Proteomics: Principles and Techniques

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Lecture No. # 29
Cell-Free Synthesis based Protein Microarrays

Welcome to the Proteomics Course. In today's lecture we will talk about microarray, which is based on the cell-free synthesis so cell-free synthesis based protein microarrays in microarrays provide high-throughput versatile platform for large scale analysis of functional proteins. These microarrays are used for various applications for example, antibody profiling, biomarker discovery, enzyme substrate identification protein-protein interaction etc.

The traditional cell based methods which were used for making the protein microarrays. They involve protein expression in heterologous system such as escherichia coli but, the protein purification is a very laborious process it involves various steps and often the protein purity protein integrity its stability and the functionality. All of this remains major issues for the protein purification.

So, if we have to generate high-throughput large number of proteins which is required for performing the protein microarray based studies. It is very tedious because one need to purify large number of proteins in the thousand scale and then maintaining the functionality and keeping them properly folded it is very tedious. So, these limitations of traditional protein purification and protein microarrays generated by using these purified proteins have been the major motivation for cell-free expression based protein microarray field.

The cell-free expression based system they overcome various limitations of protein purification and they perform in (( )) transcription and translation. During the last decade there are various methods which have emerged as very strong platform for protein microarray generation by applying this cell-free expression systems.

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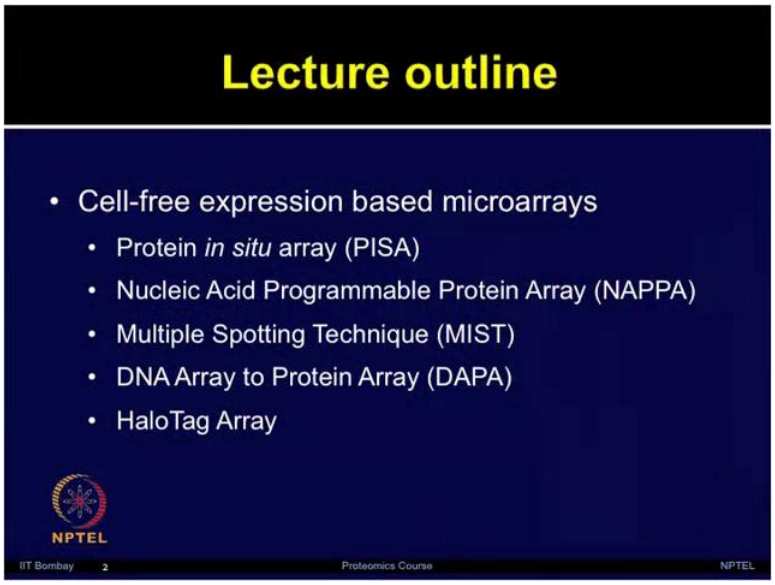

So in today's lecture, we will talk about cell-free expression based protein microarrays. I will give you an overview and the principle of various cell-free expression based protein microarrays. We will talk about protein in situ arrays or PISA, nucleic acid programmable protein arrays also known as NAPPA, multiple spotting technique or MIST, DNA arrays to protein arrays, also known as DAPA and Halo Tag arrays. These are some of the major cell-free expression based platform but, there are other cell-free synthesis based microarrays systems also exist.

So, we will discuss about the principle of each one of these protein microarray platform as well as their advantages and disadvantages. So first let us review the cell-free protein synthesis. The cell-free systems they make use of template DNA obtained from either plasmids or the PCR products. This is required for direct in vitro protein synthesis in the presence of a crude cell lysate. The cell lysate contains all the necessary machinery which is, required for the transcription and translation, by providing essential amino acids nucleotides, sads and other energy generating factors which are added exogenously in these cell free lysates.

The cell-free expression systems have been extracted from several organisms, different species and cells. Such as, wheat germ extracts rabbit reticulocyte lysate and escherichia coli. These are the majorly used systems but, there are other systems as well which include obtained from xenopus, oocytes, hybridomas insects and mammalian cells. So

there are various approaches, which have been used and demonstrated very effectively in the literature, that there are cell-free expression system which can be used to synthesize proteins.

So, let us now discuss about protein microarrays based on the cell-free expression systems. To eliminate the obstacles posed by traditional cell based methods the cell-free expression systems are increasingly adopted to generate the protein microarrays. There are several microarray generation technologies, which have been developed over the past few years.

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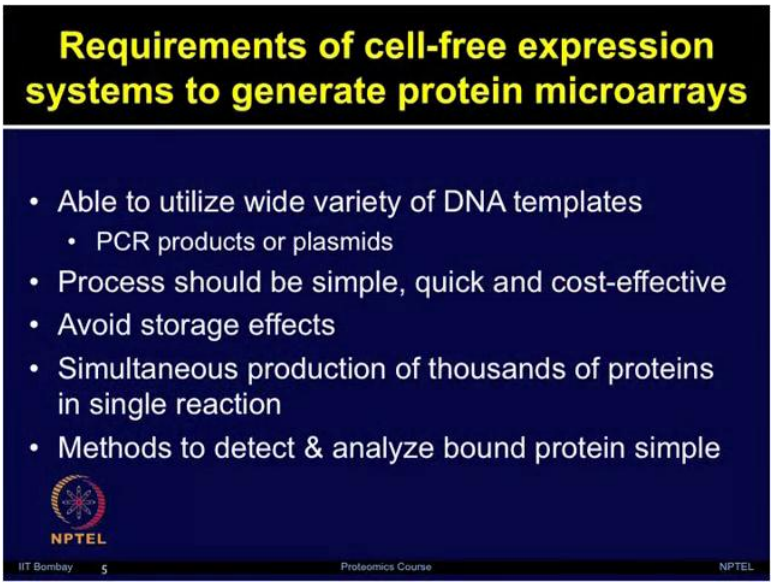

So, now let us start discussing these techniques one by one. So first of all what are the requirements of cell-free expression systems, if you want to generate protein microarrays. The cell-free expression system should meet certain requirements and these criteria's are first, it should be able to utilize wide variety of DNA templates. Because if you can synthesize proteins from variety of DNA templates whether its plasmid or a PCR products, then it gives the more versatile platform then, the system should be simple quick as well as cost effective. When we are talking about protein microarrays, the applications are usually for various clinical studies where one needs to screen for several patients or several clinical samples.

So, if the technology is very costly that becomes, one of the limiting factor for its wide spread use the microarrays should be produced on demand. So that one can avoid the

storage affects most of the purified protein arrays. They have to be stored at minus 80 degrees and storage and again thawing and rethawing. These type of effects they affect the protein integrity and protein functionality.

So, cell-free expression system, if they can be used when there is a need to perform an assay that can eliminate lot of these limitations. It can allow the simultaneous production of thousands of proteins. I think that is the common requirement for any type of microarray platform. Whether, it is DNA microarrays, traditional purified, protein microarrays or cell-free expression based protein microarrays.

The detection and analysis of bound protein should be simple, it means the assay method and the detection technology should be available and it should be very simple. So most of these assays people develop similar to what one does in the classical biochemical labs to perform the western blots, aldolase assay. So these assays are quite simple and the detection system is simple. So that redoubt can be used from the common instrumentations.

Let us, first discuss about protein in situ arrays or PISA the protein in situ array. Which is also known as in situ array technology, it provides a rapid single step method to generate protein arrays from the DNA template by using cell-free transcription and translation system. Which follows the immobilization of synthesized protein on the solid microarray surface.

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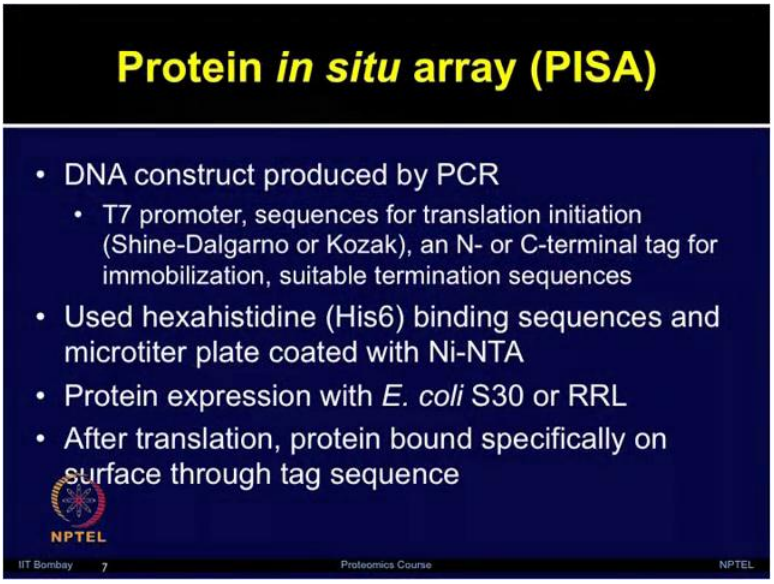

In the protein in situ arrays or PISA, there are various requirements the DNA construct can be produced by polymerase chain reaction. The construct should contain T 7 promoter the sequences which are required for translation initiation such as shine-Dalgarno or Kozak sequences an N- or C- terminal tag sequence, which is required for the immobilization of synthesized proteins the suitable termination sequences.

Now, this solid surface has to be a functionalized and add nickel NTA on that surface. So, one need to add histidine tag so, that the binding can be specific. So, use hexahistidine 6 tags binding sequence and a micro titer plate, which is coated with nickel NTA. The protein expression can be performed by using E coli based system or rabbit reticulocyte lysate or RRL. Once the translation has finished the protein which is synthesized by using the cell-free expression system specifically binds on the surface through this tag sequence.

