

# Detection of Protein-Protein Interactions Using Protein-Fragment Complementation Assays (PCA)

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**Abstract:** Protein-protein interactions play a central role in many cellular processes. Their characterisation is necessary in order to analyse these processes and for the functional identification of unknown proteins. Existing detection methods such as the yeast two-hybrid (Y2H) and tandem affinity purification (TAP) method provide a means to answer rapidly questions regarding protein-protein interactions, but have limitations which restrict their use to certain interaction networks; furthermore they provide little information regarding interaction localisation at the subcellular level. The development of protein-fragment complementation assays (PCA) employing a fluorescent reporter such as a member of the green fluorescent protein (GFP) family has led to a new method of interaction detection termed Bimolecular Fluorescent Complementation (BiFC). These assays have become important tools for understanding protein interactions and the development of whole genome interaction maps. BiFC assays have the advantages of very low background signal coupled with rapid detection of protein-protein interactions *in vivo* while also providing information regarding interaction compartmentalisation. Modified forms of the assay such as the use of combinations of spectral variants of GFP have allowed simultaneous visualisation of multiple competing interactions *in vivo*. Advantages and disadvantages of the method are discussed in the context of other fluorescence-based interaction monitoring techniques.

**Key Words:** Protein-protein interactions, green fluorescent protein, protein complementation assay, bimolecular fluorescent complementation, EGFP, two hybrid screen.

## INTRODUCTION

Many processes in living cells are controlled by the formation of stable or transient protein-protein interactions; these include transcription, translation, signal transduction and cell division. The ability to identify these interactions is crucial for the characterisation of such cellular processes. Furthermore it has been shown that data gained from protein interaction studies can also be used to determine functions of unknown proteins (Schwikowski *et al.*, 2000). There are many methods currently in use for the detection of protein interactions with new methods continually being developed (Piehler, 2005). Major existing methods, for *in vivo* detection of interactions, include the yeast two hybrid (Y2H), tandem affinity purification (TAP) tagging, fluorescence energy transfer (FRET) and more recently protein-fragment complementation assays (PCA) (Reviewed by Figeys, 2003; Meng *et al.*, 2005).

## YEAST TWO HYBRID (Y2H)

Designed and developed in 1989 by Fields and Song, the original yeast two-hybrid system detects the interaction between two proteins tagged to functionally essential domains of the yeast transcriptional factor, Gal4p. Gal4p contains an N-terminal DNA binding domain (DBD) and a C-terminal transcription activation domain (TAD) (Fields and Song, 1989). The DBD of Gal4p is fused to 'bait' protein X and

TAD fused to 'prey' protein Y. Alone TAD is inactive; however association with DBD upon X and Y interaction restores Gal4p activity (Fields and Song, 1989).

Since its invention the Y2H system has been used extensively to detect protein-protein interactions. The yeast two hybrid technology is a powerful and broadly applied system that has since been implemented in studies of interactions within yeast (Uetz *et al.*, 2000; Ito *et al.*, 2001), bacteria (McCraith *et al.*, 2000; Joung *et al.*, 2000) and the fruit fly (Formstecher *et al.*, 2006). It has the advantage of detecting interactions *in vivo* (McAllister-Henn *et al.*, 1999) in a simple and sensitive manner. A series of adaptations of the original system (Chien *et al.*, 1991; Cagney *et al.*, 2000; Uetz, 2001) has allowed for the wide-scale analysis of whole libraries of interactions in yeast permitting the investigation of a higher level of proteome organisation and the assembly of interaction network maps (Mayer and Heiter, 2000). The Y2H system has progressed rapidly over the years to become perhaps the most widely used interaction detection system. Despite its popularity with molecular biologists, the Y2H system has several limitations.

Split domains of a transcriptional factor, such as Gal4p may be the standard choice for studying protein interactions (Immink and Angenent, 2002). However Ma and Ptashne (1987), found that random 'bait' sequences when fused to the Gal4p DNA-binding domain were enough to activate the transcription factor without the need for an interaction with the activation domain or associated 'prey' protein. Autoactivation of the transcription factor gives rise to the generation of 'false positives', perhaps the most common problem with

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the Y2H system. False positives are brought about by interactions of a non-biological origin, which occur independently of any physiologically relevant protein-protein interaction; that is those which only occur in the context of the screen, and would not normally occur under normal, non-Y2H, physiological conditions (Semple *et al.*, 2002; Droit *et al.*, 2005; Fields, 2005).

The use of a transcription factor also restricts protein interactions to the nucleus (Von-Mering *et al.*, 2002). Therefore many proteins are not in their native compartments or recognized physiological environment and no information regarding interaction localization can be deduced. This shortcoming of the Y2H also makes membrane and membrane-associated proteins which account for 30% of the proteome (Piehler, 2005) inaccessible to the standard version of the screen (McAllister-Henn *et al.*, 1999).

