Protein interaction maps using yeast two-hybrid assay

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With the availability of several completed genome sequences, the analyses of large-scale interactions among the proteins encoded by them become important to decipher the complexities of biological systems involving intricate networks of macromolecular interactions. Various approaches have been developed to generate systematic genome-wide protein interaction maps (PIMs), which provide functional information of many unknown proteins. Among many methods, the yeast two-hybrid (Y2H) system is popular because it is an *in vivo* and inexpensive technique and easily amenable for genome-wide application. Analyses of proteomes of *E. coli* virus T7, vaccinia virus, yeast *Saccharomyces cerevisiae*, human pathogen *Helicobactor pylori* and nematode *Caenorhabditis elegans* have been carried out using Y2H. The resulting PIMs have identified functions of many proteins and generated several new hypotheses for further experimentation.

PROTEIN–protein interactions are essential virtually in all cellular processes ranging from DNA replication, transcription and translation to signal transduction, cell-cycle control and intermediary metabolism. In most cases, the interactions among proteins are dynamic and the knowledge of assembly and disassembly of them over time in response to complex signals helps one to perceive the processes that make up life. The detailed characterization of macromolecular interactions will also help one to identify the defective pathways of tissues in pathological state and the infective mechanisms of pathogens. This knowledge will be useful in designing more effective therapeutic approaches.

Protein–protein interactions can usually be analysed using biochemical techniques like affinity and molecular size-based chromatography, affinity blotting, immunoprecipitation and cross-linking. As the reagents used in these methods are very specific in nature, the results obtained are quite convincing in most cases. However, these traditional tools fail to analyse large-scale protein interaction networks efficiently, which is a necessity when one tries to analyse various types of protein–protein interactions occurring in an organism throughout the life cycle. In fact, with the advent of time and with upcoming knowledge of more and more completed genome sequences of different organisms, it is tempting to generate protein interaction maps (PIMs) on a genome-wide scale. This endeavour definitely encompasses a large number of protein interactions, the study of which is virtually impossible with traditional biochemical tools. Hence many relatively new technologies like yeast two-hybrid (Y2H) assay system, large-scale immuno-precipitation or glutathione S-transferase pull-down to purify the protein complexes followed by mass spectrometry to identify the interacting protein partners[1–3] are becoming increasingly important to characterize and identify a huge number of protein interactions at a time. Among these techniques, Y2H is quite popular to detect pairwise interactions between proteins[1–4], since it is an inexpensive and *in vivo* technique.

This review gives an overview of the basic concept, methodologies and new developments of Y2H assay system. Among new developments, the contribution of this technique in the formation of genome-wide PIMs is covered in more details. Recent works done in this area have been discussed to give an extensive account of the comparatively new approach.

Yeast two-hybrid system

Some modular transcription activators have a separate DNA-binding domain (BD), which binds specifically to upstream activation sequences (UAS) of a target gene and an activation domain (AD), which induces the expression of the target gene[2,5]. The BD and AD of these transcription activators can be physically separated, keeping the respective function of the module intact. Using this interesting and useful property of transcription activators, Fields and Song[2] first made a suggestion that protein interaction could be detected if potential interactors were expressed as chimeras with AD and BD in budding yeast *Saccharomyces cerevisiae*. In their experiment, a yeast protein SNF1 is fused to the BD of yeast transcription activator GAL4 and another yeast protein SNF4 is fused to the AD of the same transcription activator. When both the hybrids are present in a single cell, the interaction between SNF1 and SNF4 allows AD to come in the vicinity of BD constituting an active transcription activator, which can easily be assayed by expression of some down-

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stream reporter gene. Based on this initial experiment, the cDNA of a protein of interest is cloned in an appropriate plasmid, which will express it as a BD fusion protein in yeast cells in a two-hybrid screening. This BD fusion protein is known as bait. Similarly, a prey is prepared by cloning the cDNA of another protein of interest in an appropriate plasmid, which will express it as an AD fusion protein in yeast cells. Then both the plasmids are co-transformed into yeast cells or they are separately transformed into haploid strains of opposite mating types after which they are mated to give rise to diploid yeast cells, where interactions between prey and bait may occur. The positive clones are identified by assaying the expression of the reporter genes (Figure 1).