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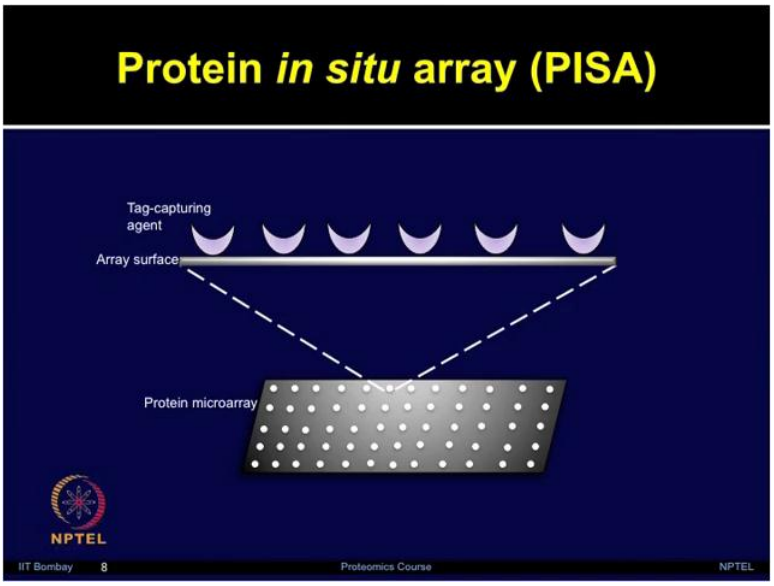

So, in PISA protein array method the surface is pre coated with the tag-capturing agent this method utilizes hexahistidine tags. Which is in the construct and the microarray titer plate which is coated with nickel nitrilotriacetic acid as shown in this slide. The DNA construct contains T 7 promoter sequence for translation initiation n terminal sequence and the termination sequence.

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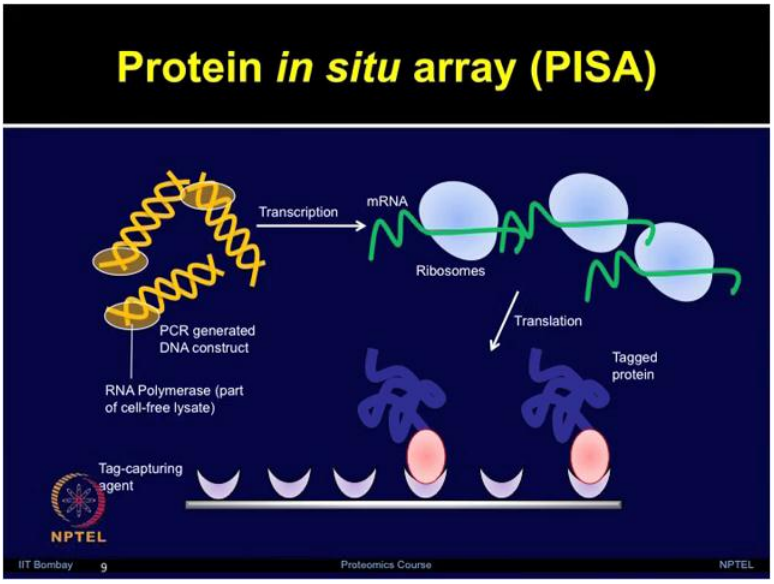

So, once the protein expression is carried out by using cell-free expression system such, as E coli s 30 or rabbit reticulocyte, lysate. The protein binds specifically on the surface through the tag sequence and unbound material can be washed off as shown in the slide by using cell-free expression system. DNA is able to produce protein by involving transcription and translation processes. And then synthesize protein which contains histidine tag gets adhered to nickel NTA surface. This is very simple concept which has been conventionally used in affinity based protein purification which we have discussed in the previous class.

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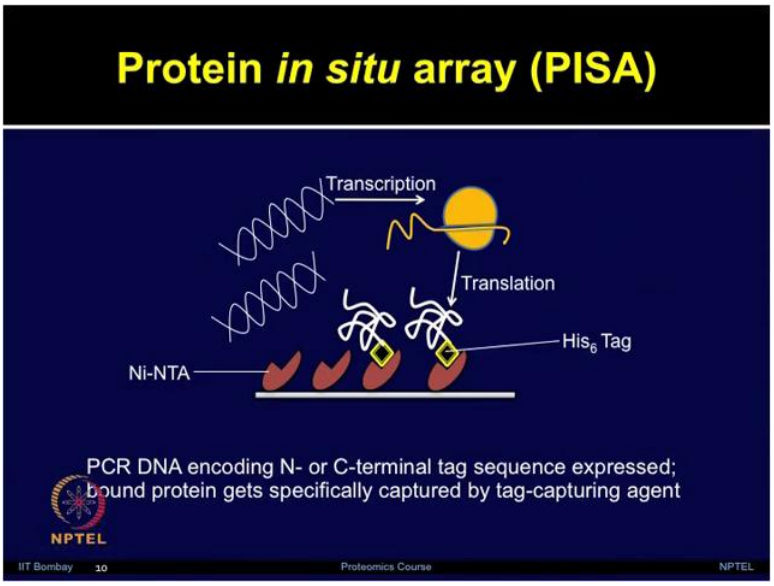

So, overall the PISA method utilizes PCR DNA which encodes N or C terminal tag sequence and then transcribes and translates. The c DNA of interest into the protein and this, proteins which is synthesized after cell free expression gets specifically captured by the tag capturing agent. So scientist Hein who have developed this method they successfully carried out the expression and functional immobilization of a fragment of human anti progesterone antibody in microtiter wells and used luciferase enzyme on nickel NTA coated magnetic beats. So, let me show you this animation and then we can discuss the working principle of protein in situ arrays or PISA in this animation protein in situ array or PISA .

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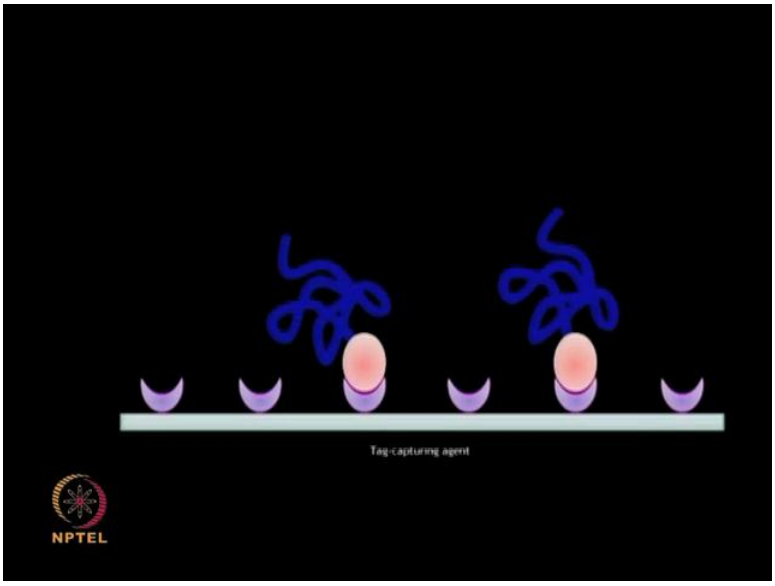

The protein microarray surface is coated with a suitable tag capturing agent that, c an immobilized the protein of interest through specific interaction once it is produced. The protein is expressed from its corresponding DNA by using cell free lysates such as E coli S 30 or rabbit reticulocyte lysate. The tag protein is captured specifically onto the array surface through the tag capturing agent. PISA method has successfully overcame drawbacks of self based techniques such as protein insolubility aggregation etcetera. So after learning the working principle of PISA let us discuss the various advantages and disadvantages of this technology.

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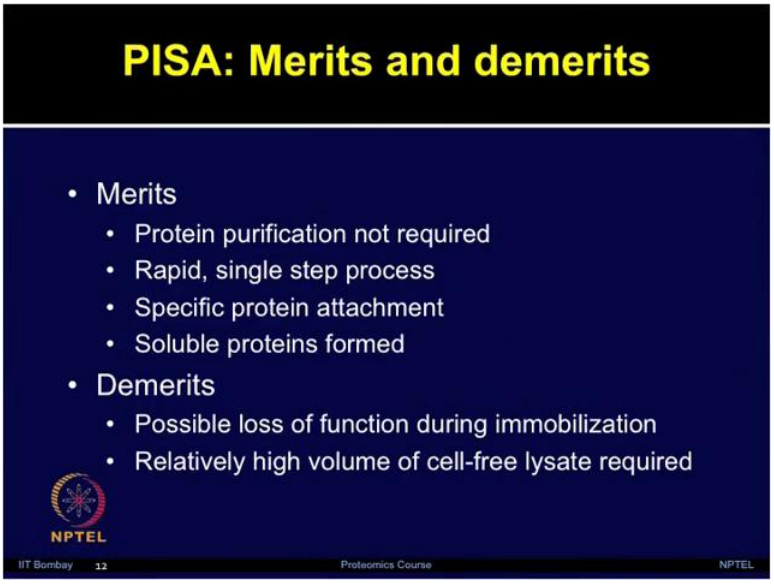

So PISA method overcomes the traditional protein purification requirements for producing the protein microarrays. So protein purification is not essential it gives rapid single step process because of histidine tag and nickel NTA interaction the specific protein attachment can be obtained or achieved by using PISA method. In this, method soluble proteins are formed, these are some of the major advantages of using protein in situ arrays. Now let us discuss the limitation of this technology so, it is possible that the function of this proteins during the immobilization step. Cell free lysates are very costly so, if the needs high volume of cell free lysate that, becomes one of the limiting factor for using this platform.

Let us, now move onto another technology nucleic acid programmable protein arrays also known as NAPPA. This technique was developed at Harvard nature of proteomics in josh labor's lab. The NAPPA process replaces the complex process of spotting the purified proteins with simple method spotting plasmid DNA. By using the recombinational cloning cell frees expression systems. The proteins are produced in vitro in the NAPPA method and this, proteins are captured on the array surface. The NAPPA technique minimized direct manipulation of proteins and it has enabled the interactions to occur in the mammalian environment by using the proper promoter sequences and the cell free expression system based on the rabbit reticulocyte lysate.

