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Applications of Display Technologies to Proteomic Analyses

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Abstract With the rapid accumulation of genetic information, development of general experimental approach suitable for large scale annotation and profiling of the whole proteome have become one of the major challenges in postgenomic era. Biomolecular display technologies, which allow expressing of a large pool of modularly coded biomolecules, are extremely useful for accessing and analyzing protein diversity and interaction profile on a large scale. Recent advances in protein display technologies and their applications to proteomic analyses have been discussed. J. Cell. Biochem. Suppl. 37: 34–41, 2001. © 2002 Wiley-Liss, Inc.

Key words: molecular interactions; affinity proteomics; phase display; genome-wide profiling

The rapid accumulation of genomic information has accelerated need for developments of general experimental methods that allow for high throughput collection of protein data with high bioinformation density. The new types of complex observational data from genome- and/or proteome-wide descriptive data promise a more comprehensive understanding of complex biological processes [Brent, 2000]. Proteomics was initially defined as the two-dimensional polyacrylamide gel technique for displaying and indexing all the proteins from a cell line or tissue [Celis et al., 1996]. Now with expansion in technology, the scope of proteomics has been extended to include more comprehensive indexing information in three areas [Pandey and Mann, 2000]: (1) micro-characterization of proteins and their post-translational modifications; (2) 'differential display' proteomics for comparison of protein levels at different developmental stage or physiological states, hence with potential application in a wide range of diseases; and (3) studies of protein-mediated molecular interactions by using various techniques. The first two areas rely more on the new development of instrumentation, such as the new improvements on protein micro-sequencing or mass spectrometry. The tools to address the third area are more challenging. The large-scale characterization and profiling of protein-mediated molecular interactions such as protein–protein interactions, which underlies the dynamic assembling and disassembling protein complexes involved in biochemical processes, will become an important data set for understanding biology. Different from the previous strategy that characterizes bio-molecular interactions individually, the proteomic approach demands to generate a variety of index profile in high throughput (HTP) format [Brent, 2000]. Biomolecular display technologies, which allow expressing of a large pool of modularly coded biomolecules, may become extremely useful for accessing and analyzing protein diversity and interaction profile on a large scale [Li, 2000]. The biological display system includes two major formats: "in vitro" and "in vivo" display. Regardless of the format, a display library normally consists of three components: displayed entities, a common linker, and the corresponding individualized codes [Li, 2000]. The core concept for display technology is that the phenotypic characters of the proteins are physically linked with their genotype. With this convenient modular feature combined with in silico technology, the genetic information encoding these proteins can be quickly decon-

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voluted without potentially labor-intensive methodologies that are directed to analyzing proteins. There are several excellent reviews focusing on different types of the display technologies [Shusta et al., 1999; Castagnoli et al., 2001]. In this review, special focus will be placed on the "in vivo" display technologies, where the modular complexes are formed within cells including prokaryotic display systems and eukaryotic display systems. Some recent advances in monitoring protein–protein interactions will be discussed.

In Vitro Display Technologies

In vitro display technologies can be featured as the biomolecular display process that can be carried out without a biological host system. Three main types of in vitro display technologies have been developed, including ribosome, mRNA display and in vitro compartmentalization methods. Both ribosome and mRNA display approaches need a separate step involving synthesis of RNA molecules in vitro combined with prokaryotic or eukaryotic translation machinery. Thus, the linked genetic codes are RNA molecules. By using water-in-oil emulsion system, in vitro compartmentalization technology can imitate the compartmentalization of living cells [Tawfik and Griffiths, 1998; Griffiths and Tawfik, 2000]. The individualized compartments function as the biological membrane enclosure, which is permeable to the small molecules for enzymetic reactions, but the large molecules, such as proteins and DNAs, can not diffuse between the droplets [Tawfik and Griffiths, 1998; Griffiths and Tawfik, 2000]. With the new techniques in making those droplets, the range of the available droplet size is wide (0.1–50 μm) [Griffiths and Tawfik, 2000]. The conditions may be adjusted so it would be suitable for containing only single gene in each droplet. In one of the recent reports, Ghadessy et al. have successfully applied this technique in Taq polymerase directed evolution [Ghadessy et al., 2001]. Through this compartmentalization, the genotype and phenotype can also be physically "linked" by so called "STABLE" method [Doi and Yanagawa, 1999], which a library of biotinylated DNA, encoding peptides fused to streptavidin, was transcribed and translated in vitro in water-in-oil emulsions. Compared with mRNA and ribosome display, the two potential advantages are: (1) the tethered genetic codes are DNAs and (2) the transcription and translation machineries are coupled in the whole process.

