LECTURE-24

CELL-FREE EXPRESSION BASED PROTEIN MICROARRAYS

Handout

PREAMBLE

Protein microarrays are miniaturized arrays containing small amounts of immobilized proteins, allowing the high-throughput study of a very large (100s-1000s) number of proteins. The biggest challenge in producing such arrays is the expression of proteins on such large-scale, without any loss in their structure and activity. Traditionally, recombinant protein expression has been carried out in systems such as *E. coli*. Despite having various advantages of expressing recombinant expression in *E. coli*, there are some inherent problems associated with this expression system. Apart from being a very long and tedious process, the protein production, protein purification and maintenance of protein structure and functionality is difficult. The expressed proteins have a short shelf life, if not stored correctly, which makes protein storage difficult. One of the other challenges of using a prokaryotic system such as *E. coli* is that post-translational modifications may not be possible. Expression of eukaryotic proteins in prokaryotic system such as *E. coli* often leads to the formation of inclusion bodies. These inclusion bodies are hard to purify and can be functionally inactive.

Cell-free expression involves the rapid, *in situ* synthesis of proteins from their corresponding DNA templates directly on the microarray surface. Protein arrays generated by this technique have shown great potential in eliminating the drawbacks of

traditional cell-based methods. These cell-free protein expression systems use the basic mechanism of protein expression in cells, but without using intact live cells. The cell-free expression system should be able to utilize a wide variety of DNA templates and be able to express proteins with high reproducibility. The most important feature is to be able to produce proteins on demand and avoid storage issues that lead to loss of activity of the produced protein. Detection and analysis of bound proteins should be simple. Although there are traditional methods for cell-free production of proteins, many novel microarray-based technologies for cell-free protein production have been developed, which will be discussed in this lecture.

OUTLINE

I. Commonly used techniques for cell-free expression

- i. *E. coli* expression system
- ii. Wheat germ extract
- iii. Rabbit reticulocyte lysate

II. Cell-free expression based protein microarrays

- i. Protein *in situ* array (PISA)
- ii. Nucleic acid programmable protein array (NAPPA)
- iii. Multiple spotting technique (MIST)
- iv. DNA array to protein array (DAPA)
- v. Halo-tag protein array

III. Applications

IV. Advantages and challenges

V. Conclusions

BOX-1. TERMINOLOGY

Cell-free expression: Expression of proteins without using intact cells but using the translation machinery such as ribosomes, promoters, tRNAs etc.

PISA: Protein microarray in which a PCR product is used as the DNA template. IVTT mixture is added, and newly expressed protein is captured on the array using a suitable capture agent.

NAPPA: Protein microarray in which the protein of interest is tagged (generally GST), and expressed using IVTT, and expressed protein is captured on slide coated with anti-capture agent (anti-GST).

DAPA: Protein microarray in which DNA template is attached to one slide, and another slide containing the capture agent is kept face-to-face with it. A permeable membrane containing the IVTT mixture is kept between the slides. Proteins are produced on the DNA slide, which is captured on the other slide.

MIST: protein microarray in which DNA is spotted on the array, while another mix containing the IVTT mixture is spotted precisely on the DNA spot. The newly expressed protein is captured on the array using a suitable capture agent.

Halolink proteins: The protein of interest is tagged with the Halotag, and the slide is coated with the Halo ligand. Thus, only the proteins containing the Halotag are bound to the slide, and desired protein microarray is formed.

I. Commonly used techniques for cell-free expression

Cell free protein expression is the expression of proteins from template DNA, which is inserted in plasmids or present as purified PCR products. The addition of a crude cell lysate containing the required cellular machinery for protein production such as enzymes, ribosomes, tRNA etc., and exogenously added co-factors - nucleotides, ATP, salts, essential amino acids is required for *in vitro* transcription and translation (IVTT) from the gene of interest. Additionally, such a system, based on cell-free expression machinery used, may also allow protein folding and post-translational modifications to produce structurally and functionally mature protein. There are different types of cell-free expression systems (Fig 1). The source of DNA for cell-free protein expression is a DNA fragment, either PCR-produced or present in a plasmid vector, that contains all the genetic information promoter, translational initiator, gene of interest, translational terminator etc., in the correct orientation and reading frame for protein synthesis.

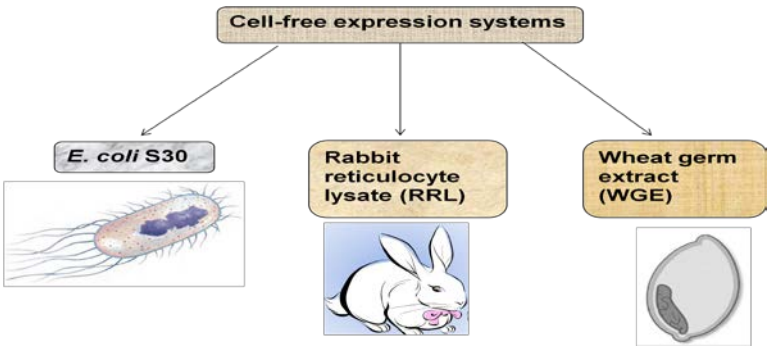

Fig 1. Different types of cell-free expression systems used for *in vitro* protein synthesis starting from DNA templates. These systems contain all the necessary components and machinery for transcription and translation. Some factors such as energy generating

components, essential amino acids etc. need to be added to the system for successful protein synthesis.

