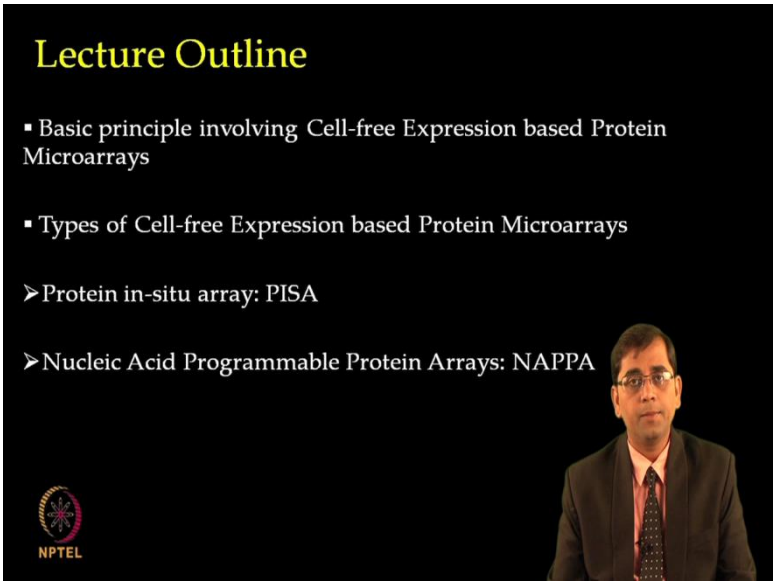

Today, we will mainly focus on two widely used cell-free expression based microarrays, namely Protein in-situ arrays PISA and Nucleic acid programmable protein arrays NAPPA. In next lecture we will expand our discussion to other techniques; we will discuss the principle of each one of these platforms, their advantages and disadvantages.

Let us first review cell free protein synthesis. The cell-free system make use of template DNA obtained from either plasmid or PCR products, this is required for direct in-vitro synthesis of proteins in the presence of a crude cell lysate. The cell lysate contains all the necessary machinery which is required for transcription and translation by providing essential amino acids, nucleotides and other energy generating factors which are added exogenously in these cell-free lysate.

The cell-free expression systems have been extracted from cells of different organisms such as wheat germ extracts, rabbit reticulocyte lysate and E. coli. These are the commonly used systems, but there are other system as well which includes cell lysates from (())(4:18), hybridomass, insects and mammalian cells. Approaches demonstrating the efficacy of cell-free expression systems to synthesize proteins in-vitro have been extensively documented in literature. Now, we will discuss about protein microarrays based on cell-free expression system. To eliminate the obstacles posed by traditional cell based methods, Cell-free expression systems are increasingly adopted to generate microarrays. There are several microarray generation technologies and those technologies have been developed over past few years. Let us discuss these techniques in detail.

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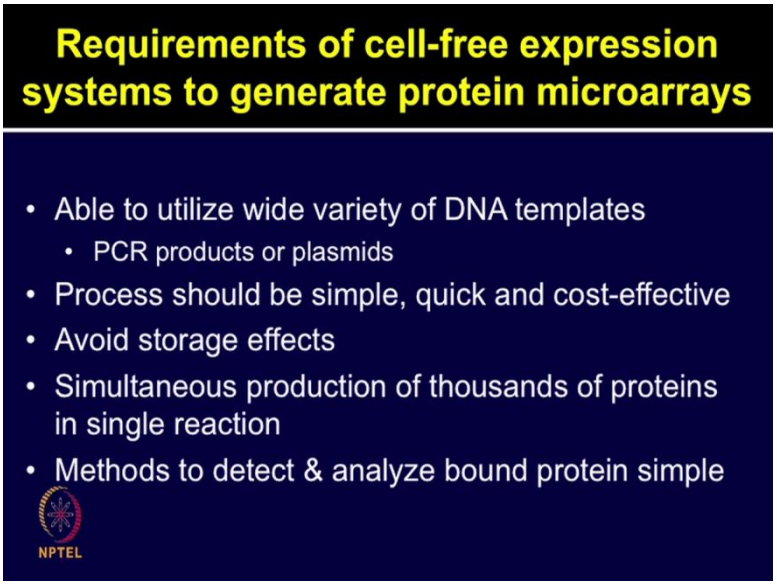

We will first talk about the requirements of cell-free expression system. Cell-free expression system should meet certain requirements and these criteria are that they should be able to utilize wide variety of DNA templates. This brings out the versatile platform. The system should be simple, quick as well as cost effective. When we are talking about protein microarrays the applications are essentially for clinical studies, where one need to study several patient (())(5:45) samples. It is an expensive technology becomes a hurdle for its wide is produced.

The microarrays should be produced on demand so that one can avoid the degradation of proteins due to storage issues. The purified arrays have to be stored at minus 80 degrees, these affects the protein integrity and protein functionality. So, cell free expression system can be used if there is an need to perform an assay that can eliminate lot of limitations. It can allow the simultaneous production of thousands of protein that is a common requirement for any type of microarrays platform whether it is DNA microarray, traditionally purified microarrays or cell-free based protein microarrays.

Detection and analysis of bound proteins should be simple; it means the assay method and detection technology should be available and very simple. Most of these assays are similar and principle to western blots or Elisa assays. So, these are quite simple, detection assays are also simple so that the read outs can be used from common instruments.

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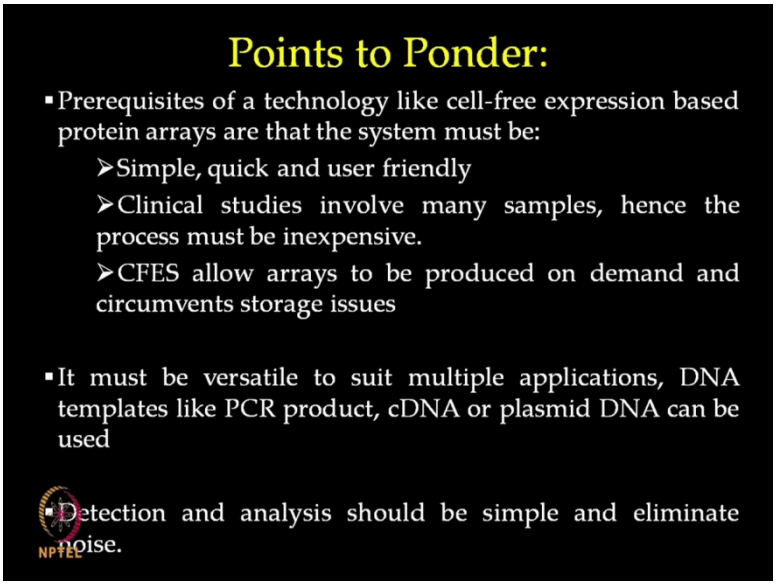

