Interactomics Basics and Applications

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Lecture – 04
Cell-free Expression Based Protein Microarrays

Hello students. I hope you remember from the previous lecture we talked about different types of protein microarray platform which includes protein purified as there as peptides or different type of antibodies printed. But more interestingly, I showed you some examples where even cDNA or unpurified PCR products could also be printed on the chip and protein could be expressed by using cell-free expression system.

In today's lecture, we are going to focus more on protein microarrays based on cell-free expression based system. The cell-free synthesis based protein microarrays provide high throughput, versatile and large scale platform for analysis of proteins in a very very functional manner. These microarrays are used for various applications for example, antibody profiling, biomarker discovery, enzyme substrate identification, protein protein interactions etcetera. The traditional cell based methods which were used for making the protein microarrays involved protein expression in at another system such as E coli.

The protein purification is very laborious process. It involves various steps such as the protein purification, protein integrity, its stability and functionality. So, if one has to generate high throughput large number of proteins which is required for printing high density arrays to perform the protein microarray studies it is going to be very tedious, because one need to purify large number of proteins in the scale of thousands and then maintaining the functionality and keeping them properly folded which is very very tedious process. Therefore, these limitations of traditional protein purification and protein microarrays generated by these purified proteins have been the major motivation for the cell-free expression based protein microarrays.

The cell-free expression based system overcome various limitations of protein purification and they perform in situ transcription and translation. During the last decade various methods have emerged as a strong platform for protein microarrays generated by applying the cell-free expression based systems.

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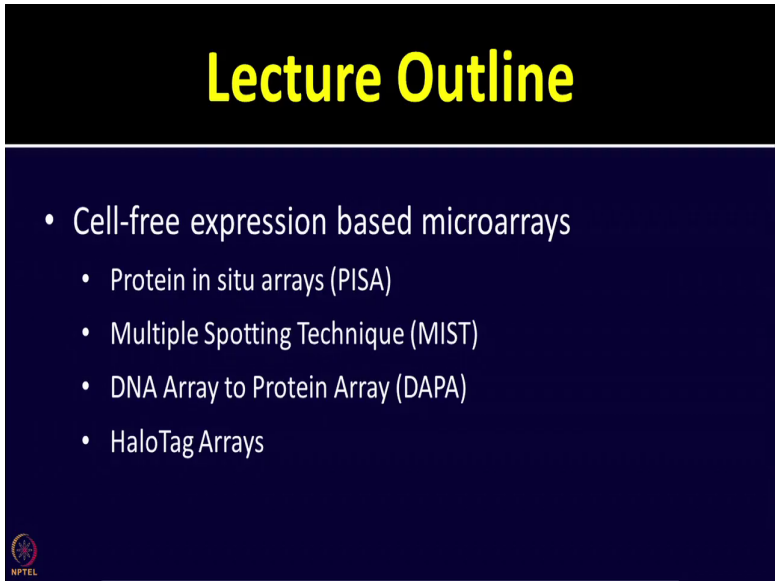

Today I am going to talk more about the cell-free expression based protein microarrays, provide your overview and the basic principle involved in performing different type of cell-free expression based protein microarrays. However, before I start about using the cell-free expression systems for protein microarray. Let us first talk about what is cell-free protein synthesis.

The cell-free systems make use of template DNA obtained from either plasmids 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 the cell-free lysate.

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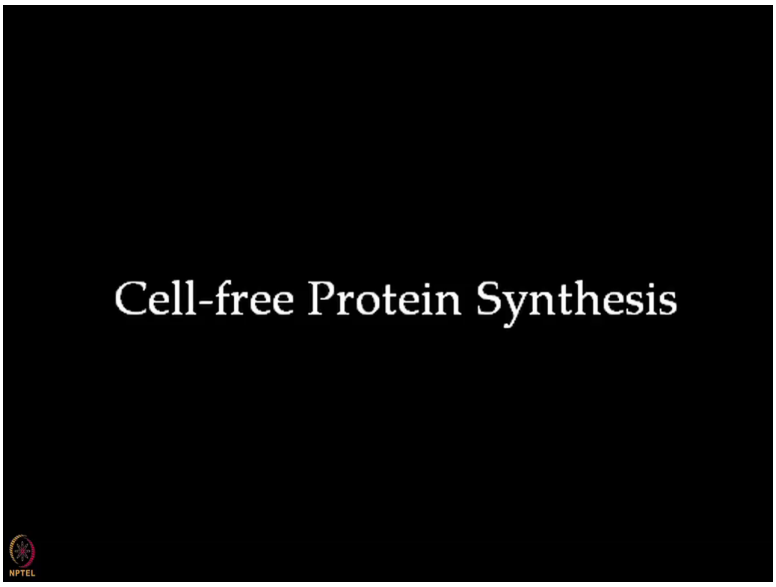

The cell-free expression systems have been extracted from cells of different organisms, such as wheat germ extracts, rabbit reticulocyte lysate and a Escherichia coli. These are commonly use systems. But there are other systems as well which includes cell lysates from the (Refer Time: 04:30) site, hybridomas, insects and mammalian cells.

Approaches demonstrating the efficacy of cell-free expression systems to synthesize protein in vitro have been extensively demonstrated in literature. So, now, we will talk about protein microarrays based on cell-free expression systems. To eliminate the obstacles posed by the traditional cell based method cell-free expression systems are increasingly adopted to

generate microarrays. There are several microarray generation technologies which have been developed over the past few years. Let us discuss some of these technologies one by one today. Let first talk about what are the requirements of cell-free expression system.

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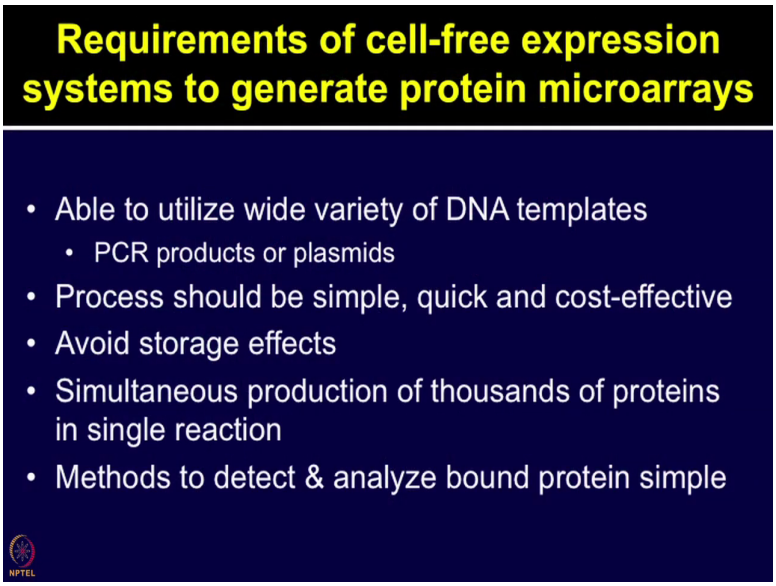

The cell-free expression system should meet certain requirements and these criterias are that they should be able to utilize wide variety of DNA templates. This brings out a versatile platform, the system should be simple, quick and cost effective. When we are talking about protein microarrays the applications are essentially for clinical studies or various biological applications, where one need to look at very pressure samples in a very very small volume. Thus an expensive technology becomes a hurdle for the wide spread use of the potential of this technology for clinical applications or other high throughput biological applications.

The microarrays should be produced on demand, so that one could avoid the degradation of proteins due to their storage issues. Once you have made these purified printed arrays, the array should be stored in minus 80 degrees and properly stored because otherwise the protein integrity and protein functionality will be compromised. So, cell-free expression system can be used if there is a need to perform an assay that can eliminate many of these limitations. It can allow the simultaneous production of thousands of protein that is the common requirement for any type of microarray platform, whether it is DNA microarrays, traditionally purify microarrays or the cell-free expression based protein microarrays.

The 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 in principle like western blots or elise analysis. So, these are quite simple production assays are also simple, so that they read out could be used by using the common instruments available in the labs.

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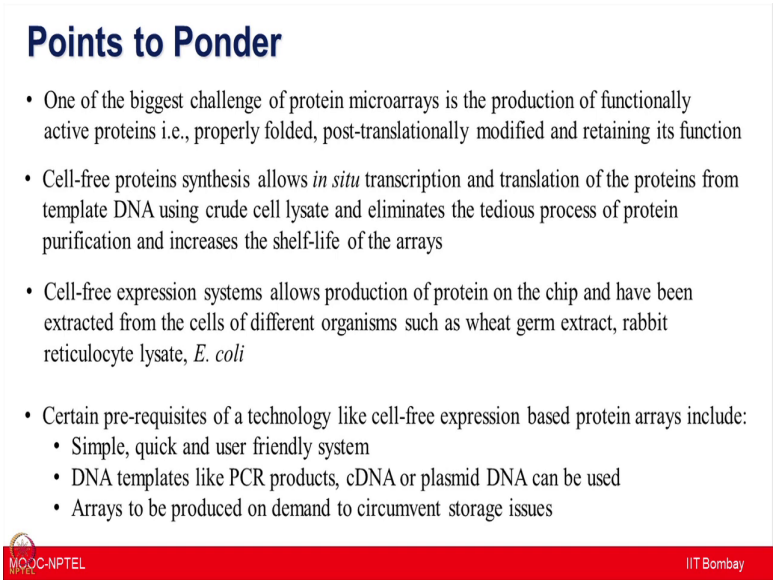

Let us now focus on few of the cell-free expression based protein microarray system. It goes back the first development of technology which was PISA or protein in situ array and let us first talk about this.