Illustration: Commonly used cell-free systems

Cell-free systems are used for in vitro protein synthesis starting from DNA templates. These systems contain all the necessary components and machinery for transcription and translation. Some factors such as energy generating components, essential amino acids etc. need to be added to the system for successful protein synthesis. Commonly used cell-free systems are illustrated in following animations.

The following methods have been used for *in vitro* protein expression:

i. *E. coli* extract (ECE)

The *E. coli* extract system is in use for over the past 50 years. It consists of the crude cell free extract – consisting of ribosomes, tRNAs, translation machinery. *E coli* S30 extracts are most commonly used (Fig 2). This is the one of the best systems for translation of protein from DNA templates as the source.

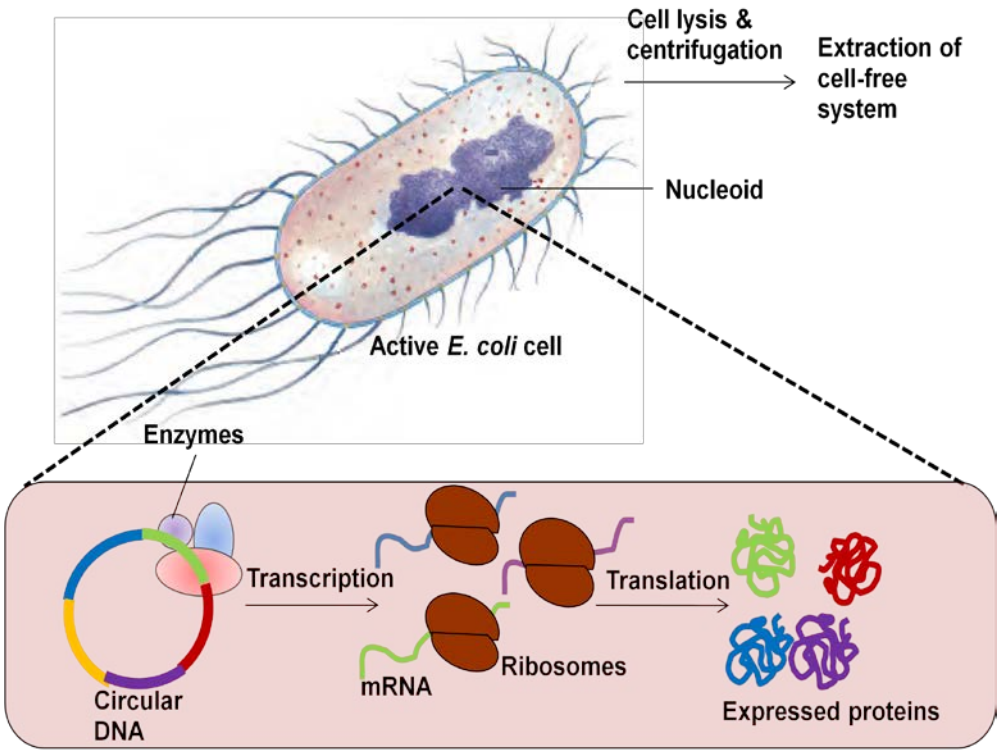

Fig 2. *E. coli* S30 is a commonly used bacterial expression system; however, it is not capable of carrying out post-translational modifications (PTMs) of proteins due to the absence of required machinery for this process and very often produces incomplete protein chains. The DNA templates obtained from bacterial sources are commonly used with cell-free lysate for *in vitro* transcription and translation of proteins.

Illustration: E. coli S30 extract

Actively growing and replicating E. coli cells can be used for extracting cell-free lysates. These cells that are in the process of growth and division are constantly producing proteins and other factors required for various cellular processes. Co-factors and enzymes such as RNA Polymerase, peptidyl transferase are available in significant quantities due to cellular processes of transcription and translation taking place in the cell. The cells are lysed with a suitable buffer and then centrifuged at 30,000g to collect the supernatant containing the extract. Lysate that is extracted from such actively growing and dividing cells will contain all required cellular machinery to carry out in vitro protein synthesis and requires addition of essential amino acids, nucleotides, salts and other energy generating factors.

ii. Wheat Germ Extract (WGE)

The wheat germ extract is also used for *in vitro* translation. The extract is from plant embryo and it contains all the machinery for protein synthesis such as initiation factors, translation factors etc. In addition to having all the components needed for protein expression, wheat germ extract has very low levels of endogenous mRNA, which aid in reducing the background translation by a considerable amount (Fig 3). WGE has been used for efficient translation of exogenous RNA from a variety of different organisms.

