LECTURE-29

Cell-free synthesis based protein microarrays

TRANSCRIPT

Welcome to the proteomics course. In today's lecture we will talk about protein microarrays based on cell-free synthesis. So cell-free synthesis based protein microarrays provide high throughput, versatile platform for large-scale analysis of functional proteins. These microarrays are used for various applications for e.g., antibody profiling, biomarker discovery, enzyme-substrate identification, protein-protein interaction etc. the traditional cell based methods which were used for making the protein microarrays involve protein expression in heterologous system such as *E. coli*. But the protein purification is a very laborious process. It involves various steps and of the protein purity, protein integrity, its stability and the functionality. All of these remain major issues for protein purification. So if we have 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 in the scale of thousands and then maintaining the functionality and keeping them properly folded is very tedious. So these limitations of traditional protein purification and protein microarrays generated by these purified proteins have been the major motivation for cell-free expression based microarray field. The cell-free expression based system overcome various limitations of protein purification and they perform in situ transcription and translation. During the last decade there are various methods, which have emerged as very strong platform for protein microarray generation by applying these cell-free expression systems.

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 (PISA), nucleic acid programmable protein arrays (NAPPA), multiple spotting technique (MIST), DNA array to protein array (DAPA) and HaloTag array. These are some of the major cell-free expression based microarray platforms but there are other cell-free based microarray systems also exist.

So we will discuss the principle of each one of these protein microarray platform as well as their advantages and disadvantages.

So first, let's review 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 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 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 *E. coli*. These are the majorly used systems. But there are other systems as well which include obtained from *Xenopus* oocyte, hybridomoas, insects and mammalian cells. So there are various approaches, which have been used and demonstrated effectively in the literature that there are cell-free expression systems which can be used to synthesize proteins.

Let's now discuss about protein microarrays based on cell-free expression systems. To eliminate the obstacles posed by traditional cell-based methods the cell-free expression systems are increasingly adopted to generate microarrays. There are several microarrays generation technologies, which have been developed over the past few years. So let's 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. Cell-free expression systems should meet certain requirements and these criteria are first it should be able to utilize wide variety of DNA templates. Because if you can synthesize proteins from wide variety of DNA templates whether it's plasmid or PCR products then it gives 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 study several patients samples. So if the technology is expensive 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 effects. Most of the purified arrays have to be stored at -80°C and storage, thawing and re-thawing. These types of effects affect the protein integrity and protein functionality. So cell-free expression system can be used if there is a need to perform an assay that can eliminate lot of limitations. It can allow the simultaneous production of thousands of proteins. That is the common requirement for any type of microarray platform whether DNA microarrays, traditionally purified microarrays or cell-free 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 should be very simple. Most of these assays people develop similar to what one does in the classical biochemical labs to perform the western blots or ELISA assays. So these assays are quiet simple and the detection assays are simple so that the read-out can be used from the common instrumentations.

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

In the protein *in situ* arrays or PISA there are various requirements. The DNA construct can be produced by PCR. The construct should contain T7 promoter, sequences required for translation initiation such as Shine-Dalgarno or Kozak sequences, n 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 add Ni-NTA on that surface. So one need to add Histidine tag so that binding can be specific. So use Hexa-histidine or His6 tag binding sequence and a microtitre plate, which is coated with Ni-NTA. The protein expression can be performed by using *E. coli* based system or rabbit reticulocyte lysate (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.

In PISA protein array method the surface is coated with a tag-capturing agent. This method utilizes hex-histidine tags which is in the construct and the microarray titre plate which is coated with nickel nitrilo triacetic acid (Ni-NTA) as shown in this slide.

The DNA construct contains T7 promoter sequence for translation initiation, N-terminal sequence and the termination 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 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 by involving transcription and translation processes and then synthesize proteins which contain Histidine tags get adhered to the Ni-NTA surface. This is a very simple concept, which has been conventionally used in affinity based protein purification which we have discussed in the previous class.

So overall the PISA method utilizes PCR DNA, which encodes N- or C-terminal tag sequence and then transcribes and translates the cDNA of interest in to the protein and this protein which is synthesized after the cell-free expression gets specifically captured by tag-capturing agent. Scientists He and Tausig, who have developed this method, successfully carried out the expression and functional immobilization of a fragment of human anti-progesteron antibody in microtitre wells and used luciferase enzyme on Ni-NTA coated magnetic beads. So let me show you this animation and then we can discuss the working principle of protein *in situ* arrays in this animation.

Animation - In PISA, the protein microarray surface is coated with a suitable tag-capturing agent that can immobilize the protein of interest through specific interactions once it is produced. The protein is expressed from its corresponding DNA using cell-free lysates such as *E.coli* S30 or rabbit reticulocyte lysate (RRL). The tagged protein is then captured specifically onto the array surface through the tag-capturing agent. PISA successfully overcame drawbacks of cell-based techniques such as protein insolubility, aggregation etc.