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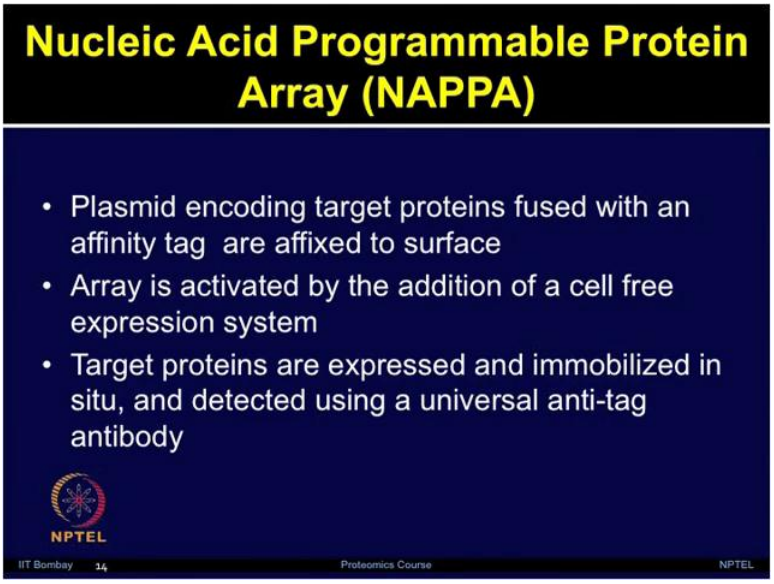

So, let us discuss the principle of nucleic acid, programmable protein arrays. How these arrays are generated so, plasmid which encodes target proteins, these are fused with an affinity tag and are affixed to the microarray surface. The microarrays are activated by the addition of a cell free expression system. Generally, rabbit reticulocyte lysate the target proteins after the expression is immobilized in situ and detected by using a universal anti-tag antibody so in this case mostly anti GST tag is used but, one can use any other tag and the corresponding antibodies can be used. So, in NAPPA method a glass slides is first coated with aminosilane reagent. Once this functionalization is done then a master mix sprinted on the chip surface.

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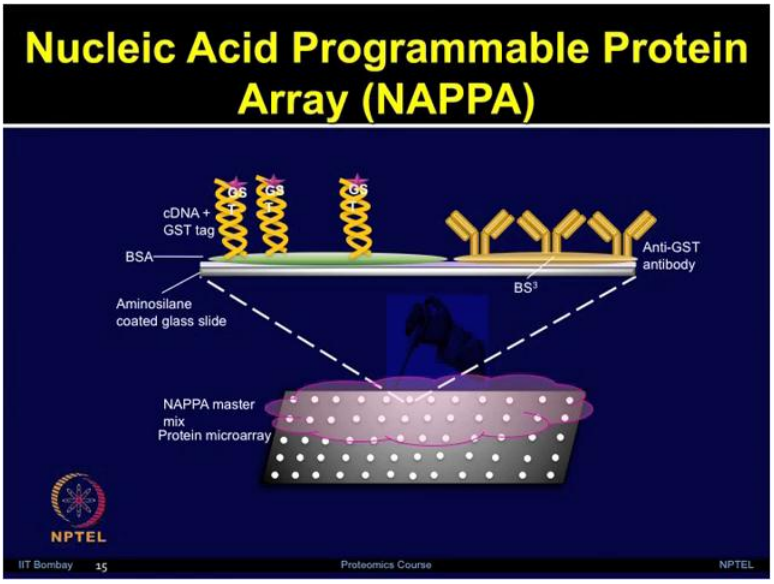

The master mix consist of few components which includes a cDNA containing GST tag BSA or bovine serum albumin protein BS 3 cross linker and anti-GST capture antibody. So, the cDNA containing gene to be expressed as its glutathione as transfer rays GST fusion is immobilized on the array surface master mix protein bovine serum albumin that is added as a part of the master mix, which improves binding efficiency of cDNA. Although, the exact mechanism is not yet clear BD 3 that is a cross linking agent which facilitates immobilization of capture antibody. Which in this case is anti-GST antibody and that enable the immobilization on the array surface.

Anti-GST antibody, which binds the synthesized protein, containing GST tag. As I mentioned earlier one can use any tag here but, only requirement will be corresponding antibodies for example, you can use anti flag antibody if you have used flag tag in your cDNA. Similarly, myc tag and corresponding anti-myc antibodies can be used. So once this spot the master mix which contains the mixture of BSA, BS 3 capture antibodies and the c d n of interest that is printed on the chip surface. The next requirement is the, activation of this by adding cell free expression system.

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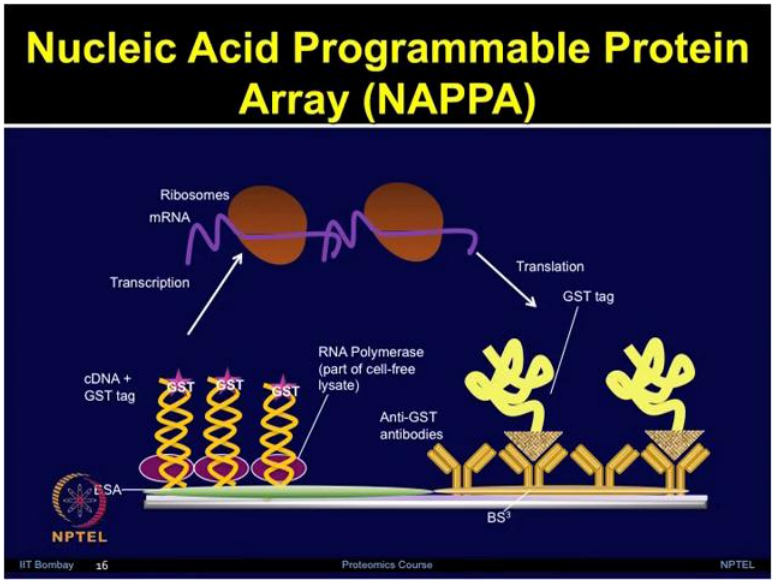

So, the cell free expression system such as rabbit reticulocyte lysate , amino acid mixtures, T 7 polymerase, RNA's inhibitor etc are added on the chip surface which contains these spots. And as I mentioned each spot contains four components now after incubating this arrays at 37 degrees for 90 minutes followed by lower down the temperature at 15 degrees. This process helps the protein expression and protein capture. So, first step is as you can see in the slide the transcription which is happening on the Ribosome's and mRNA followed by the translation to synthesize the protein. These proteins contain GST tag because each of the cDNA clones has cDNA tag. Now these tags are immobilized the capture antibody which is immobilized in the chip surface contains anti-GST antibody.

So, now these proteins will go and add here to this anti-GST capture antibodies very specifically. So problem expression in NAPPA is carried out by using cell free mammalian system, such rabbit reticulocyte lysate. Therefore, the conditions are excellent for mammalian protein expression and folding. Which is often the limitation of using wheat germ extracts or E coli based methods.

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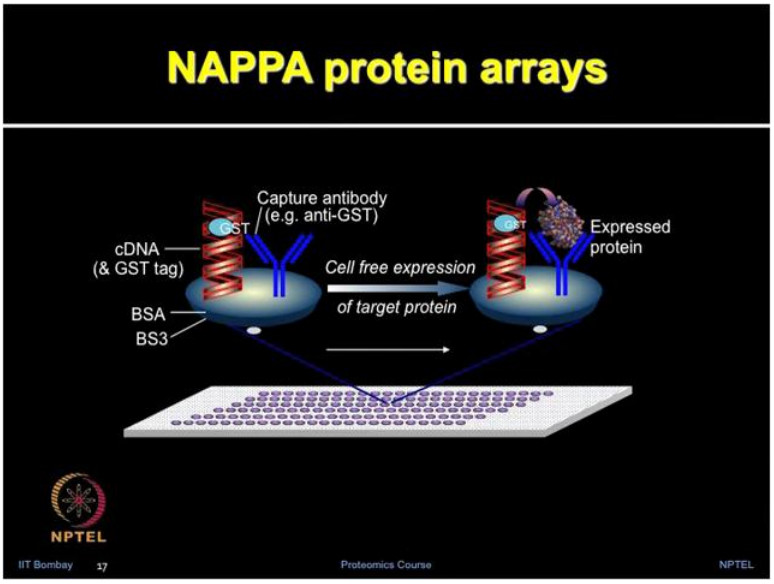

The NAPPA arrays the master mix contains DNA and other components. Which can be stored at, the room temperature. So the storage form of NAPPA microarray contains only DNA. Therefore, shelf life or a storage of these arrays is not a major concern actually transport is one of the major advantage and milestone achieved from using the NAPPA method. So, in this slide I have shown the protein expression how it can be detected on the NAPPA arrays.

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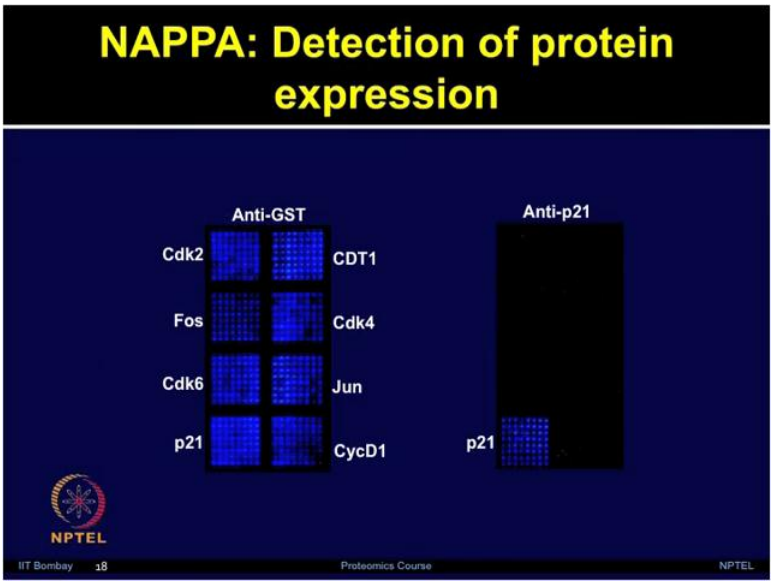

So, left image is showing the a proof of concept where eight genes such as Cdk 2, CDT 1, Fos Cdk 4, Cdk 6, Jun p 21, Cyclin D 1 these genes or the cDNA are printed on the chip surface by using NAPPA chemistry each spot is printed 64 times in the 8 by 8 block. By using anti-GST antibody one can detect that proteins are synthesized those are expressed. But, how specific the protein expression is to achieve this or to ensure the specific protein expression one can use a protein specific antibody. Such as one which is shown in the right panel where and anti-p 21 antibody is used and only p 21 printed spots are showing signal and rest other seven proteins are not showing any signal. So this shows that proteins are expressed and specific proteins can be detected.