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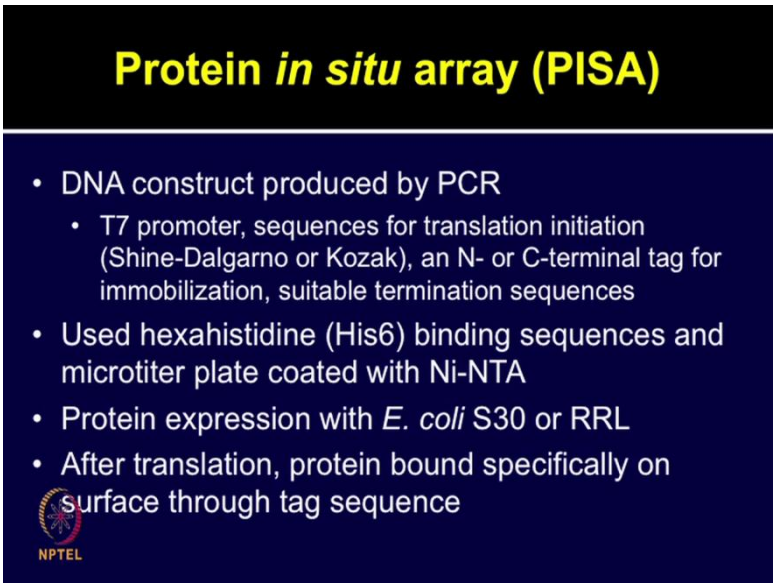

Let us first discuss Protein in-situ Array or PISA. It is also known as (())(7:52) array technology, which provides rapid single step method to generate protein arrays from the DNA template by using cell-free transcription and translation system which allows immobilization of synthesized protein on solid microarray surface. In PISA methods, there are various requirements. The DNA construct can be produced by PCR as listed here in the slide, the construct should contain T7 promoter, sequence require for translation initiation such as shine-Dalgarno or Kozak sequences, N- or C-terminal tag sequence required for the immobilization of synthesized proteins and suitable termination sequences. The solid surface has to be functionalized and nickel NTA to be added on the surface.

So, one need to add histidine tag so that binding can be specific. Hexa-histadine probability histidine 6 tag binding sequence and microtiter plate which is coated with nickel-NTA is used for this purpose. The protein expression can be performed by using E. coli based system or rabbit reticulocyte lysate. Once the translation has finished, the protein which is synthesized by using cell-free expression system specifically binds on the surface through the tag sequence.

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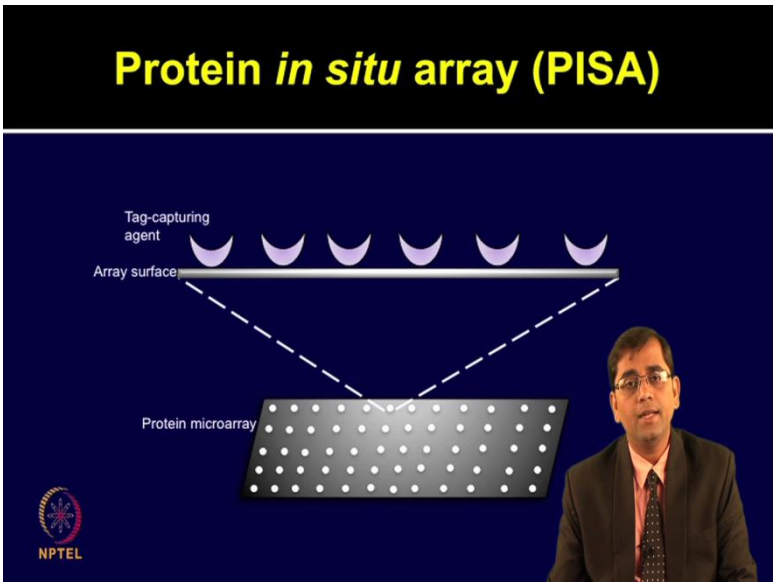

In PISA protein array method, this surface is coated with a tag capturing agent. This method utilizes hexa-histidine tags which is in the construct and the microarray titer plate, which is coated with nickel nitrilotriacetic acid or in nickel-NTA as shown in this slide.

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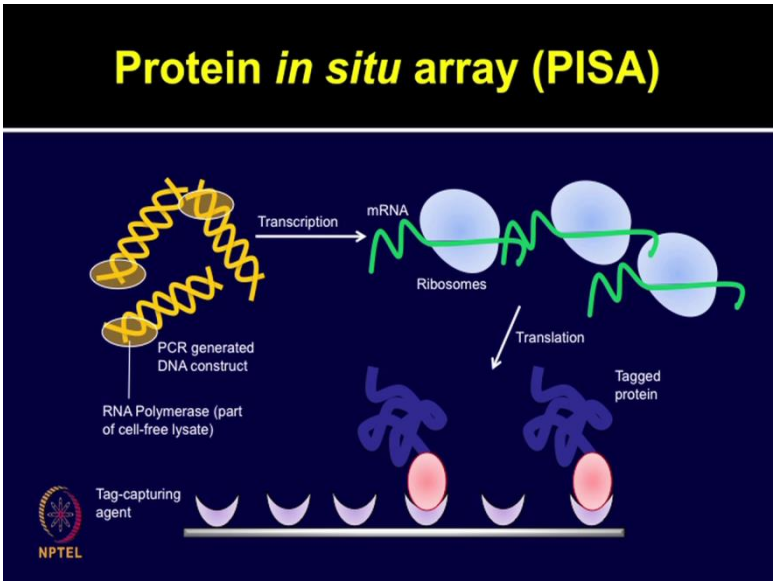

The DNA construct contains T7 promoter sequence for translation initiation internal sequence and the termination sequence. Once, the protein expression carried out by using cell-free expression system such as E. coli S30 or rabbit reticulocyte lysate. The protein binds specifically on the surface to the tag sequence and the unbound material can be washed off. As shown in this slide by using cell-free expression system DNA is able to produce protein through transcription and translation processes containing histidine tags which get adhere to nickel-NTA surface.