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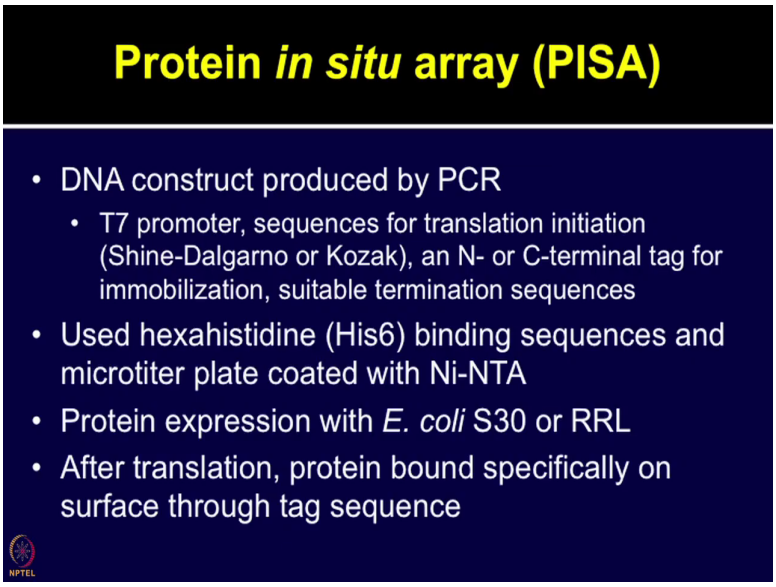

So, PISA technology also known as distant array technology provides rapid single step method to generate protein arrays from the DNA template by using cell-free transcription and translation system which allows the immobilization of synthesized protein on solid microarray surface. For performing a PISA array there various requirements. The DNA construct can be produced by PCR. The construct should contain T 7 promoter, sequences which are required 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 sequence

The substrate or 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 the binding can be specific. The hexahistidine or histidine 6 tag binding sequence and a microtiter plate which is coated with

nickel NTA is used for this purpose. The protein expression can be performed by using E. coli base system or rabbit reticulocyte lysate RRL.

Once the translation has finished the protein which is synthesized by using the cell-free expression system is specifically binds on the surface through this tag sequence. In PISA array method the surface is coated with a tag capturing agent. This method utilizes hexahistidine tags which is in the construct and the microarray titer plate which is coated with nickel, metal or tri acetic acid and INTA as shown as the slide.

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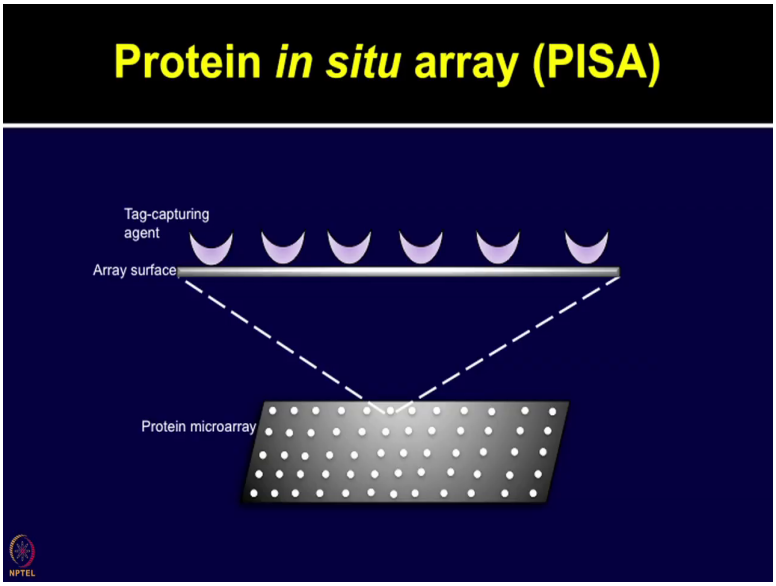

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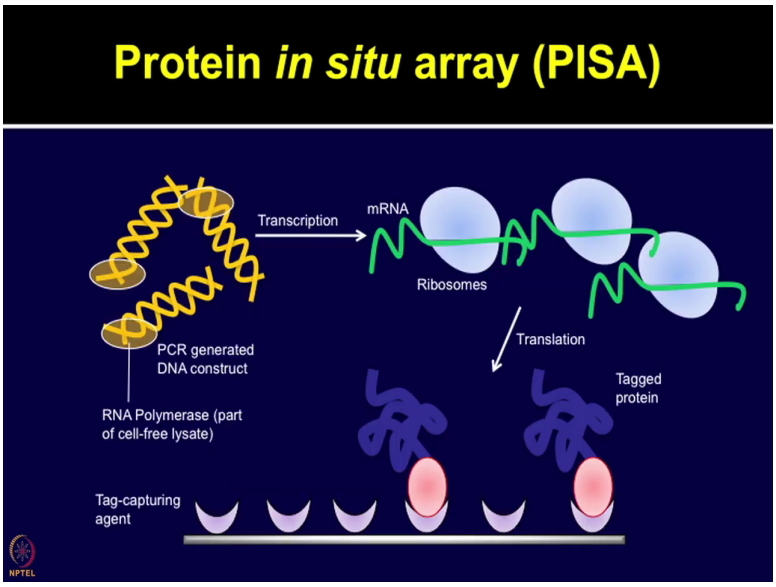

The DNA construct contains T 7 promoter sequence for translation initiation n terminal sequence and the terminational sequence. Once the protein expression is carried out by using cell-free expression system such as E coli S30 or rabbit reticulocyte lysate. The protein binds is specifically on the surface 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 using transcription and translation processes containing histidine tags which gets adhere to the nickel NTA surface.

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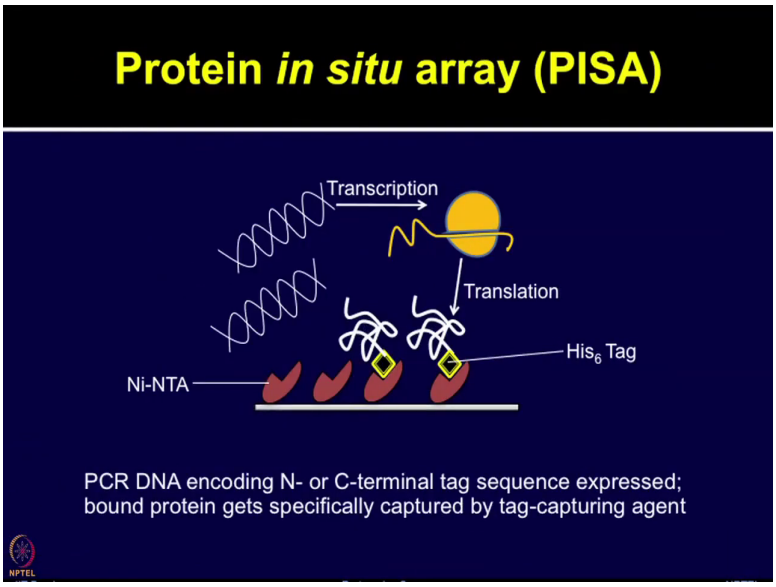

So, overall PISA method utilizes the PCR DNA which encodes N- or C- terminal tag sequences and then transcribes and translates the cDNA of interest into the protein. The protein which is synthesized after the cell-free expression gets is specifically captured by tag capturing agent.

Scientist (Refer Time: 11:23) who have developed this method successfully carried out this experiment, the expression and functional immobilization of a fragment of human n-type electron antibody, in microtiter wells and used luciferase enzyme on nickel NTA coated magnetic beads. Let me now show you this animation where we can discuss the working principles of protein in situ arrays.

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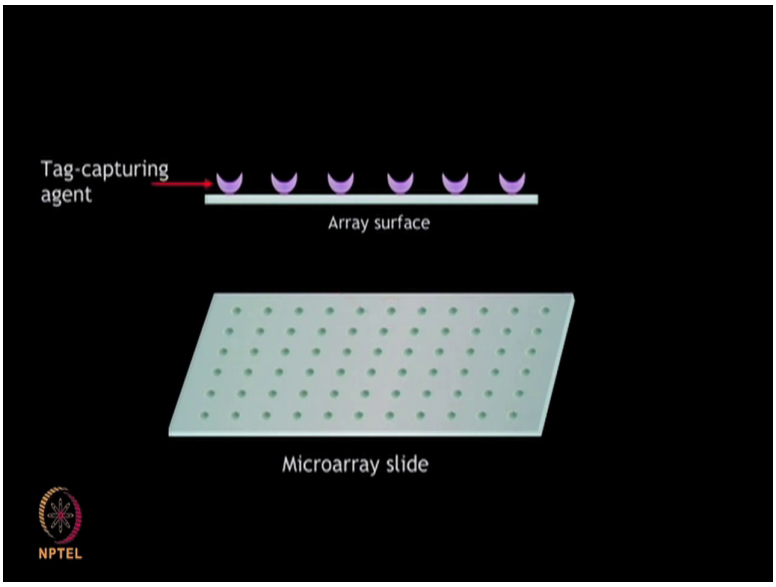

Protein in situ array or PISA. In PISA the protein microarray surface is coated with a suitable tack capturing agent that can immobilize the protein of interest through a specific interaction once it is produced.

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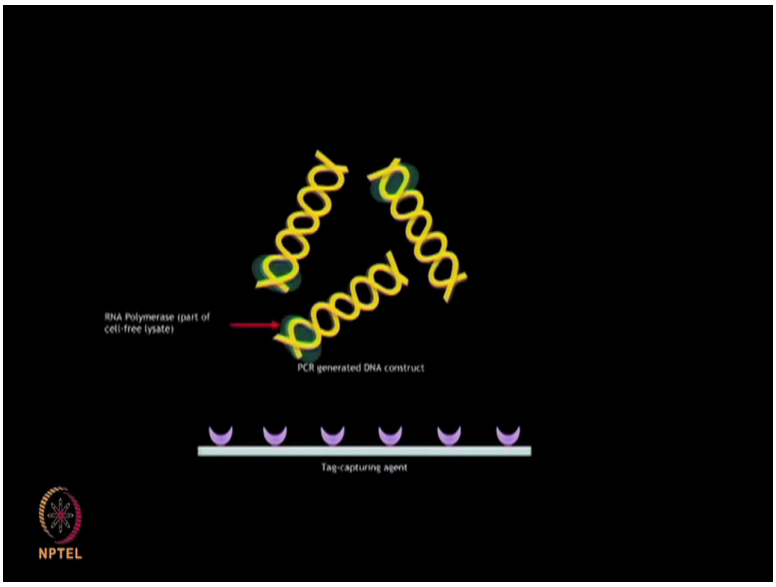

The protein is expressed from its corresponding DNA by using cell-free lysates.