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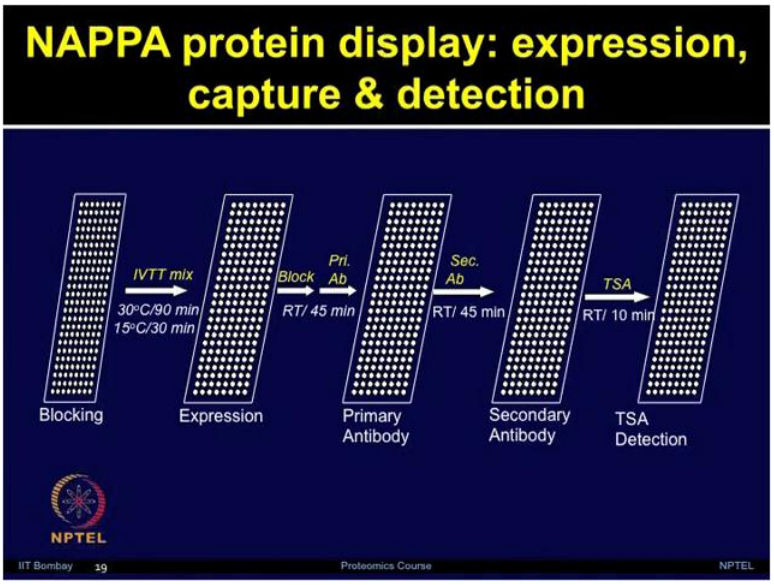

Now, NAPPA method is very simple and it is similar to the western bolt assays. Very first step here once you have printed these chips one need to do the blocking. So that all the surface which do contain the spot can be blocked, by using BSA or milk in the p b s. After blocking is done then in vitro transcription and translation mix can be added on this chip surface incubation involves two steps incubation at 30 degrees for 90 minutes followed by 15 degrees for 30 minutes. It has been observed that lowering the temperature from 30 degree to 15 degrees help in protein capture.

So, there are two steps involved here one was the protein expression and then once the proteins are expressed in that a cell free environment after that protein has to capture with the capture antibody. So this lowering temperature helps in that step once the

proteins are expressed then you need to wash away all the lysate such as rabbit reticulocyte lysate which was added in the mixture. Again you can add milk in p b s or BSA, once this blocking is done at the room temperature then primary antibody can be added if you experimental aim is to just do the quality control check of protein expression you can use entire GST antibody or if your aim is to look for certain of auto antibody markers, then at this stage one can use serum from various patients.

So, once this incubation is done, then corresponding secondary antibodies can be used. After that either the secondary antibody can be tagged with cy 3 or cy 5 conjugated antibodies for detection or one can use tyramide signal amplification system. Which achieves superior signals, they will just a schematic showing various steps involved in performing protein display protein expression capture and detection.

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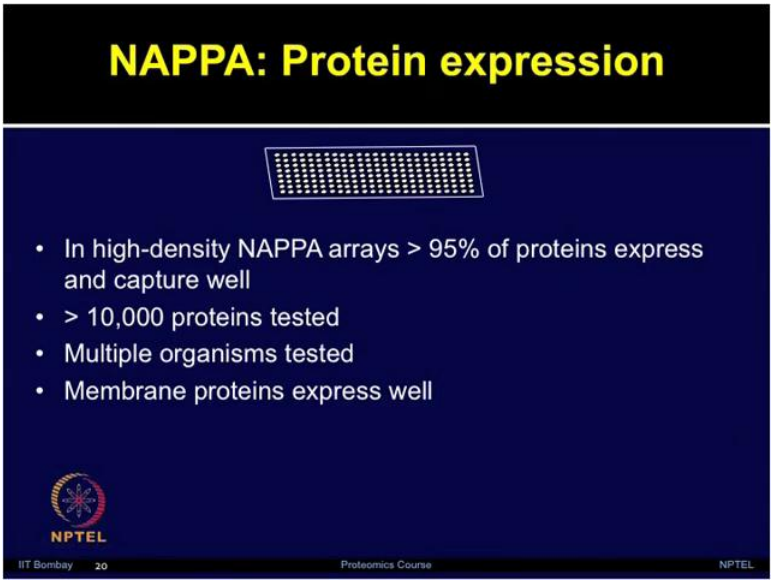

So, the NAPPA arrays have now achieved the high density and over all greater the 95 percent of proteins on these arrays express and capture very well. Over 10000 human proteins have been tested on these array surfaces. NAPPA arrays have also been applied on multiple organisms. Because the concept is very simple once you have access to the clone cDNA containing. This GST tag or any other tag one can make use of those cDNA repositories

So, now NAPPA approach haven applied to multiple organisms. NAPPA approach do not have a bias for specific group of proteins or class of protein for efficient expression, it

can also express even a membrane protein kinases transcription factors so there is no bias for specific class of the proteins a valuable aspect of NAPPA lyes in its flexibility to allow a broad range of target immobilization and query detection schemes.

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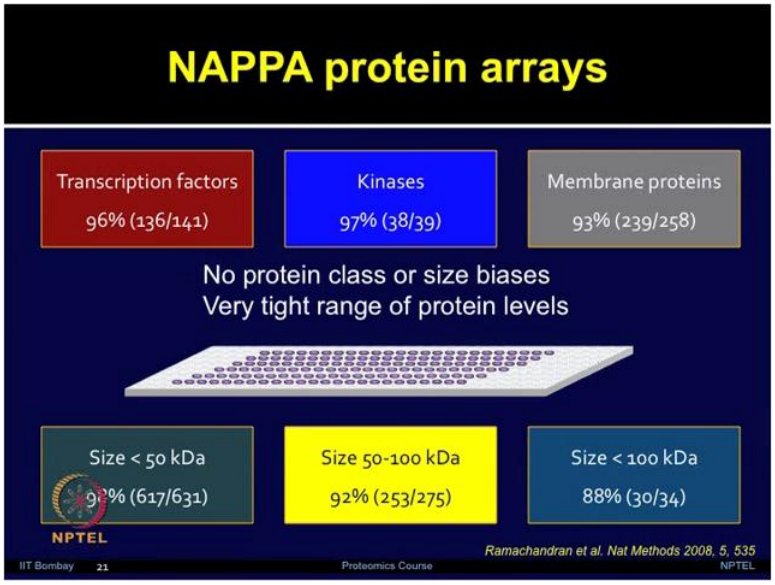

So, as I mentioning in the NAPPA protein arrays there is no protein class or size bias. Such as transcription factors it has been, shown that over 96 percent expression efficiency can be achieved when 141 proteins were tested which belongs to the transcription factor 136 protein showed protein expression in kinases out of 39 kinase 38 expressed well on the array surface over 97 percent efficiency. The membrane proteins 258 proteins which, were printed on the chip surface out of those 239 expressed well showing 93 percent of efficiency.

Similarly, there is no bias for the size of the protein for example, proteins below 50 killo dalton or between 50 to 100 killo dalton or more than that all of these proteins can be synthesized expressed well by using NAPPA chemistry. when proteins less than 50 killo dalton 631 proteins are tested 617 were expressed which shows 98 percent efficiency between 50 to 100 killo dalton proteins out of 275 253 expressed well showing 92 percent efficiency greater than 100 killo dalton 34 out of those 30 proteins expressed well which shows 88 percent efficiency.

So, by testing different class of proteins often considered very difficult one's. It was demonstrated that NAPPA approach can be used for protein synthesis, were in the

difficult situations, such as a very small protein or very large protein as well as membrane proteins. So NAPPA method has many merits there is no need to express and purify the proteins separately because one can use in vitro transcription and translation system.

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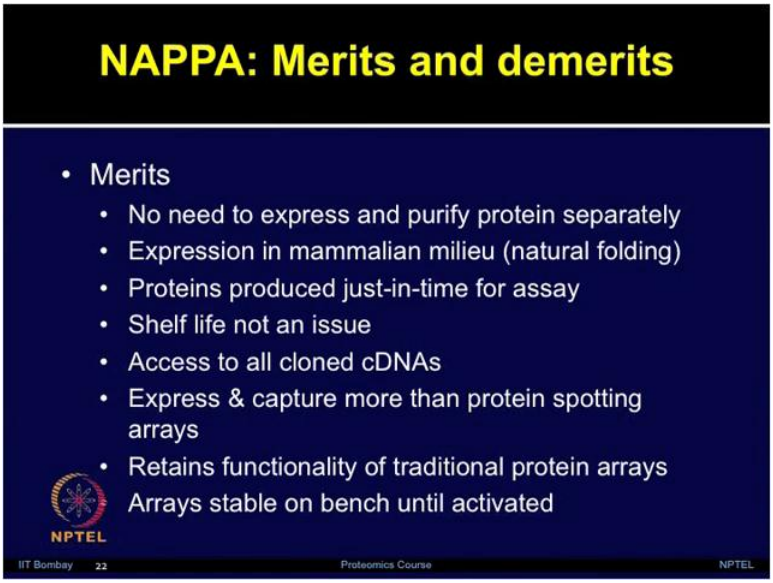

The expression is performed in the mammalian environment by using rabbit reticulocyte lysate, which provides or helps in the natural folding of mammalian proteins. Proteins can be produced just in the time where do you want to perform an assay because cDNA's are quiet stable to store on the room temperature.

Therefore shelf life is not an issue in this case. If you purify a protein print on the chip surface than you have to ensure that proteins are stored in the cold condition often at minus 20 or minus 80 but, here you can store the cDNA at the room temperature. Now cDNA which, you have access which contains, the tags that can be used efficiently here by using NAPPA chemistry and proteins can be synthesized. Now comparison of a protein synthesis from NAPPA chemistry with the traditional purified protein 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 by using traditional protein arrays and adding strength to the system the storage on the bench is one of the very major achievement of NAPPA approach.