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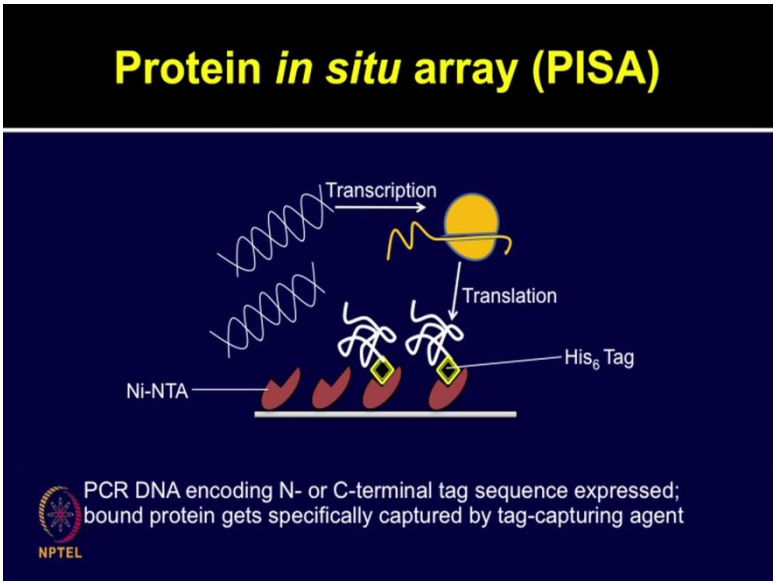

Overall PISA method utilizes PCR DNA, which encodes N- or C- terminal tag and then transcribes and translates the c-DNA of interest into the protein. This protein which is synthesized after the cell-free expression gets specially captured by tag capturing agent. Scientist (())(11:00) have developed this method successfully and carried out the expression and functional immobilization of a fragment of human anti-progesterone antibody in microtiter wells and used luciferin enzyme on nickel-NTA coated magnetic bits. Let me show you this animation where we can discuss, the working principle of protein in-situ arrays.

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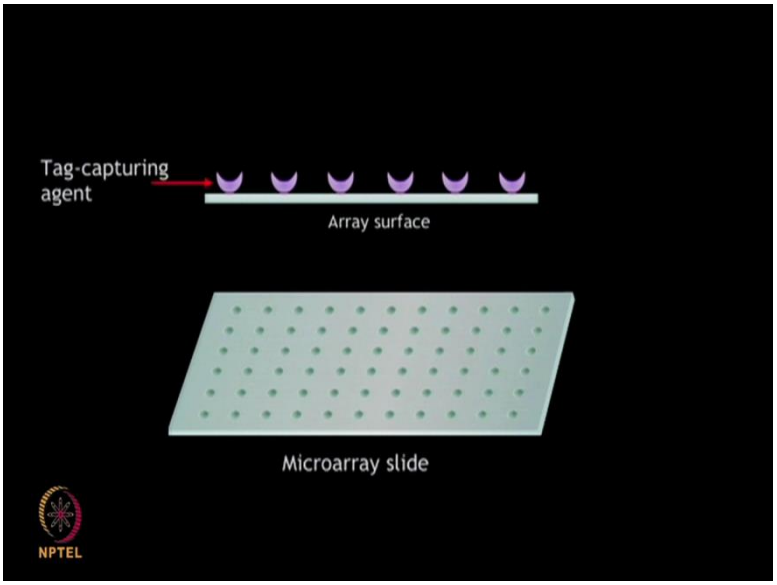

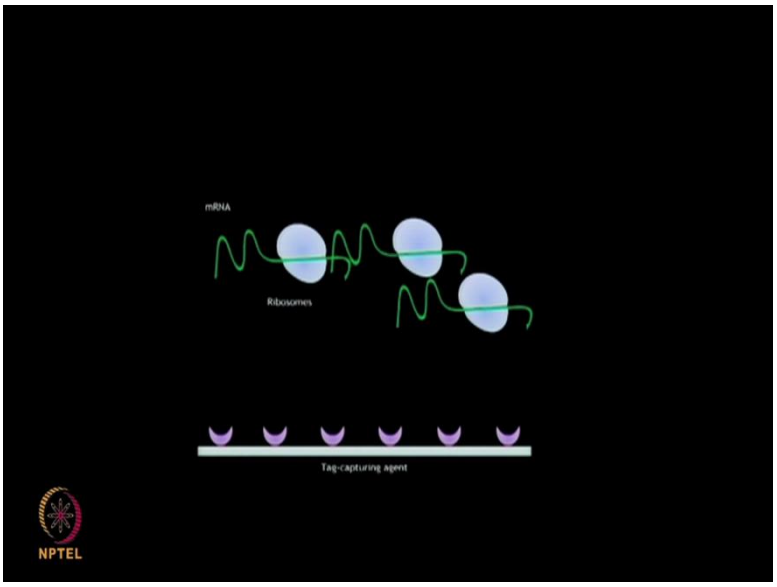

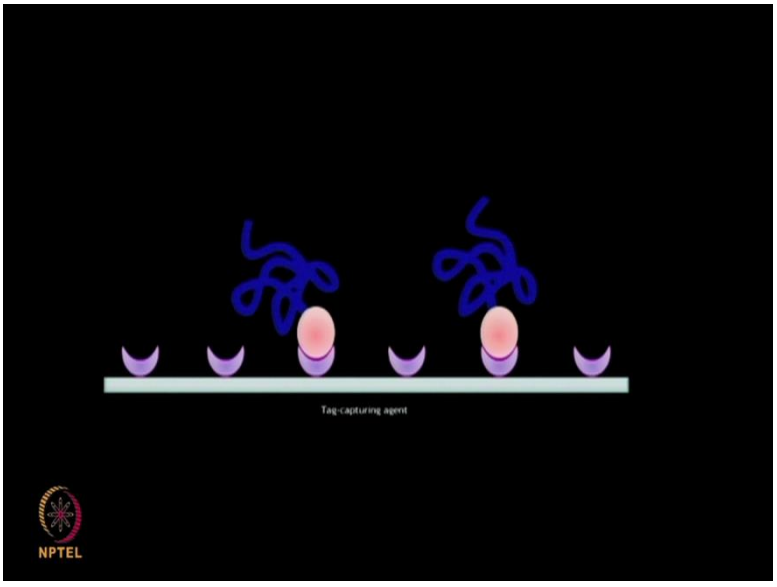

Protein in-situ array or PISA, in PISA the protein microarray surface is coated with a suitable tag capturing agent that can immobilize the protein of interest through a specific interaction once it is produced. The protein is expressed from its corresponding DNA by using sell-free lysates such as E. coli S30 or rabbit reticulocyte lysate. The tag protein is captured specifically on to the array surface through the tag capturing agent. PISA method has successfully overcome drawbacks of cell based techniques such as protein, insolubility, aggregation, etc.

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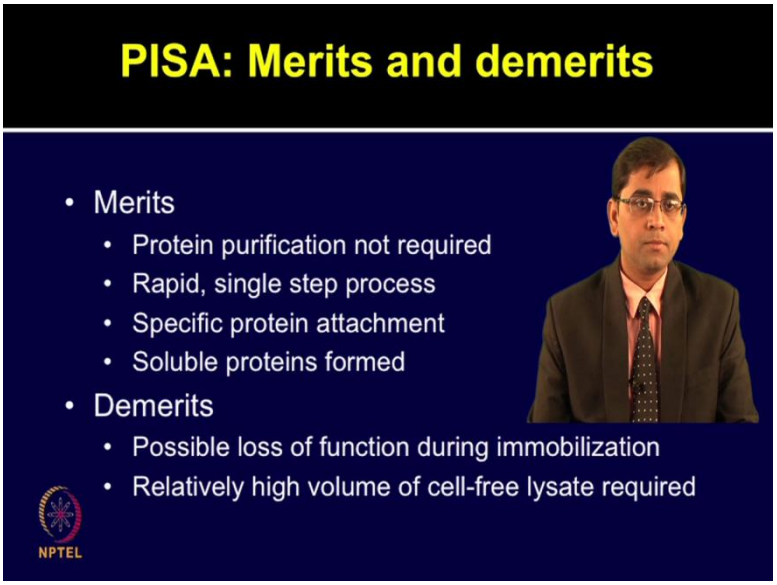

After learning the working principle of PISA, let us discuss various advantages and disadvantages of this technology. PISA method overcomes the traditional purification requirements for producing the protein microarrays. Protein purification is not essential, it gives rapid, single step process because of histidine tag and nickel-NTA interaction, this specific protein attachment can be achieve by using PISA method. In this method, soluble proteins are formed. These are some of the major advantages of using PISA technology. Let us also discuss the limitations of this method. It is possible that there is loss of function these proteins during the immobilization step. Cell-free lysates are very costly. So, if one needs high volume of cell-free lysate that becomes one of the limiting factors for this technology.