Although initially designed to predict single protein–protein interactions, the Y2H system soon emerged to be a more dependable and consistent technique to predict protein interactions on a large-scale. In fact, the approach is more extensively used to screen a prey cDNA library with single bait, yielding a method to identify multiple interacting preys of a single bait protein. Recently, the method has been further extended to examine interactions among two separate pools of bait fusion proteins and prey fusion proteins[6] (discussed later). Hence, the most important fact about this methodology is its ability to scan a large number of protein interactions at a time, which is perhaps impossible to do with other classical biochemical or physical methods.

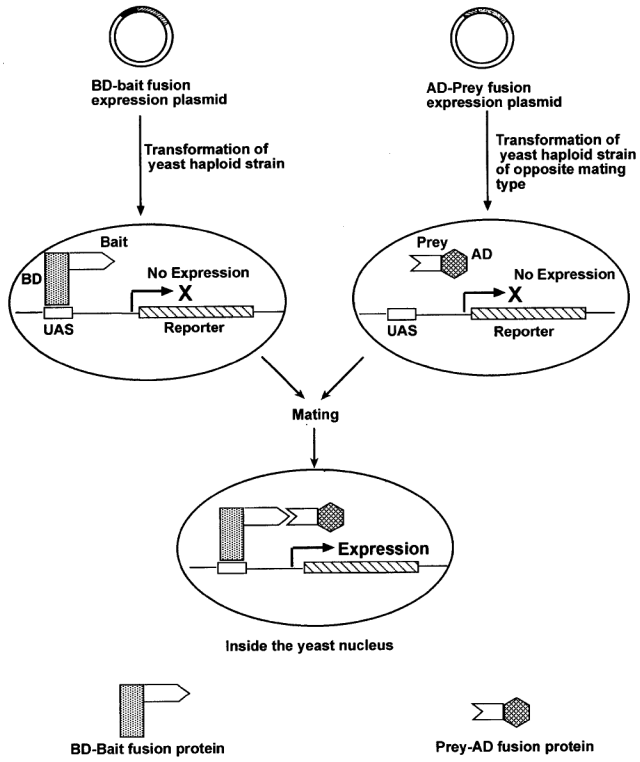


Figure 1. Overview of yeast two-hybrid assay system. BD, binding domain; AD, activation domain.

The most popular system used in two-hybrid assay technique is based on transcription activator GAL4 which is involved in expressing genes encoding enzymes required in galactose utilization in budding yeast, *S. cerevisiae*[7]. This factor consists of two separate domains – an N-terminal UAS-binding domain (BD) and a C-terminal transcription activation domain (AD), each of which can separately form fusion proteins with interactors. Another two-hybrid assay system is based on prokaryotic lexA protein, which normally functions as a repressor of SOS genes in *E. coli* by binding to lexA operator sequences. It was first reported by Brent and Ptashne[8] that the lexA protein when bound to upstream operator sequences of reporter genes in yeast, behaved as a DNA-binding domain only and did not act as a repressor anymore. On the other hand, GAL4 AD-fused prey protein when hybridized with this lexA BD-fused bait protein, constitutes a lexA-GAL4 hybrid which can act as a transcription activator in yeast. However, in lexA-based systems, the reporter gene that is to be induced must have an upstream lexA operator sequence for binding of lexA protein. In both the systems, multiple reporter genes are used to select the positive interactors.

The advantage of Y2H assay is that it is an eukaryotic *in vivo* technique. Unlike most of the biochemical approaches where high quantities of purified proteins or good quality antibodies are required, only a bait protein and a prey cDNA library are needed to initiate a screening in Y2H assay. Also, weak and transient interactions, often the most interesting ones in signalling cascades can be detected by this system[9]. One of the most appealing features of the system is that the identification of an interacting protein implies the cloning of at least part of the corresponding gene, which can be utilized subsequently in many other studies.

