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PERSPECTIVE

Emerging technology of *in situ* cell free expression protein microarrays

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Recently, *in situ* protein microarrays have been developed for large scale analysis and high throughput studies of proteins. *In situ* protein microarrays produce proteins directly on the solid surface from pre-arrayed DNA or RNA. The advances in *in situ* protein microarrays are exemplified by the ease of cDNA cloning and cell free protein expression. These technologies can evaluate, validate and monitor protein in a cost effective manner and address the issue of a high quality protein supply to use in the array. Here we review the importance of recently employed methods: PISA (protein *in situ* array), DAPA (DNA array to protein array), NAPPA (nucleic acid programmable protein array) and TUSTER microarrays and the role of these methods in proteomics.

INTRODUCTION

Protein microarrays represent a high-throughput technology to study thousands of protein functions simultaneously on a solid substrate, often a surface modified microscopic slide. The fundamental principle of protein microarrays was first put forward by Ekins (1989). The protein microarrays started out by printing tiny spots of purified proteins or other macromolecules on a slide, and provided better sensitivity than other conventional immunoassays. Protein microarrays gained popularity through large-scale genome sequencing projects and DNA microarray technologies (Pease et al., 1994). The development of DNA microarray technologies in global gene expression profiling (Morley et al., 2004) could be standardized and implemented into several areas of functional genomics yet biological functions are carried out by protein rather than nucleic acids (Gygi et al., 1999). Protein microarrays are a great tool for the large-scale analysis of both functional genomics and proteomics (Predki, 2004; Lueking et al., 2005). Currently protein arrays are extensively used for various applications such as antibody profiling, biomarker identification, protein–protein interactions, and enzymatic assays (Kawahashi et al., 2003; Feilner et al., 2005; Ramachandran et al., 2008b; Anderson et al., 2011; Hu et al., 2011; Ramani et al., 2012).

Conventional protein microarrays require purified protein for printing on the array, which remains a major challenge. Recombinant expression of proteins, in the numbers needed for functional protein microarrays, relies on the availability of large collections of cDNAs in readily expressible formats. However, some proteins may be insoluble or toxic to the expressing organism. Moreover, the high throughput expression and purification of thousands of proteins remain outside the realm of most laboratories. Another requirement is a collection of soluble, purified proteins, which are covalently or non-covalently attached onto suitable surfaces such as derivatized glass slides or beads (Stevens, 2000; Murthy et al., 2004). There have been significant developments in cell free expression systems where proteins are synthesized by means of cell extracts (lysates) containing all the essential elements for transcription and translation. Such expression systems have been made from several different organisms, of which *Escherichia coli*, rabbit reticulocyte and wheat germ are commonly used (Endoh et al., 2006; Langlais et al., 2007)

Four main methodologies for *in situ* protein microarray technologies have been developed in the past 10 years (described below). These are arrays of proteins generated by immobilized cDNA or PCR fragments containing a C or N terminal fusion tag along with a capturing agent on a solid support coupled with commercially available cell free expression systems (He et al., 2008b). These microarrays are being used to investigate the functions of proteins on the array or of biomolecules in solution. Recently, these microarrays have gained popularity in the field of proteomics because of their shelf life, relative low cost and the potential functional applications in the fields of protein–protein interaction, biomarker,

and drug discovery among others.

CELL FREE EXPRESSION BASED *IN SITU* PROTEIN MICROARRAY

PISA

PISA (protein *in situ* array) was the first well known cell free based *in situ* protein microarray technology that provided a rapid, single-step approach for the generation of a protein array from DNA fragments coupled with cell-free expression system followed by immobilization onto a solid support (He and Taussig, 2001). In this technique, the DNA constructs encoding the protein of interest contain a T7 promoter and sequences for translation initiation (Shine-Dalgarno or Kozak), an N- or C-terminal tag sequence for immobilization along with suitable termination sequences. The DNA constructs are produced by PCR amplification using a high fidelity TAQ polymerase. The surface is precoated with a tag capturing agent and the protein expression is carried out, commonly with *E. coli* S30 or rabbit reticulocyte lysates. After transcription/translation, the expressed proteins bind specifically onto the surface through the tag sequence and the unbound material could be washed away. The authors of this technology made use of 6X histidine tag and a microtiter plate having 24 wells coated with nickel nitrilo-triacetic acid (Ni-NTA). They successfully achieved the expression and functional immobilization of a fragment of the human antiprogesterone antibody in microtiter wells and that of luciferase enzyme on Ni-NTA-coated magnetic beads. Quantification by Western blot analysis unveiled that 120 ng of protein was produced (He and Taussig, 2001). PISA was the first technique and its success opened the door for other similar technologies in cell free based protein *in situ* arrays.