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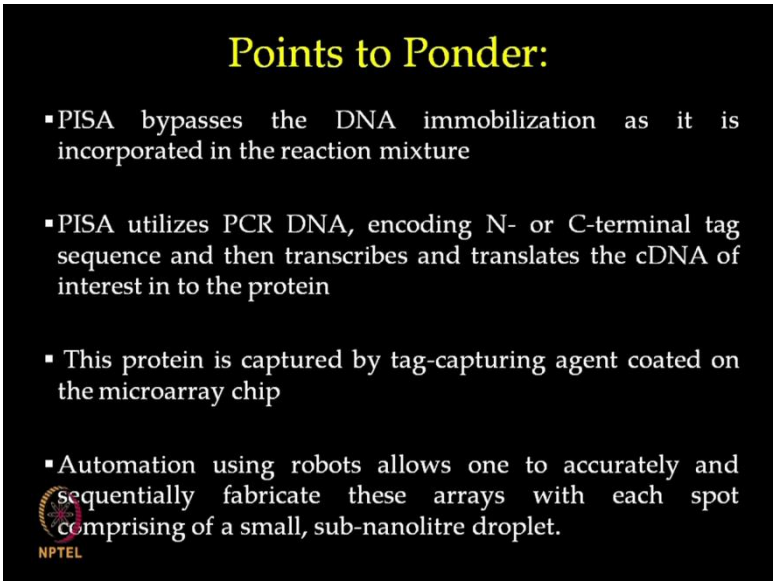

Let us now discuss another promising technology Nucleic acid programmable protein array developed at Harvard institute of proteomics by Dr. George Laberts lab. NAPPA process replaces the complex process of spotting the purified proteins with simple method of spotting with plasmid DNA. By using the recombinational cloning and cell-free expression system proteins are produced in-vitro in NAPPA method and these proteins are captured on array surface. NAPPA technique minimizes direct manipulation of proteins and has a enabled interactions to occur in mammalian (())(15:01) by using the proper promoter sequence and cell-free expression based on rabbit reticulocyte lysate.

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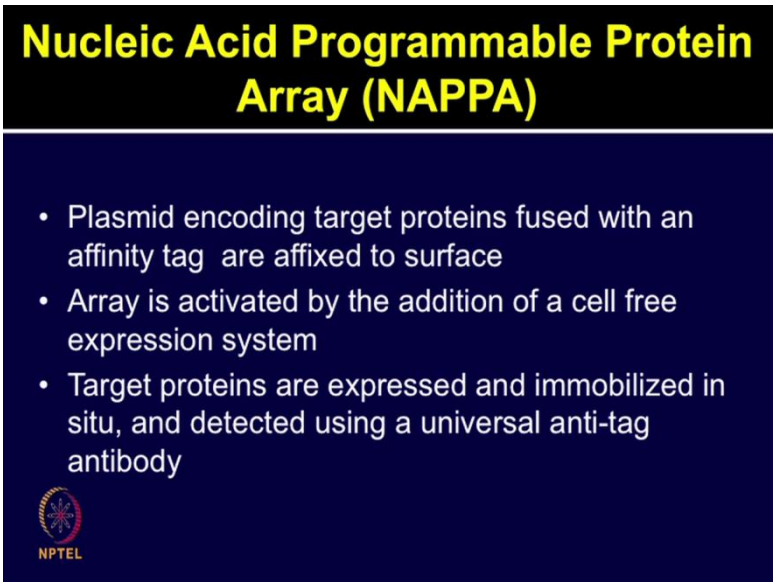

Let us discuss the nucleic acid programmable protein array to see how these arrays can be generated. Plasmid such encode target proteins are fused with an affinity tag and are fixed to the microarray surface. The microarrays are activated by the addition of cell-free expression system, generally rabbit reticulocyte lysate. The target proteins after the expression are immobilized in-situ and detected using a universal anti-tag antibody. In this case, mostly anti-GST tag is used but one can use any other tag and corresponding antibodies as we can see here, in NAPPA method, the glass slide is first coated with aminosilane reagent, its functionalization is done then master mix printed on the chip surface.

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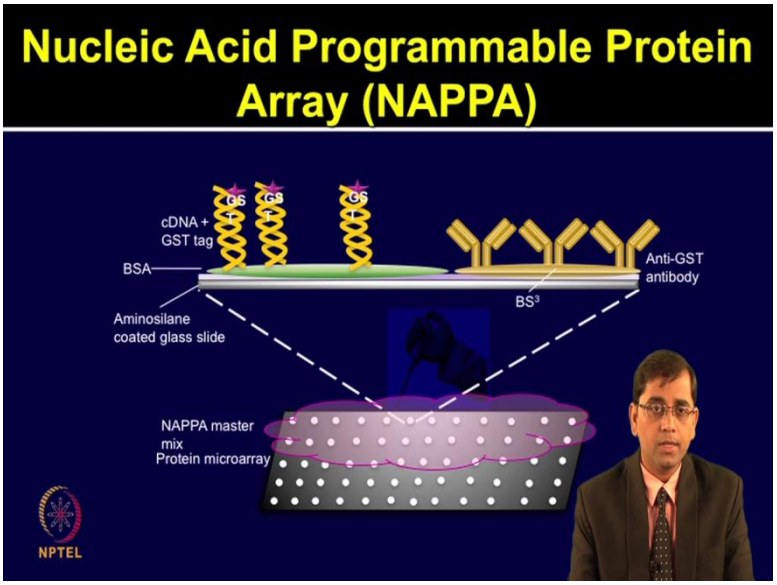

The master mix consists of few components which includes a DNA containing GST tag, bovine serum albumin BSA, BS3 cross linker and anti-GST capture antibody. So, the DNA containing gene to be expressed as it Glutathione S-transferase fusion is immobilized on the array surface in master mix. Protein bovine serum albumin is added as part of the master mix, which improves binding efficiency of c-DNA although, the exact mechanism is not yet clear.