Ribosome display technology was initially introduced for the identification of receptor peptide ligands [Mattheakis et al., 1994]. Later it has been expanded for the selection of folded proteins [Hanes and Pluckthun, 1997; He and Taussig, 1997]. Through the non-covalent ternary complexes of mRNA, ribosome and nascent polypeptide, the genotype and phenotype are coupled. Although it is non-covalent linkage, the ternary complexes are pretty stable [Amstutz et al., 2001]. Ribosome display has been successfully applied for screening high affinity single chain antibodies through directed evolution [Hanes et al., 2000]. Recently, Bieberich et al. also reported the cDNA cloning of sialyltransferase II by ribosome display [Bieberich et al., 2000].

The puromycin-linked mRNA display method was originally developed by taking advantage of the fact that puromycin, attached at the 3′-end of the synthetic mRNA, can mimic the aminoacyl end of tRNA, and enters the proceeding peptidyl transferase site to form a covalent linkage with the nascent peptide [Monro and Marcker, 1967; Monro and Vazquez, 1967; Roberts and Szostak, 1997]. Hence, the genotype and the displayed polypeptide are connected through the puromycin molecule. Because the mRNA hybrid and the synthesized polypeptide are covalently coupled together, it has some obvious advantages over the standard ribosome display. Now, the protocol for this method has been further improved in several aspects, such as using the psoralen-containing DNA linker to photo-crosslinked to the 3′ end of the mRNA, replacing the mRNA molecule after the polypeptide synthesis with mRNA-cDNA hybrid or its double-stranded cDNA [Kurz et al., 2000; Hammond et al., 2001], and improving the library quality by preselection procedures [Cho et al., 2000; Keefe and Szostak, 2001].

In summary, the "in vitro" display technologies can be operated in the total of absence of a living cell. Theoretically, there are no limitations posed on these systems providing several unique advantages. Firstly, the library size can be extremely large (up to 10^{11}–10^{13}) without subcloning step, that often limits the complexity of library. Secondly, the methods reduce negative selection posed by host system as seen in "in vivo" display system. The target molecules can be selected in variety of conditions including

biologically incompatible conditions. Therefore, "in vitro" display systems among other applications are particularly suitable for the directed molecular evolution and protein engineering.

Prokaryotic Systems

There are at least four types of prokaryotic display systems, including phage display, peptides-on-plasmid display, bacterial periplasmic display, and bacterial cell wall display. Phage display is the most commonly used method in display technology. Several extensive reviews on phage display technology are available [Rader and Barbas, 1997; Griffiths and Duncan, 1998; Rodi and Makowski, 1999]. The common feature for phage display technology is that the bacteriophage coat protein genes were used as a carrier to display foreign peptide sequence. Phage display technology was first introduced in the middle of the 80s by Smith using *Escherichia coli* filamentous bacteriophage M13 [Smith, 1985]. Although several alternative phage display systems, such as λ-phage, T4 and T7 phage [Santini et al., 1998; Ren and Black, 1998; Houshmand et al., 1999], have been developed for specific applications, M13 phage display is still the most robust method particularly applicable to small peptide and antibody engineering [Rader et al., 1998; Schmitz et al., 2000]. Applications of phage display technology in characterization of interactions between peptide ligands and ion-channel receptors have also been reported [Li, 1997].

Bacterial periplasmic display, also called periplasmic expression with cytometric screening (PECS) method, is a new alternative of phage display technology [Chen et al., 2001]. This approach takes advantage of the observation that under certain conditions bacteria permitmolecules as large as about 10-kDa to equilibrate through periplasmic space, but still retains the periplasmically localized bacterial proteins [Chen et al., 2001]. Thus, this system allows for the proteins, which are intrinsically incompatible with the bacteriophage display system, to be properly expressed. By using fluorescence conjugated ligands, the positive binding bacterial cells can be efficiently selected by fluorescence-activated cell sorting (FACS) approach without traditional panning and intermediate phage infection process.