NAPPA

NAPPA (nucleic acid programmable protein microarray) was developed by LaBaer and colleagues (Ramachandran et al., 2004). Unlike PISA, NAPPA uses immobilized plasmid DNA. These vectors encode the proteins of interest with a C-terminal GST fusion tag, for *in vitro* transcription and translation (IVTT). In NAPPA, the plasmid DNA is biotinylated (UV crosslinked through psoralen) and immobilized onto an amino fabricated glass slide via avidin along with anti-GST antibodies as a capturing agent. Protein expression is then carried out by coupling the microarray with rabbit reticulocyte lysate. The newly synthesized proteins can reach levels ranging from 4 to 29 fmols (Ramachandran et al., 2004). In a follow up article (Ramachandran et al., 2008a), this method was further improved for the generation of high-density protein microarrays. In this method, the authors found that BSA improved the binding efficiency of DNA onto the surface and they were able to bind around 97% printed DNA tested by PicoGreen. A test array of cDNAs for 96 genes along with a negative control of non-expressing plasmid DNA and a concentration series of recombinant, purified DNA were printed and they were able to detect 99% of protein signals of these 96 genes by anti-GST antibody. The success of this technique was proven when used to array up to 1000 unique human cDNAs, with an average protein yield of ~9 fmol per feature. NAPPA was found to be a cost-effective technique due to the small volume of cell-free extract required for protein expression as compared to PISA.

DAPA

This innovative technique was developed by He et al. (2008a). DAPA makes it possible to repeatedly use the same DNA template slide for printing multiple protein arrays. It starts by spotting PCR amplified DNA fragments encoding the tagged protein on one slide, which are then assembled face-to-face with another slide with Ni-NTA as a tag-capturing agent. In between the two slides, a permeable membrane with the cell-free lysate was placed. Protein synthesis takes place from the immobilized DNA spots and the newly synthesized proteins penetrate the membrane and bind to the surface of the capturing slide. DAPA is particularly advanced as it could be used to generate multiple copies of pure protein microarrays with the reuse of preprinted DNA array, and the immobilized DNA could be stored and reused after prolonged periods of time. However, the DAPA technique is limited by the possibilities of protein diffusion during membrane penetration, especially regarding larger multimeric proteins.

TUS-TER microarray

T-T microarray was developed by Chatterjee et al. (2008). In this technique, the printed expression plasmids serve a dual function as an encoder as well as a capturing agent through a high affinity DNA–protein interaction. The TUS protein exploits the high-affinity binding (~$3–7\times10^{13}$ mol/L) with Ter, a 20-bp DNA sequence. In this system, the microarray plasmid is designed for expression of the protein of interest with a C-terminal Tus fusion protein. This allows the capturing of the expressed protein by embedded Ter DNA sequences within the plasmid. In the TUS-TER microarrays the plasmid DNA is the only material immobilized onto the nitrocellulose glass slide and the protein expression is carried out by coupling the microarray with rabbit reticulocyte lysates. The advantage of T-T microarray is that it removes the need of capturing agent along with the DNA. Like other DNA microarrays, the T-T microarray can be stored for longer periods of time and be used when needed.