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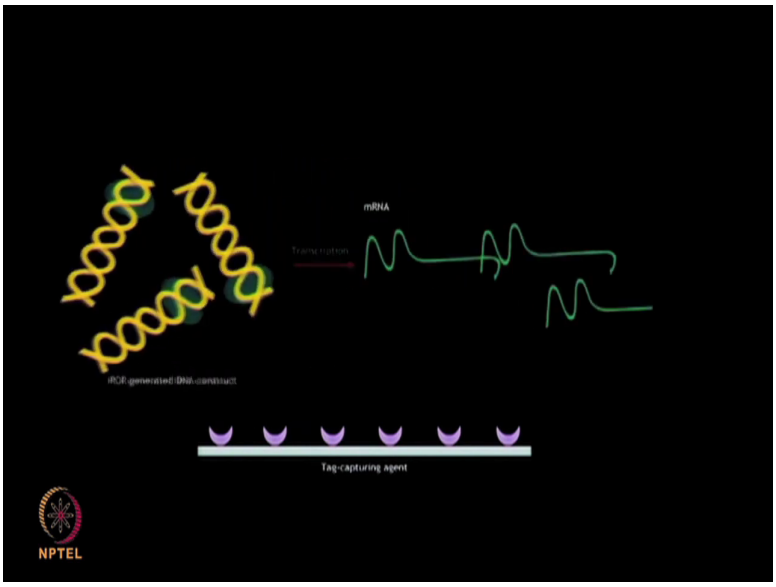

Such as E coli.

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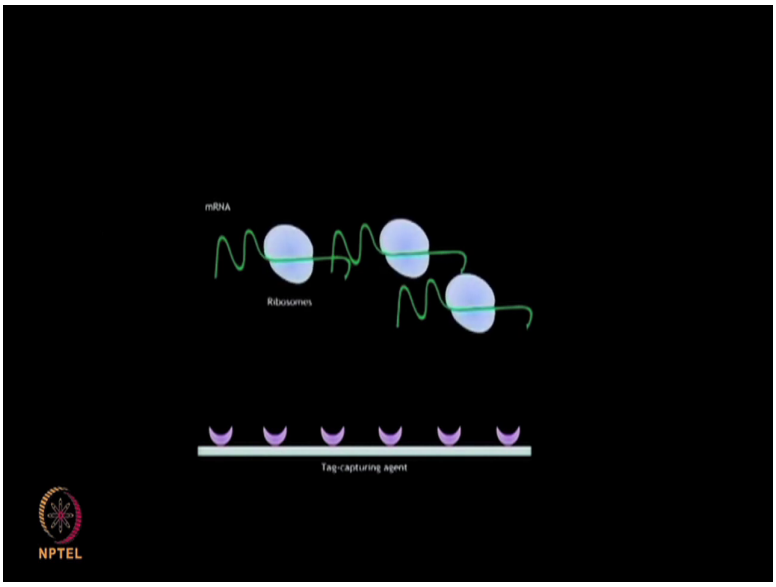

S30 or rabbit reticulocyte lysate.

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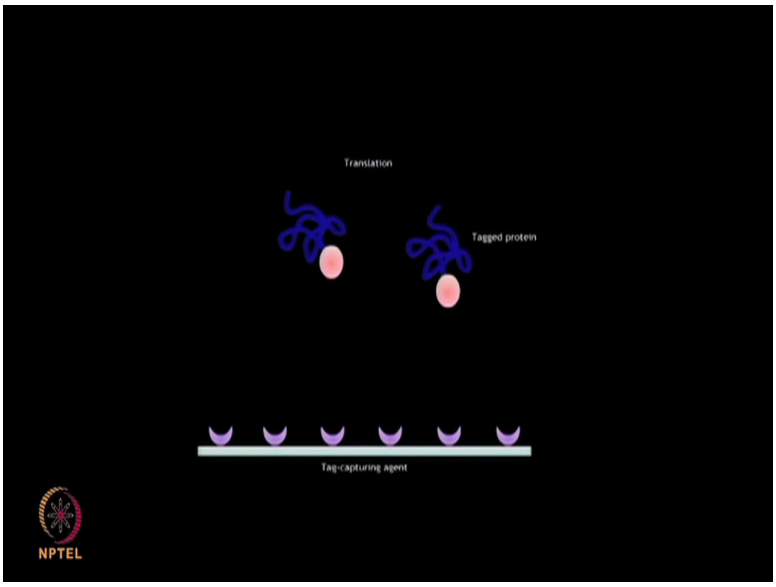

The tagged protein is captured a specifically onto the array surface through the tag capturing agent.

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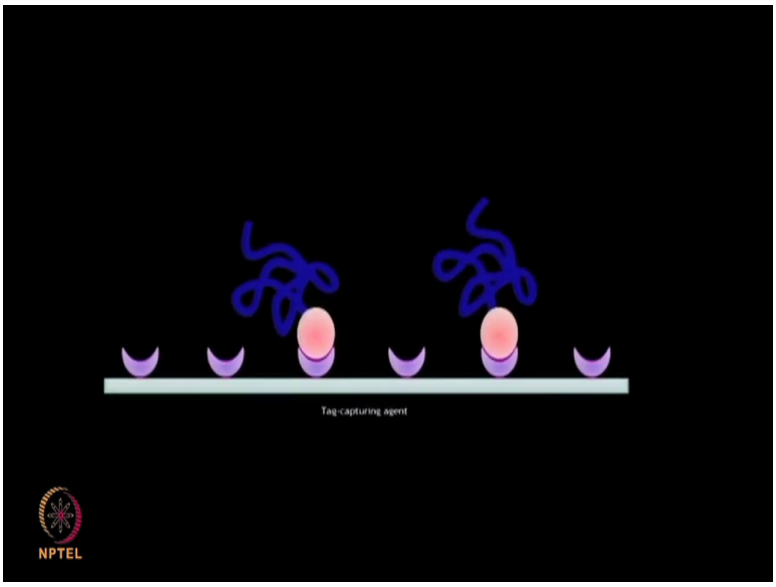

PISA method has successfully overcame drawbacks of cell based techniques such as protein insolubility, aggregation etcetera. After learning the working principle of PISA, let us discus various advantages and disadvantages of this technology.

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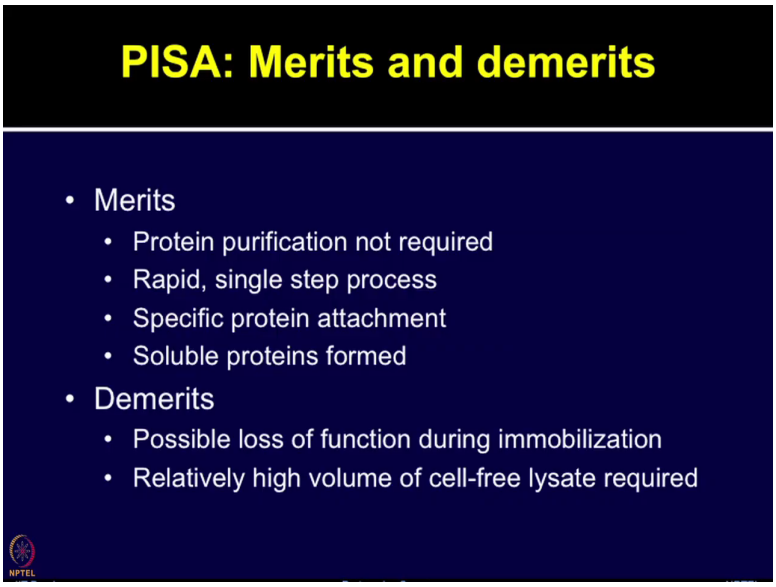

PISA method overcomes the traditional purification requirements for producing the protein microarrays. So, protein purification is not essential it gives rapid single a step process because of histidine tag and nickel NTA interaction the specific protein attachment could be achieved by using PISA method. In this method solving proteins are formed. These are some of the major advantages of using protein in situ arrays

Let us now discuss the limitations of this technology. It is possible that there is loss of function of these proteins during the immobilization a step. The cell-free lysates are very costly. So, if one needs high volume of cell-free lysate that becomes one of the cost base limiting factors for this platform.

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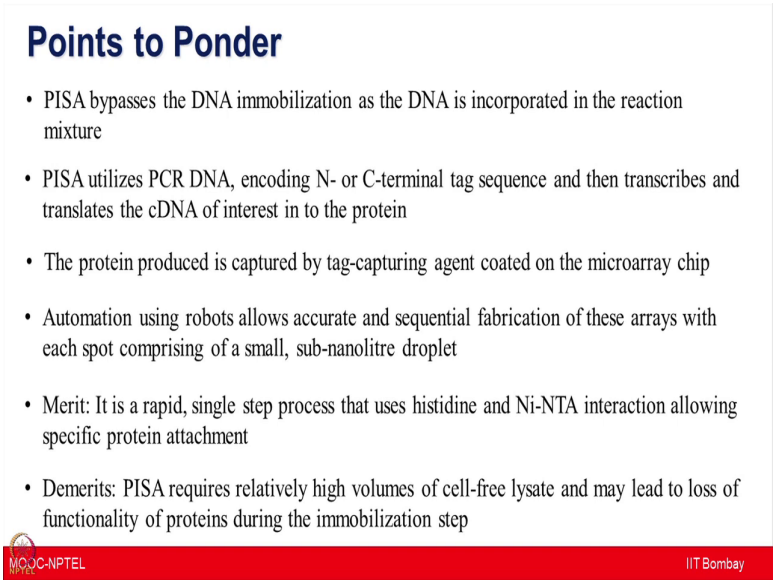

Let us now move on to another cell-free expression based protein microarray system which is MIST or a multiple spotting technique. This is another approach which facilitates generation of high density protein microarrays by using cell-free expression system. In the MIST approach, both DNA and the cell-free expression system are printed on the chip surface.