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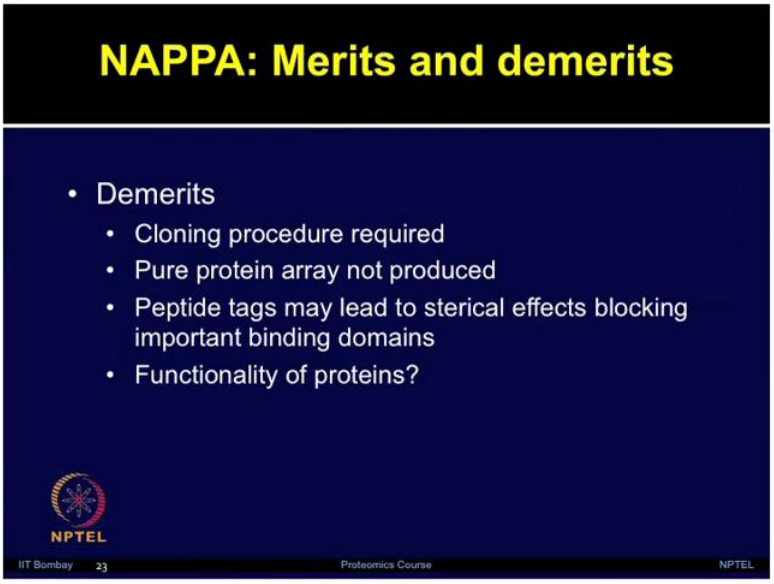

But, NAPPA approach also have certain limitations you need to rely on cloning procedure which is very tedious or you have to rely on the those clones which are available in the cDNA clone repositories. Now, there is always a concern where there pure proteins are produced because you have printed the cDNA on the chip which is synthesizing, the protein so the co existence of both DNA and the protein can be one of the limitation .The peptide tags which are added for the capturing procedure, that may lead to the sterical effects blocking which could be important for binding domains and functionality of proteins, that always remains a question for any of these cell free expression base methods.

So, NAPPA method provides various applications similar to other test protein arrays including detection of interactions with proteins nucleic acids lipids a small, molecules antibodies and enzymes.

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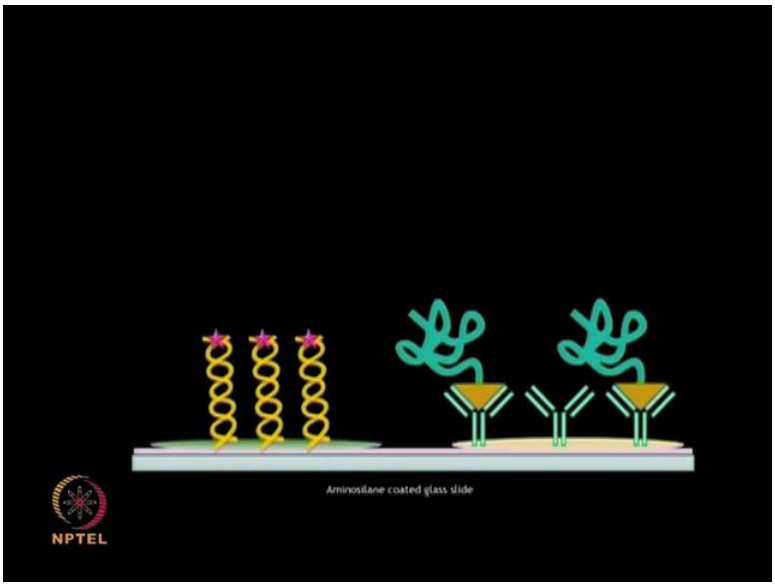

So let us, now discuss the working principle of NAPPA in this animation nucleic acid programmable protein arrays or NAPPA. in NAPPA method an aminosilane coated glass slides forms the array surface for NAPPA .To this surface the NAPPA master mix is added is consist of BSA BS 3 GST tags cDNA and anti GST capture antibodies. The BSA improves, the efficiency of immobilization of cDNA on to the array surface while BS 3 cross linker facilitate the binding of capture antibody.

The cDNA, I expressed by using cell free lysate to give the corresponding protein with its GST tag paused after transcription and translation the protein is synthesized and tag enables the capture of protein on to the slide by using anti GST antibody. Let us now move on to multiple spotting techniques also known as MIST. This is another approach, which facilitates generation of high density protein microarrays by using cell free expression system.

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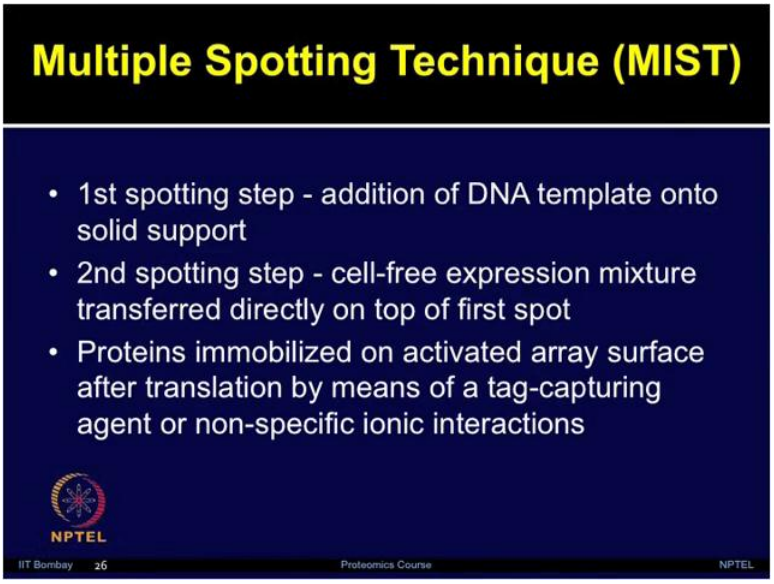

So, unlike NAPPA approach in which printing hours performed by adding a mixture of cDNA containing GST tag BSA BS 3 and the capture antibody in the MIST approach both DNA as well as the cell free expression system these are printed on the chip surface. So, here two rounds of spotting is performed in the first spotting step the addition of DNA template on to the microarray solid support and then second printing is perform where cell free expression mixture is transferred directly on top of the first spot. Which, contains DNA so in this method aim is to print the DNA as well as the cell free expression system. So that after incubation protein can be directly synthesized on the same feature and one do 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 a tag capturing agent or non specific ionic interactions.

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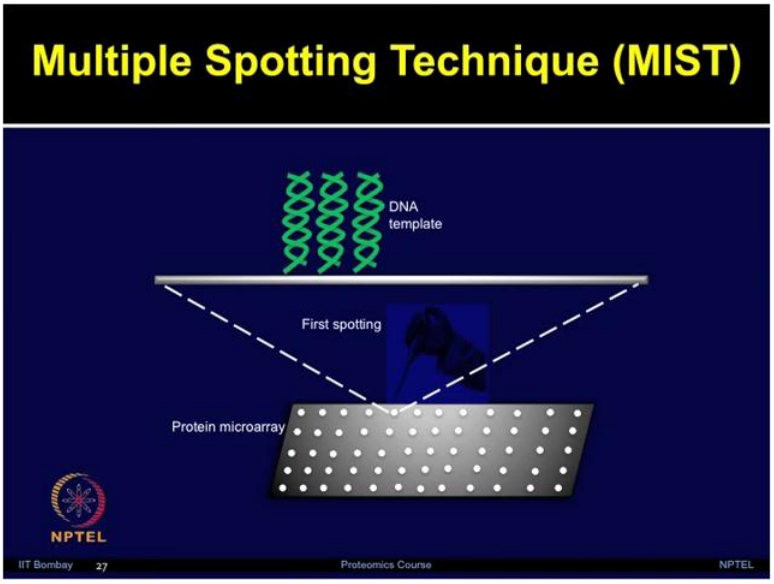

So in multiple spotting techniques or MIST the first spotting step as shown in this slide here it involves addition of DNA template on to the solid microarray support after the first spotting.

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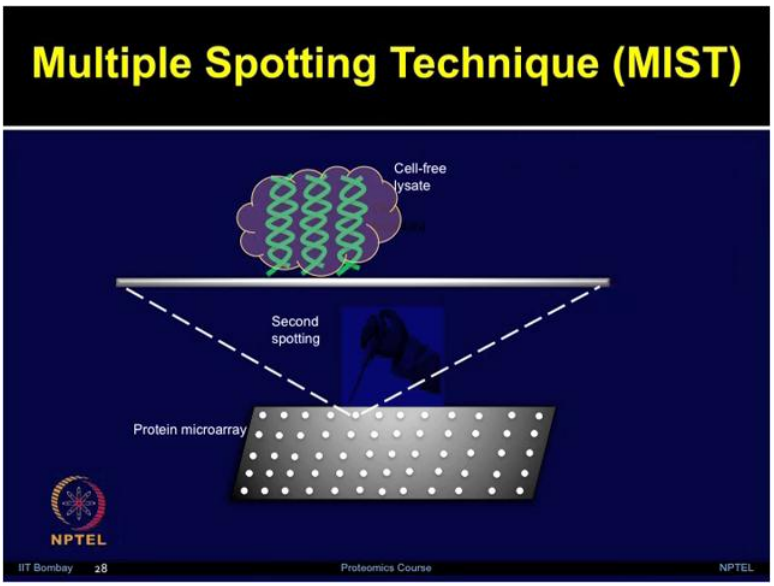

The second spotting is performed where cell free expression mixture is transferred directly on top of the first spot.

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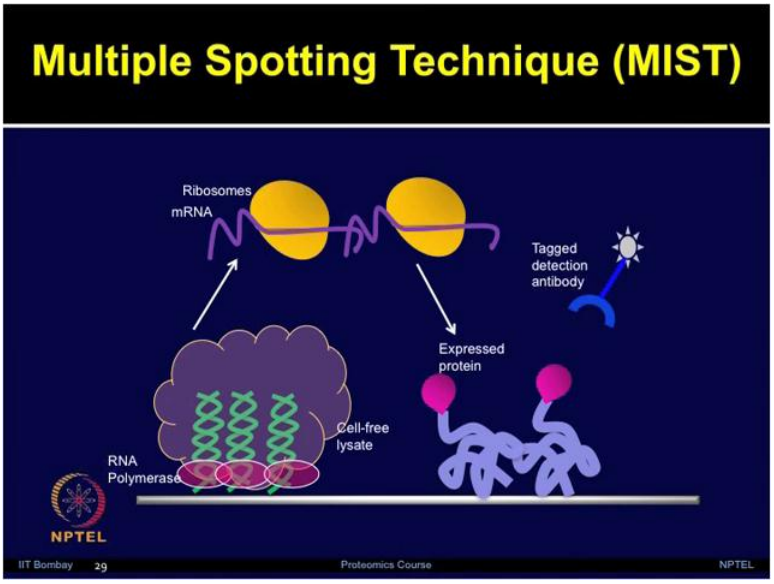

So, in this way where two steps printing are involved on top of each DNA template the cell free expression system is also printed so after incubation both transcription and translation process happen and then proteins are synthesized which can be detected by using detection antibodies.