Even though there are many advantages, certain aspects should be considered to obtain reliable results from a two-hybrid screening. Since the system is based on reconstitution of a functional transcription activator, checking the autoactivation capacity of bait fusion is crucial for overall feasibility of the interaction assay. The use of artificially-made fusion proteins always embodies a potential risk of altered function of the bait and/or the prey due to incorrect conformation of the chimeras. This can be avoided in some possible cases by performing test interaction assay using known positive interactors of the respective proteins. Also, it is important to verify the interactions identified by Y2H using other biochemical approaches. Another requirement, apart from correct folding of the fusion proteins and inability of the bait to initiate autonomous transcription, is the ability of the fusion protein to get localized in the yeast nucleus. This is important because all transcription events are dedicated nuclear processes. It is found that GAL4 BD has its own nuclear localization signal (NLS) within its N-terminal 74 residues[10], but lexA being a bacterial protein contains no NLS. Therefore,

NLS of SV40 large T antigen is fused in frame with lexA to ensure its nuclear localization. The technique may not be suitable for interactions involving membrane proteins, because the membrane proteins may not be folded properly in the cytoplasm and nucleus due to the presence of a large number of hydrophobic patches.

But an important fact about the system is that the functional information is not lost during the assay unlike the other large-scale approaches, including 2D gel electrophoresis. Therefore the assay might render a functional clue about an interacting protein, if at least one of its interacting partners has a known function in an well-understood pathway. In some cases, the outcome of a screening often results in many new hypotheses and in generating preliminary idea about new undiscovered pathways, which can be validated by other methods.

The most convincing argument in favour of the two-hybrid interaction assay is the number and speed in which many biochemical pathways can be resolved in molecular details[11]. In fact, due to this advantage, the two-hybrid interaction assay is being extensively used nowadays to generate genome-wide PIMs of various organisms.

Genome-wide protein interaction maps – A new application of yeast two-hybrid system

With the evolution of Y2H technique during the past decade, application of different types like molecular dissection of known protein–protein interactions and the identification of new potential interacting partners became evident. However, with the availability of complete DNA sequences for many prokaryotic and eukaryotic genomes, it has become important to develop reliable genome-wide approaches for a better understanding of protein function. To fulfil this requirement, Y2H assay system has been upgraded in several ways so that it becomes appropriate for the establishment of large-scale PIMs of an organism. These maps may consist of many possible protein interactions that occur during the entire lifespan of a cell. Several types of information can be obtained from these PIMs, including revelation of functions of newly identified proteins, characterization of different signal transduction and metabolic pathways, identification of new drug targets and generation of insight in the complexity of a biological process. In short, it can be said that the time has come when one should look at all sorts of protein interactions taking place in order to get an overall view of living processes, rather than concentrating on a single interaction or a small subset of interactions at a time. Till now, genome-wide PIMs have been obtained for *E. coli* virus T7 (ref. 12), vaccinia virus[13], budding yeast *S. cerevisiae*[6,14,15], a human pathogen *H. pylori*[16] and *C. elegans*[17] using Y2H assay. The methods adopted to produce these interaction maps could be broadly classified into two types[18] – the matrix approach and the library screening approach.

Matrix approach

The matrix method was first described by Russel *et al.*[19] while exploring the interactions among *Drosophila* cell-cycle regulators. In their work, a number of individual protein–protein interactions among cyclin-dependent kinases and potential partners were tested. The results of these tests were then displayed as two-dimensional arrays, which were called 'interaction matrices'. After this first small-scale array experiment, the method was applied at a genome-wide scale to determine protein–protein interaction networks for yeast and some of the prokaryotic and eukaryotic viruses[6,12–17].

The first genome-wide two-hybrid study was carried out on bacteriophage T7. Bartel *et al.*[12] screened a library of BD fusions of T7 protein fragments against a random library of T7 AD fusions. Among the 55 phage proteins, they found 25 interactions, including four interactions that had been described previously.