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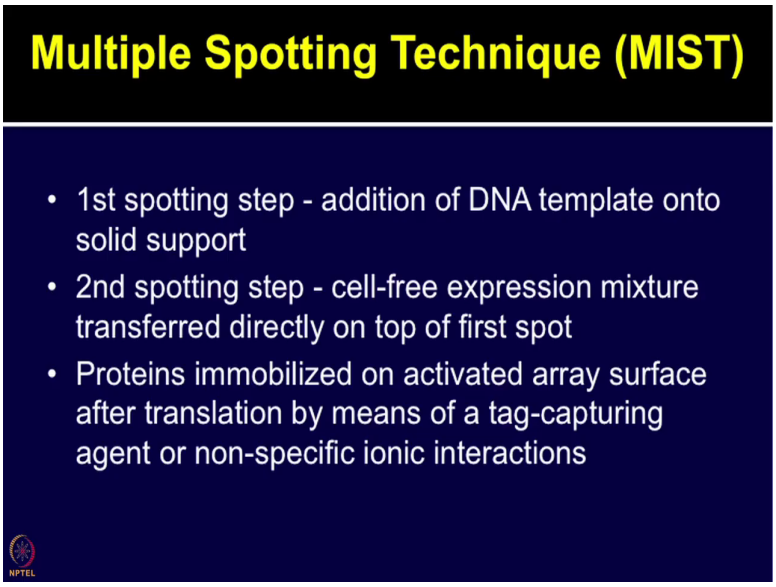

Here two rounds of a spotting is performed. In the first spotting step, the addition of DNA template to the microarray solid support is performed. In second spotting is performed where cell-free expression mixture is transferred directly on top of the first spot which contains DNA. Aim is to print DNA as well as cell-free expression system, so that after incubation protein can be directly synthesized on the same feature. Anyone does not need to add or do a separate step of cell-free expression system addition.

So, the proteins which are immobilized on the activated array surface after translation by means of attack capturing agent or nonspecific ionic interactions. So, the multiple spotting technique or MIST the first spotting step as shown in the slide involves addition of DNA template onto the solid microarray support.

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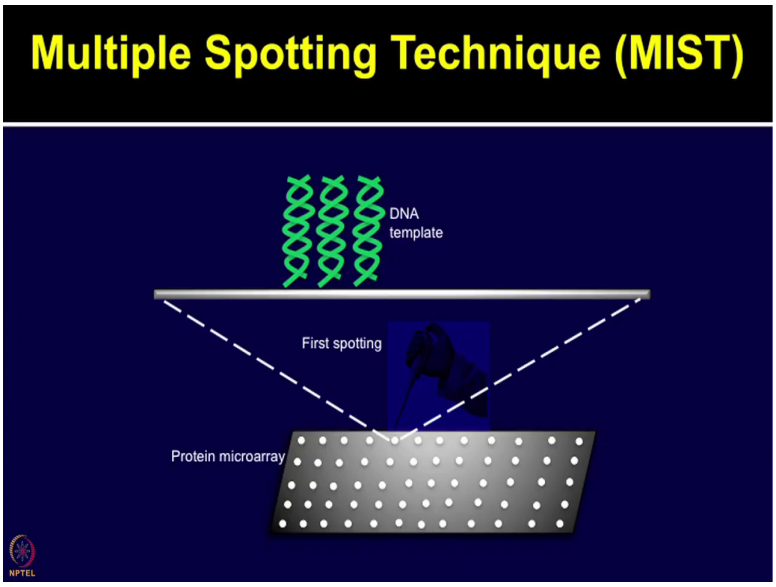

After the first spotting is performed where cell-free expression mixture is transferred directly on top of the first spot. In this way where two printing steps are involved on top of each DNA template cell-free expression system is also printed.

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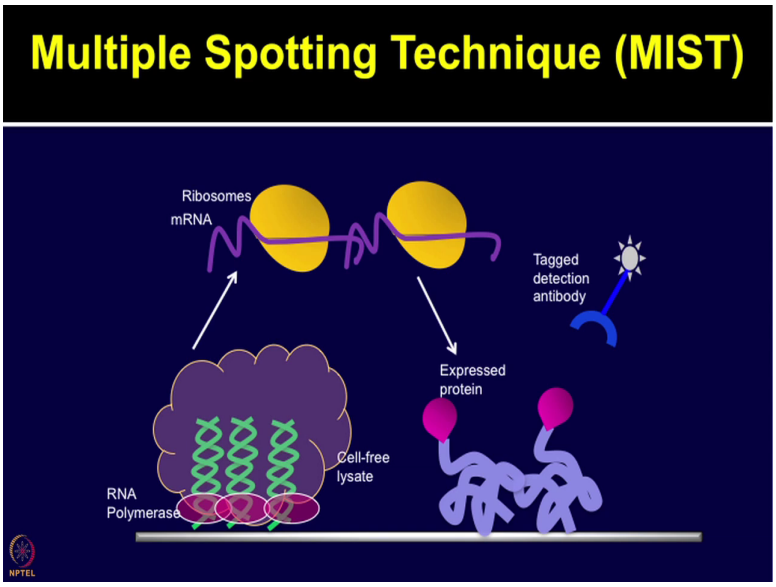

After incubation both transcription and translation processes happen and the proteins are synthesized which could be detected by using detection antibodies. So, over all in MIST technique the DNA template is spotted in the first step followed by the cell-free lysate in the second step which is directly added on top of the first spot.

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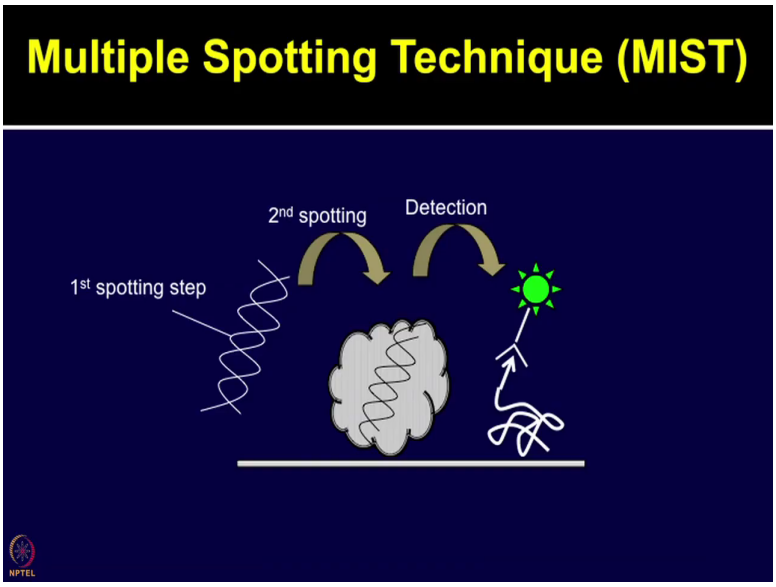

The expressed protein is detected by using fluorescently tagged antibody. So, the inventors of MIST technology reported that even 35 femtogram of PCR product was sufficient for expression and detection of wild type in fluorescent protein. The high density array containing 13000 spots per slide could be achieved by using MIST technology. Let us now discuss the working principle of MIST by showing this animation.

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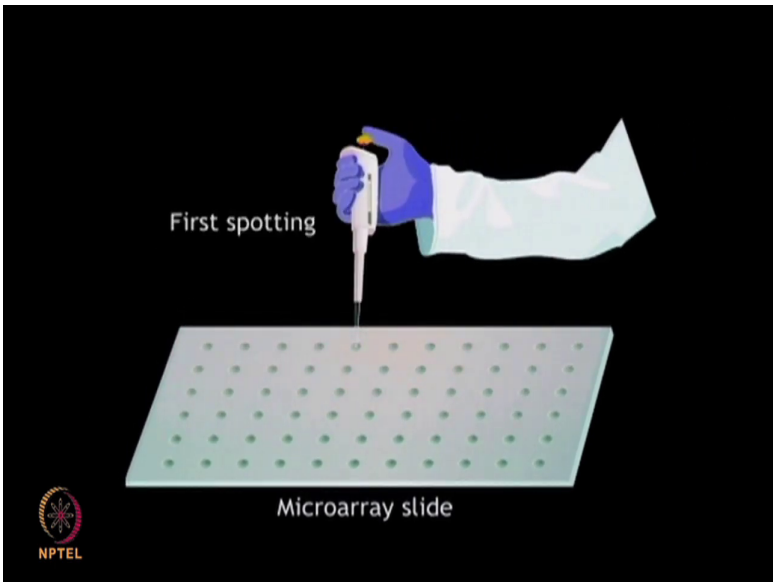

Multiple spotting technique or MIST, in this technique the first spotting it chip of the multiple spotting is capable of producing high density arrays.

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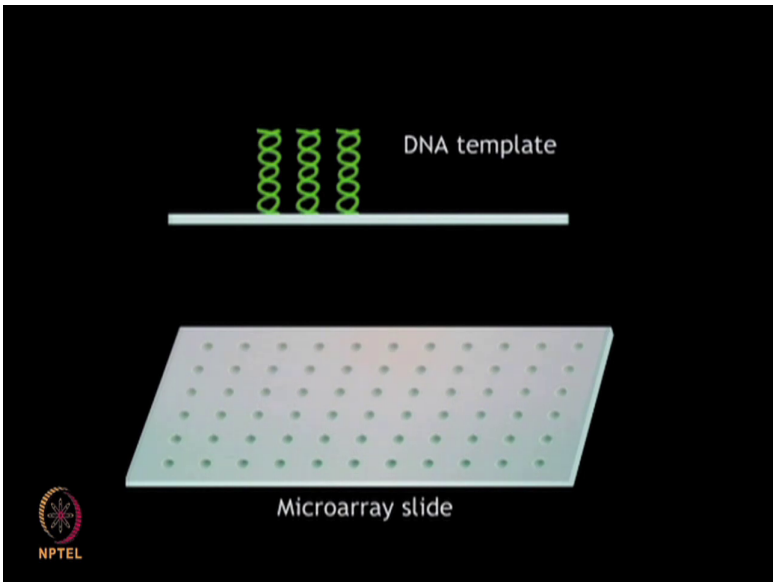

It involves addition of template DNA onto the solid array support. The template DNA can even be in the form of unpurified PCR product which is one of the major advantage of using this MIST technique.