The peptides-on-plasmid display comes from the application of controllable binding between *lac*I and the *lac* operator (*lac*O). The plasmid used in peptides-on-display contains a *lac*I open reading frame and also the DNA sequence of *lac*O [Cull et al., 1992]. In this system, the random peptides can be fused to the C-termini of *lac*I protein. After the protein synthesis in bacteria, the *lac*I fusion proteins will form a tetramer and specifically bind to the plasmid encoding itself. Herein, the genotype and phenotype are linked together. This display format has been successfully applied for the identification of binding partner for PDZ domains in PSD-95 and nNOS [Stricker et al., 1997; Stricker et al., 1999].

Polypeptides can also be displayed on the bacterial cell surface as a fusion protein [Georgiou et al., 1997]. Using this technology and fluorescence resonance energy transfer (FRET) substrate, they developed a high throughput approach to screen large libraries on the basis of catalytic turnover [Olsen et al., 2000].

Eukaryotic Systems

Prokaryotic display systems have been proven to be enormously useful for antibody engineering, detection of protein-ligand interactions, as well as directed protein evolution. The applicability to eukaryotic proteins has been limited because of several factors including differences in codon bias, folding apparatus, and post-translational machinery. With the large data set from human genome sequencing project, the high-throughput (HTP) identification of protein function becomes a major challenge in the post-genomic era. The development of eukaryotic display systems may greatly facilitate this effort.

Mammalian cell based cDNA expression cloning approach has been successfully applied in cDNA library screening to clone the cell surface receptor genes. This cloning method takes advantages of the abilities of cell surface expression of receptor genes, so that they can be easily detected by specific monoclonal antibody or ligands without affecting the viability or integrity of the cell. This approach needs several rounds of plasmid DNA transfection to mammalian cells, panning or sorting selection, and plasmids recovery from selected cells, till the positive clone is identified. In order to efficiently recover enough plasmids for each round of panning selection, the cell lines that support active replication of episomal plasmid DNA were often chosen for plasmid based expression

cloning. Several genes encoding for cell receptor or transmembrane protein have been cloned by this way, including brain injury-derived neurotrophic peptide (BINP) binding receptor [Hama et al., 2001] and CD34 [Simmons et al., 1992]. Although the cell based plasmid expression cloning method has been mainly applied in cloning protein genes localized on the cell surface, with the application of direct fluorescence-activated cell sorting of permeabilized cells, this method can also be applied for cDNA cloning of the intracellular antigens [Horst et al., 1991].

One of the major challenges for expression cloning is that the low efficiency of clonal delivery of cDNA library delivery into eukaryotic cells. This technical problem has somewhat restricted the complexicity of the expression cloning cDNA library. Furthermore, different from bacterial transformation, most of the current DNA transfection method can not guarantee one gene per cell. Therefore, it compromises the effectiveness of the subsequent genetic decoding process. Although bacteria protoplast fusion method or DNA electroporation can greatly reduce the number of different genes introduced into one cell, it is still relatively cumbersome comparing with phage display or bacteria based expression cloning method [Mougneau et al., 1995]. Yeast is a eukaryotic system, which in many ways shows high similarities to bacterial cells. Dane Wittrup's group [Boder and Wittrup, 1997] reported a yeast display system, where a foreign gene can be fused to the C-terminus of the membrane-associated alpha-agglutinin adhesion receptor (*Aga2*). Following the transformation of yeast, the *Aga2* gene fusion product can form two disulfide bonds to the Aga1 cell-wall protein. This yeast surface display has been used to engineer high affinity single chain T-cell receptors (TCRs) of nanomolar binding affinity [Holler et al., 2000].