Recently, two publications by Ito *et al.*[6] and Uetz *et al.*[14] have described Y2H-based large-scale approaches to detect genome-wide protein–protein interactions in *S. cerevisiae*. In the approach followed by Ito *et al.*[6], about 6000 possible yeast open reading frames (ORFs) were cloned individually as BD fusion baits in an yeast strain and as AD fusion preys in another yeast strain of opposite mating type. Subsequently, these two collections of BD and AD fusion clones were clustered separately into 65 pools, each containing 96 clones. Out of possible 4225 (65×65) mating reactions, the authors conducted 430 of them covering about 10% of all possible combinations. They selected the positive clones using four different reporter gene expressions to maintain a high selection pressure. Through the procedure, about 4×10^6 different combinations of interaction were examined, which account for about 10% of the total proteome analysis (4×10^7). As a result of this screening, 866 positive clones were obtained. Their analyses resulted in identification of 183 independent interactions, 12 of which were previously known. Furthermore, the result of these systematic Y2H screens enabled the authors to characterize many complex interaction networks, including the one that explained a previously unsolved mechanism for the connection between distinct steps of vesicular transport[6].

The work done by Uetz *et al.*[14] is the most extensive and systematic study of protein–protein interactions in yeast published so far. In their approach, the authors took two strategies – a time and labour-intensive one-by-one array approach and a high-throughput one.

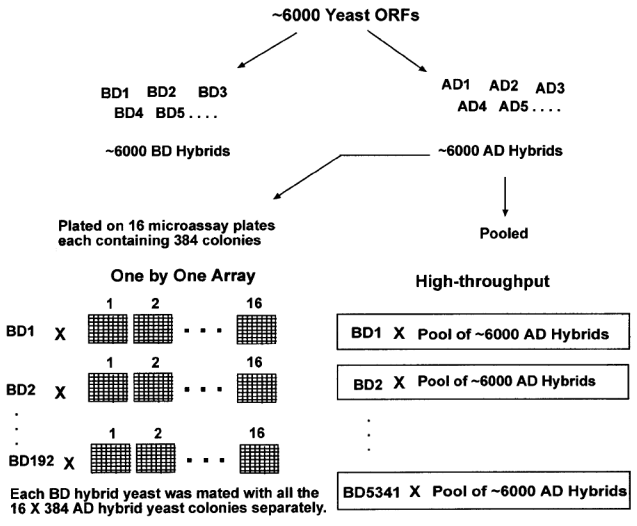
One-by-one array approach: In this method, about 6000 yeast transformants were generated, each containing one

possible ORF cloned into GAL4 activation domain vector to express the hybrid protein. The transformants were distributed among 16 micro-assay plates, each containing 384 colonies. Thus a total of 6144 (384 × 16) types of ORFs were expressed as AD fusion proteins (prey). Simultaneously, a set of 192 GAL4 BD hybrids (bait) was prepared in yeast of opposite mating types following similar steps.

Now, to screen for the positive interactions, individual BD hybrid was mated with all the transformants of the array (Figure 2). The diploids obtained after mating were selected by the reporter gene expression. The use of this array technique did not require further classification of the interactor proteins, because just by looking at the position of the array one could easily assign the prey and bait engaged in the interaction.

The range of positives in each mating was found to be from 1 to 30. However only 87 out of 192 baits used gave any reproducible positive interactions (i.e. reproduced also in a second screening). Out of all the positives tested, 281 interactions were found to be discrete, suggesting a mean value of 3 interactions per protein.

High-throughput approach: The array approach described above yielded definite results, but was very laborious and time-consuming. Hence, as an alternative, the authors had also described a high-throughput approach. In this approach, transformants containing about 6000 potential ORFs fused to GAL4 AD were pooled together to make a library. At the same time, a separate transformant containing each of about 6000 ORFs as GAL4 BD fusion proteins was prepared. To screen for positive interactions, individual BD hybrid transformant was mated with AD library and selected for the expression of the reporter genes. In this approach, out of 6144 potential budding yeast ORFs, 5345 (87%) could be successfully cloned into both plasmids (bait and prey). Out of this, 5341 were pooled together to form the AD library. 817 of yeast ORFs were found to give positive interactions, 692 of which were that due to discrete interacting protein pairs.