There are other problems associated with the Y2H screen. The original system cannot detect interactions between three or more proteins at any one time; some interactions therefore may be missed if a third 'bridge' protein is involved. An adapted system developed by Zhang and Lauter (1996), overcame this problem and allowed for detection of interactions within a ternary complex. The two proteins are fused to DBD and TAD domains while the third protein is fused to a nuclear localisation signal. This modification also carries its own problems such as the detection of interactions that don't require the third protein (Causier and Davies, 2002). Furthermore the third protein may promote or inhibit formation of the two-hybrid interaction complex (Drees, 1999).

A chief concern when choosing any approach to explore an interactome is its reliability. Two large Y2H screens carried out in 2000 to analyse the *Saccharomyces cerevisiae* proteome resulted in remarkably different sets of data, which in turn triggered a considerable amount of criticism. A screen carried out by Ito *et al.* (2000) generated 842 interactions of high relevance, while a similar screen by Uetz *et al.* (2000), resulted in 641 interactions of which only 141 overlapped with the former study. However before being too dubious about the method one must bear in mind its value in answering the fundamental question, 'does A interact with B?' Its ability to do just that has allowed the yeast two hybrid screen to remain a strong favourite with molecular biologists.

### TANDEM AFFINITY PURIFICATION (TAP)

First described by Rigaut *et al.*, 1999, the TAP tagging method is based on affinity purification of a protein of interest and its associated interacting partners. The original TAP method utilized two tags – the immunoglobulin (IgG) binding domains of *Staphylococcus aureus* protein A and a calmodulin binding peptide (CBP). In the sequence joining the tags is a tobacco etch virus (TEV) protease cleavage site. The gene encoding the protein of interest is fused to a sequence encoding the TAP tag. Tagged proteins are expressed at native levels under normal physiological conditions. Interacting proteins form a complex which is then subject to a two-step affinity purification under non-denaturing conditions. The first purification step involves the binding of the *S. aureus* protein A to an IgG matrix. Incubation with TEV

protease cleaves the tag and elutes the bound protein complex. The second purification step is carried out with calmodulin agarose beads which remove contaminants and remaining protease. Finally the protein complex is eluted using a calcium ion chelating agent such as EGTA. Recovered complexes are then separated by SDS-PAGE and analysed by mass spectrometry (Puig *et al.*, 2001; Bauer and Kuster, 2003). Variations of TAP have since been developed using successive rounds of purifications with several other tags. This 'multiple affinity purification' or MAFT produces even purer preparations of protein complexes (Honey *et al.*, 2001).

As well as having the advantages of allowing the detection of interactions at their native levels (Suter *et al.*, 2006) the TAP method also permits purification of very large complexes - as shown in comprehensive yeast proteome studies by Gavin *et al.* (2002) and Ho *et al.*, (2002) and Krogan *et al.*, (2006). In the study by Gavin *et al.* (2002), 78% of 589 tagged proteins were purified along with associated partners, whilst the remaining 22% were unable to be purified or identified. Gavin *et al.* (2002) deduced that the tagging can impair protein function or localization and interfere with complex formation due to the 20 kDa tag size. In a similar study by Ho *et al.* (2002), 493 complexes were purified, of which 93 overlapped with the Gavin study, 48 (52%) of these complexes contained interacting partners detected by both groups and the remaining 45 (48%) complexes showed no similarities. The study by Krogan *et al.* (2006) detected over 7,000 interactions involving 2,708 proteins. Each protein complex was prepared using two independent methods and analysed by mass spectrometry. The need for mass spectrometry to analyse the complexes retrieved by affinity purification biases the results found in the studies towards long lived and stable complexes (Ito *et al.*, 2002). Krogan *et al.* (2006) describe the results from the study as 'a snapshot of interactions and complexes in that particular yeast strain subjected to particular growth conditions'. Some complexes may not have been present under those given conditions and some less stable or loosely-associated complexes or those with short life times may not have endured such time consuming purification steps.

Like the yeast two hybrid system, the original TAP method designed for yeast has seen many refinements allowing its use to study interactions within bacteria (Escuela *et al.*, 2006), plants (Rohila *et al.*, 2004, 2006; Rubio *et al.*, 2005) and mammalian cells (Knuesel *et al.*, 2003; Li *et al.*, 2004).

The TAP tag system may well be the most accurate and efficient system for the detection of multiprotein complexes (Von-Mering *et al.*, 2002). However it requires much experimental effort and extensive data analysis for each detected interaction complex and cannot detect transient interactions. It also provides no information regarding interaction localisation.

### FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

FRET is a biophysical phenomenon in which energy is transferred between two fluorescent molecules. For this transfer to take place, the molecules must be close in space

(less than the so-called Förster distance of 5-6 nm), correctly orientated and the emission spectrum of one molecule must overlap with the absorption spectrum of the other (Förster, 1959). Typically, the system consists of a mixture of fluorescent molecules which is irradiated with light at the lower of the two absorption wavelengths (ie the higher energy). If the molecules do not interact, then the emission spectrum will be characteristic of the irradiated molecule (the donor). However, if they do interact then the emission maximum will match that of the second molecule (the acceptor). Note that the process involves radiationless transfer of energy between the two molecules rather than emission by the donor and reabsorption by the acceptor.