So 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. So protein purification is not essential. It gives rapid, single step process. Because of histidine tag and Ni-NTA interaction the specific protein attachment can be 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's discuss the limitations of this technology. It is possible that there is lot of loss of function of 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 platform.

Let's now move on to another technology, nucleic acid programmable protein array (NAPPA). This technique was developed in Harvard institute of proteomic at Dr. Joshua LaBaer's lab. The 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 systems, the proteins are produced *in vitro* in the NAPPA method and these proteins are captured on array surface. The NAPPA technique minimized the direct manipulation of the proteins and has enabled the interactions to occur in the mammalian environment by using the proper promoter sequence and the cell-free expression based on rabbit reticulocyte lysate.

So let's discuss the nucleic acid programmable protein array and how these arrays are generated. So plasmids which encodes target proteins those are fused with an affinity tag and are fixed 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 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 the corresponding antibodies can be used.

In NAPPA method a glass slide is first coated with aminosilane reagent. One its functionalization is done then a master mix is printed on the chip surface. The master

mix consists of few components which includes a cDNA containing a GST tag, bovine serum albumin (BSA) protein, BS3 cross linker and anti-GST capture antibody. So the cDNA containing the gene to be expressed as its glutathione S-transferase fusion is immobilized on the array surface in the master mix. Protein bovine serum albumin is added as a part of the master mix which improves binding efficiency of cDNA, 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, and that enables the immobilization on the array surface. Anti-GST antibody, the capture antibody, binds to the synthesized proteins containing GST tag. As I mentioned earlier one can use any tag here but the only requirement will be the 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. Once this spots the master mix which contains the mixture of BSA, BS3, capture antibodies and cDNA of interest that is printed on the chip surface then the next requirement is the activation of this by adding cell-free expression system.

So the cell-free expression system such as rabbit reticulocyte lysate, amino acid mixtures, T7 polymerase, RNase inhibitor, etc are added on the chip surface which contains these spots. And I mentioned each spot contains four components. After incubating these arrays at 37°C for 90 min the temperature is lowered 15°C. This process helps the protein expression and protein capture. So the first step is, as you can see in the slide, the transcriptions which is happening on ribosomes and mRNA followed by the translation to synthesize the proteins. These proteins contain GST tag because each of the cDNA clone has cDNA tags. These tags are immobilized. The 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 limitation of using wheat germ extract or *E. coli* based methods. In NAPPA arrays the master mix contains DNA and other

components which can be stored at room temperature. So the storage form of NAPPA microarray contains only DNA, therefore the shelf-life or storage of these arrays is not a major concern. Actually this is one of the major advantages and major milestone achieved using NAPPA method.

In this slide I have shown the protein expression, how it can be detected on the NAPPA arrays. So the left image is showing the proof of concept where cDNA of 8 genes such as Cdk2, CDT1, Fos, Cdk4, Cdk6, Jun, p21 and CycD1 are printed on the chip surface by using NAPPA chemistry. Each spot is printed 64 times in 8x8 block. By using anti-GST antibody, one can detect that the proteins are synthesized those are expressed. But specific the protein expression is to achieve this, how to ensure the specific protein expression, one can use a protein specific antibody such as one which is shown in the right panel where an anti-p21 antibody is used and only p21 printed spots are showing signal and the rest of the 7 protein are not showing any signal. This shows that the proteins are expressed and specific proteins can be detected.

NAPPA method is very simple and similar to the western blot assay. The first step here, once you have printed these chips, one needs to do the blocking so that all the surface which does not contain the spots can be blocked by using BSA or milk in PBS. 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°C followed by 15°C for 30 min. It has been observed that lowering the temperature from 30°C to 15°C helps in protein capture. So there are two steps involved here. One is the expression where the proteins are expressed in the cell-free environment and after that protein has to capture the capture-antibody. So this temperature lowering helps in that step. Once the proteins are expressed then you need to wash away all the lysate such as rabbit reticulocyte lysate added in the mixture. Again you can add milk in PBS or BSA. Once the blocking is done at the room temperature then primary antibody can be added. 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 for certain autoantibody markers then at this stage one can use serum from various patients. Once this incubation is done, corresponding

secondary antibodies can be used. After that either the secondary antibody can be tagged with Cy3 or Cy5 conjugated antibodies for detection or one can use tyramide signal amplification (TSA), which achieves superior signals. This is just schematic representation showing various steps involved in performing protein display, protein expression, capture and detection.