BS3 the cross linking agent, which facilitates the immobilization of capture antibody which is anti-GST antibody in this case enables the immobilization on the array surface. Anti-GST antibody, the capture antibody binds to the synthesized proteins containing GST tag, one can use any tag here but the only requirements will be availability of corresponding antibody, for example, you can use anti-flag antibody if you have used flag tag in your c-DNA. These are added to the master mix, which contains the mixture of BSA, BS3, capture antibody and c-DNA of interest that is printed on the chip surface. The next requirement is activation of the chip by adding cell-free expression system.

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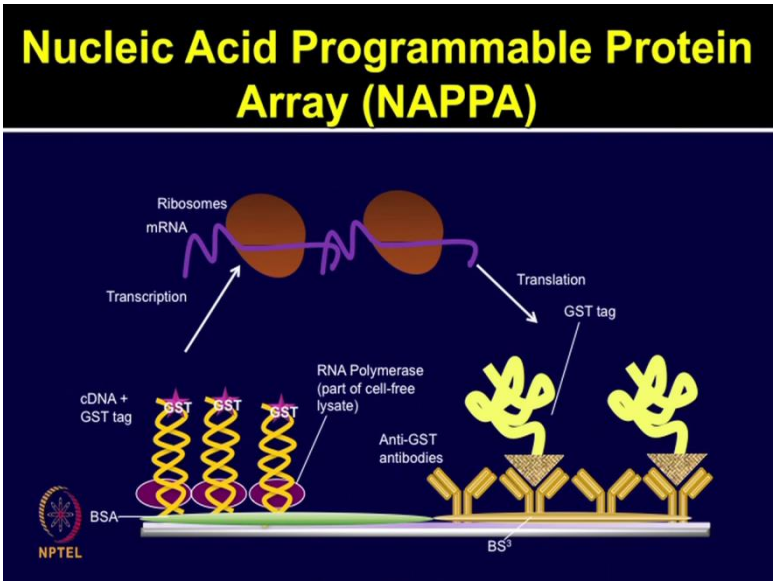

The cell-free expression system such rabbit reticulocyte lysate, amino acid mixtures, T7 polymerase, RNAs inhibiter excetra are added on the chip surface which contains these spots. After incubating these arrays at 37 degrees for 90 minutes, the temperature is lowered to 15 degrees; this process helps the protein expression and protein capture. So, the first step as you can see in the slide is the transcription is happening on ribosomes and m-RNA followed by translation to synthesize the proteins. These proteins contain GST tags because each of the c-DNA clones possesses GST tag. These tags are immobilized, they capture antibody which is immobilized on the chip surface contains anti-GST antibody.

So, now these proteins will go and adhere to these anti-GST antibodies very specifically. So, protein expression in NAPPA is carried out by using cell-free mammalian system such as rabbit reticulocyte lysate. Therefore, the conditions are excellent for mammalian protein expression and folding which is often the limitations of using wheat germ extract or E. coli based methods.

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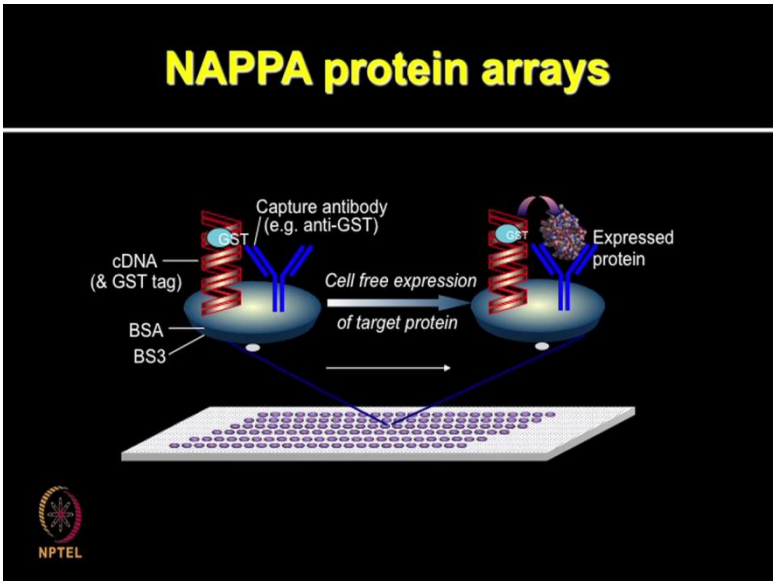

In NAPPA arrays, the master mix contains DNA and other components which can be stored at room temperature. So, the storage for NAPPA microarray contains only DNA. Therefore, their self-life or storage of these arrays gel is not a major concern. Actually, this is one of the major advantages and major mild-stone achieved using NAPPA method.

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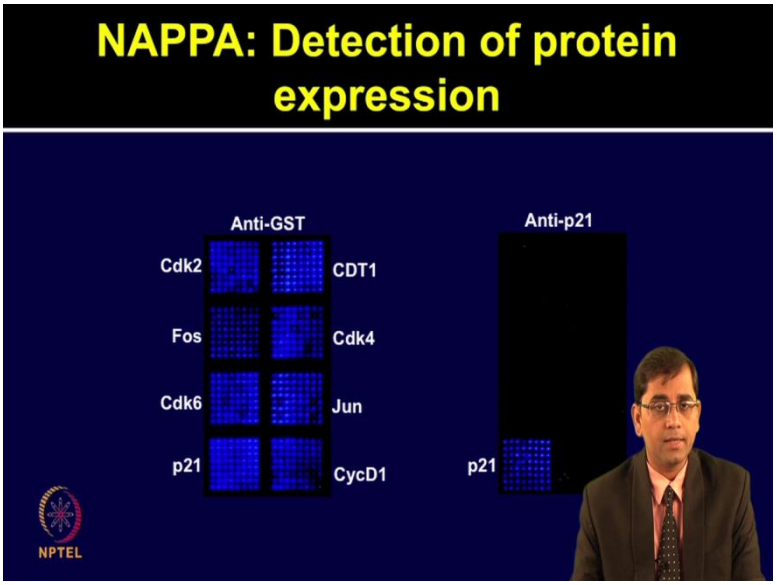

In this slide, I have shown you how protein expression can be detected on NAPPA arrays. The left image shows a proof of concept study, where c-DNA of 8 genes are printed on chip surface using NAPPA chemistry, each spot is printed 64 times in 8 by 8 block, by using ant-GST antibody one could detect the proteins that are synthesized and expressed, but it is important to ensure that the protein is captured specifically using protein specific antibodies.

In this experiment, anti-P21 antibody is used to capture P21 protein which shows signal while the rest of the 7 proteins do not show any signal. It just shows that the proteins are expressed and specific protein expression can be detected. NAPPA method is very simple and similar to the western blot assays.

