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Bimolecular fluorescence complementation (BiFC) analysis: advances and recent applications for genome-wide interaction studies

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Abstract

Complex protein networks are involved in nearly all cellular processes. To uncover these vast networks of protein interactions, various high-throughput screening technologies have been developed. Over the last decade, bimolecular fluorescence complementation (BiFC) assay has been widely used to detect protein-protein interactions (PPIs) in living cells. This technique is based on the reconstitution of a fluorescent protein *in vivo*. Easy quantification of the BiFC signals allows effective cell-based high-throughput screenings for protein-binding partners and drugs that modulate PPIs. Recently, with the development of large screening libraries, BiFC has been effectively applied for genome-wide PPI studies and has uncovered novel protein interactions, providing new insight into protein functions. In this review, we describe the development of reagents and methods used for BiFC-based screens in yeast, plants, and mammalian cells. We also discuss the advantages and drawbacks of these methods and highlight the application of BiFC in large-scale studies.

INTRODUCTION

Genetic interactions and phenotypes associated with genetic perturbations provide important information in understanding biological processes. For example, a genome-wide study in budding yeast examined 5.4 million gene-gene pairs for synthetic genetic interactions, uncovering a global network of genetic interaction profiles [1]. These genetic networks are enriched in interactions involving protein complexes, suggesting that protein complexes play a central role in the genetic landscape [2]. Indeed, nearly all biological processes in any living cell are mediated by complex interactions of proteins. It has been suggested that the

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human interactome may contain about 130,000 binary protein interactions [3]. These protein-protein interactions (PPIs) are often densely connected with each other and are organized in networks. Aberrant PPIs and disturbance of protein networks are associated with various diseases including cancer and Alzheimer's disease [4, 5]. Mapping PPIs thus provides important clues for our understanding of how proteins mediate normal biological processes and how perturbations of PPIs or viral protein interactions are implicated in diseases.

A large number of high-throughout studies have uncovered complex protein interactomes in diverse organisms, owing to development of various technologies including the yeast two-hybrid system (YTH), co-immunoprecipitation (co-IP), fluorescence resonance energy transfer or Förster resonance energy transfer (FRET), tandem affinity purification (TAP), and protein fragment complementation assays (PCAs) [6-8]. Since its invention over 25 years ago [9], the yeast two-hybrid analysis has been widely used to map protein binding partners from diverse cell types [10]. YTH has also contributed to the development of variant technologies for mapping PPIs *in vivo*, such as membrane yeast-two hybrid assay (MYTH) [11]. MYTH is a split ubiquitin-based two-hybrid analysis, which is designed to overcome limitations of the original YTH system for application to membrane proteins [12-14]. YTH and the related methods have been widely used for high-throughout studies of PPIs [15-18]. FRET refers to an energy transfer process from an excited donor molecule to another nearly acceptor molecule, and is very sensitive to the distance between the donor and acceptor molecules [19]. Recent advances in microscopy and spectroscopy make FRET a powerful technique to monitor spatiotemporal changes in PPIs, albeit some inherent limitations. FRET-based methods are also used for high-throughput screening of PPIs using protein microarrays [19-21].

In PCAs, a reporter (such as a fluorescent protein or an enzyme) is truncated and fused to two proteins of interest. If these two proteins interact with each other, the complementary fragments are capable of proper folding and assembly, reconstituting the reporter activity. One of the PCA methods is bimolecular fluorescence complementation (BiFC), which is based on the reconstitution of a fluorescent protein *in vivo*. Fluorescence complementation via reassembly of GFP from its truncated N- and C-terminal fragments was first reported by Regan and colleagues [22]. Since Kerppola and colleagues successfully reconstituted YFP in mammalian cells using fragmented YFP fused to interacting transcription factors [23], the technique has been more widely used in biological research. A BiFC assay is unique among the protein fragment complementation assays; it allows direct visualization of PPIs in living cells. Two proteins of interest are fused to N-terminal or C-terminal non-fluorescent fragment of a fluorescent protein and then expressed in living cells. If an interaction takes place between the two proteins of interest, this will facilitate reconstitution of the fluorescent protein, forming a fluorescent complex (Figure 1) [24]. The BiFC signal can then be visualized by fluorescent microscopy or analyzed by flow cytometry without any other special treatment to cells.