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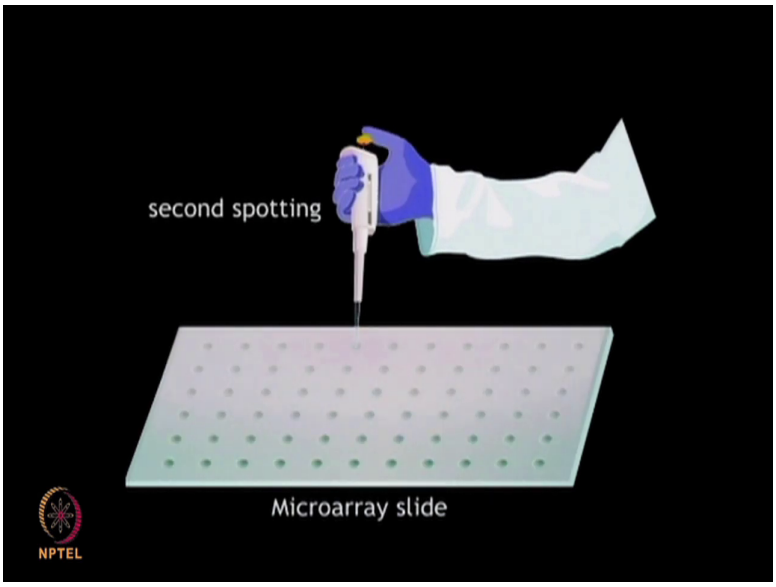

The second is spotting strip involves addition of cell-free lysate directly on top of the first spot.

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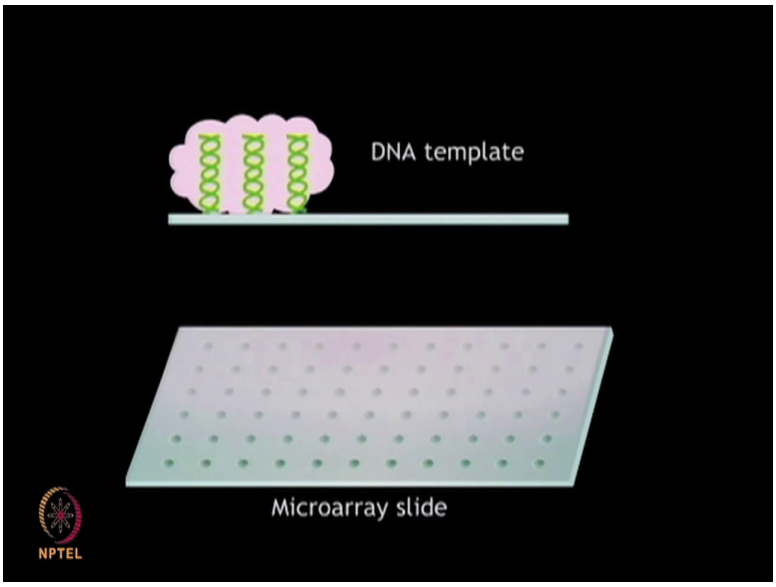

The transcription and translation can begin only after the second spotting step.

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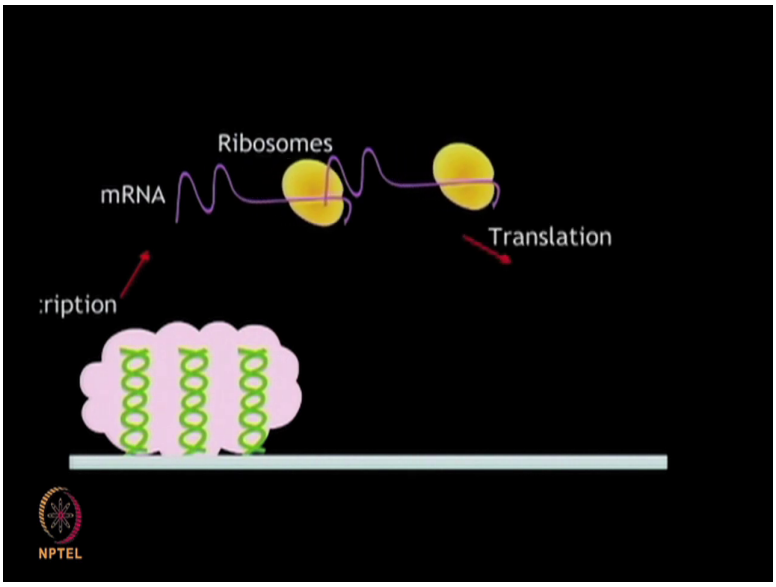

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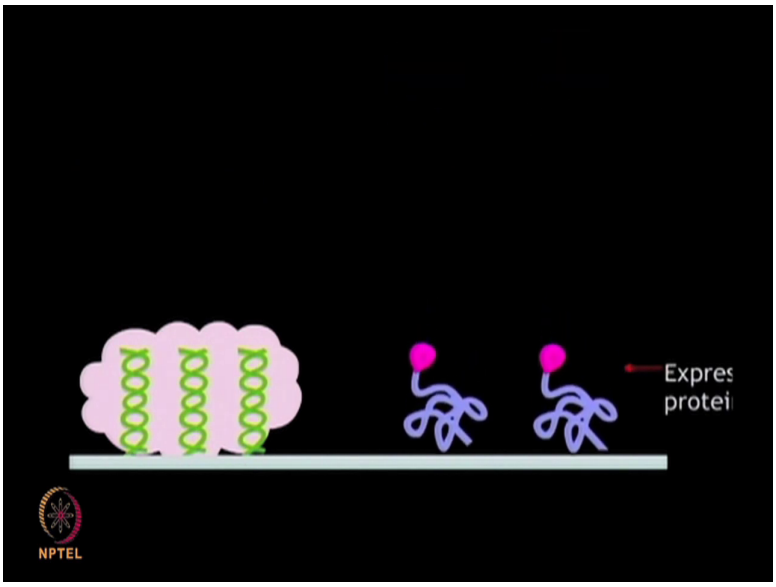

The protein expressed from the template DNA binds to the array surface by means of nonspecific interactions which is one of the drawbacks of this procedure.

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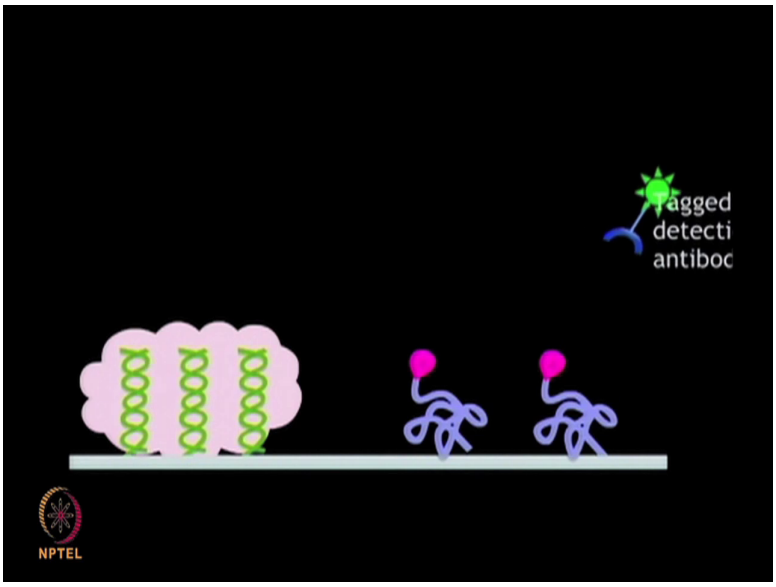

A detection antibody is specific to the protein of interest is added which indicates that protein expression levels by using suitable fluorophore

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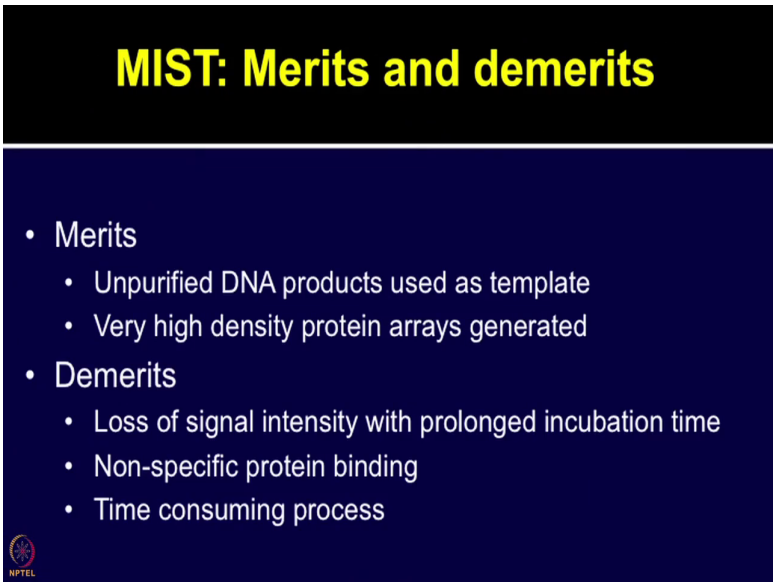

Let us now talk about the merits and demerits of using MIST technology. It involves unpurified DNA products that can be used as template source which was not the case of other method which we have discussed so far. Now, in this method very high density protein can be generated because a spot chemistry is not very complicated. Limitation of using this technique is there is a loss of signal intensity with prolonged incubation time of the arrays.

Since, in this case even the cell-free expression system are printed on the top of arrays the stability could be one of the major issues. The nonspecific protein binding as well as overall process is more time consuming. So, some of these are limitations of best technique.