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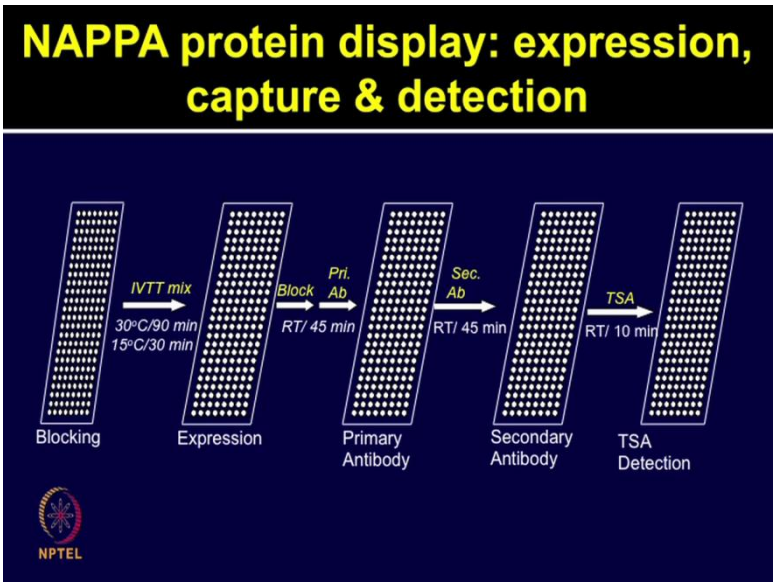

The first step here after printing these chip is blocking using BSA or milk in PBS to avoid non-specific binding. After blocking is done then in-vitro transcription and translation mix can be added on this chip surface, incubation involves two steps, incubation at 30 degrees followed by 15 degrees for 30 minutes. It has been observed that lowering the temperature from 30 to 15 degrees helps in the protein capture process.

There are two steps involved here; one is the expression where the proteins are expressed in the cell-free environment and then protein capture on antibody which is facilitated by lowering the temp. Once, the proteins are expressed then you need to wash, all the lysates away from the chip, you can add milk in PBS or BSA. once the blocking is done at the room temperature then primary antibody can be added as you can see the slide, you can follow this workflow if your experimental aim is just to do the quality control check of protein expression, you can use anti-GST antibody or if your aim is to look at certain auto-antibody profiling then you can use, serum from various patients.

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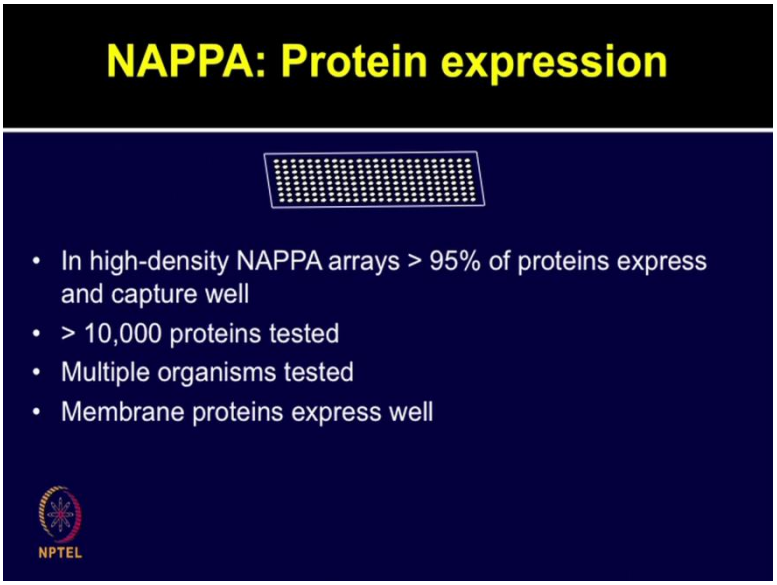

After incubation is done, corresponding secondary antibodies can be added. The secondary antibodies can be tagged with Cy3 Cy5 or tiramide (())(22:30) signal amplification system. The schematic representation shows the various steps involved in performing protein display, which includes protein expression, capture and detection. NAPPA arrays are good platform for achieving high density arrays and successfully enable capture of over 95 percent of proteins; over 13 thousand human proteins have been tested on this array surface.

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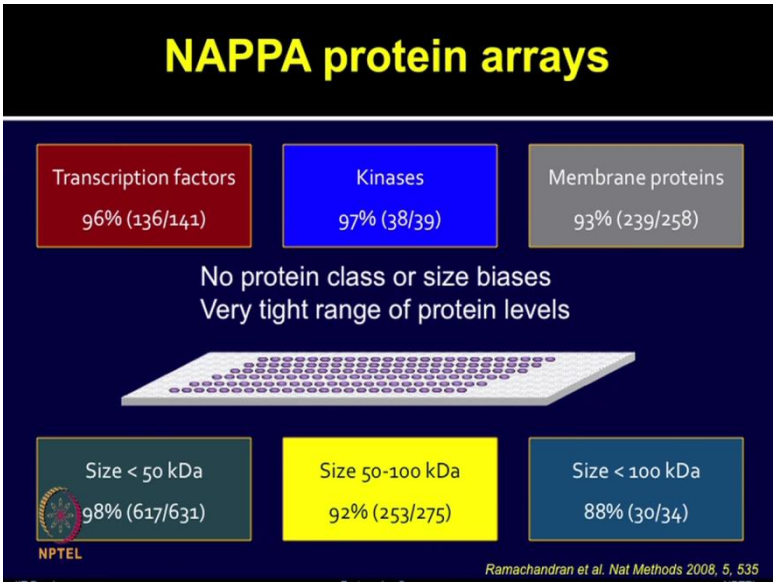

NAPPA arrays have also been applied on various organisms because the concept is very simple, once you have access to the clone c-DNA containing GST tag or any other tag, you can make use of the c-DNA repositories. NAPPA approach does not have a bias for a specific

group of proteins or a class of proteins for efficient protein expression. It can also express membrane proteins, kinases and transcription factors. Similarly, there is no bias for the size of the proteins, for example, proteins below 50 kilo Dalton or between 50 to 100 kilo Dalton or even more size, they could also be synthesized, expressed using NAPPA chemistry.