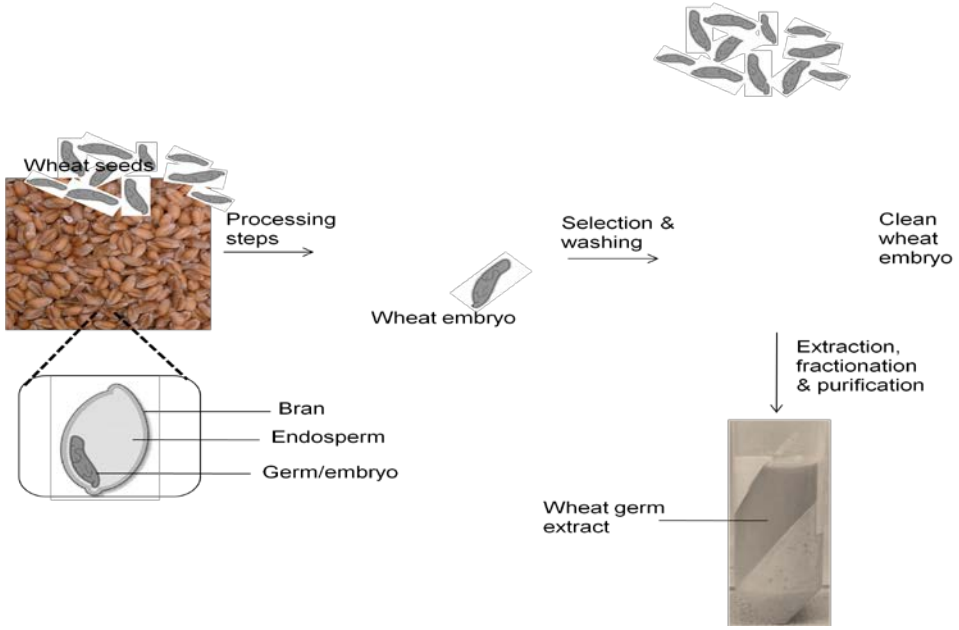

Fig 3. Wheat germ extract (WGE): This is a cell-free expression system that is capable of producing full-length proteins with correct folding and PTMs from bacterial, plant or animal sources. Yields obtained in this system are however slightly lower than the *E. coli* and RRL.

Illustration: Wheat germ extract (WGE)

One of the most commonly used eukaryotic cell-free expression systems is obtained from the embryo of the wheat seeds. The seeds are grinded and then sieved to remove their outer coating fragments. The embryos and other small particles are floated in an organic solvent like cyclohexane. The floating embryos are quickly removed and dried to avoid any damage from the organic solvent. The dried embryos are then carefully sorted out such that only the good embryos without any endosperm coating are selected. The endosperm contains certain inhibitors of protein synthesis that must be removed. The selected embryos are washed thoroughly with cold water after which they are mixed with the extraction buffer and grinded. This solution must be centrifuged at 30,000g at 4°C which results in the wheat germ extract forming a layer in between the top fatty layer fraction and pellet at the bottom. This fraction can be separated and then purified by chromatographic methods to remove any components of the extraction buffer. This cell free lysate is capable of synthesizing full-length eukaryotic proteins.

iii. Rabbit reticulocyte lysate (RRL)

Another commonly used system for *in vitro* translation of proteins is the rabbit reticulocyte lysate. *In vivo*, the reticulocytes have the entire translation machinery as they produce hemoglobin. Thus, the reticulocyte lysate is ideal for translation of proteins, as it contains all the requirements for protein expression – initiation factors ribosomes etc. Addition of micrococcal nuclease to the extract results in degradation of the endogenous mRNA (Fig 4).

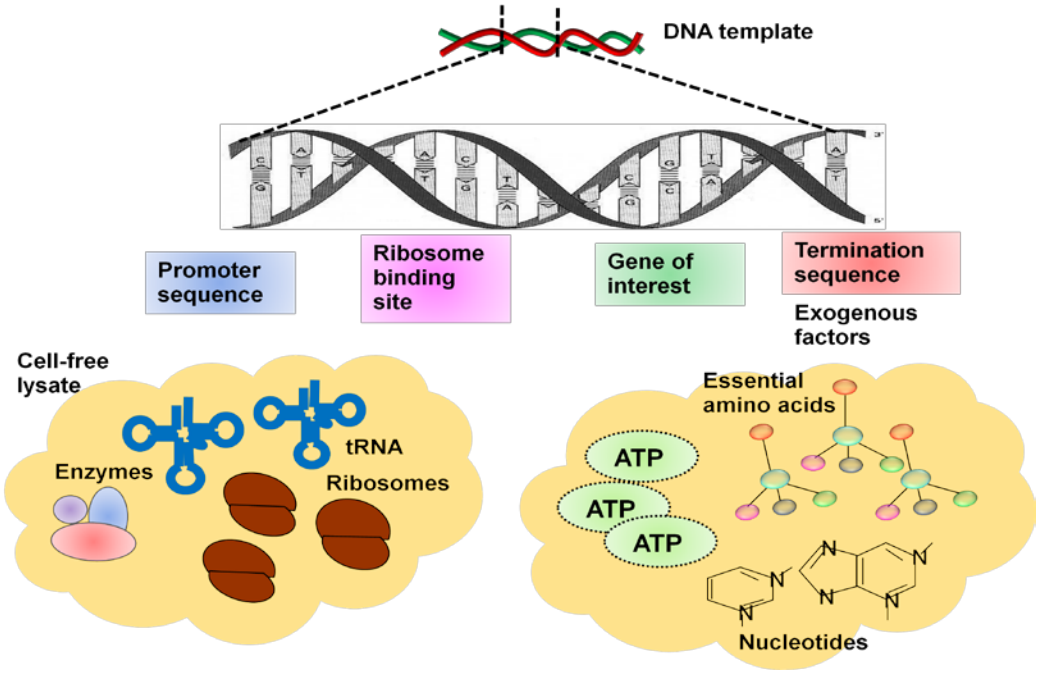

Fig 4. Rabbit reticulocyte lysate (RRL) is a mammalian cell-free system, which is more suitable for expression of full-length eukaryotic proteins that require proper folding and PTMs.