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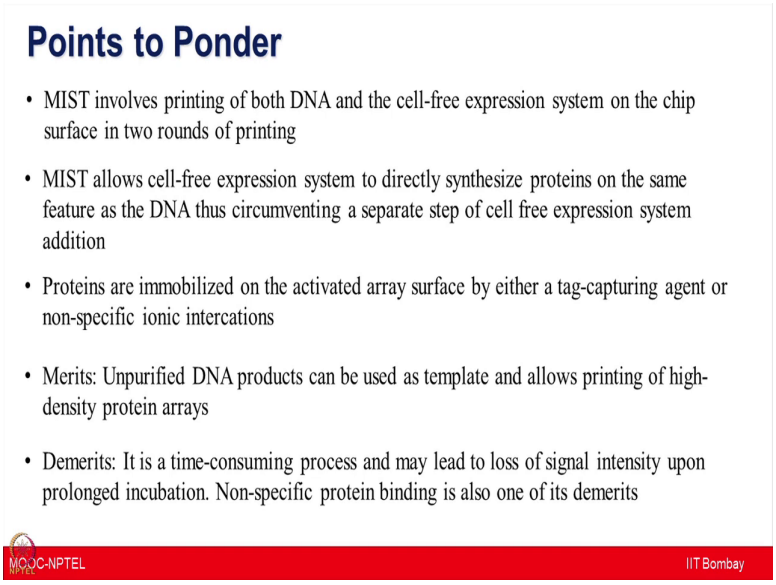

Another technique which requires more detailed discussion is NAPPA or nucleic acid programmable protein array, but I would like to talk about this technology in the next lecture, when I am going to talk to you about the working principle and the workflow involved in doing the NAPPA based experiments, but let us continue on another cell-free expression based system which is DAPA. So, DNA array to protein array is another technique which makes possible the repeated use of same DNA template slide for printing multiple rounds of protein arrays.

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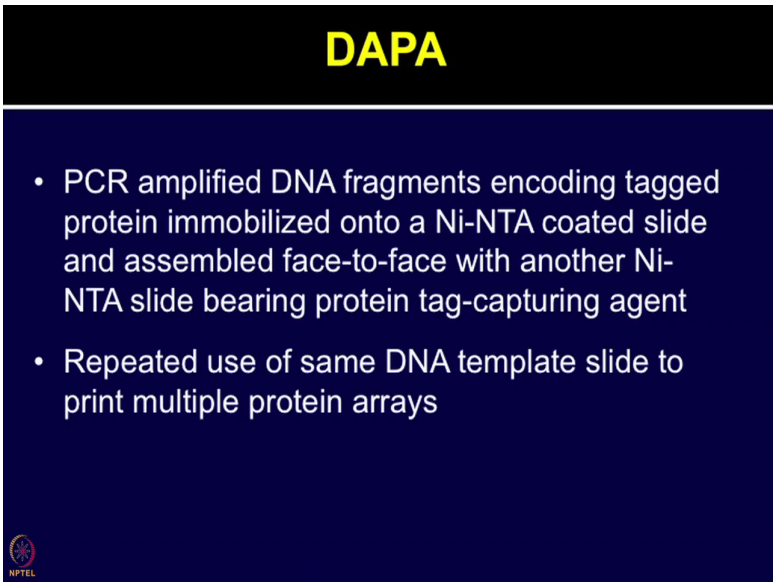

In DAPA technique, the PCR amplified DNA fragments which encode tagged proteins immobilized onto a nickel NTA coated slide an assembled face to face with another nickel NTA slide bearing the protein tag capturing agent is utilized. The repeated use of same DNA template can be performed here and multiple protein arrays can be generated by using DAPA method.

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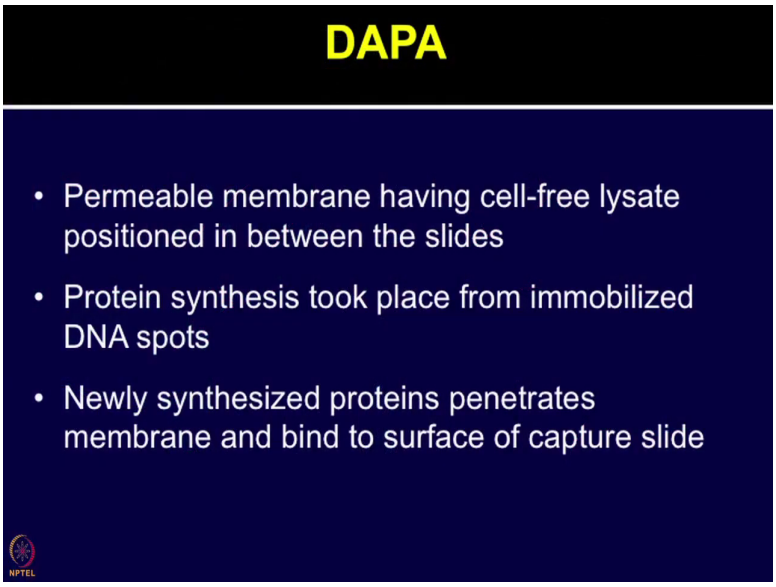

In this method, the permeable membrane which contains cell-free lysate which is positioned between two slides for these proteins to be diffused. Protein synthesis takes place on this membrane and then the synthesized protein diffuse from the membrane and move on to the other slide for capture. Newly synthesized proteins penetrate the membrane and bind to the surface of captured slide.

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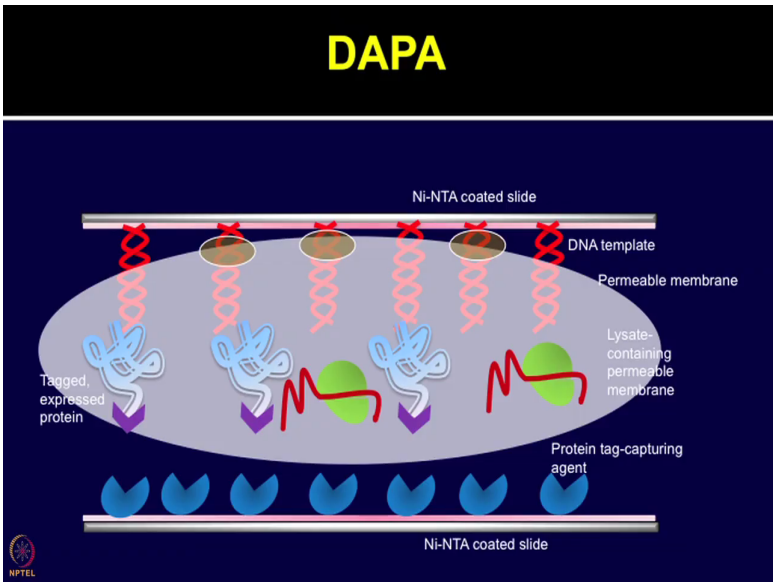

As you can see the slide, in the DAPA the PCR amplified DNA fragments encoding the track proteins are immobilized onto a nickel NTA coated slide and assembled face to face with another nickel NTA containing protein tag capturing agent. In between these two slides a permeable membrane containing cell-free lysate is placed.

The protein synthesis takes place from the immobilized DNA spots. The newly synthesized proteins can penetrate this membrane and binds to the surface of captured slide. In DAPA approach the investigators produced an array of double hexahistidine tag GFP and data was found to be comparable with existing protein array technologies. With DAPA it is possible to use same DNA template repeatedly to print multiple protein arrays. It has been shown that one can use this template for printing almost 20 arrays. Let me now show you the working principle of DNA arrays to protein arrays in following animation.

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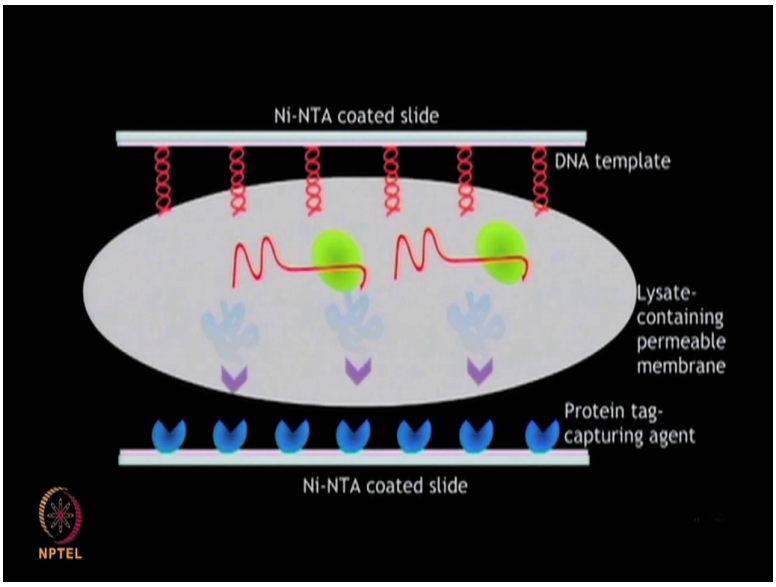

DNA array to protein array known as DAPA. In DAPA, the slides bearing the DNA template and the protein tag capturing agent are assembled face to face with a lysate containing permeable membrane placed in between the expressed protein slowly penetrates the membrane and gets immobilized on the slide surface through its capture agent. The DNA template array can be reused several time by using this method.

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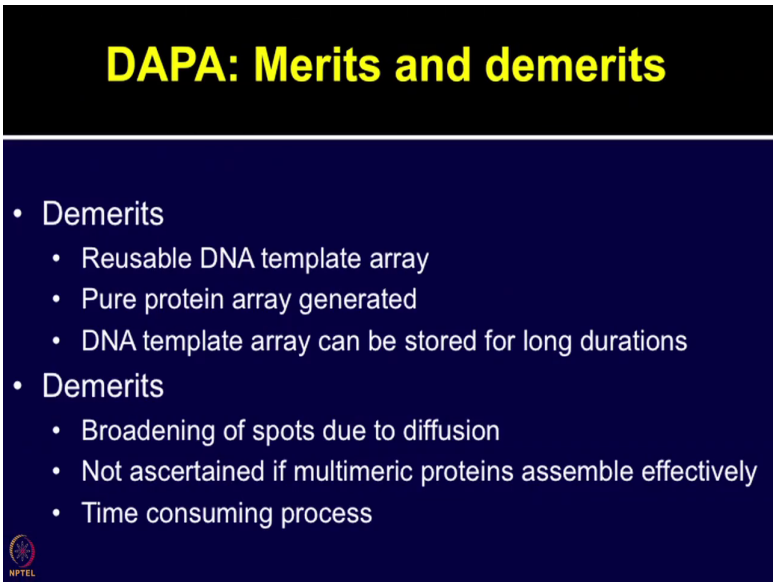

Let us not talk about the merits and demerits of DAPA. DAPA method provides few advantages as compared to the previously described methods. One can get the pure protein because the protein is diffused from the membrane, so we are not having any traces or remnants of DNA in the protein array slide.