Recently, BiFC assays have been utilized in high-throughput screens, uncovering novel PPIs in yeast, plant and mammalian cells. BiFC has allowed the systematic analysis of protein interactomes, while also revealing the subcellular localization of protein interactions and

thereby providing new insights into protein functions. In this review, we first describe the development of reagents and methods for BiFC analysis. We then discuss recent large-scale studies that have applied BiFC assays in yeast and other cell types. For comprehensive discussion of BiFC analysis, we refer readers to excellent reviews by Kerppola and his colleagues [24-26].

ADVANTAGES OF BiFC ANALYSIS AND CRITICAL CONSIDERATIONS

BiFC analysis is highly sensitive with minimum background because fluorescence recovery requires interaction of two proteins, each of which is fused to non-fluorescent, truncated fluorophores [24]. BiFC thus allows direct visualization of the protein complex in live cells. This is a major advantage compared to other fluorescence-based techniques, such as FRET, which requires image capturing at two different wavelengths and FRET measurement as well as computation [27]. While FRET analysis is powerful for detecting changes in PPIs instantaneously, this method has relatively low sensitivity because small fractions of interacting protein partners often associate with each other at any given time. In addition, the fluorophores of the two interacting partners are required to be closer in proximity (less than 10 nm apart) in FRET analysis compared to BiFC. Since protein fragment association can be facilitated when two fusion proteins are in the same macromolecular complex, BiFC assays may generate positive signals when two proteins fused to the fluorescent protein fragments are present within the same macromolecular complex, even when there is no direct contact between the two proteins. The association between protein fragments is, however, inefficient unless covalent or non-covalent interactions are present between the two proteins [25]. Since the presence of proteins in the same complex often suggests their involvement in the same biological process, this capacity of BiFC analysis provides important information about protein function.

Because of the intrinsic fluorescence of the complemented protein complex, BiFC does not require any special treatment of cells with exogenous reagents. The procedure does not require cell fixation or lysis, and thus BiFC enables visualization of the subcellular locations of specific protein interactions with minimum perturbation of the normal cellular environment. BiFC assays can also be readily adapted for different cellular conditions. The procedure is relatively simple, and the imaging can be performed using a standard epifluorescence microscope. For example, two proteins fused to the N-terminal or C-terminal fragment of YFP (yellow fluorescent protein) can be co-expressed by mating two yeast strains expressing each fusion protein. The diploid cells are then imaged to examine the interaction between two proteins. This assay thus allows rapid visualization of the compartment-specific interactions of a protein complex, and PPIs *in vivo* can be easily quantified.

Despite these advantages, there are some potential problems and critical factors that one should consider for a BiFC assay. To apply BiFC assays, fragmented fluorescent proteins should not be capable of folding together spontaneously unless they are brought into close proximity by the interaction between two fused proteins. Another important issue of BiFC includes functionality of fusion proteins, as in the case of other techniques using fusion or tagged proteins. In addition, fluorescence intensity of reconstituted fragments should be

comparable to that of intact fluorescent protein and be bright enough to be distinguished from background signals. Nevertheless, false positive fluorescent signals can still be detected. To ensure a positive interaction, it is essential to have a proper control such as a binding partner carrying a mutation in the binding interface [26]. Finding an appropriate negative control can often be challenging when one examines an interaction between two novel proteins without biochemical or structural information. In such cases, it is desirable to do BiFC competition analysis by overexpressing one of the binding partners as an untagged protein and/or to have other independent assays to validate the interactions.