Illustration: In vitro protein synthesis

The DNA template is thawed and then placed on ice during the preparatory process. For in vitro protein synthesis to take place, the DNA template must contain the gene coding for the protein of interest. In addition to this, there must be a promoter sequence, which can initiate the transcription process, a translation initiation sequence for binding of the ribosome as well as suitable termination sequences to correctly synthesize only the protein of interest. The thawed cell-free lysate containing the essential cellular machinery for protein synthesis is added to the DNA template followed by the other exogenous factors that are required for the process. All these are done while storing the template on ice to ensure that there is no loss of activity. The tube containing all the required components is then incubated at $30^{o}C$. Enzymes for transcription bind to the promoter sequences and in the presence of other factors like ATP and nucleotides, they carry out synthesis of the mRNA transcript. This mRNA is then translated into the corresponding protein with the help of ribosomes, tRNA, enzymes and other factors required for the process.

II. Cell-free expression based protein microarrrays

Apart from the traditional methods for *in vitro* translation, new methods are being developed for cell-free protein expression. Many of these methods include production of proteins on a solid substrate – on a microarray. The following are some of the methods for *in situ* protein microarray production:

i. Protein *in situ* array (PISA)

PISA was the first technique of its kind to be developed. This technique was developed by He *et al.* 2001*,* which is also called as the DiscernArray. In this technique, a PCR product is used for translation. The PCR template has the sequence encoding the protein of interest, a T7 bacteriophage-derived promoter, a ribosome binding sequence for translation initiation, like a Shine-Dalgarno or Kozak sequence, an N- or C-terminal tag sequence and termination sequences. This template is added as a free molecule onto the solid support - microtiter wells. The N- or C terminal tag is used for immobilization of the expressed protein onto the array wells. This technique avoids DNA immobilization, but is instead designed to immobilize the *de novo* synthesized protein. The DNA fragment is translated using any one technique like WGE or RRL and protein is thus produced on the array. There are many methods to capture the bound proteins, e.g. the protein is engineered to contain a hexa-histidine tag, while the support pre-coated with the tag-capturing agent, nickel nitrilo-triacetic acid (Ni-NTA) is captured on the solid substrate. Thus the target protein gets captured on the microarray, while the non-specific, unbound materials can be removed by subsequent washing steps (Fig 5). Thus, a protein microarray of desired proteins can be produced.

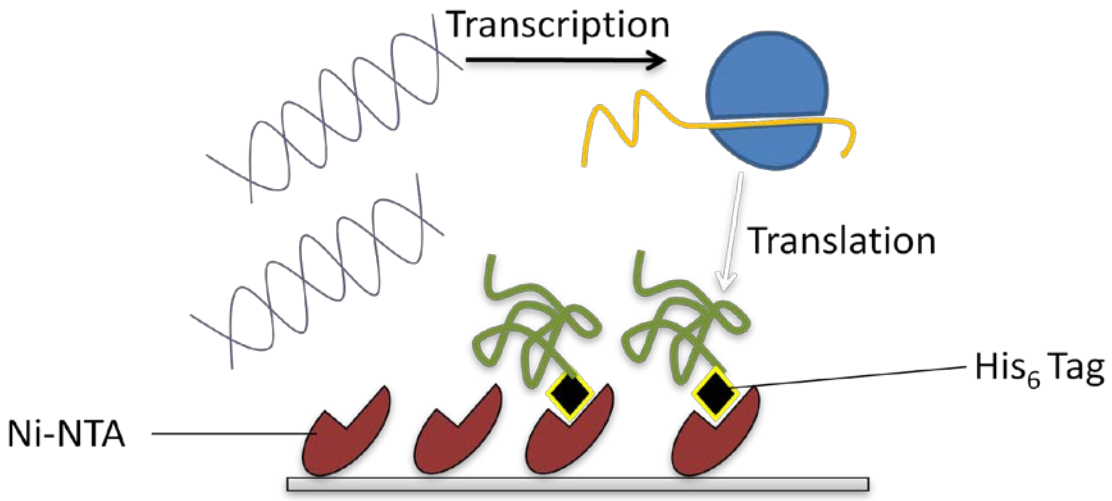

Fig 5. In PISA method, PCR DNA encoding N- or C-terminal tag sequence expressed; bound protein gets specifically captured by tag-capturing agent.

Merits

- Protein purification not required
- Rapid, single step process
- Specific protein attachment
- Soluble proteins formed

Demerits

- Possible loss of function during immobilization
- Relatively high volume of cell-free lysate required

Illustration: Protein In Situ Array (PISA)

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 and aggregation.

ii. Nucleic acid programmable protein array (NAPPA)