FRET has been exploited in *in vitro* studies of protein-protein interactions for many years. Generally this has been achieved by labelling two potentially interacting proteins with different fluorescent probes with overlapping absorption and emission spectra. Careful measurement of the efficiency of transfer can lead to the computation of molecular distances and the orientations between molecules (for examples see Trayer and Trayer, 1988; Soumillion *et al.*, 1998). In more recent years, FRET has been developed as a tool for monitoring protein-protein interactions *in vivo*. This has been largely driven by the discovery of green fluorescent protein (GFP, see below) and the engineering of spectral variants of this molecule. Genetic manipulation techniques can be used to direct the expression of potentially interacting proteins fused to variants of GFP (reviewed by Hailey *et al.*, 2002). Live cells expressing these proteins can then be examined either under a fluorescence microscope or in a fluorimeter. The occurrence of FRET between the GFP variants is strong evidence for the close association of (although not, necessarily, the direct interaction between) the two proteins. The technique has been successfully applied in bacterial, fungal, plant and animal cells (for example: Sourjik and Berg, 2004; Warren *et al.*, 2002; Adjobo-Hermans *et al.*, 2006; Amiri *et al.*, 2003). It is particularly powerful when used in organisms where direct manipulation of the genome is possible. For example, in *S. cerevisiae* it is possible to insert the coding sequences for GFP variants immediately 3' to the genes of interest so that the fusion protein is expressed from its wild type promoter sequences (Sheff and Thorn, 2004). As in TAP-tagging in this organism (see above), this strategy reduces the possibilities of artefacts due to altered expression levels of the protein compared to wild type. *In vivo* FRET offers advantages over Y2H and TAP-tagging in that it is potentially applicable in any cellular compartment and it monitors interactions in real-time. An advantage of GFP-based FRET is that the fluorescence signal is generated immediately on association of the tagged proteins (Heim *et al.*, 1994). However, there is a risk of false-negatives if the two fluorescent tags attached to interacting proteins are positioned beyond the Förster distance. False positives can arise if two proteins are part of a larger complex, but do not directly interact. Of course, this risk applies to many different methods of protein-protein interaction detection *in vivo*.

### PROTEIN-FRAGMENT COMPLEMENTATION ASSAYS (PCA)

Protein-fragment complementation assays (PCA) were implemented as a means to detect interactions of biological

relevance in intact living cells (Michnick, 2003; Remy and Michnick, 2004a). The underlying principle of a PCA is that a protein, quite often an enzyme or fluorescent protein, is split into two fragments which cannot function alone (Michnick, 2003; Remy and Michnick, 2004a). These fragments are fused to potentially interacting protein partners and complementation upon interaction leads to restored function, detected either by reconstituted enzyme activity or fluorescence. Protein-fragment complementation assays benefit from the co-operative, all or nothing, nature of protein folding (Michnick, 2003). Polypeptide sequences encode all the necessary information required for protein folding (Anfinsen *et al.*, 1961); therefore when split polypeptide fragments come together, between them they contain enough information to drive protein folding. Reconstitution of these fragments can therefore be assisted by interacting proteins.

Complementation of proteins and peptides has long been employed in molecular biology since the introduction of  $\beta$ -galactosidase ( $\beta$ -Gal) complementation by Ullmann *et al.* (1965) more than 40 years ago. Designed as a reporter system for cloning in *E. coli*, the original protocol involves two inactive mutant proteins with deletions within separate domains essential for activity. This type of complementation, termed  $\alpha$ - or intracistronic complementation, is due to two interacting mutant proteins which are specified by the same cistron. In the case of  $\beta$ -galactosidase, it is the *lacZ* gene (Eglen and Singh, 2003). Enzyme activity can be restored and detected by sharing active domains brought about by protein fragment complementation (Ullman, 1992; Rossi *et al.*, 1997; Eglen and Singh, 2003). Having been originally developed for use in *E. coli*, it has been shown to act as a marker for *in vitro* studies in yeast (Abbus-Terki and Picard, 1999). The *lacZ* gene is also extensively used to detect successful DNA insertion during gene cloning using the widely used blue-white colony screening assay (Ullman *et al.*, 1967). Modified forms of the original screen have brought in to play the use of split  $\beta$ -galactosidase enzyme to detect protein-protein interactions (Rossi *et al.*, 1997, 2000). The introduction of the split  $\beta$ -Gal fragment complementation assay in detecting protein-protein interactions launched the implementation of other split proteins that could be used in similar assays.