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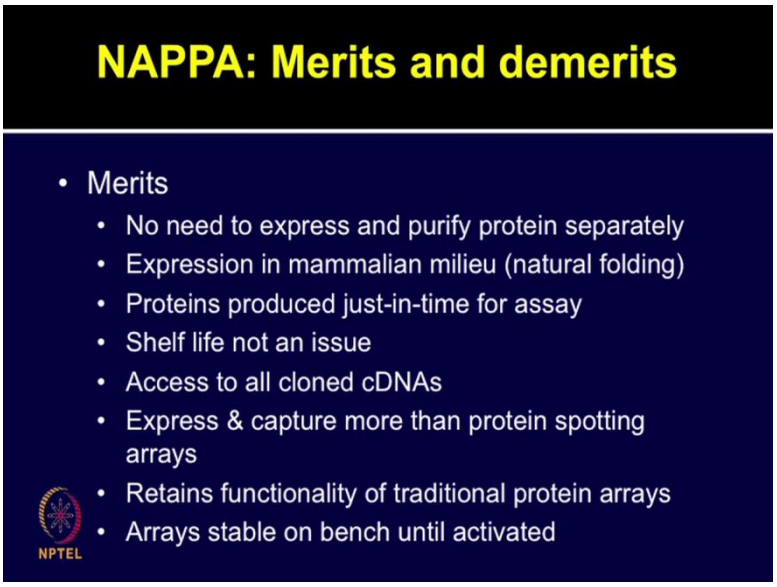

NAPPA method has many merits, there is no need to express and purify the proteins separately because one could use in-vitro transcription and translation system. The expression is performed in mammalian environment involvement by using rabbit reticulocyte lysate, which helps in the natural folding of mammalian proteins. Proteins could be produced just-in-time when you want to perform an assay because c-DNA are quite stable to store at room temperature therefore, self-life is not an issue in this case. If you purify your protein print on a chip surface then you have to ensure the proteins are stored in cold condition often at minus 20 or minus 80 degrees but here you can store your printed slide at room temperature.

Any c-DNA which you have accessed, which contains tag that can be used efficiently here by using NAPPA chemistry and proteins can be synthesized. Comparison of protein synthesis from NAPPA chemistry with traditional protein purified arrays have demonstrated that expression and capture can be achieved more by using NAPPA chemistry. The NAPPA approach retains all the functionality which one can use for traditional protein arrays and adding strength to the system, storage of protein arrays printed using NAPPA chemistry at room temperature is one of the major advantage of this chemistry.

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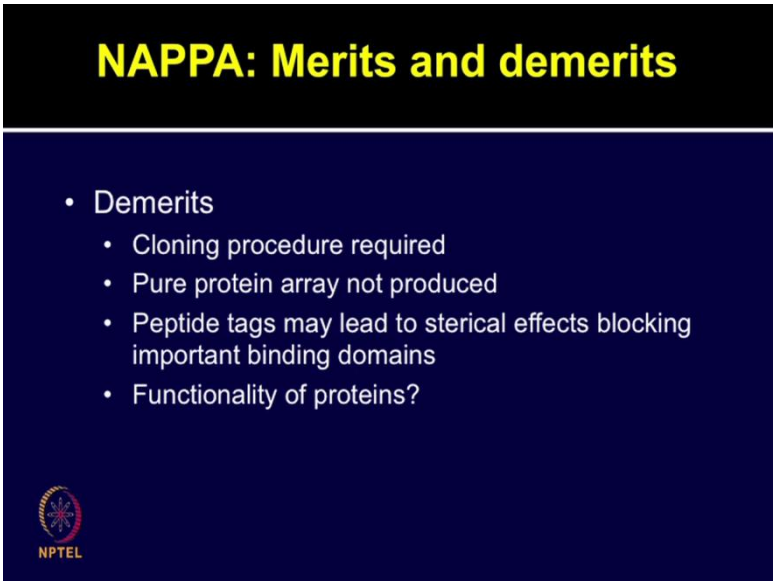

Despite several advantages NAPPA approach also has certain limitations you need to rely on cloning procedure which is very tedious or you may have to obtain the clones from the repositories. There is also concern with a pure protein is produced because you printed c-DNA on the chip which is synthesizing the protein, so there is a co-existence of both DNA and proteins on the chip. The peptide tags which are added for the capturing procedure may lead to steric hindrance blocking which could be important for binding domains. The functionality of the proteins is a still remains a question for any of the cell-free expression based methods. So, NAPPA method provides various applications similar to other test protein arrays including detection of interaction with proteins and other biomolecules. Let us now discuss the working principle of NAPPA in this animation. Nucleic acid programmable protein arrays or NAPPA.

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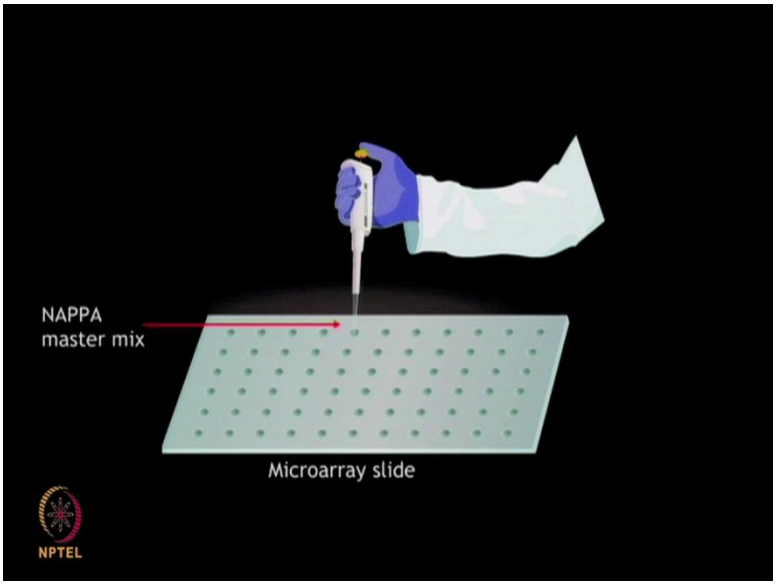

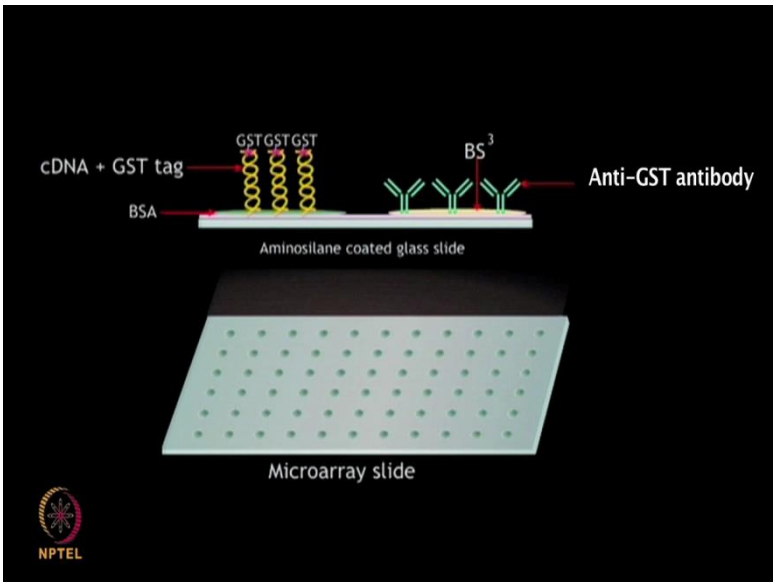

In NAPPA method, an aminosilane coated glass slide forms the array surface for NAPPA, to this surface the NAPPA master mix is added which is consist of BSA, BS3, GST tag c-DNA and anti-GST capture antibody. The BSA includes the efficiency of immobilization of c-DNA on to the array surface while BS3 cross linker facilitates the binding of capture antibody.