This technique was developed by Labaer and colleagues (Ramachandran *et al.* 2004). NAPPA combines recombinant cloning technologies with cell-free protein expression. It replaces the cumbersome process of spotting the synthesized protein with simpler process of spotting purified plasmid DNA. In this method, cDNA encoding a fusion of protein of interest with a tag (usually glutathione-S-transferase, GST) is expressed in plasmids by recombinant cloning. To prepare the microarray slides, a master mix, consisting of 4 components, namely plasmid-borne cDNA encoding a transcript for protein of interest fused with the GST tag protein, Bovine Serum Albumin (BSA) to improve binding efficiency of cDNA, anti-GST antibodies and amine-amine cross-linker, BS3, is spotted onto the array chip. The array surface is coated with APTES (aminopropyltriethoxysilane), which helps in binding of anti-GST antibodies cross-linked by BS3. The cDNA immobilization on the array surface is facilitated by BSA. The array is then 'activated' by adding RRL supplemented with T7 polymerase, RNase inhibitors and essential amino acids. Following transcription and translation off the ribosomal complex, the newly synthesized protein fused to the GST tag is then captured by the anti-tag (GST) antibodies attached to the array (Fig 6). Thus, a protein microarray is obtained. This technique has been used to express proteins from over 10,000 unique cDNAs and has been used to express proteins from a variety of organisms. Furthermore, it is not confined to any particular class of proteins and has been efficiently (> 90%) used to express a wide variety of proteins, including transcription factors, kinases and transmembrane proteins. Moreover, a wide range of sizes (from <50 to >

100 kDa) can be efficiently expressed, making this a versatile tool for protein expression.

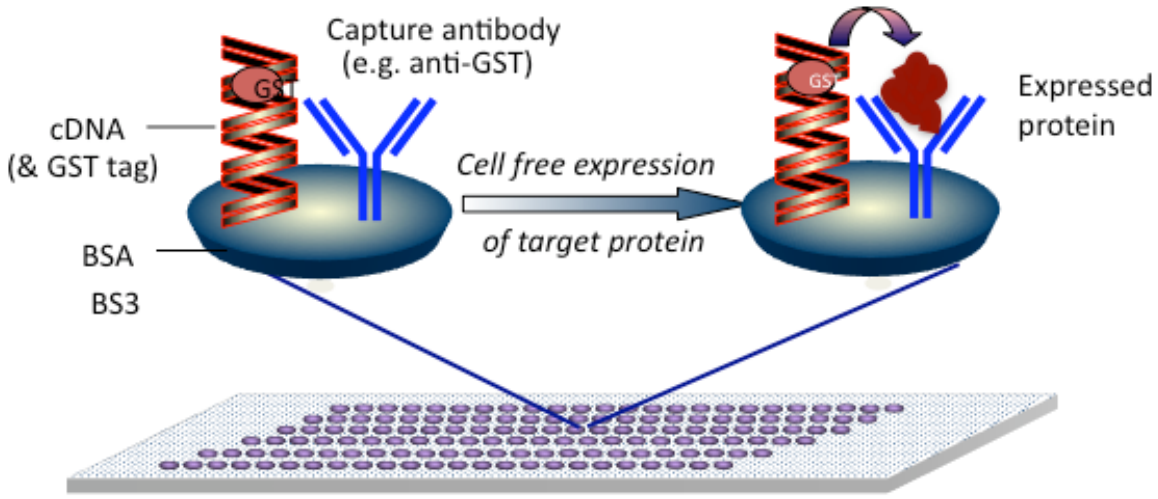

Fig 6. Protein microarray in which cDNA containing protein of interest is tagged (generally GST), and expressed using IVTT. The expressed protein containing GST tag is captured on slide coated with anti-capture agent (anti-GST antibody).

Merits

- No need to express and purify protein separately
- Expression in mammalian milieu (natural folding)
- Proteins produced just-in-time for assay
- Shelf-life not an issue
- Access to all cloned cDNAs

- Express & capture more than protein spotting arrays
- Retains functionality of traditional protein arrays
- Arrays stable on bench until activated

Demerits

- Cloning procedure required
- Pure protein array not produced
- Peptide tags may lead to sterical effects blocking important binding domains
- Functionality of proteins

Illustration: Nucleic Acid Programmable Protein Array (NAPPA)

An aminosilane-coated glass slide forms the array surface for NAPPA. To this, the NAPPA master mix is added which consists of BSA, BS3, GST-tagged cDNA and the anti-GST capture antibodies. The BSA improves efficiency of immobilization of the cDNA onto the array surface while the BS3 cross-linker facilitates binding of the capture antibody. The cDNA is expressed using a cell-free extract to give the corresponding protein with its GST tag fused to it. This 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 cDNA.

iii. Multiple Spotting Technique (MIST)

Another method of making a protein array is the multiple spotting technique (MIST), which was developed by Angenendt *et al*. (Angenendt *et al*. 2004). Here, the support-like a slide is pre-coated with a protein capture agent. The first spot printed on slide consists of the DNA template. The second spot printed contains the *in vitro* translation mixture, which is added exactly on top of the spot containing the DNA template (Fig 7). The translation of the proteins takes place, and protein-capture agent captures newly formed protein on slide, and an array is constructed.