Although the Y2H screen shares some similarities with PCAs, it is not strictly one as it requires the reconstitution of separate, structurally undefined subunits of a transcription factor. The various limitations of this screen as discussed earlier sparked an interest in extending the complementation approach to other proteins. The first true protein-fragment complementation assay was the split-ubiquitin screen (Johnsson and Varshavsky, 1994). The range of proteins used has expanded considerably since then to include  $\beta$ -lactamase (Galarneau *et al.*, 2002; Wehrman *et al.*, 2002), luciferase (Kaihara *et al.*, 2003), dihydrofolate reductase (DHFR) (Pelletier and Michnick, 1997; Pelletier *et al.*, 1998; Remy and Michnick, 1999), green fluorescent protein (GFP) (Ghosh *et al.*, 2000; Park and Raines, 2000; Wilson *et al.*, 2004; Magliery *et al.*, 2005; Magliery and Regan, 2006) and its associated variants such as yellow fluorescent protein (YFP) (Westwick and Michnick, 2005). The key features of each are illustrated in Fig. (1).

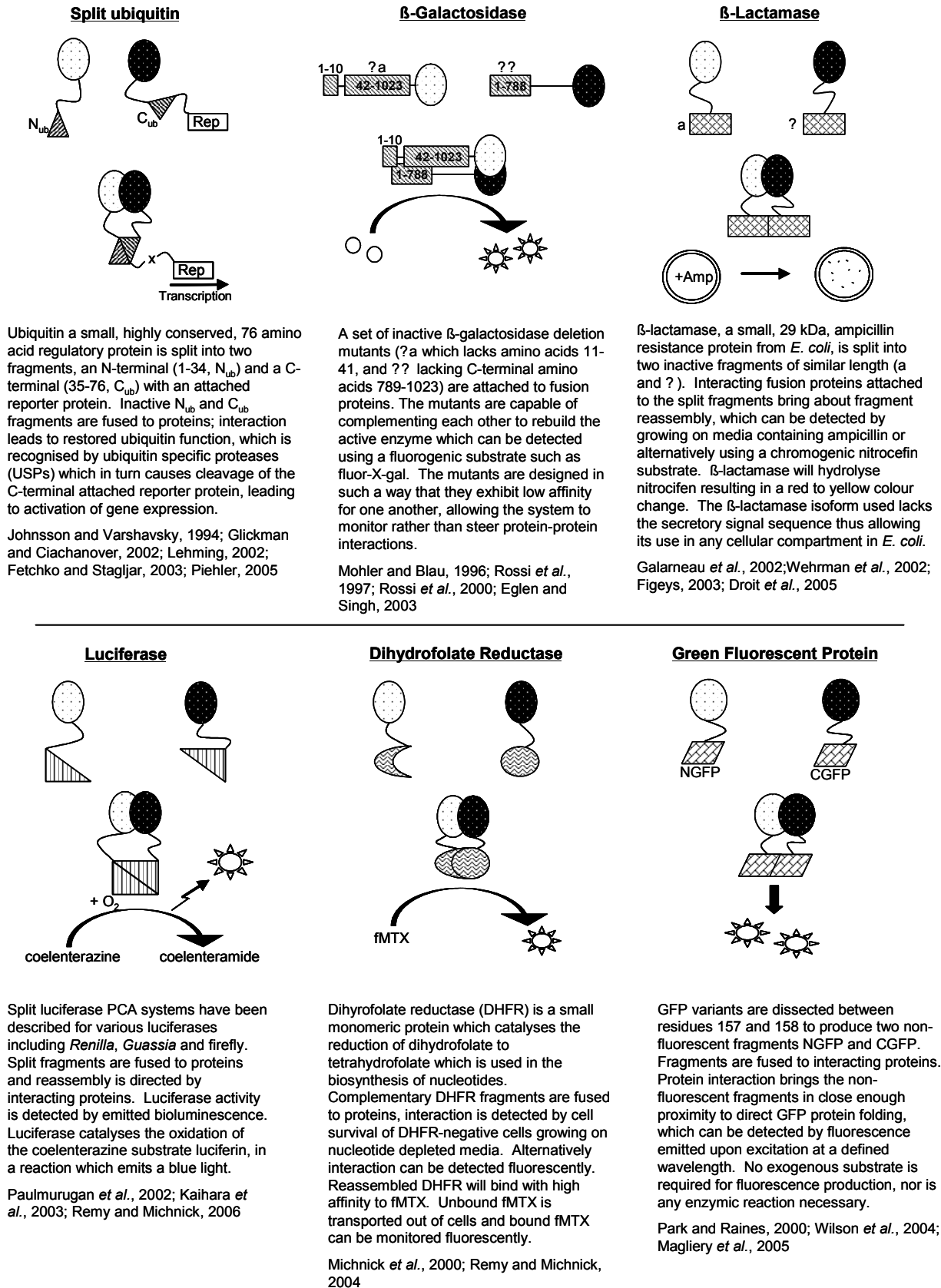


Fig. (1). Different proteins implicated in protein-fragment complementation assays and their characteristic features.