The reusable DNA template which is able to print multiple chips by using this chemistry and now the source template which is DNA that array can be stored at room temperature for long duration. When there is a need for making a the protein arrays, one can use the membrane with the lysate and then followed by generation of multiple protein arrays.

However, there are certain limitations of using DAPA method including the broadening of the spots because of the diffusion. It is not ascertained if multimeric proteins assembly effectively forms and it is also the time consuming process.

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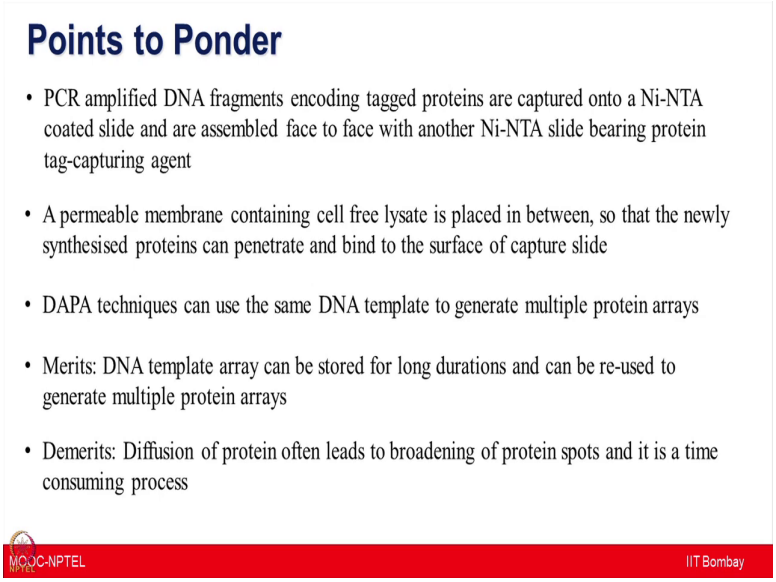

Now, let us take one more last technique for the day which is Halo Tag Arrays. The halo link protein array systems are developed by Company Promega which combined few technologies together to create the protein microarrays.

First of all it uses the cell-free expression transcription and translation system. It uses Halo Tag which is mutated hydrolysed protein that forms a covalent bond with the Halo Tag ligands. Third, it uses polyethylene glycol coated glass slights activated with Halo Tag ligand

for a specific capture of the proteins which are expressed by using cell-free expression system.

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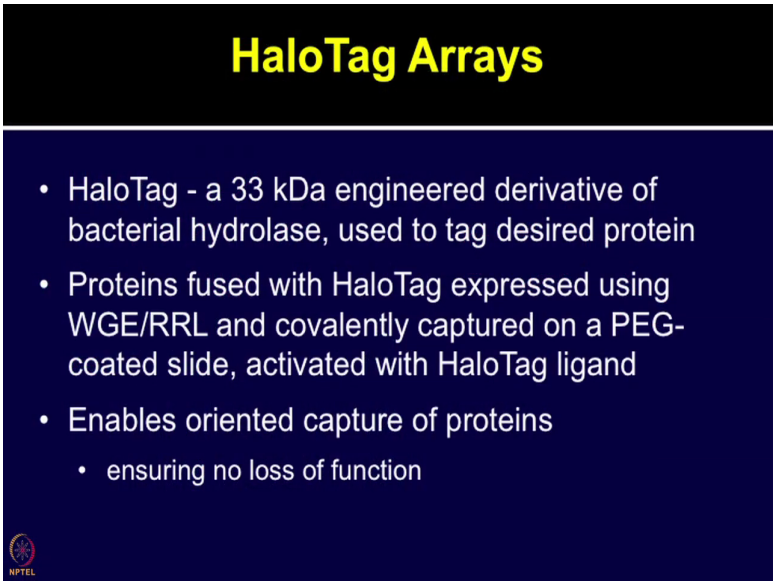

So, what is Halo Tag? It is a 33 kilo Dalton engineer derivative of bacterial hydrolase which is used to tag desired proteins. The proteins which are fused with hello tag are expressed by using, we germ extract expression system or rabbit reticulocyte lysate and covalently captured on to peg containing slides. These are then activated with Halo Tag ligands. These Halo Tag arrays achieve oriented capture of proteins and thereby ensures no loss of function or minimal loss of function.

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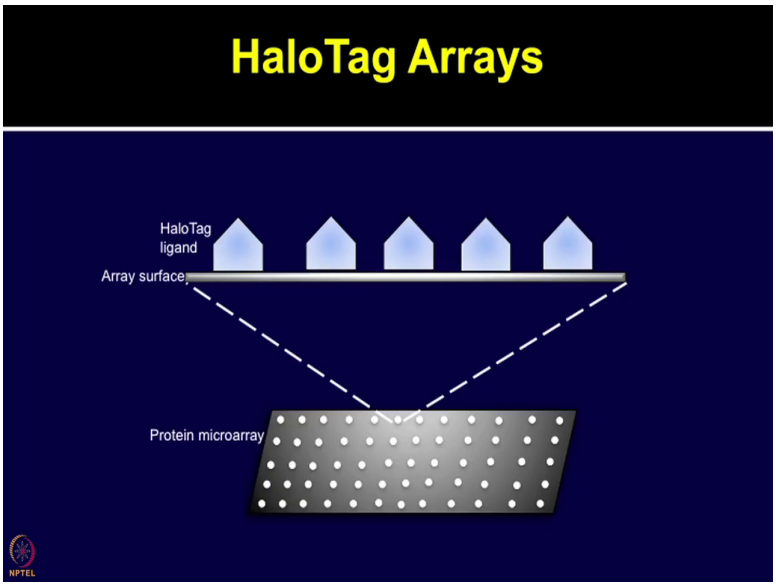

As shown in this slide the polyethylene glycol coated slides can be activated using Halo Tag ligands.

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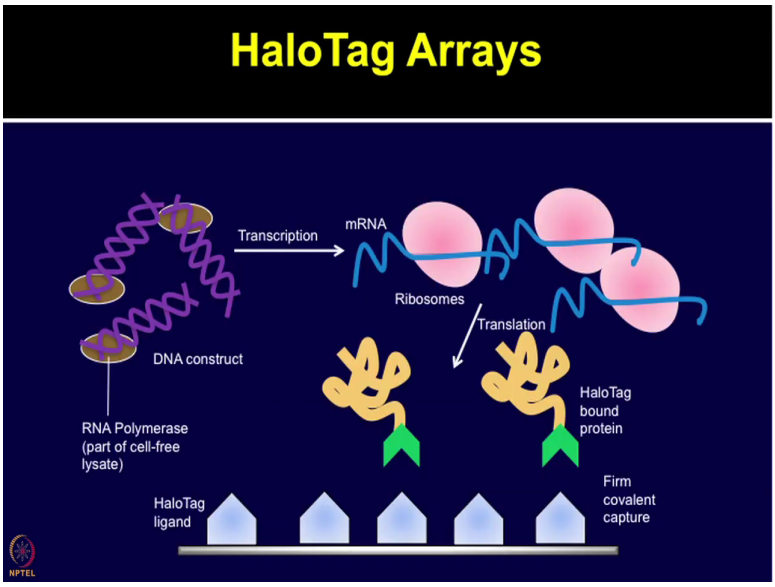

The proteins are fused with hello tag are expressed by using cell-free expression system and are covalently captured on polyethylene glycol coated slides. So, Halo Tag method enables the oriented capture of the proteins.

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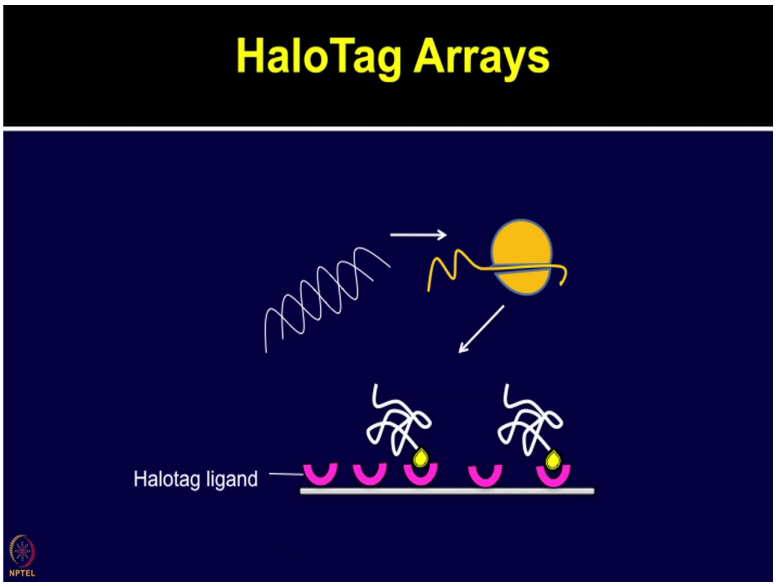

In nut shell, the hello tag fused protein is expressed and covalently captured on peck coated slide and activation is performed by using Halo Tag ligand. This provides very strong covalent interaction and minimizes loss of synthesized proteins which usually occurs in other protein microarray platforms.

In protein arrays, one need to perform a lot of washing steps. If the protein or the molecules are bound on the surface, but very strong interaction then there will be a minimal loss from the surface which can be achieved in this case by using Halo Tag system.

So, Halo Tag arrays the capture chemistry which is based on the binding of Halo Tag protein with synthetic ligand that enables covalent and oriented capture of proteins on solid surface directly from the cell-free expression based system. This method not only overcomes the limitations of protein purification, but also overcomes several other limitations which are

commonly observed in other protein microarray technologies. That will discuss the working principle of Halo Tag arrays in this animation.