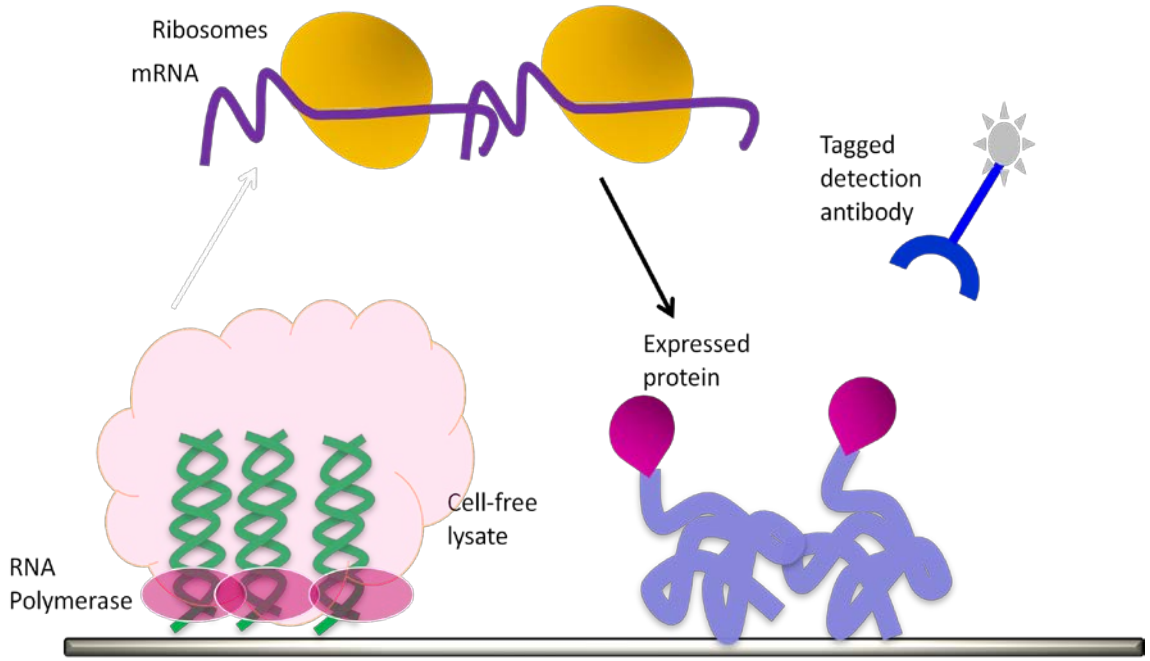

Fig 7. Multiple spotting technique (MIST). First spotting step involves the spotting of DNA template coding for the protein of interest in as little as fg quantities, onto the solid array surface. Second spotting: After the template DNA is spotted on to the array surface, the cell-free lysate is then transferred exactly on top of the first spot. This

second spotting step marks the beginning of expression of the template DNA. The proteins produced by cell-free expression from the corresponding DNA templates are immobilized on the array surface either through a tag-capturing agent or more commonly, by means of non-specific interactions. These proteins can then be detected by suitably tagged antibodies.

Merits

- Unpurified DNA products used as template
- Very high-density protein arrays generated

Demerits

- Loss of signal intensity with prolonged incubation time
- Non-specific protein binding
- Time consuming process

Illustration: Multiple Spotting Technique (MIST)

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, 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, 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.

iv. DNA Array to protein array (DAPA)

This concept was developed by He *et* al. in 2008 (He *et al.* 2008). In the DAPA concept, two different slides are used. One of the slides coated with Ni-NTA has the PCR-amplified DNA fragments, which encode the protein of interest fused with a tag immobilized onto it; while the other Ni-NTA slide has the protein-tag capturing agent. The slides are placed face-to-face and a permeable membrane is kept between them. The permeable membrane has the cell-free lysate, and thus protein expression is initiated in between the two slides. The newly synthesized proteins are produced on the slide with the DNA template, which then penetrate the membrane and get immobilized on surface of the slide bearing the tag capture reagent. Thus, a replica of the DNA array is formed on the capture slide (Fig 8). DAPA leads to the construction of a pure protein array i.e. an array with no DNA contamination, as the two are kept separate throughout the experiment. Another important advantage of DAPA is that the DNA template slide is reusable i.e. the proteins can be printed many times from the same DNA, making the process less time-consuming and cost-effective.

Demerits

- Reusable DNA template array
- Pure protein array generated
- DNA template array can be stored for long durations

Demerits

- Broadening of spots due to diffusion
- Not ascertained if multimeric proteins assemble effectively
- Time consuming process

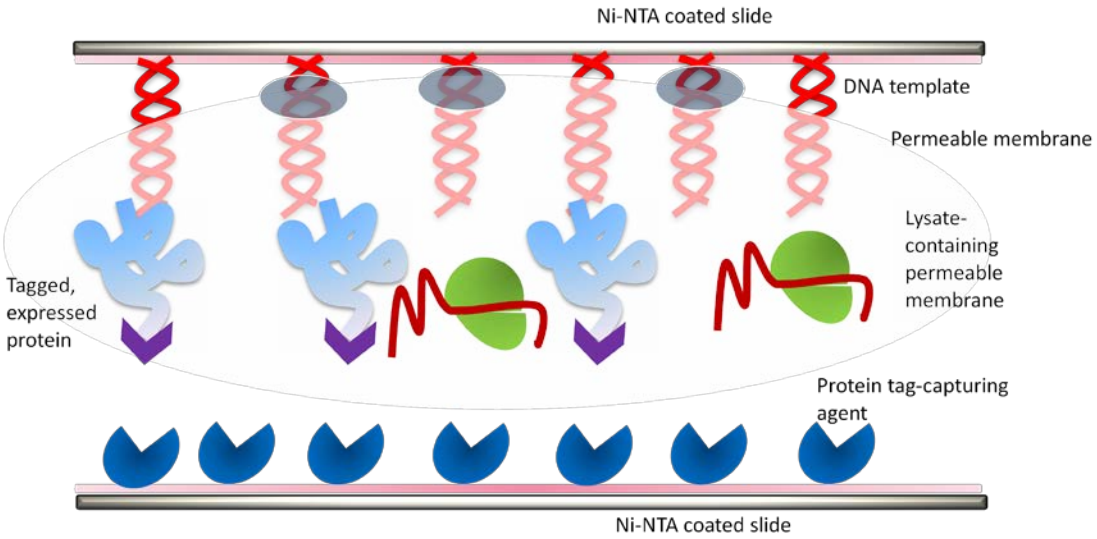

Fig 8. The microarray slide surface is coated with Nickel-nitrilotriacetic acid (Ni-NTA), which acts as a useful capture agent. PCR amplified DNA that codes for the protein of interest is immobilized on a Ni-NTA coated slide. A permeable membrane that is soaked with the cell-free extract is placed in between the immobilized DNA template slide and a slide having the protein tag-capturing agent. The newly expressed proteins penetrate the membrane and bind to the protein purification slide.