FUTURE PERSPECTIVE

Protein microarrays based on the cell free system are a cost

effective technology which allows the availability to create a protein microarray on demand. These microarrays could be used for high throughput study of proteins, which are hard to express and purify in a living organism. However not all issues concerning these methodologies have been solved. Fusion tags which are being used in NAPPA and TUS-TER are at least 30 kDa of molecular weight and leaky His tags being used for PISA and DAPA in themselves create problems. To address this issue, new small molecular weight fusion tags with high binding affinity or modification of previous strategies are being developed. Another potential issue is the proper folding of the synthesized proteins. As expected, not all the expressed proteins by cell free expression system are properly folded which includes oxidative folding pathways to allow for isomerization and formation of disulfide bonds. Considerable progress has been made towards enhancing the folding of eukaryotic proteins with multiple disulfide bonds (Carlson et al., 2011). In addition to this, new advances have been made to form and isomerize disulfide bonds by using natural enzymes (e.g. the Dsb system in *E. coli* and other chaperones). The simple addition of these macromolecules has been important for the generation of complex proteins *in vitro* (Katzen et al., 2005). More recently, eukaryotic chaperones such as Hsp70 or BiP (Welsh et al., 2011) have also being used. Bundy and Swartz (2011) have shown that it is possible to establish an oxidizing environment in the cell lysates that promotes disulfide bond formation through balancing the redox potential reaction.

Another issue of the current cell free expression systems is the density of protein. More recently, higher protein levels have been achieved by supplying extraenergy for protein biosynthesis in cell free expression system (Kim et al., 2011). Other attempts deal with scaling up the cell extract production (Zawada et al., 2011), using diverse fusion tags (Kralicek et al., 2011), a cell mimic device (Siuti et al., 2011) or using microporus microfluidic devices that will give a cleaner and high density protein microarray. Another approach that facilitated the creation and functionality of the protein microarrays is the use of 3 dimensional surface chemistries. The use of a 3D surface chemistry (Ma et al., 2010) will not only give high density of protein but also lower the non-specific binding or non-relevant proteins and improve the sensitivity. These new generation of protein microarrays will become the norm for future interaction screens especially for small compounds (e.g. drug profiling). All the cell free expression based microarray technologies described here are based on fluorescence detection, which can be limiting. Fluorescent labeling can affect the activity or function of a protein and fails to give kinetic parameters and real time information. An attractive alternative to traditional fluorescence-based microarray detection methods is the surface-sensitive optical technique of surface plasmon resonance imaging (SPRi) (Hickel et al., 1989). SPRi detects the binding of label-free molecules onto the surface of a gold thin film by measuring changes in the local refractive index upon adsorption (Ro et al., 2005). The integration of this technology with *in situ* expression arrays could be a major advancement in this area. By using SPRi one can analyze protein microarray quantitatively (Lausted et al., 2008) and can study the kinetics parameters of protein–protein interaction, protein–DNA/RNA, protein–drug interaction and therapeutic antibody profiling. Ideally, the next generation of protein microarrays will have a high density of expressed and purified protein on the chip without any contaminating DNA or large immobilization tags or anchoring macromolecules. These represent ambitious challenges that scientific communities are currently working on from different angles.

Multiplex *in situ* protein microarray will become more widely accepted and implemented not only in proteomic research but also in drug discovery and diagnostics areas. The detection of autoantibodies patterns as a response to a condition or progression of a disease is gaining momentum (Wang et al., 2005; Chatterjee et al., 2006; Järås and Anderson, 2011). By testing multiple protein variants generated by SNPs (single nucleotide polymorphism), one can also start thinking about applying these microarrays to personalized medicine. The advanced development of genomic and proteomic information about pathogens can also open the door for a series of multiplex screens with innate immune proteins as well as antibodies. This will have a positive outcome for the generation of new vaccines in the future. Another major application is the selection of better therapeutic antibodies from libraries of single chain antibodies (Carlson et al., 2011). Antibody profiling is another major area where these technologies can make a real difference. We believe that there is a bright future for *in situ* protein microarrays and we foresee many developments in this field that will change the areas of biomarker and drug discovery in the near future.

ABBREVIATIONS

DAPA, DNA array to protein array; GST, glutathione s-transferase; NAPPA, nucleic acid programmable protein array; PISA, protein *in situ* array; SNPs, single nucleotide polymorphism; SPRi, surface plasmon resonance imaging; T-T microarray, Tus-Ter microarray

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