The differences in a variety of aspects between the single cell organisms and mammalian cells remain to be a factor that limits the potential usage of this system for functional genomics. Viruses have evolved effective ways to overcome their trophic host defense for delivery of their own genes for expression. This has been inspiring the idea of using them for foreign gene display. Baculovirus is a well-known eukaryotic protein expression system for its robust protein expression level and high titers attainable. The work from Ian Jones' group has showed that Autographa californica Nuclear Polyhedrosis Virus (AcNPV) can be used to present distinct proteins on the viral surface [Boublik et al., 1995]. In this system, the AcNPV surface glycoprotein, gp64 was chosen as the carrier to present the foreign gene product. The genes encoding glutathione-S-transferase (GST) and HIV gp120 were successfully incorporated into amino terminus of gp64. Because of "in vivo" recombination processes required for generating recombinant baculoviruses, the system is more applicable to a specific gene display than library display.

The introduction of retroviral expression cloning approach has made the expression cloning to become a reliable approach for functional genomics. Comparing with the above-discussed cDNA mammalian cell based expression cloning methods, the retroviral expression cloning exhibits several special advantages. Firstly, the retrovirus can be stably introduced into a wide range of mammalian cells, including primary cell culture at nearly 100% efficiency. This feature would make the retrovirus expression system possible to clone surface protein genes that could not be cloned in COS cells or other large T-antigen containing cell lines, because of the presence of cross-reactive molecules or the absence of auxillary molecules on these cell lines [Kitamura et al., 1995]. Secondly, the gene expression is controlled in cellular environment with less cytopatholic effects, since the foreign genes are stably integrated into the chromosomal of the host cell line. Thirdly, the positive clones that express the desired surface protein can be enriched by several methods including FACS sorting and affinity panning. By using separate packaging cell line, the representation of the cDNA library can also be well preserved. With this highly effective transfection method, one can readily investigate protein functions in a HTP manner. For example, retroviral technology has been applied to map the signal sequence in the whole proteome of a cell. This approach was designated as signal sequence trap method by retrovirus-mediated expression screening (SST-REX). In a pilot experiment, a total of 150 known and 48 novel cDNA clones were successfully isolated, and all the known cDNA clones were found to encode secreted and cell-surface proteins [Kojima and Kitamura, 1999].

In addition to the successful application of retrovirus for functional genomic screening, several other previously developed virus vectors were also adapted for functional genomic analysis, especially those once used as vaccine vectors for expressing diverse antigens, such as adenovirus [He et al., 1998], reovirus [Roner and Joklik, 2001], poliovirus [Andino et al., 1994], and alphavirus [Koller et al., 2001]. Among these viruses systems, Sindbis virus, a member of the alphavirus class of single-stranded positive-polarity RNA in a protein-lipid coat, has been modified as a HTP expression cloning system for rapid identification of genes encoding a functional activity [Koller et al., 2001]. This newly developed alphavirus expression system shows several features that might have overcome several drawbacks in other expression cloning system. (1) With the self-duplication ability within the cytoplasmic compartment, high gene expression level can be easily obtained at low multiplicities of infection, leading to one gene per cell. (2) The number of primary clones from cDNA library can be generally $> 10^6$. Besides this, the cDNA library can be cloned using standard procedures and does not involve the in vivo homologous recombinantion process, which is the necessary case for DNA-viruses, such as vaccinia-, baculo-, or adenovirus [Wong et al., 1994; Granziero et al., 1997; He et al., 1998]. (3) The alphaviruses have broad range of host cells including insect, mammalian, or primary cell cultures. (4) The positive cells can be sorted by FACS and the infected cells contained a large number of recombinant virus RNA. Therefore, the selected positive cells can be directly proceeded for cDNA reverse-transcription and sequencing without subcloning. (5) The secreted, intracellular, and membrane proteins can be readily detected by an effective eukaryotic plaque-lift assay [Koller et al., 2001]. (6) Alphavirus vectors have been successfully used for expression of many topologically different proteins, such as nuclear, cytoplasmic, membrane-associated and secreted proteins [Liljestrom and Garoff, 1991], G protein-coupled receptors and ligand-gated ion channels [Lundstrom et al., 1995; Lundstrom, 2000].