Illustration: DNA Array to Protein Array (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.

v. Halo-link protein array

This is an amalgamation of technologies developed by Promega and is useful in generating tightly immobilized arrays. The Halolink array consists of a DNA construct encoding the gene of interest fused with the 'Halotag', a 33 KDa mutated bacterial hydrolase. The protein is constructed using a cell free expression system (WGE or RRL). The newly formed proteins are captured on a polyethylene glycol coated glass slide, which has been activated by HaloTag ligands. The HaloTag fused to the protein of interest binds the Halo ligand on the slide by covalent bonding, thus enabling capture of the desired protein. The strong bond afforded by the covalent linkage prevents material loss during washing, which is always a concern for any microarray. It also allows oriented capture of proteins, hence keeping the protein activity unaffected.

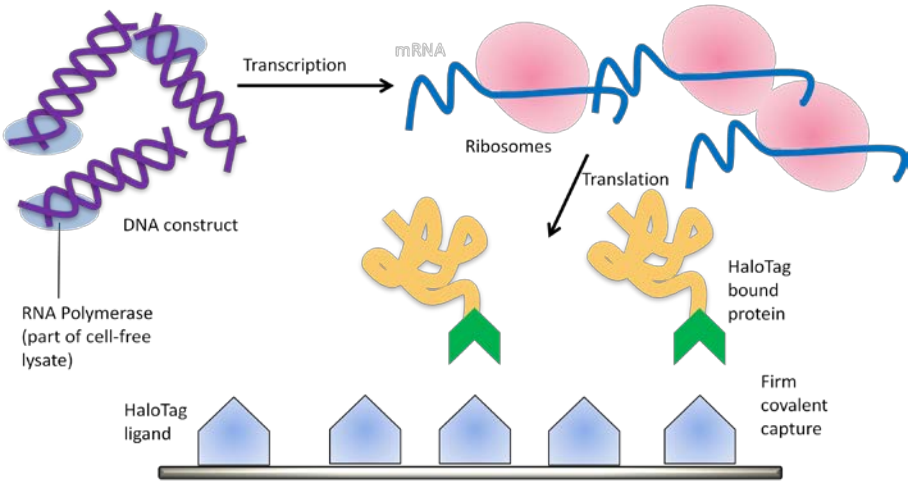

Fig. 9. The template DNA coding for the protein of interest along with the HaloTag. The mode of interaction between HaloTag and its ligand is through covalent bonding, thereby ensuring firm capture of the protein on the array surface without any material loss during washing.

Merits

- Strong covalent bond between protein and ligand
- No material loss during washing
- Oriented capture of protein
- No non-specific adsorption
- Easy quantification
- No need for a microarrayer printer

Demerits

- Possible loss of function on binding to Halotag
- HT application will require optimization of printing

Illustration: HaloTag technique

The slide is activated with the HaloTag ligand, which captures the expressed protein through firm covalent interactions thereby preventing any material loss and ensuring oriented capture of the protein. The HaloTag fused protein is expressed using lysates like RRL or WGE and 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.

III. Applications

The microarrays spotted with proteins expressed using cell-free expression system have variety of applications such as:

- Biomarker discovery
- Immunogenicity studies
- Protein-protein interaction studies
- Post-translational modification studies
- Simultaneous screening of a large number of proteins

Illustration: Application – protein-protein interaction study

Ramachandran et al., tested the use of NAPPA microarrays by immobilizing 29 sequence-verified human genes involved in replication initiation on the array surface and then expressing them in duplicate with RRL. The expressed proteins bound to the anti-GST antibodies present on the array surface. The authors made use of each of these expressed proteins to probe another duplicate array of the same 29 proteins thereby generating a 29 x 29 protein interaction matrix. 110 interactions were detected between proteins of the replication initiation complex, of which 63 were previously undetected ones.

IV. Advantages and challenges

Technique	Advantages	Challenges
PISA	• Creates soluble proteins *in situ*. • Overcomes common problems (Protein insolubility, degradation and aggregation issues) endemic during protein expression in prokaryotic systems. • Only tagged protein remains on the array.	• Requires immediate utilization of PCR-produced DNA. • Not cost effective as a relatively large volume of cell-free lysate is required. • Hexa-histidine tag may interfere with proper protein folding.
NAPPA	• Use of mammalian expression systems allows efficient folding. • Access to a wide variety of cloned cDNAs. • Shelf-life not an issue: cDNA arrays stable for long periods at RT, and more difficult-to-store	• Need for time-consuming cloning before generating array, or alternately dependent on available clones. • Need to clone gene of interest as its GST fusion • Pure protein arrays not produced: expressed

	proteins produced just before assay. • Cost effective: low required volume of cell free lysate. • Effective process: over 95% of proteins tested express and capture well. Discrete spots are obtained.	protein remains co-localized to cDNA. • Peptide tags may produce steric hindrance while studying protein interactions. • Correct functionality of proteins always remains in doubt during cell-free expression.
MIST	• Non-purified PCR product can be used as DNA template. • Very high-density protein arrays generated.	• Non-specific protein binding can occur. • Time consuming. • Loss of signal intensity occurs with prolonged incubation.
DAPA	• A pure protein array, free of DNA is generated. • Allows the generation of multiple protein arrays from a single DNA template.	• Broadening of spots may occur due to protein diffusion. • Multimeric proteins may not assemble efficiently. • Time consuming process.