PCAs have many advantages over previously described technologies. The ever-increasing choice of enzymes and proteins implemented in the screen provide many benefits when choosing a PCA over other methods for detecting protein-protein interactions. The split ubiquitin assay has been shown to be successful at identifying membrane protein interactions (Stagljar *et al.*, 1998; Miller *et al.*, 2005), and modifications of the system have been applied to allow for large-scale identification of membrane and membrane associated protein interactions in the yeast *S. cerevisiae* (Miller *et al.*, 2005). The split  $\beta$ -galactosidase assay has several advantages: as the reconstitution occurs with low efficiency (Eglen, 2002), protein interaction is necessary to direct enzyme reassembly; the system is based on an enzymatic reaction and so the signal is amplified providing an extremely sensitive assay (Eglen and Singh, 2003); the assay also allows for the detection of interactions, including those between membrane and membrane associated proteins, in the cellular location in which they occur (Rossi *et al.*, 2000). Like the  $\beta$ -galactosidase assay, the  $\beta$ -lactamase assay also exhibits signal amplification. However  $\beta$ -lactamase as an assay protein has the added advantage of having no orthologues in eukaryotic and most prokaryotic cells (Galarneau *et al.*, 2002), thus reducing background signal and having immense potential as an assay to detect interactions in mammalian cells (Droit *et al.*, 2005). The *Renilla* luciferase assay has the advantage of emitting a bioluminescence signal only at the sites and times of interaction occurrence in cells and the protein is easily expressed in mammalian cells (Kaihara *et al.*, 2003). The dihydrofolate reductase and GFP assay systems are also capable of interaction localisation determination (Remy and Michnick, 2004a).

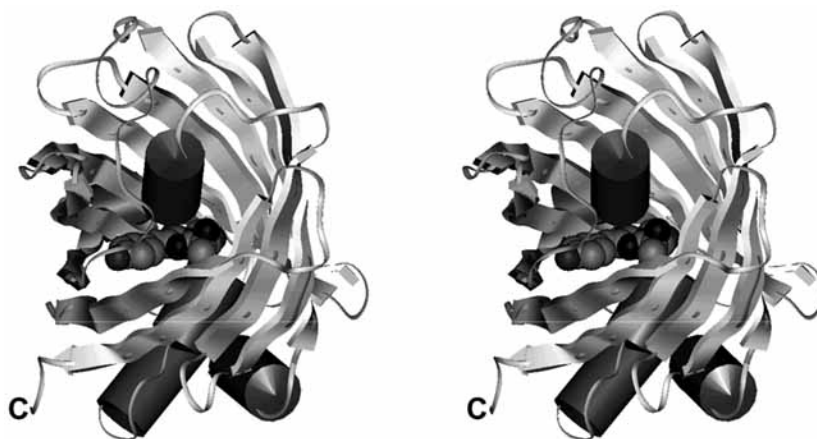
When choosing which protein to employ when developing a protein-fragment complementation assays, there are certain factors one must take into consideration. A key feature of PCAs is that the split fragments do not fold spontaneously; therefore knowledge of the protein of choice is required for rational design of fragments. A common feature of proteins implemented in PCAs is the small monomeric nature of the protein to be split. Proteins must also be well characterised with a wealth of structural and functional in-

formation available. Different proteins can be implemented in different screens; enzymatic proteins give an amplified signal which can be beneficial for detecting weak interactions, whereas fluorescent proteins can give interaction localisation information. However, the most popular choice for PCAs is the use of fluorescent proteins requiring no exogenous fluorogenic or enzymatic substrate for fluorescence, such as that of Green Fluorescent Protein (GFP).

## GREEN FLUORESCENT PROTEIN

GFP is a small protein of 238 amino acids (Prasher *et al.*, 1992) isolated from coelenterates such as the jellyfish *Aequorea victoria*. Discovered by Shimomura *et al.* in 1962, GFP has the ability to fluoresce spontaneously without the need for additional enzymes or cofactors (Chalfie, 1995). GFP owes its fluorescence to its intrinsic chromophore formed from cyclisation and oxidation reactions involving three amino acids; Ser65, Tyr66 and Gly67, found in the  $\alpha$ -helix which is buried in the centre of the characteristic cylindrical can shape made up of 11  $\beta$ -strands (Fig. 2), (Cody *et al.*, 1993; Yang *et al.*, 1996). Mature GFP exhibits great stability. It retains fluorescence at temperatures of up to 65°C and up to pH 11, and it resists proteolytic digestion for hours (Ward and Bokman, 1982; Cubitt *et al.*, 1995). This stability is maybe due to the protection of the central chromophore provided by the  $\beta$ -strands (Ormo *et al.*, 1996; Yang *et al.*, 1996).

The cloning of the *Aequorea* GFP gene in 1992 by Prasher *et al.*, led to multiple genetic manipulation attempts to enhance and alter the fluorescent properties of the protein. Wild type GFP has an excitation peak at 395nm and also a minor peak at 475nm. The emission peak is at 508 nm. Spectral variants caused by mutations have led to the generation of different colours of fluorescent proteins. The Y66W mutation gives rise to a shifted wavelength generating a blue fluorescent protein (BFP) (Heim *et al.*, 1994), allowing the protein to be used along with GFP in multicolour analysis. The blue variant was not as bright as GFP, a problem that was solved when a second mutation of BFP, Y145F, resulted in a blue fluorescent protein with almost twice the brightness of