BD Tested	192	5341
AD Tested	6144	5341
Baits giving positive results	87	432
Discrete interacting protein pairs	281	692
Average interactors per baits	3.3	1.8

Figure 2. Overview of the matrix approach as performed by Uetz *et al.*[14].

Comparison of the two approaches: When the high-throughput approach is compared with the array method, it was observed that the two methods gave different experimental results. 45% of 192 proteins used as bait yielded interactions in the array method, but only 8% of 5345 potential ORFs yielded interaction in high throughput method (Figure 2). Some of the differences in the number of interactions may have resulted due to non-random choice of proteins for the array approach. Some categories of proteins like membrane proteins and metabolic enzymes are omitted during bait selection in the array approach, since they are less likely to yield interactors compared to proteins of signalling pathways. Apart from this, the application of stringent selection pressures in the case of high-throughput method may partially account for less number of positives. However, even after considering these points, one can say that the array method yields much more candidate interactors than the high-throughput method. Array method gave an average of 3.3 interacting partners per bait protein compared to 1.8 in case of high-throughput approach. In spite of this, the most important advantage of the high-throughput method over the array method is the involvement of less time, money and labour. The array method is systematic, but at the same time it is time-consuming and laborious, which severely limits the number of baits tested at a time (192 baits tested in array method compared to 5345 baits tested in high-throughput method). Thus, one can take recourse to the high-throughput approach for having a rough idea of probable interactions after which more rigorous studies can be done by the array method using selective baits/targets.

Altogether, both approaches resulted in 957 interaction pairs of which 109 were known before[14]. Results of the screen could place functionally unclassified proteins into appropriate pathways in the cellular context. As examples, identification of proteins involved in arginine metabolism and vacuolar protein transport could be mentioned. Insight into biological processes like RNA splicing had also been gained from the generated PIM.

Library screening approach

Y2H assay technique was primarily designed to detect a physical association between two known proteins in a cell. But with time, the technique rapidly became the most widely used method to screen libraries for identification of interacting protein pairs. Usually cDNA library is prepared in AD fusion vector and screened with a single BD fused bait in the interaction assay. This concept of library screening through Y2H assay technique is nowadays applied to obtain genomewide PIMs of prokaryotes and lower eukaryotes. In this case, instead of a cDNA library, a genomic DNA library is used. Since the library is generated by random fragmentation of genomic DNA, some of the identified prey interactors may contain intergenic, non-coding regions. To generate the final PIM, these nonsense interactors and other possible false positives are eliminated by adopting suitable statistical approaches.

This type of library approach was first adopted a few years ago by Fromont-Racine *et al.*[15] to generate a PIM of *S. cerevisiae*. The authors generated a highly complex library of random yeast genomic fragments consisting of about 5×10^6 clones. This was then fused to AD vectors to prepare a library of prey proteins and transformed into yeast haploid cells. In parallel, some selective baits were chosen and following the same procedure these were transformed into yeast haploid cells of opposite mating type. An efficient mating strategy between the prey library and each of the baits was followed. After this, positive diploid clones were selected and the prey fragments were characterized by sequencing. All positive prey candidates were classified according to distinct heuristic value and the prey candidates having higher heuristic values were chosen as baits for a second round of screening procedure. This procedure of screening the library with potential preys of the previous screens chosen as baits was repeated several times. In this particular attempt, the authors were able to characterize new interactions between known splicing factors, identify new yeast splicing factors and reveal novel potential functional links between cellular pathways.

The library approach was further examined in *C. elegans* starting with only 27 proteins involved in vulval development[17]. Though *C. elegans* is an organism with less complexity than higher eukaryotes, still the validity of the library screening approach in determining precise PIM was checked using this system. Also, most of the protein interactions involved in vulval development of the organism were previously reported and well-studied. Hence the result obtained from the library screening method could be verified using already known interaction pathways. Apart from this verification, the resulting map also revealed the existence of some new interactions, which could not be there due to false positives. With the help of this approach, functional annotation of approximately 100 uncharacterized gene products was possible. Hence it became obvious that the library screening approach can be a feasible method to generate PIM on a genome-wide scale.