Slow maturation of a chromophore [28] is another limiting factor for its application for visualization of transient or dynamic interactions in cells. Thus the subcellular location where a bimolecular fluorescent complex is observed may not always be the site of interaction of two proteins. Related to this issue is the fact that the BiFC complex formation is irreversible *in vitro* [29]. Although this is often also the case in *in vivo* studies, some studies suggest otherwise [30-33] (see below, *Yeast*). This potential irreversible nature of BiFC requires careful evaluation of the site of interaction of two proteins and limits the use of the assay in monitoring dynamic PPIs. However, this irreversibility can help with the detection of transient or weak interactions, benefitting high-throughput screening [34-36] (see below, **LARGE-SCALE APPLICATIONS OF BiFC**). A number of different approaches have been developed for the characterization of multimeric protein complex dynamics by combining BiFC with other techniques such as FRET or FRAP (fluorescence recovery after photobleaching) [37, 38].

FLUORESCENT PROTEINS AND THEIR APPLICATIONS FOR BiFC ASSAY

More than ten fluorescent proteins have been developed for BiFC assays to date, as summarized in Table 1.

Green fluorescent protein (GFP)

GFP is the first fluorescent protein applied for a BiFC assay by Regan and colleagues [22]. Two fragments of GFP (NGFP and CGFP), split in a loop between residues 157 and 158, did not associate to form reassembled GFP when produced in bacteria. In contrast, when strongly interacting antiparallel leucine zippers were fused to the C terminus of NGFP and N-terminus of CGFP, these split GFP fragments recovered proper folding and fluorescence [22]. Using a library of antiparallel leucine zippers that differ in their dissociation constants, this BiFC-based screen was able to detect weak interactions with the minimum estimated $K_D \approx 1$ mM [39]. This screen was further used to detect PPIs in bacteria, expanding its scope beyond peptide-peptide interactions that were addressed previously.

A GFP-based BiFC assay was also used to screen for proteins interacting with protein kinase B (PKB)/Akt using a human brain cDNA library [34]. The human brain cDNA library was used to express “prey” fusions to the N-terminal fragment of GFP (amino acids 1-158), and the full-length PKB cDNA was fused to the C-terminal fragment of GFP (amino acids 159-238) to generate a “bait”. After cotransfection of the bait and cDNA library prey fusions into COS-1 cells, a physical interaction between the bait and a prey protein were screened by fluorescence-activated cell sorting (FACS), which lead to successful identification of a

novel PKB binding protein [40]. Unlike these two cases, the GFP truncations generated between residues 154 and 155 have not been successfully applied for BiFC assays in mammalian cells [41] or in the budding yeast, *S. cerevisiae* (M. K. Sung and WKH, unpublished observation), although the reason is not clear.

A coral fluorescent reporter protein [monomeric Kusabira-Green (mKG)] with spectral characteristics similar to those of GFP has been developed for a BiFC assay [42]. mKG protein (excitation and emission maximums at 494 and 507 nm, respectively) was derived from monomeric Kusabira-Orange protein (excitation and emission maximums at 548 and 559 nm, respectively) [43] by introducing 7 mutations. mKG protein fragments split at 168-169 were tested for BiFC assays by fusing them to leucine zipper proteins. This mKG-based BiFC system was then used to detect phox protein interactions in macrophages. This study uncovered an intramolecular interaction within p40^{phox} as well as interactions between p67^{phox} and adaptor proteins, p47^{phox} and p40^{phox} [42]. The mKG-based BiFC system has also been used in high-throughput screening for PPI inhibitors using a large collection of natural product library [44]. By screening 123,599 samples of the natural product library in a 1536-well format, a specific inhibitor for PPI of PAC3 homodimer was identified. This *in vitro* PCA system demonstrates application of a BiFC-based assay to the high-throughput screening for PPI inhibitor drugs.