**Fig. (2).** Stereodiagram of GFP. Antiparallel sheets are shown as ribbons and helices shown as cylinders. The fluorophore is depicted in space-filling format. C denotes the carboxy terminus of the crystal structure (1w7s) (van Thor *et al.*, 2005) – residue 231 in this case. The amino terminus is obscured at the bottom of the  $\beta$ -can structure in this view.

the single mutant (Heim and Tsien, 1996). After the generation of BFP alternative fluorescent proteins were developed, including cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (Patterson *et al.*, 2001; Lippincott-Schwartz and Patterson, 2003). Efforts to shift the wavelengths of GFP to produce a red fluorescent protein have been futile. However a red fluorescent protein from *Discosoma*, described as a GFP homologue (Wall *et al.*, 2000) has extended the range of fluorescent proteins consequently broadening the applications of the GFP-family.

Mutagenesis experiments have also generated some variants that enhance the properties of wild type GFP. As *A. Victoria* lives in the sea, wild type GFP has evolved to fold and mature at temperatures around 25°C. However, most applications require it to fold at 37°C. Improved folding at this temperature is often at the expense of efficient maturation (Tsien, 1996) and so wildtype GFP can take several hours to fold. Several mutations have been identified which help overcome this problem. The S65T mutant produces the fluorophore four times faster than wild type and also increases photostability of the protein (Heim *et al.*, 1995). Enhanced GFP (EGFP) is commonly used in most GFP applications; the F64L-S65T double mutant exhibits a 35-fold increase in fluorescence and is more resistant to photobleaching when compared to wild type (Cormack *et al.*, 1996). More recently a superfolder GFP has been developed which is more resistant to denaturants and has superior folding kinetics (Pedelacq *et al.*, 2006). An S30R mutation was considered responsible for the improved folding and robustness of the GFP variant (Pedelacq *et al.*, 2006). Superfolder GFP fusions are more soluble than wildtype or enhanced GFP fusions, increasing the range of proteins that can be fluorescently labelled (Pedelacq *et al.*, 2006).

The cloning of the GFP gene also opened many avenues for its exploitation - including protein tagging and localisation (Feilmeier *et al.*, 2000) and as a marker of gene expression (Gerdes and Kaether, 1996; Misteli and Spector, 1997). GFP has also been shown to act as a reporter for protein-protein interactions using fluorescence resonance energy transfer (FRET), a radiationless energy transition between donor and acceptor fluorophores (Selvin, 2000; Jares-Erijman and Jovin, 2003), and more recently its application in protein-fragment complementation assays (Wilson *et al.*, 2004; Magliery *et al.*, 2005) also known as bimolecular fluorescence complementation (BiFC).

## **BIMOLECULAR FLUORESCENT COMPLEMENTATION (BiFC)**

BiFC assays exploit the reconstitution of fragmented GFP, and its spectral variants, to screen protein-protein interactions. The split fragments alone are non-fluorescent. However when fused to interacting partners the fragments reassemble, autocatalytically generating the fluorophore. The direct readout of fluorescence emitted from the reassembly of non-fluorescent GFP fragments coupled with intrinsic fluorescence of GFP has made it an ideal candidate in BiFC analysis. Furthermore GFP can be expressed and mature in many cell types, including bacterial, mammalian and nematode cells (Magleiry and Regan, 2006). The potential use of split GFP to detect protein protein interactions was first de-

scribed by Ghosh *et al.* in 2000. In this study GFP was split between residues 157 and 158 to form N-terminal and C-terminal fragments denoted NGFP and CGFP respectively. Antiparallel leucine zippers were designed and attached *via* a linker sequence to the NGFP and CGFP fragments. A key requirement in BiFC design is that fragments should not recombine independently of the protein interaction, nor should they drive the interaction forward. Cotransformation experiments by Ghosh *et al.* (2000), demonstrated the requirement for peptide zippers to guide reassembly of GFP, suggesting that fragments alone were not enough to produce fluorescence. This system was shown to work *in vitro* and in *E coli* cells. Following this, Hu *et al.*, (2002), demonstrated a similar system for the detection of interactions in mammalian cells, using transcription factors from the bZIP and Rel families. In their study non-fluorescent YFP fragments were covalently linked to C-terminal ends of Fos and Jun. When expressed alone, no chimeric proteins exhibited fluorescence, but on co-expression of the two chimeric proteins, fluorescence was detected. Further investigations by Hu *et al.* (2002) demonstrated that the interactions between Fos and Jun were not driven by YFP reassembly. Alternative interaction partners were able to compete with the chimeric proteins and prevent fluorophore formation. However YFP reassembly did result in enhanced stability of protein complexes, making them more resistant to competing interactions. This characteristic of the BiFC system allows for the detection of weakly associated and short-lived interactions. The sensitivity of the GFP screen was also shown by the construction of a group of antiparallel leucine zippers with different affinities for each other and it was found that the screen can detect interactions between weakly associating peptides, with a  $K_D$  of approximately 1 mM (Magleiry *et al.*, 2005). However this same characteristic of trapping transient interactions prevents the use of BiFC in detecting interactions as and when they occur, due to the irreversibility of the formed fluorophore (Kerppola, 2006). Also upon GFP/YFP reassembly the stabilised interaction is no longer subject to displacement by competing proteins which affects the compartmental complex equilibrium (Hu *et al.*, 2002; Kerppola, 2006).