Recently, the protein–protein interaction map of human gastric pathogen, *Helicobactor pylori*, has been published by Jean *et al.*[16] using the same library screening approach (Figure 3). They first constituted an activation domain fusion library of random genomic fragments of *H. pylori* strain 26695, whose whole sequence was previously known. This library was then introduced into yeast by transformation. In parallel, yeast transformants of opposite mating type were prepared with 261 different ORFs as BD fusion baits. After this, mating was done and positive clones were isolated. Prey fragments from these positive clones were then sequenced and identified by comparing with the genome database previously available.

With the help of bioinformatics tools, positive prey fragments (sequenced earlier) were clustered into families of overlapping fragments. The common sequence shared by these fragments is referred to as the selected interacting domain (SID). SID is basically defined as the smallest docking site of the prey protein required to interact with the bait. After clustering of the prey fragments into several SIDs, the ones which did not code for part of *H. pylori* ORF were discarded. Then for each remaining SID, the authors computed a statistical expected value (*E*-value) ranging from 0 (for a most probable interacting pair) to 1 (probable artifact). Through this kind of analysis, experimental artifacts, which give rise to false positives, were warded off and construction of PIM could be

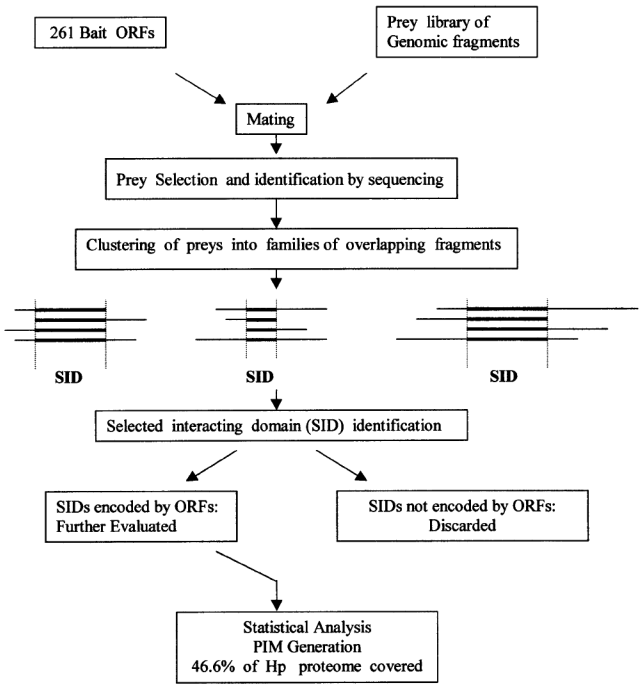


Figure 3. Outline of the strategy for building *H. pylori* proteome-wide interaction map.

done confidently. Thus in total, 46.6% of *H. pylori* proteome were connected through PIMs. The resulting PIM revealed many biological pathways and helped in prediction of functions of many proteins. A few examples include the components involved in chemotaxis, urease complex and DNA replication. The results also identified complexes in *H. pylori* that had been shown or postulated to be present in other bacteria like *E. coli*, validating the strategy adopted in the study.

Conclusion

The technology based on Y2H assay system revealing protein–protein interactions has already made a huge impact on basic and applied biological research. However, its highest achievement is in the production of genome-wide PIMs. As whole genome sequences of organisms ranging from prokaryotes to human are being added to the database, opportunities for Y2H assay system are increasing by leaps and bounds. Till date, attempts have been made to generate exhaustive PIMs of prokaryotes (e.g. *H. pylori*) and lower eukaryotes (e.g. *S. cerevisiae*). The comprehensive version of Y2H assay technique is also being applied to generate interaction maps in multicellular organism like *C. elegans*[20]. Certain aspects of higher eukaryotic genomes like huge number of gene products and profuse presence of introns between the coding sequences make the experiments more difficult. However, if analyses are done with cDNA libraries from mRNA pools of specific tissues or organs using random primers, then the number of expressed gene products reduces considerably. Such libraries can be used to perform extensive Y2H screens following the library screening method. Also, keeping in view the progress made in preparing intact ORFs and long-insert cDNA cloning technologies, it is quite possible that Y2H assay technique may serve as a prototype for studies of protein interactions in cells with much larger genomes. A recent announcement by a consortium of four biotechnology companies to generate extensive PIMs of human proteome using both Y2H and mass spectrometry methods, justifies the statement.