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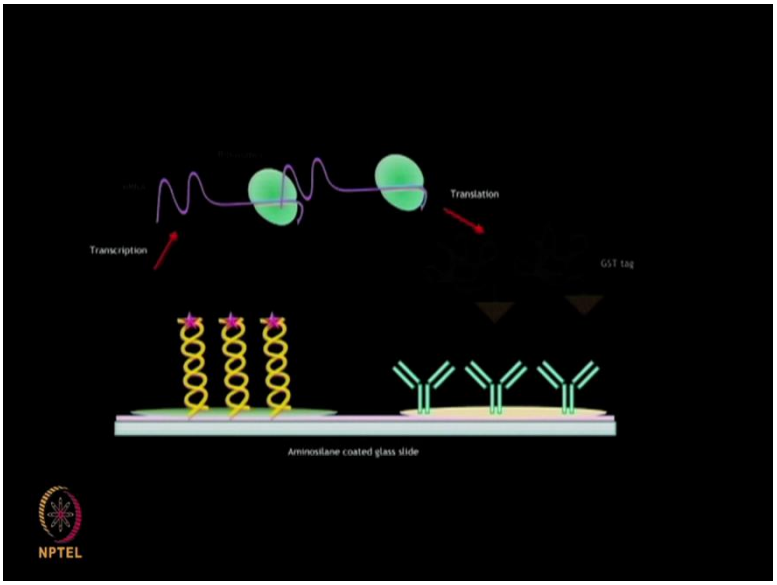

The c-DNA is expressed by using cell-free lysate to give the corresponding protein with its GST tag fused, after transcription and translation the protein is synthesized and tag enables the capture of protein on to the slide by using anti-GST antibody.

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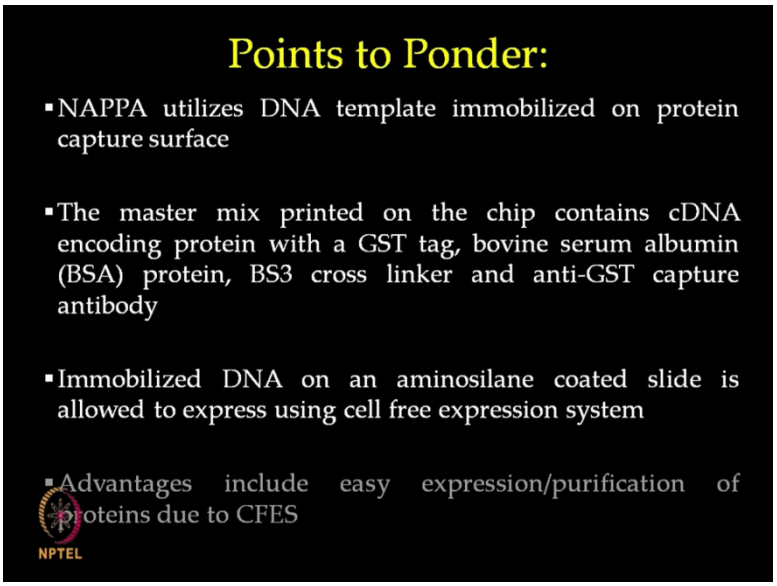

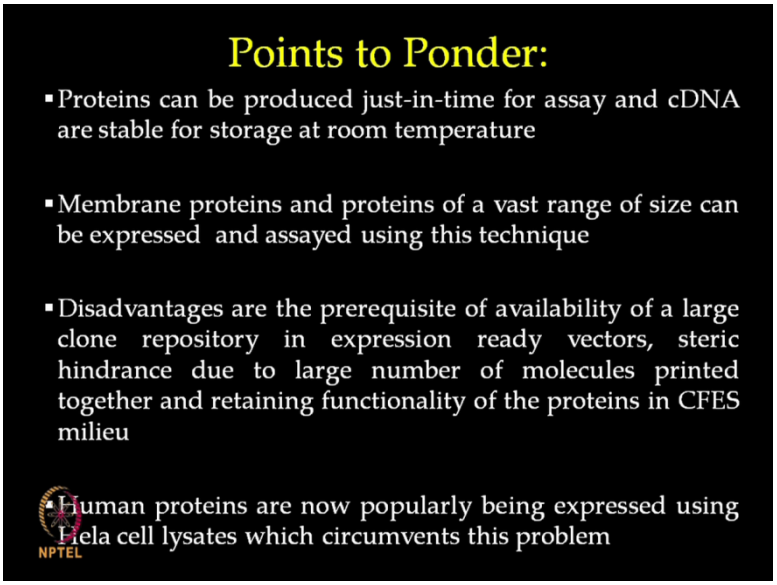

In the past decade the biological research has witness a paradigm shift from focused reductionist approaches to a greater dependence on data provided by large industrial size proteins. These high throughput projects can capture data at a scale of entire organisms and provide inside into the biological system as well as organization of physiological networks. The development of cell-free expression microarrays have overcome several limitations of cell based protein microarrays and revolutionized the ability to simultaneously study thousands of proteins. In today's lecture, we have tried to understand the (()(29:31) for arrays like Protein in-situ array PISA and Nucleic acid programmable protein arrays NAPPA. In the next lecture, we shall overview other cell-free protein microarrays, Thank you.

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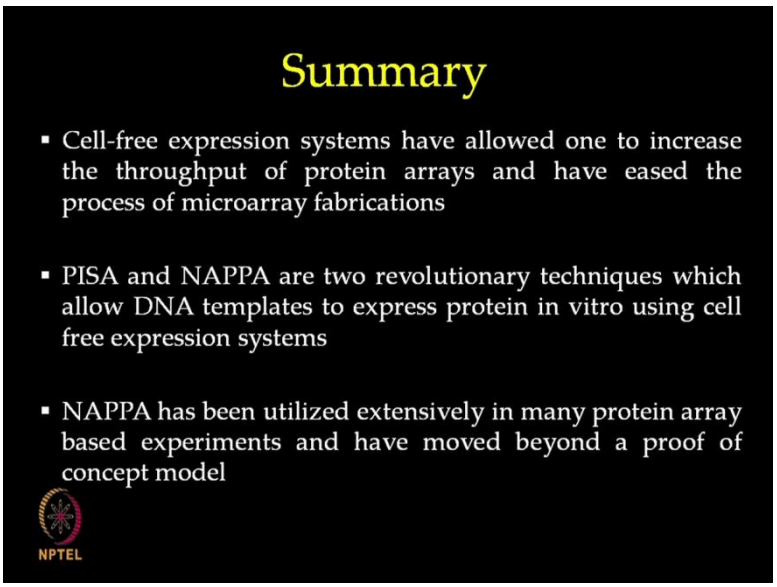

References

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