Studies by Ghosh *et al.* (2000), Hu *et al.* (2002) and Magliery *et al.* (2005) all employ the use of a short glycine-rich peptide linker sequence between proteins to be investigated and the split GFP fragment. The purpose of the linker is to allow a degree of flexibility so that the fragments can be correctly orientated, in order to reconstitute fluorescence. Magliery *et al.*, (2005) tested various linker lengths ranging from 4-15 amino acids; they concluded that linker length had little bearing on the reassembly reaction of GFP fragments. Linkers of varied lengths were tested with each other and it was found that reassembly will still occur if one linker is even 7 amino acids longer than the other. As linkers are used in BiFC analysis, little structural information of interacting proteins is required. In theory, the association between fragments can still occur regardless of their orientation. Furthermore peptide linker sequences can also overcome the problems of steric hindrance that may prevent GFP fragment reconstitution (Kerppola, 2006).

Other groups have developed an alternative split GFP screen for detection of protein protein interactions that in-

volve the splicing of an intein. In their original splicing screen Ozawa *et al.* (2000), exploit the post-translational splicing event of an intein sequence of the yeast *S. cerevisiae*, termed VMA1. N- and C- terminal fragments of the VMA1 intein sequence are fused to N- and C-terminal fragments of GFP respectively. Each fusion protein is attached to two potentially interacting proteins. Upon protein interaction the inteins are brought close together and undergo folding which induces the splicing event, subsequently leaving the GFP fragments covalently attached to one another, ultimately resulting in fluorescence. The original screen which suffered from weak fluorescence was later improved by replacing the VMA1 intein with a smaller intein, dnaE, from bacteria (Ozawa *et al.*, 2001). This modification resulted in a much faster system, taking four hours for fluorescence formation as compared to three days for the VMA1 system.

Since its introduction the BiFC assay has undergone modifications and improvements to broaden its application. Hu and Kerppola (2003) describe the application of BiFC for simultaneous visualisation of protein interactions by employing a variety of split fluorescent variants of GFP. The multicolour complementation analysis utilises spectrally different fragments for analysis of protein complexes and interactions within the same cell. A combination of YFP, BFP, CFP and GFP fragments were fused to domains of transcriptional proteins Fos and Jun. Fragments of different fluorescent proteins did not affect the dimerization between the proteins, and complementation efficiencies of different fluorescent proteins were similar, thus allowing multicolour BiFC to be used to investigate and compare efficiencies of interactions between competing and multiple interacting proteins (Hu and Kerppola, 2003).

Further mutational studies were done on yellow fluorescent protein to enhance its use in BiFC screens. Griesbeck *et al.* (2001) modified YFP with a single Q69M point mutation. The mutated protein which they called Citrine, has better expression in *E. coli* cells at 37°C, increased resistance to halides and is twice as photostable. Another YFP variant was described a year later. Modified YFP, named Venus, had an extra point mutation, F46L, resulting in an increase in maturation of fluorescence by accelerating the rate limiting oxidation step and also improved resistance to acid treatment (Nagai *et al.*, 2002). Citrine and Venus fluorescent variants were tested in a multicolour BiFC screen, which found that Venus showed a 12-15 fold increase in fluorescence and Citrine a 4-5 fold increase when compared to the previous YFP used for BiFC analysis (Yu *et al.*, 2003; Nyfeler *et al.*, 2005; Shyu *et al.*, 2006).

BiFC systems can provide a great deal of information regarding interaction localisation. The use of the stable green fluorescent protein allows for simple detection of interaction. Most cells have little if any background fluorescence at the excitation wavelength of GFP, therefore allowing direct visualisation of subcellular interaction localisation. Unlike the yeast-two-hybrid screen, PCAs are not restricted to any one cellular compartment, allowing interactions to be investigated whilst in their native context, under their normal physiological conditions. This was demonstrated using the DHFR-based PCA - see Fig. (1) - by Remy and co-workers (Remy and Michnick, 1999, 2001; Remy *et al.*, 1999). Using

a GFP-based BiFC, the transcription factors, Fos and Jun, were shown to interact in the nucleus (Hu *et al.*, 2002). Hu *et al.* (2002), also monitored interactions between ATF2 and Jun which were shown, as expected, to be perinuclear. However in response to SAPK the ATF2-Jun dimers translocate to the nucleus (Hu *et al.*, 2002). Identification of interaction localisation can also provide information about the functions of the protein complexes formed. Sub-nuclear Fos and Jun heterodimers have different regulatory functions than Jun and ATF2 heterodimers, which were found to be situated around the nucleus of the cell (Hu *et al.*, 2002).