Recently, the Y2H system has been modified by several groups to enable genetic selection against a specific protein–protein interaction[21,22]. This modified system is called reverse two-hybrid technique and it makes use of yeast strains in which the interaction of hybrid proteins increases the expression of a counterselectable marker that is toxic under a particular condition. In this situation, the dissociation of interacting partners provides a selective advantage, facilitating detection. This system is useful in identification of mutant gene product affecting a particular protein–protein interaction, by screening pools of randomly-generated mutants. Most importantly, it enables the identification of proteins or small molecules responsible for dissociating a protein complex. As protein–protein interactions are becoming prospective drug targets, reverse two-hybrid assay can be applied to identify compounds inhibiting these interactions[23].

For various reasons, large-scale Y2H screens sometimes produce false positives. Few strategies are adopted to avoid false positives. In their exhaustive analyses of yeast proteomes, Uetz *et al.*[14] have carried out each screen in duplicate and considered only the reproducible interactors for further analyses. To reduce the occurrence of non-specific interactors, Serebriiskii *et al.*[24] have developed a dual-bait Y2H system where two unrelated baits are simultaneously used. Specific interaction with one bait will give rise to the activation of a set of reporter genes, but non-specific interactions, which usually give rise to false positives, will have greater probability to bind to both the baits, resulting in the activation of different sets of reporter genes. Use of such methods helps in avoiding false positives in Y2H screens.

With the development of high-throughput approaches for analysing proteomes like pull-down assays, mass spectrometry, etc. it is reasonable to question the strength of Y2H assay technique compared to the others. Mass spectrometry, with its recent innovations and extensions can now analyse and characterize a large number of proteins in a short duration with great precision. Also, this technology has been found to be useful in identification of proteins in a complex and in estimation of expression of a gene[25]. However, in spite of the development of this outstanding method to analyse the proteome, the Y2H assay technique remains the method of choice to interpret the protein networks funtionally[3]. This is primarily because Y2H assay is an *in vivo* technique and is a direct method to screen binary, ternary protein complexes and even weak interactions occurring in a living cell. The recent development of three-hybrid system to look for molecules mediating a particular protein–protein interaction can be mentioned in this context[26]. In contrast, mass spectrometry methods generally involve purification of protein complexes from living cells and subsequent identification of their components. Due to these reasons, mass spectrometry studies often require significant amount of purified proteins. Hence, the studies involving mass spectrometry have concentrated on relatively abundant complexes like ribosomes or spliceosomes[27, 28]. In addition, pairwise connectivity of protein components remains unclear by this analysis. Hence two-hybrid studies, which identify both weak and strong pairwise interactions in a living cell, can allow the reconstruction of higher resolution PIMs. Moreover, Y2H technique is less expensive and technically simpler to handle compared to mass spectrometry.

Using a proteomics workstation consisting of two-dimensional gel electrophoresis followed by mass spectrometry, one can separate and characterize specific proteins responsible for a particular pathological state of a

tissue or for an infective stage of a pathogen. However, due to the use of denaturing condition in gel electrophoresis, the information about the interacting partners is lost in this analysis. This information can easily be acquired by trying out Y2H screens using the particular proteins as baits. Hence, the techniques can be combined effectively to render proteome-wide information of a wide variety of organisms. In this context, we have to consider the fact that not every ORF is suitable for Y2H analysis. Hence other proteomics approaches like large-scale immunoprecipitation or glutathione S-transferase pull-down to purify protein complexes together with mass spectrometry to identify the interacting partners or any other strategies should be implemented as alternative approaches to generate near-complete interaction maps. Furthermore, the Y2H assay system needs to be supported to a large extent by bioinformatics tools, which are essential requirements in handling huge data sets and to extract some biologically relevant information from them. All things considered, Y2H assay system with all the advantages and new developments is definitely going to reveal interesting new frontiers of research in coordination with other functional and structural genomics approaches.

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