So the NAPPA arrays have now achieved the high density and overall greater than 95% of proteins on these arrays express and capture very well. Over 10,000 human proteins have been tested on this array surface. NAPPA arrays have also been applied on various organisms. Because the concept is very simple, once you have access to the clone cDNA containing GST tag or any other tag, one can make use of those cDNA repositories. So now NAPPA has been applied to multiple organisms. NAPPA approach does not have a bias for a specific group of proteins or a class of proteins for efficient expression, it can also express even membrane proteins, kinases, transcription proteins. So there is no bias for specific class of the proteins. A valuable aspect of NAPPA lies in its flexibility to allow a broad range of target immobilization and query detection schemes.

So as I was mentioning in the NAPPA protein arrays there's no protein class or size bias such as transcription factors. It has been shown over 96% expression efficiency can be achieved when 141 proteins were tested which belong to the transcription factor, 136 proteins showed protein expression. In kinases, out of 39 kinases 36 were expressed on the array surface with over 97% efficiency. The membrane proteins, 258 proteins which were printed on the chip surface. Out of those 239 expressed well showing 93% expression efficiency. Similarly there was no bias for the size of the proteins. For example, proteins below 50 kDa or between 50 to 100 kDa or more than that, all of these proteins can be synthesized, expressed well by using NAPPA chemistry. When proteins less than 50 kDa, 631 proteins were tested 617 were expressed which shows 98% efficiency. Between 50 t o100 kDa proteins, out of 275, 253 expressed well showing 92% efficiency. For greater than 100 kDa, 30 out of 34 proteins expressed well showing 88% efficiency. So by testing different class of

proteins, often considered very difficult ones it was demonstrated that the NAPPA approach can be used for protein synthesis even in difficult situation such as very small or very large protein as well as membrane proteins.

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. The expression is performed in the mammalian environment by using rabbit reticulocyte lysate which helps in the natural folding of mammalian proteins. Proteins can be produced just-in-time when you want to perform an assay because cDNA are quiet stable to store at room temperature. Therefore shelf life is not an issue in this case. If you purify your protein, print on the chip surface then you have to ensure that proteins are stored in cold condition often at -20°C or -80°C. But here you can store cDNA at room temperature. Any cDNA to which you have access, which contains tags that can be used efficiently here by using NAPPA chemistry and proteins can be synthesized. Comparison of protein synthesis from NAPPA chemistry with the 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 by using traditional protein arrays. And adding strength to the system the storage on the bench is one of best achievements of NAPPA approach.

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

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

antibodies and enzymes. Let's now discuss the working principle of NAPPA in this animation.

Animations - In NAPPA method, an aminosilane-coated glass slide forms the array surface for NAPPA. To this, the NAPPA master mix is added which consists of BSA, BS^3, GST-tagged cDNA and the anti-GST capture antibodies. The BSA improves efficiency of immobilization of the cDNA onto the array surface while the BS^3 cross-linker facilitates binding of the capture antibody.

The cDNA is expressed using a cell-free lysate to give the corresponding protein with its GST tag fused to it. After transcription and translation the protein is synthesized and the tag enables capture of the protein onto the slide by means of anti-GST antibodies. NAPPA technique can generate very high density arrays but the protein remains co-localized with the cDNA.

Let's now move on to multiple spotting technique (MIST). This is another approach which facilitates generation of high-density protein microarrays by using cell-free expression system.

So unlike NAPPA approach in which printing was performed by adding a mixture of cDNA containing GST tag, BSA, BS^3 and the capture antibody. In the MIST approach both DNA and the cell-free expression system are printed on the chip surface. Here two rounds of spotting is performed. In the first spotting step, the addition of DNA template to the microarray solid support and then second spotting is performed where cell-free expression mixture is transferred directly on top of the first spot which contains DNA. So in this method, the aim is to print 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.

So in the multiple spotting technique or MIST, the first spotting step, as shown in this slide, involves addition of DNA template on to the solid microarray support. After the first spotting is performed where cell-free expression mixture is transferred directly on top of the first spot.

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

So overall 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 first spot. The expressed protein is detected by using floutescently tagged antibody.

So the inventors of MIST technology reported that even 35 fg 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 this MIST technology. So now let's discuss the working principle of MIST by shoing this animation.

Animation - The first spotting step of the multiple spotting technique, which is also capable of producing high density arrays, involves the addition of template DNA on to the solid array support. The template DNA can even be in the form of unpurified PCR product, which is one of the major advantages of this technique.

The second spotting step involves the addition of the cell-free lysate directly on top of the first spot. 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 specific to the protein of interest is then added which indicates protein expression levels by means of a suitable fluorophore.

There are various merits and demerits of using MIST method. It involves unpurified DNA products that can be used as template source which is not the case using NAPPA. Now in this method very high-density protein arrays can be generated because the spot chemistry, the master mix is not very complicated. So higher density can be achieved in this case. The limitations of using this technique of using this technique is, first of all there's loss of signal intensity with prolonged incubation time of the arrays. Since 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. The non-specific protein binding as well as the overall process is more time consuming. These are limitations of MIST technique.