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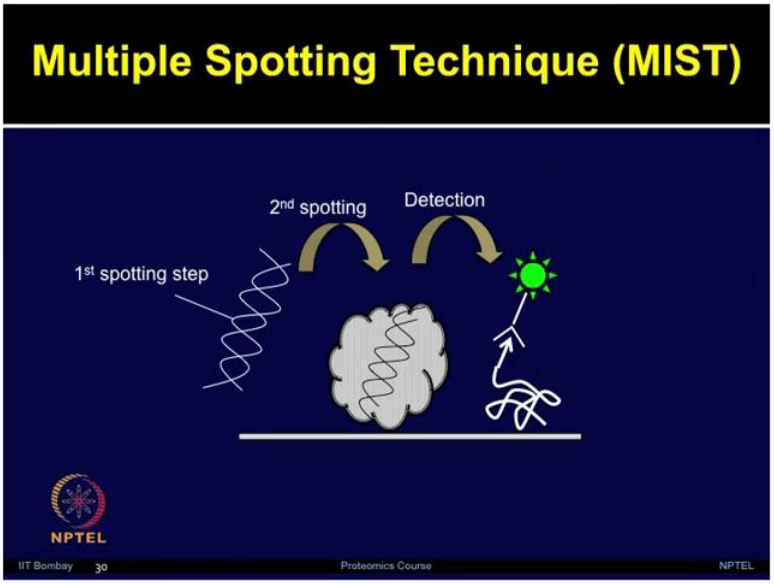

So, over all in MIST technique that DNA template is spotted in the first step followed by the cell free lysate in second step which is directly added on top of the first spot the express protein is detected by using fluorescently tagged antibody. So, inventors of MIST

technology they reported that even 35 femtogram of PCR product was sufficient for expression and detection of wild type green fluorescent protein the high density arrays containing 13000 spots per slide can be achieved by using thickness. MIST technology so let us now discuss the working principle of MIST by showing this animation multiple spotting technique or MIST in thickness technique.

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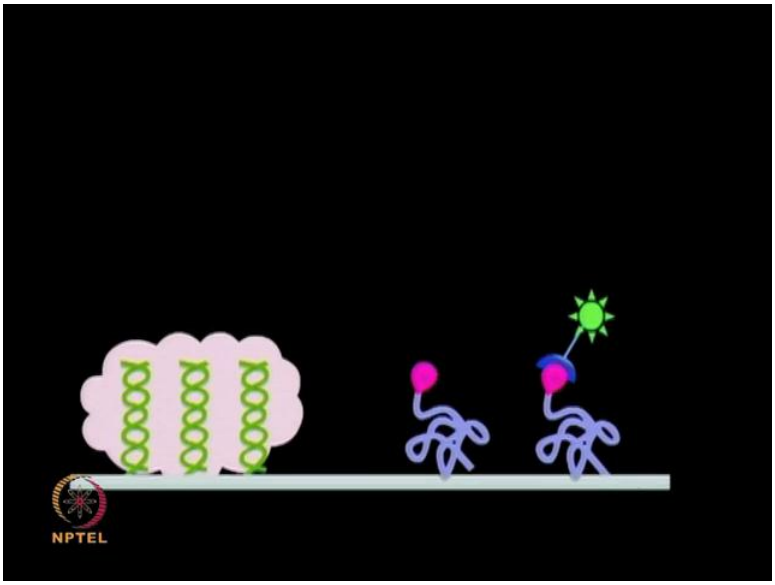

The first spotting step of the multiple spotting is capable of producing. Producing high density arrays it involves addition of template DNA onto the solid array support the template DNA can even be in the form unpurified PCR product which is one of the major advantage of using this MIST technique.

The second spotting strip involves addition of sulphuric acid directly on top of the first spot. The transcription and translation can begin only after the second spotting step the protein expressed from the template DNA binds to the array surface by means of non specific interactions. Which is one of the drawbacks of this procedure 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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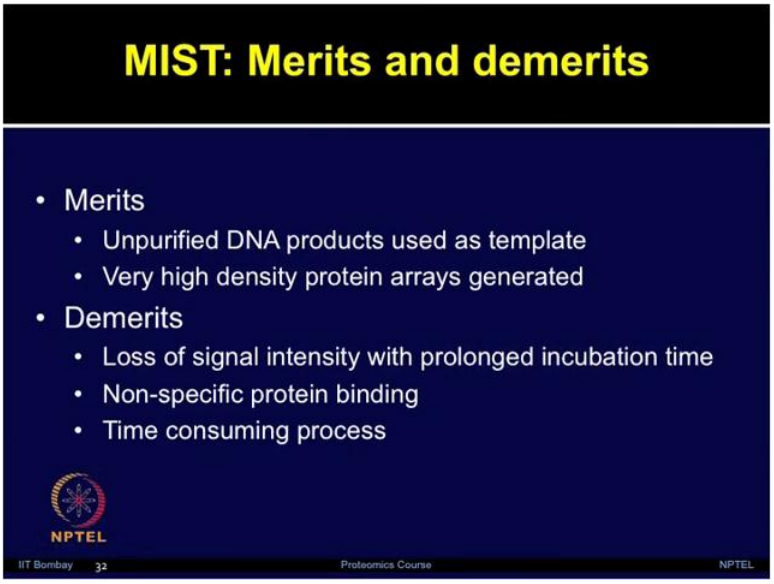

The difference merits and demerits of using MIST method. It involves unpurified DNA products which could be used as a template source which is not the case for using NAPPA now. In this method, very high density protein arrays can be generated because the spot chemistry the master MIST is not very complicated. So more high density can be achieved in this case the disadvantage or limitations of using MIST technique is first of all this loss of signal intensity with prolonged incubation time of the arrays. So as in this case even the cell free expression systems are printed on the top of arrays, the stability could be one of the major issues. A non specific protein binding as well the overall process is more time consuming these are some of the limitations or demerits of MIST technique. Let us, now move on to DNA arrays to protein arrays or DAPA. The DAPA technique makes possible the repeated use of same DNA templates like for printing multiple rums of protein arrays.

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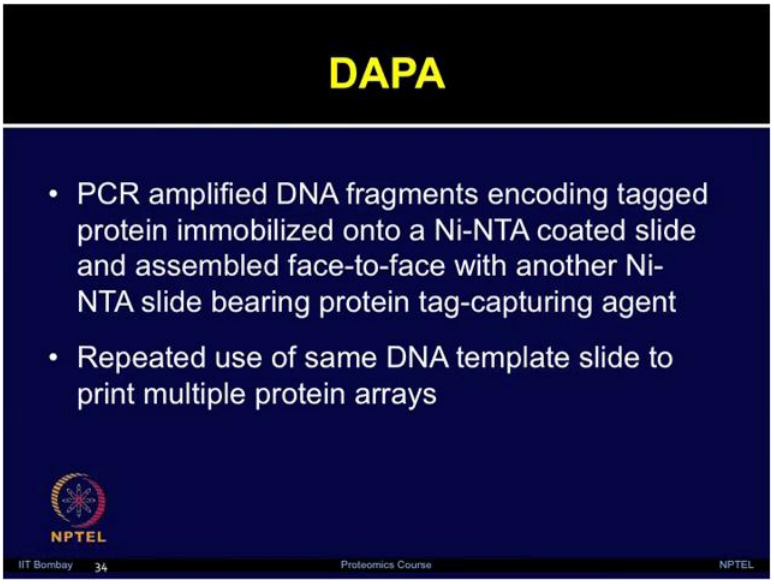

So, in DNA arrays to protein arrays the PCR amplified DNA fragments which encode tagged proteins immobilized onto a nickel NTA coated slide and assembled face to face with another slide also with the nickel NTA bearing protein tag capturing agent. The repeated use of same DNA template can be performed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

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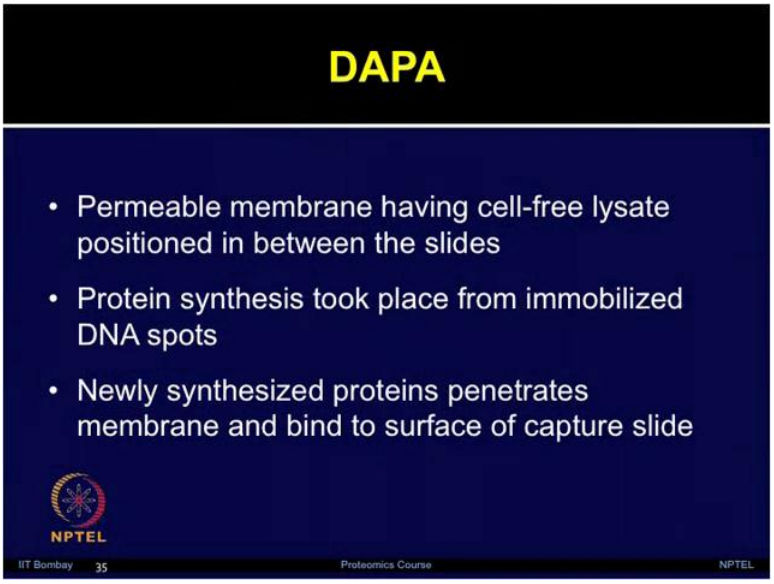

In this, method the permeable membrane, which contains, cell free lysate that is positioned in between two slides. Which are face to face for this proteins, to be diffused

now protein synthesis takes place on this membrane and then, the synthesized proteins diffuses from the membrane and moves onto the other slide for capture. So newly synthesized proteins then penetrates the membrane and binds the surface of captured slide.

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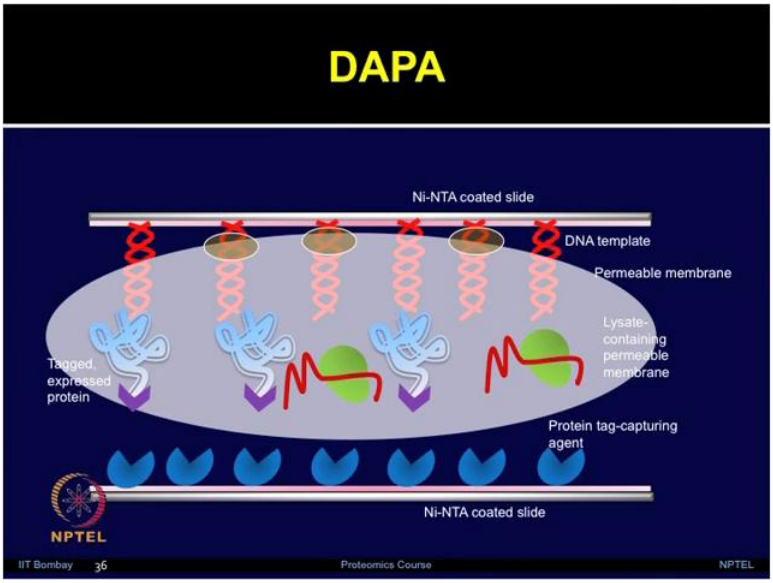

So, as you can see in this slide in DAPA, the PCR amplified DNA fragments encoding the tapped proteins are immobilized onto a nickel NTA coated slide and assembled face to face with another nickel NTA slide, containing the protein tag capturing agent. In between these two slides a permeable membrane 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.