The assay has also been shown to identify the sub cellular translocation of unknown proteins. Using Ozawa *et al.*'s (2000) intein splicing-based system, proteins have been shown to be located in the mitochondria. The C-terminal GFP fragment was fused to a mitochondrial targeting signal (MTS) and a cDNA library of proteins were fused to the N-terminal GFP fragment. Proteins containing an MTS were translocated to the mitochondria, resulting in protein splicing and reassembly of GFP. Ozawa *et al.*, (2003) analysed 258 proteins of which seventy proteins were suspected mitochondrial proteins, these included proteins known to be present in the mitochondria as well as those with previously unknown subcellular location and those with unknown functions. By replacing the MTS signal with signals appropriate for other subcellular compartments including the golgi and nucleus, it may be possible to identify the localisation of unknown proteins in cells (Ozawa *et al.*, 2003).

Less than a decade from its introduction, the BiFC system has been applied to a range of proteins within different organisms including bacteria (Magliery *et al.*, 2005; Aparicio *et al.*, 2006) mammalian cells (Hu *et al.*, 2002), fungal cells (Hoff and Kuck, 2005; Park *et al.*, 2007) and plant cells (Brachi-Drori *et al.*, 2004).

In addition to detecting protein-protein interactions, the bimolecular fluorescent complementation system has also been described for use in other applications. Jeong *et al.*, (2006) describe the use of split GFP fragment reconstitution in monitoring conformational changes within maltose binding protein (MBP). The characteristic hinge-twist movement of MBP upon binding to its substrate maltose (Sharff *et al.*, 1992) can be readily detected by the movement of split GFP fragments into close enough proximity with one another to rebuild the fluorescent protein. The same group later used this technology to detect the conformational changes in the glycolytic enzyme hexokinase induced by binding glucose (Jeong *et al.*, 2007). Cabantous *et al.*, (2005), describe the use of the split fluorescent technology in overcoming protein misfolding. Target proteins are tagged with a small GFP fragment and complementation with the large fragment of GFP can detect the tagged fusion protein. GFP fluorescence can therefore act as a sensor of correct folding of the tagged protein. The split GFP screen was also shown to be effective at increasing protein solubility for otherwise insoluble and unmanageable proteins, thus providing sufficient quantities of soluble proteins for interaction and crystallization studies.

A major application of BiFC is its potential to detect unknown interactions. Remy and Michnick (2004c), demonstrate the capability of BiFC to detect interacting partners of the protein kinase PKB/Akt using a cDNA screening ap-

proach. Ft1 was identified from a library of labelled brain cDNA as a binding partner of PKB from the GFP BiFC library screen (Remy and Michnick, 2004b; 2004c). Interaction between PKB/Akt and Ft1 which expressed complementary GFP fragments induced GFP reconstitution which generated a fluorescence signal.

PCAs also have potential to be applied to drug discovery and targeting research. Protein-protein interactions may be implicated in the development of some pathological diseases (Archakov *et al.*, 2003). Indeed the DHFR-based PCA has already been applied (Remy and Michnick, 2001; MacDonald *et al.*, 2006). An improved knowledge of protein interactions and changes in protein interactions upon drug binding can be gained through BiFC assays. The system may also be useful in the identification and/or development of inhibitors and activators of protein-protein interactions. As the reassembly of the fluorescent protein is irreversible, after interaction the stable complex cannot be disrupted nor enhanced by inhibitors or activators. This property can be exploited to screen for possible inhibitors/activators. Potential inhibitors/activators added at the start of cell growth may compete for the proteins thus preventing interaction and GFP reassembly (Magliery and Regan, 2006).

PCA (including BiFC) systems have many advantages over the conventional two-hybrid approach to interrogating protein protein interactions. PCAs overcome many of the limitations of by the yeast two hybrid screen. The unique characteristics of the green fluorescent protein such as its ability to form a readily detectable intrinsic fluorophore coupled with the wide variety of spectral variants available, allow for a simple and speedy detection of protein-protein interactions both *in vivo* and *in vitro*. It provides information on interactions including location. BiFC can also provide a better understanding of interaction networks within cells and heighten the development of proteome interaction mapping. The further development of this technology is thus likely to provide yet more tools for interrogating proteomes.

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#### ABBREVIATIONS

β-Gal	=	β-galactosidase
BFP	=	Blue fluorescent protein
BiFC	=	Bimolecular fluorescent complementation
CBP	=	Calmodulin binding peptide
CFP	=	Cyan fluorescent protein
DBD	=	DNA binding domain
EGFP	=	Enhanced green fluorescent protein
EGTA	=	Ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EYFP	=	Enhanced yellow fluorescent protein
FRET	=	Fluorescence resonance energy transfer
GFP	=	Green fluorescent protein

IgG	=	Immunoglobulin G
K <sub>D</sub>	=	Dissociation constant
MBP	=	Maltose binding protein
MTS	=	Mitochondrial targeting signal
PCA	=	Protein-fragment complementation assay
TAD	=	Transcription activation domain
TAP	=	Tandem affinity purification
TEV	=	Tobacco etch virus
Y2H	=	Yeast two hybrid
YFP	=	Yellow fluorescent protein

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