Let's move on to DNA array to protein array (DAPA). The DAPA technique makes possible the repeated use of same DNA template slide for printing multiple rounds of protein arrays.

So in DNA array to protein array, the PCR amplified DNA fragments which encode tag proteins immobilized onto a Ni-NTA coated slide and assembled face-to-face with another Ni-NTA slide 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.

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 proteins diffuse from the membrane and then move on to the other slide for capture. Newly synthesized proteins penetrates the membranes and bind to the surface of capture slide.

As you can see in this slide, in DAPA the PCR amplified DNA fragments encoding the tag proteins are immobilized on to a Ni-NTA coated slide and assembled face-to-face with another Ni-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 capture slide.

In DAPA approach the investigators produced an array of double hexa histidine tag GFP and data wasfound to be comparable with existing protein array technologies. With 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 arrays to protein arrays in this animation.

Animation 3:- 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 in this method.

The DAPA method provides few advantages as compared to the previously described methods. One can get pure protein because the protein is diffused from the membrane. The reusable DNA template which is able to print multiple chips by using this chemistry and now the source temple which is DNA, that array can be stored at room temperature for long duration. When there is a need for making the protein array one can use the membranes 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 spots due to diffusion. It is not ascertained if multimeric proteins assemble effectively and it is also time consuming process.After discussing PISA, NAPPA, MIST and DAPA techniques now let's move on to the latest addition, HaloTag arrays. The Halo link protein array systems are developed by Promega company which combined 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 HaloTag which is mutated hydrolase protein that forms a covalent bond with the HaloTag ligands. Third, it uses polyethylene glycol coated glass slide activated with HaloTag ligand for specific capture of proteins which are expressed by using cell-free expression system.

The HaloTag is a 33 kDa engineered derivative of bacterial hydrolase which is used to tag desired proteins. The proteins which are fused with HaloTag are expressed by using wheat germ extract expression system or rabbit reticulocyte lysate and covalently captured on to PEG containing slides. These are then activated with HaloTag ligands. These HaloTag arrays achieve oriented capture of proteins and thereby ensures no loss of function or minimal loss of function.

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

The proteins are fused with HaloTag are expressed by using cell-free expression system and are covalently captured on polyethylene glycol coated glass slides. So HaloTag method enables oriented capture of proteins.

As you can see, in a nutshell the HaloTag fused protein is expressed and covalently captured on PEG-coated slide and activation is performed by using HaloTag ligand. This provides very strong covalent interaction and minimizes loss of synthesized proteins which usually occurs in the other protein microarray based methods. In protein arrays one needs to perform lot of washing steps. If the proteins or the molecules are bound on the surface with very strong interaction, then there will be minimal loss from the surface which can be achieved in this case by using HaloTag system.

So in HaloTag arrays the capture chemistry which is based on binding of HaloTag 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 microarray technology. So let’s discuss the working principle of HaloTag arrays in this animation.

Animation - In HaloTag technique the slide is activated with the HaloTag ligand which captures the expressed protein through firm covalent interactions that prevents any material loss and ensures oriented capture of the protein.

The HaloTag fused protein is expressed using lysates like RRL or WGE and the synthesized protein is covalently captured on to the array surface through the HaloTag ligand. The specific interaction ensures oriented capture of the protein thereby preventing any possible functional loss.

HaloTag arrays have various advantages such as the strong covalent binding between the 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-specfic adsorption due to PEG-coating. The quantification is easy and one do not need a microarrayer printer to the print the proteins on this chip because the commercial kit of HaloTag arrays provide the gasket which can be used for printing the arrays. However there are certain limitations of using HalTag arrays such as it has not been shown that a system can not be used for high-density arrays. Only the proof-of-concept studies have been shown with few spots by using commercial gaskets which can do 50 or so proteins. So high-density arrays in theory are possible but one needs to ensure that even at the high-density these arrays function properly. Then this possibility of loss of function on binding to the HaloTag. So these types of quanlity control checks have already been performed but still more biological questions need to addressed on these arrays before one feels confident on applying these for various clinical applications.

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 size proteins. These high-throughput projects can capture data at a scale of entire organism and provide insight into the biological systems as well as organization of physiological networks. The development cell-free expression microarrays have overcome several limitations of cell-based protein microarrays and revolutionized the ability to simultaneously study thousands of proteins.

So protein microarrays offer a range of diverse 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 concerns of storage and protein stability. Several cell-free systems have been used, developed in 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 systems including protein *in situ* arrays, nucleic acid programmable protein arrays, multiple spotting techniques, DNA arrays to protein arrays and HaloTag arrays. There are other promising approaches as well which also involve similar principles.

Thank you.