So in DAPA approach or DNA arrays to protein arrays the investigators produced an array of double heads histidine tagged g s p and data was found to be comparable with existing protein array technologies. So by using DAPA it is possible to use same DNA template repeatedly to print multiple protein arrays and it has been shown that one can use this template for printing almost 20 arrays. So let me show you the working principle of DNA array to protein arrays in this animation, known as DAPA.

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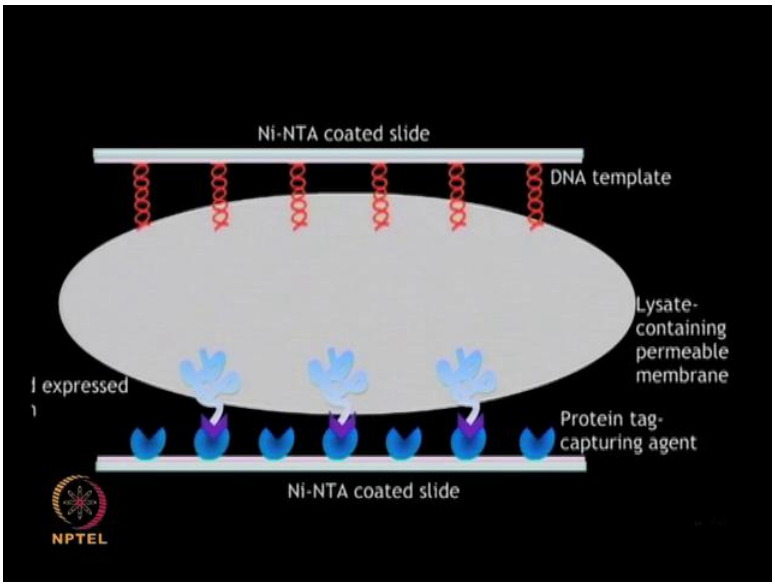

In DAPA the slides bearing the DNA template and the protein tag capturing agent are assembled face to face, with a lysate containing permeable membrane placed in between. The expressed protein slowly penetrates the membrane and gets immobilized on the slide surface through its capture agent. The DNA template array can be reused several times by using this method. The DAPA approach provides few advantages as compared to the previously described method. One can get pure protein because the protein is diffused from the membrane.

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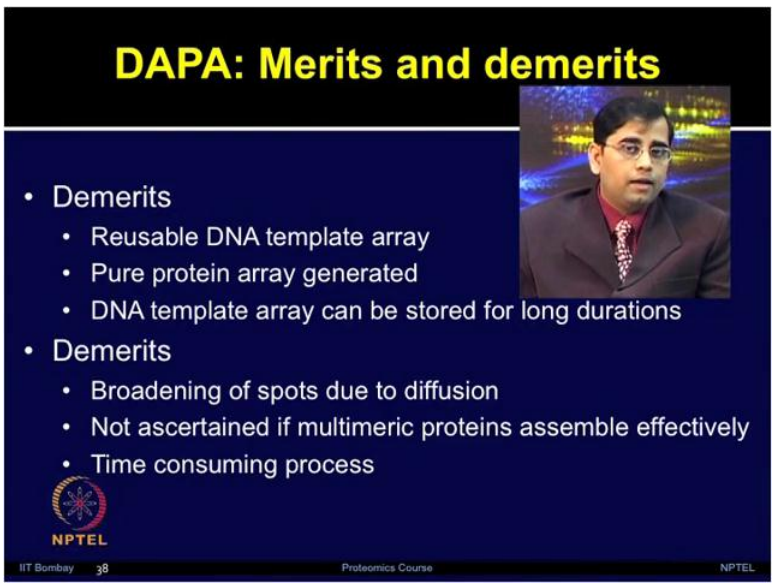

The reusable DNA template which is able to print multiple chips by using this chemistry and now the source template which is DNA that array can be stored at the room temperature for long duration and when there is a need for making the protein arrays one can use the membrane with the lysate and then followed by generation of multiple protein arrays. However there is certain limitation of using DAPA method, including the broadening of spots due to the diffusion it is not ascertained, if multimeric proteins assemble effectively it is also a time consuming process.

So far, we have been discussing PISA, NAPPA, MIST and DAPA techniques. Now let us move on to the latest addition halo tag arrays the halo link protein array systems are developed by promega company, which combines few technologies together to create protein microarrays. First of all it uses cell free expression transcription and translation system based on wheat germ extract it uses a hallow tag, which is mutated hydrolase protein that forms a covalent bond with the halo tag ligands and third it uses a polyethylene glycol coated glass slide activated with halo tag ligand for specific capture of proteins which is expressed by using cell free expression system.

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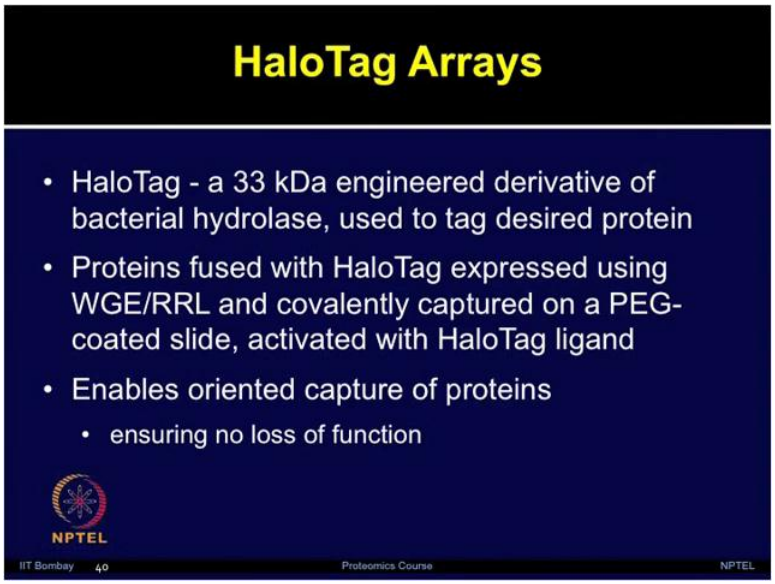

Halo Tag arrays, the halo tag is a 33 kilo dalton engineered derivative of bacterial hydrolase which is used to tag desired protein. The proteins which are fused with the halo tag are expressed by using wheat germ expression system or rabbit reticulocyte lysate and covalently captured onto peg containing slides. These are then activated with

halo tag ligands. So this halo tag array achieves oriented capture of the proteins and thereby ensures no loss of function or minimal loss of function.

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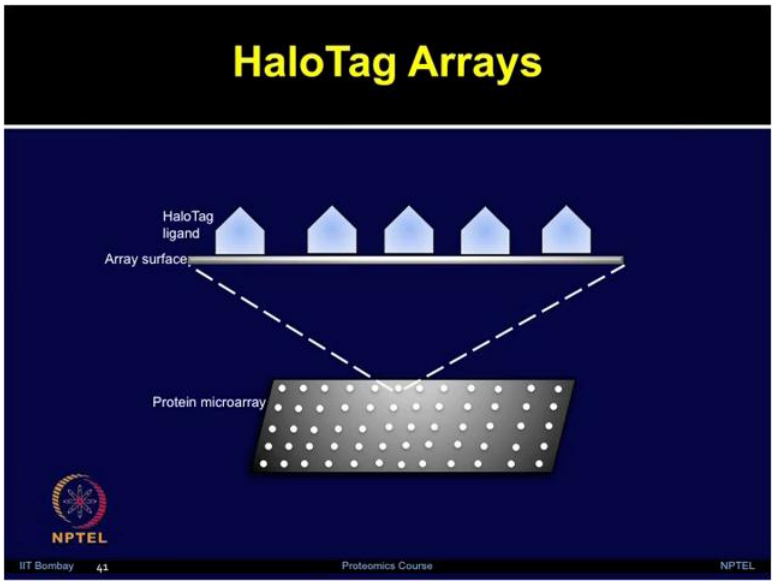

So, as shown in the slide the polyethylene glycol coated glass slides can be activated by using Halo Tag ligand. The proteins are fused with halo tag are expressed by using cell free expression systems and or covalently captured on polyethylene glycol coated glass slide.

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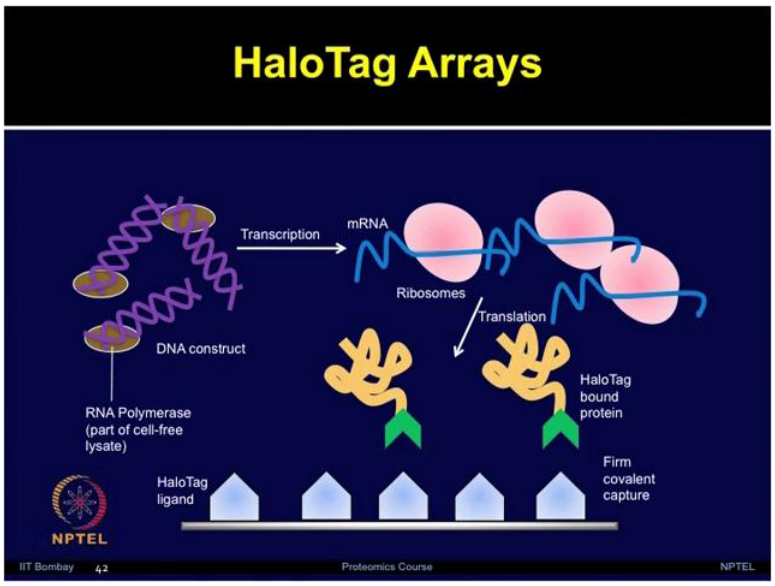

So Halo Tag array method enables oriented capture of proteins. So as you can see in the nutshell the halo tag fused protein is expressed and covalently captured on the spec coated slide and activation is performed by using halo tag ligand.