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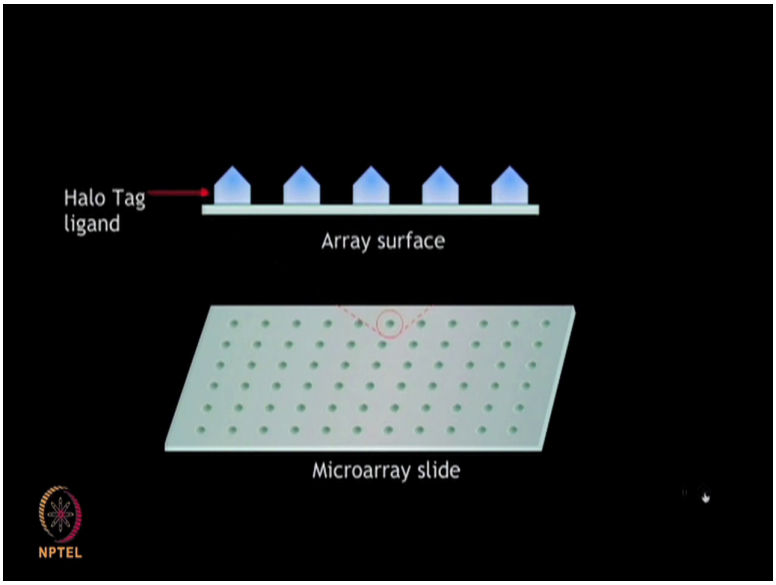

Halo tag technique. In Halo Tag technique, the slide is activated with the Halo Tag ligand which captures the expressed protein through firm covalent interaction which prevents any material loss and ensures oriented capture of the protein.

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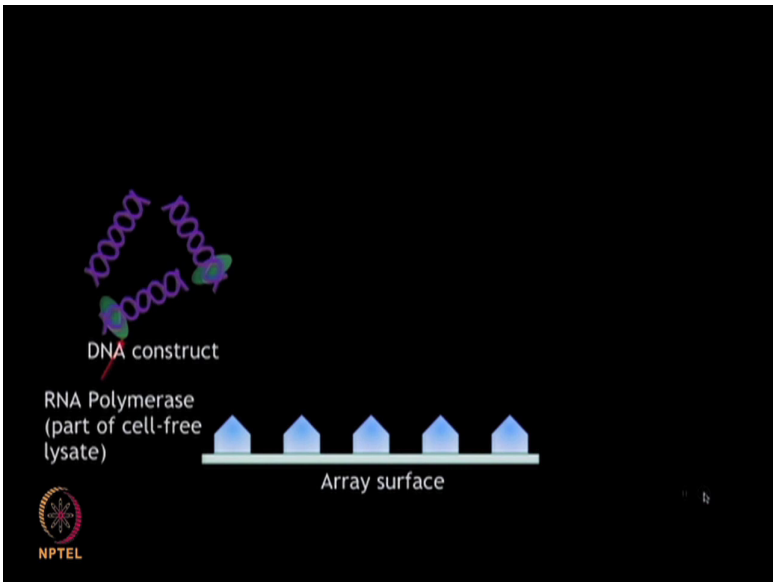

The hello tag fuse protein is expressed by using lysates such as Rabbit Reticulocyte Lysate, RRL or wheat germ expressional system.

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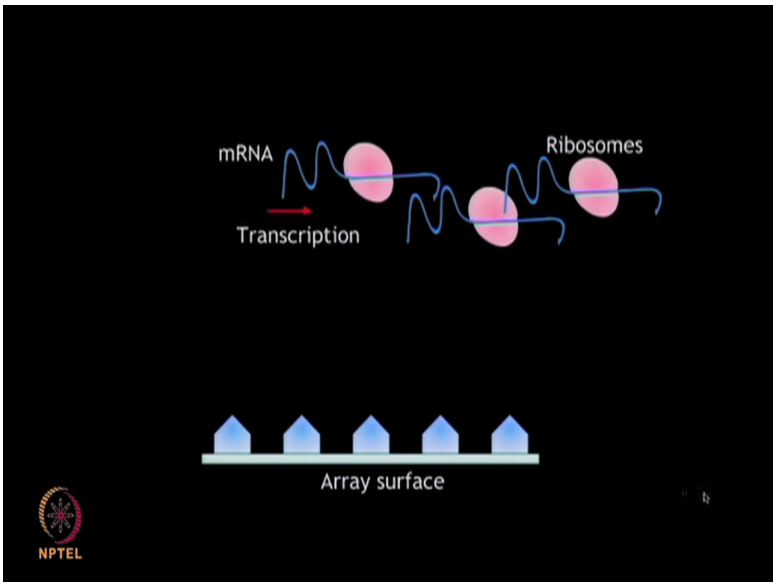

And synthesized protein is covalently captured onto the array surface through the Halo Tag ligand.

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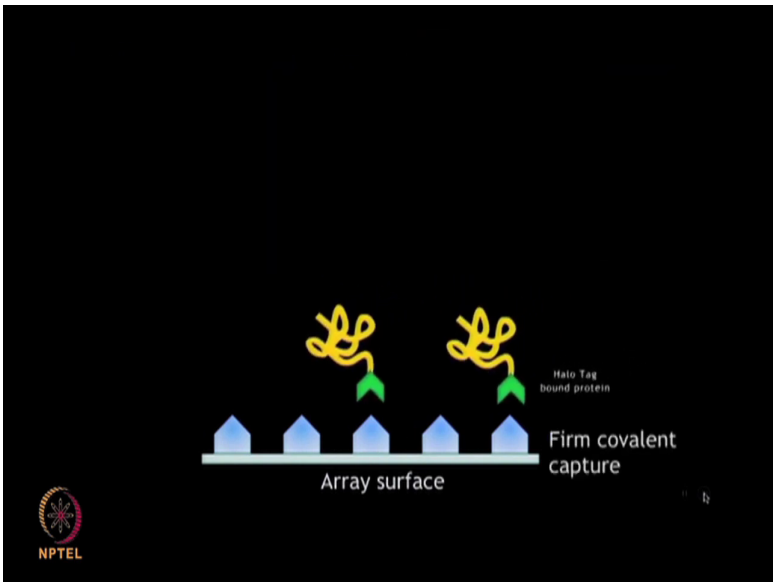

The specific interaction ensures the oriented capture of protein and prevents any possible functional loss.

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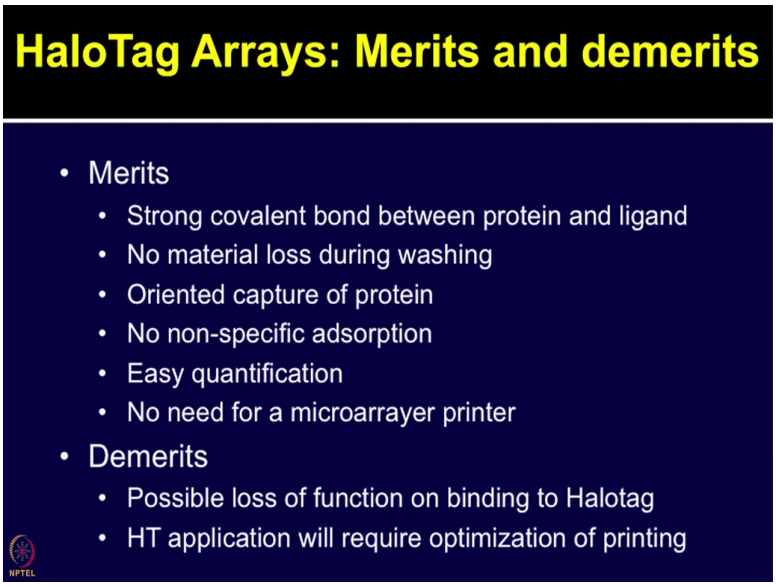

Let us now talk about some merits and demerits of using Halo Tag arrays. Halo Tag arrays have various advantages such as the strong covalent bonding between the protein and the ligand. No material loss occurs during the washing steps because of the strong interaction. The proteins are captured, oriented and there is no nonspecific adsorption due to the peck coating. The quantification is easy and one do not need a microarray printer to print the proteins on the chip because the commercial kit off Halo Tag arrays provide the gasket which can be used for printing the array.

However, there are several limitations of using Halo Tag arrays such as it has not been shown that a system cannot be used for high density array. Only a proof of concept a study has been shown with few spots using commercial gasket. So, utility of the arrays for high density applications must be utilized to really show this application for high throughput applications.

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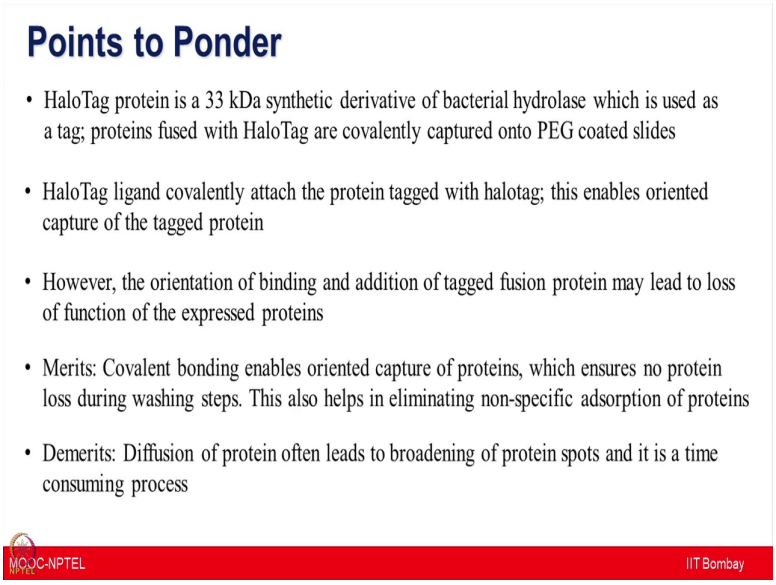

Since summary, today we talked about four different type of cell-free expression based methods, PISA, MIST, DAPA and Halo Tag arrays. Of course, I have not discussed to you a very important technology which is NAPPA, which will talk in the next class. But I hope you got in an understanding that protein microarrays offer a range of diverse applications because the different type of arrays could be produced is starting from antibodies, purified proteins, peptides and cell-free expression based systems.

The cell-free expression system facilitates synthesis of several proteins in single reaction and produce proteins on demand and eliminate the constraints of the storage and protein stability. Several cell-free systems have been used developed in the past decade and over the last, in the previous lecture and today you at least got some idea that how scientists are started using

making use of these cell-free expression systems for generating the contents which was otherwise not possible if you are following the standard procedure of protein purification.

In the coming lecture, I will talk more about the NAPPA based protein microarray and the workflow involved in performing such a microarray based experiment.

Thank you.