Monitoring the Protein–Protein Interactions

Genome sequencing projects of different organisms have provided a great potential to unveil the complex biology. To decipher such information and put them in the appropriate biological context will be a major task. In eukaryotic cells, signaling pathways or a variety of intracellular processes were formulated through the interaction network of different biological molecules. Hence, the association of unknown gene products with known gene products will provide critical information useful for establishing the function of a gene. Yeast two-hybrid system is one of the well-established methods for identification of protein–protein interactions. Several excellent reviews about yeast two-hybrid system are available [Legrain and Selig, 2000; Walhout et al., 2000a]. Recently, yeast two-hybrid screening strategies have been applied to investigate the protein–protein interactions across the entire genomes [Uetz et al., 2000; Walhout et al., 2000b; Rain et al., 2001]. Because the yeast two-hybrid system requires nuclear transcription, there has been a need for a technique that can monitor the protein interactions in specific subcellular compartments native to where biological processes normally take place. Dihydrofolate reductase complementation assay (DHFR-PCA) and β-galactosidase complementation assay are two newly developed protein interaction detection systems that can monitor protein interaction in real time without requirement of nuclear localization. The general idea for these two systems is based on the same strategy that any reporter enzyme can be rationally dissected into two fragments and the fragments are fused to two test proteins. The binding of the test proteins to each other will bring together of the two separate fragments, thereby allowing for detection of the reconstituted enzyme activity [Michnick, 2001]. For DHFR-PCA, the selection criterion is the survival ability in the presence of the anti-folate drug trimethoprim. The improved system has shown high specificity for the detection of the protein–protein interactions [Pelletier et al., 1999]. Recent report has shown to use DHFR-PCA to map the biochemical pathways in living cells [Remy and Michnick, 2001; Subramaniam et al., 2001]. Another special feature for DHFR-PCA is that this technology can be used as a molecular ruler to measure the allosteric transitions in dimeric or multimeric protein interfaces. In addition, DHFR-PCA has been successfully applied to monitor the conformational change of erythropoietin receptor (EpoR) with or without ligand binding [Remy et al., 1999].

Comparing with other protein–protein interactions monitoring methods, the β-galactosidase based intracistronic complementation methodology shows several advantageous properties [Rossi et al., 2000]. (1) It works in live mammalian cells; (2) it provides a quantitative readout, allowing the monitoring of interaction kinetics. Thereafter, this method might be amenable to high throughput screening; (3) the β-galactosidase enzymatic reactions can amplify the signal without the requirement of overexpression. For example, when the FRAP and FKBP12 were fused with the β-galactosidase deletion mutants respectively, the rapamycin-induced chimeric FRAP/FKBP12 protein complex could be monitored in a time- and dose-dependent manner [Rossi et al., 1997]. 'Ubiquitin split protein sensor' (USPS) is another protein complementation method that has been demonstrated some special ability in monitoring the transient protein–protein interactions and protein conformational changes [Johnsson and Varshavsky, 1994; Raquet et al., 2001].

CONCLUSIONS AND FUTURE PERSPECTIVES

Biomolecular display technology was reported more than a decade ago. With the development of display technology, the complexity, diversity, and quality of the modularly coded biomolecules have improved enormously. "In vitro" display technologies showing the ability of forming extremely large size library without any biological selection bias have been specifically adapted in directed antibody and protein engineering. Different from the "in vitro" display technology, the primary objective for "in vivo" display technology is mainly focused on how to display every genetically coded biomolecule in its original and natural status. The introduction of virus-based expression cloning system has greatly facilitated both development and application. However, the potential cytopatholic effects accompanied with over-expression of foreign protein need to be optimized for functional genomic application. Due to the difficulty of functional expression of eukaryotic proteins, previous display technologies are restricted within the scope of directed protein evolution or functional cloning. The biological pathways and metabolic processes are consisted of a totally integrated the molecular interaction networks within living cells. Identification of protein–protein interactions will greatly help us dissect and understand these biological pathways. Several reported efforts have showed the power of using display technologies in monitoring protein–protein [Zozulya et al., 1999; King et al., 2000] or chemical–protein interactions [Sche et al., 1999]. Although the yeast two-hybrid system has been shown for the ability to display folded protein molecules for identification of protein–protein interactions in eukaryotic system, it is still an inconvenient way to functionally screen the protein–protein interactions of mammalian cell proteome. Now with the new improvements in display technologies, especially viral expression cloning system, it is possible to display and detect the protein–protein interactions of mammalian cell proteome in its original context.

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