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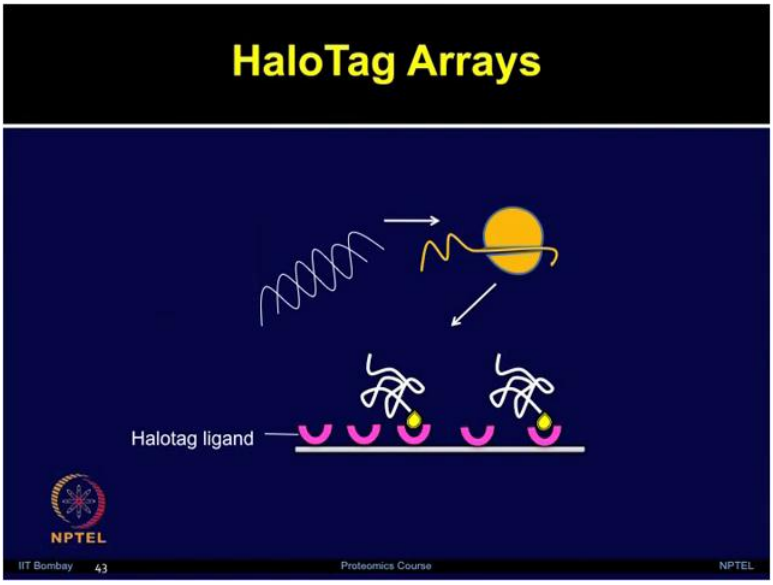

This provides very strong covalent interaction and minimizes loss of the synthesized protein which, usually occurs in the other protein microarray based methods. So, in protein arrays, one needs to perform not washing steps. So if the protein or the molecules are bind on the surface, with very strong interaction then there'll be minimal loss from the surface. Which, can be achieved in this case by using Halo Tag system. So in halo tag arrays the capture chemistry. Which, is based on 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 so this method not only overcomes limitation of protein purification but, also overcomes several limitations which are commonly observed in any protein array technology. So let us discuss the working principle of halo tag arrays in this animation.

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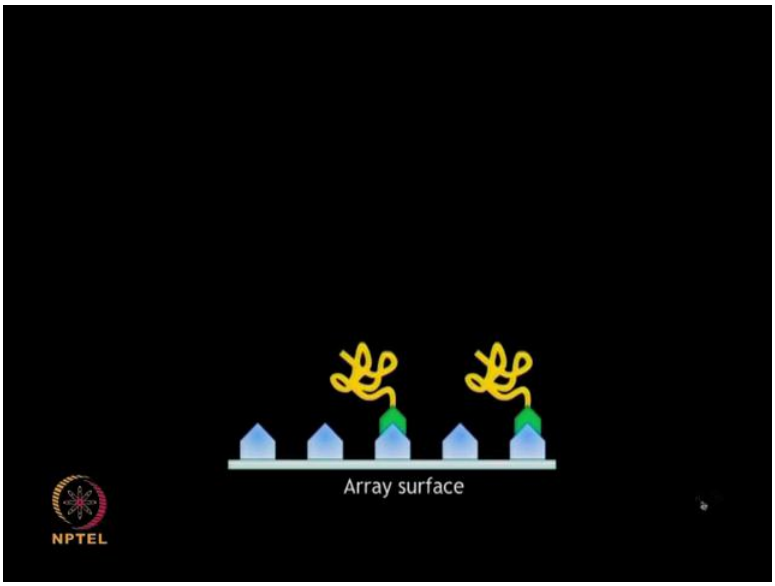

Halo tag technique, in halo tag technique the slide is activated with the Halo Tag ligand. Which captures, the expressed protein through firm covalent interaction which prevents any material loss and ensures oriented capture of the protein? The halo tag fused protein is expressed by using lysates such as, rabbit reticulocyte, lysate RRL or wheat germ expression system and synthesized protein is covalently captured onto the array surface through the Halo Tag ligand, the specific interaction ensures. The oriented capture of protein and prevent any possible functional loss.

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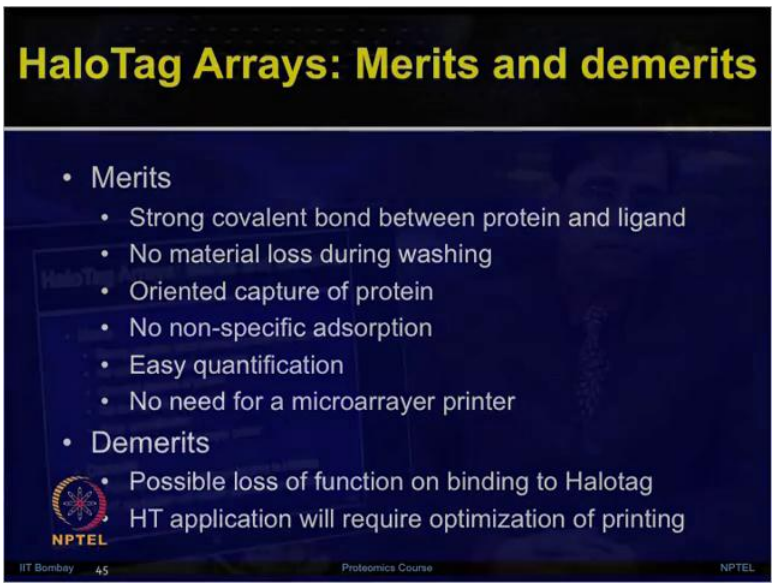

Halo tag arrays have various advantages such as, the strong covalent binding between protein and the ligand. No material loss occurs during the washing steps because, of this strong interaction the proteins are captured oriented and there is no non specific adsorption due to the peg coating. The quantification is easy and one do not need a microarrayer printer to print the proteins on this chip because, the commercial kit of halo tag arrays they provide the gas kit which can be used for printing the arrays.

However, there are certain limitations of using halo tag arrays such as it cannot at least it is has not been shown that system can be used for the high density arrays so only the proof of concept studies have been shown with spots by using commercial gas kits which can do 50 or so protein so high density arrays in theory is possible but, one need to ensure that even at the high density these array functions properly. Then there's possibility of loss of function on binding to the halo tag. So these type of quality control checks have already been performed but, there's still more biological questions need to be addressed on these arrays before one feel confident on applying these for various clinical applications.

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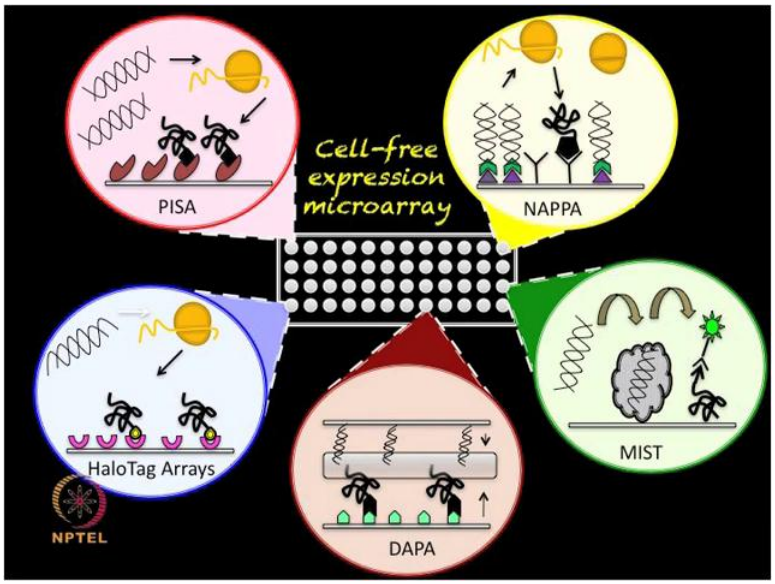

So, in summary in the past decade the biological research has witnessed a paradigm shift from focused reductionist approaches to a greater dependence on data provided by large industrial sized proteins. These high throughput projects they capture data at the scale of an entire organism and provide insight into the biological systems as well as organization

of physiological networks. The development of cell free expression base microarrays have overcome several limitations of cell based protein microarrays and revolutionized the ability to simultaneously study 1000's of proteins.

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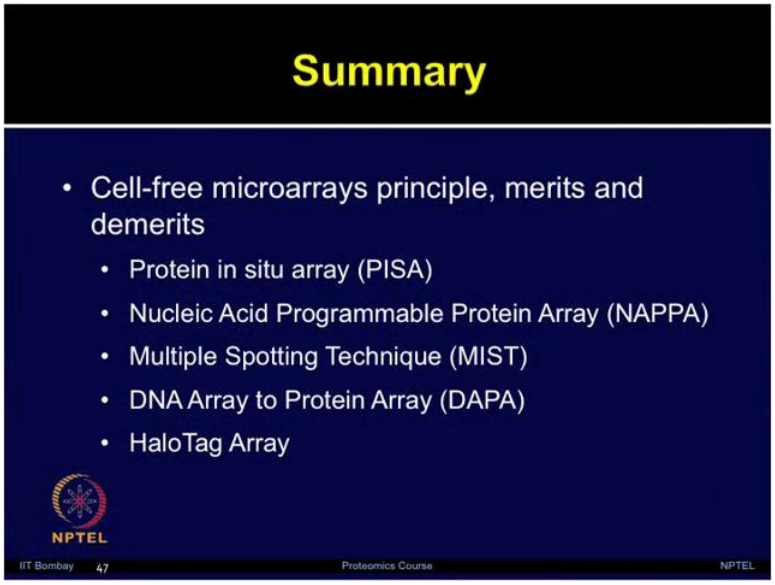

So, protein microarrays they offer a range of diverge applications and are being adopted extensively for clinical as well as non clinical studies. The cell free expression systems facilitate synthesis of several proteins in single reaction and produce proteins on demand and eliminate concerned of storage and protein stability. So, several cell free expression systems have been used have been developed during the past decade and today. We have discussed the working principle merits and demerits of some of the promising cell free expression based protein microarray system including protein in situ, arrays, nucleic acid programmable protein arrays, multiple spotting techniques, DNA arrays to protein arrays and Halo Tag arrays. There are other promising approaches as well, which also involve similar principle. Thank you.