	• The DNA template array can be stored for long periods.	
Halo-link Protein arrays	• Covalent bond allows firm immobilization of proteins. • Proteins are captured in an oriented manner with no non-specific adsorption. • Little functional or quantitative losses of materials during washing steps. • Accurate quantification of protein possible. • No requirement for microarray printer as gaskets for printing are provided.	• Has not been validated for high density-large protein number arrays, although theoretically possible. • Loss of protein function may occur due to binding to the Halotag.

V. Conclusions

The usage of protein microarrays have made it possible to screen a large number of proteins simultaneously, leading to high-throughput studies. Many types of cell-free expression based microarrays have shown promising results (Fig 10).

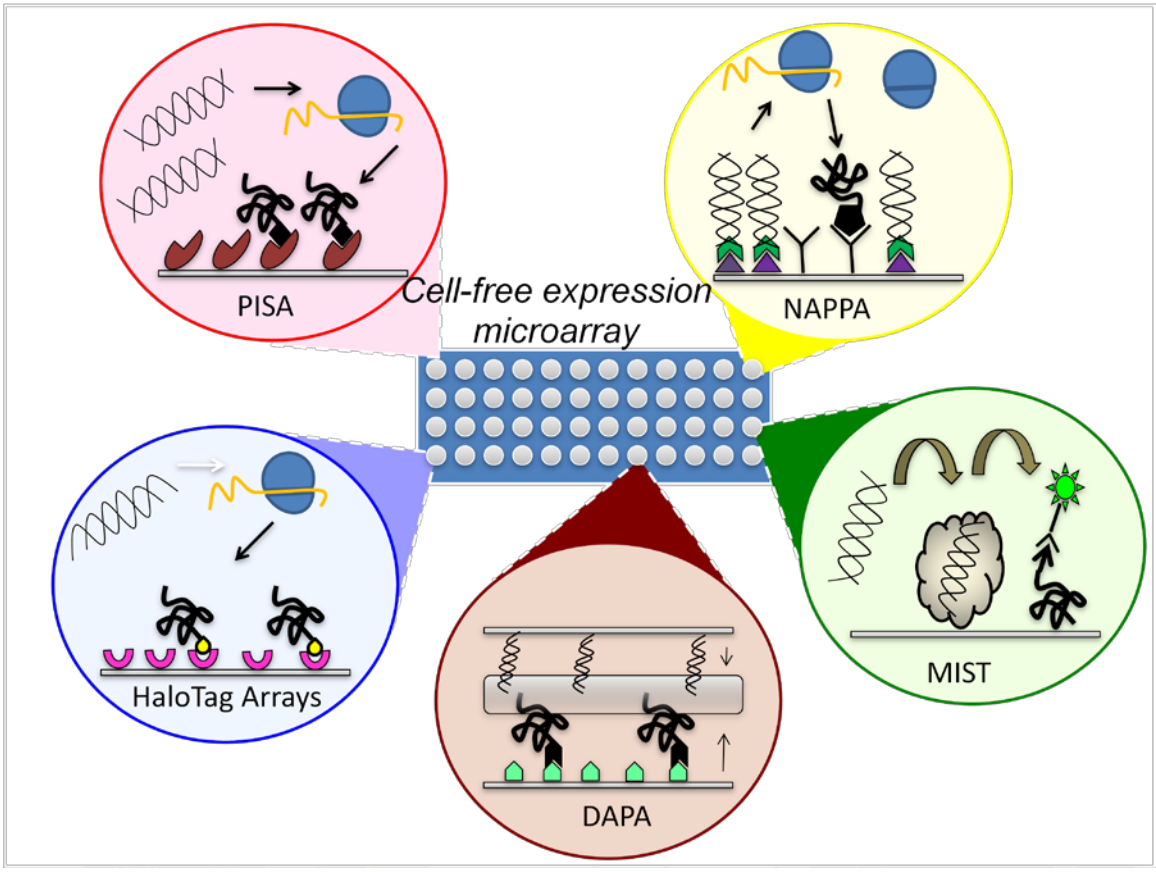

Fig 10. An overview of cell-free expression based microarrays.

NAPPA and DAPA aid in building pure protein arrays with no DNA contamination. Proteins have relatively shorter shelf lives and protein microarrays help in increasing the shelf life of the proteins. Making protein arrays is also cost-effective, as very small amounts of reagents are used. Moreover, the DNA templates are reusable, which

reduces the cost further. The production of proteins becomes much easier, and storing of DNA arrays helps in reproducing the arrays when required. Microarrays have tremendous potential in clinical applications as well as non-clinical research such as detection of protein-protein interaction and biological screening.

VI. References

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