Yellow fluorescent protein (YFP) variants

The validity of YFP fragments for a BiFC assay was first demonstrated in a study to visualize Ca²⁺-dependent PPIs in living cells [45]. In this study, the N-terminal fragment (amino acids 1-144) of EYFP (V68L, Q69K), a YFP variant [46], was fused to calmodulin, and the C-terminal fragment of EYFP (amino acids 145-238) was fused to M13 calmodulin binding peptide. When co-expressed in HeLa cells, the two fusion proteins produced fluorescent signals that reflected the reversible association between calmodulin and M13 peptide in response to changes in intracellular Ca²⁺. Kerppola and colleagues divided EYFP (S65G, S72A, T203Y) into two fragments at several non-conserved amino acid residues located in loops connecting β -barrels and fused these fragments to Jun257-318 (bJun) and Fos118-210 (bFos) [23], the basic leucine zipper (bZIP) domains of Jun and Fos [47]. Among these variants, YFP fragments that were split between amino acids 154 and 155 (YN155 and YC155) showed the highest fluorescence and had excitation/emission spectra similar to those of intact YFP [23]. Deletion of bZIP domains of fused proteins led to the disappearance of fluorescence signal, indicating that the fluorescence did not result from self-assembly between YFP fragments.

Although EYFP shows bright fluorescence, it has drawbacks such as sensitivity to pH and requirement for pre-incubation at low temperatures before visualization [48]. A few YFP variants have been developed to make up for the weak points of EYFP: Citrine, a YFP variant carrying Q69M mutation, shows relieved pH sensitivity [49], and Venus, a YFP variant carrying F46L mutation, folds well even at 37°C [50]. Both Citrine and Venus have been successfully applied for BiFC assays in COS-1 cells [51]. The split Citrine at D155 and split Venus at D173 show about 4 - 5 fold and 12 - 15 fold increased BiFC efficiency, respectively, compared to EYFP. Moreover, Citrine and Venus-based BiFC assays do not

require pre-incubation at temperatures lower than 37°C. Indeed, Venus is the most widely used fluorescent protein for BiFC assays under physiological conditions.

Cyan fluorescent protein (CFP) variants

A series of efforts have improved CFP variants for use in BiFC assays. The N- and C-terminal fragments of CFP split between amino acids 154 and 155 (CN155 and CC155) show fluorescence complementation when fused to bJun and bFos [41]. Interestingly, CC155 can complement with YN155, and the fluorescent spectrum of this pair is different from those of YN155-YC155 and CN155-CC155. In addition, the C-terminal fragment of EYFP comprised of residues 173-238 (YC173) can complement with the N-terminal fragment of CFP comprised of residues 1-172 (CN173). In the case of CC155, it could produce fluorescence with YN173 and CN173. Localization and selectivity of bJun-bFos interaction were consistent in all combinations, indicating that they are not affected by different kinds of fused fluorescent protein fragments [41]. This finding opened up the possibility to use CFP and YFP together for a multicolor BiFC assay, a method for simultaneous visualization of multiple protein interactions in living cells [41]. An improved CFP variant with point mutations S72A, Y145A and H148D, designated Cerulean, shows increased quantum yield [52] and has also been successfully applied for BiFC assay [51]. The N-terminal fragment of Cerulean comprised of residues 1-172 and CC155 could complement with each other and strongly fluoresced when fused to bJun and bFos, respectively, in COS-1 cells.

Since many proteins often have several binding partners, multicolor BiFC analysis provides an effective assay to compare the subcellular distributions of protein complexes formed with different binding partners. Multicolor BiFC can also allow analysis of the competition between different binding partners for binding a shared partner [53]. Although multicolor BiFC assay has not been applied for high-throughput screen yet, several studies demonstrate its utility as a tool to investigate complex protein networks in living cells. For example, in a study of Ypt/Rab GTPase involved in intracellular trafficking in budding yeast, multicolor BiFC analysis was performed to test multiple PPIs including Ypt1, Trs85 (a Ypt1 activator subunit), and Atg11, which is the preautophagosomal structure (PAS) organizer [54]. To distinguish different PPIs, multi-color BiFC assays were performed using Y/CFP-C (the C-terminal amino acids 155–238 of CFP) fused to Ypt1, Trs85 fused to CFP-N (the N-terminal 172 amino acids of Cerulean), and Atg11 fused to YFP-N (the N-terminal 172 amino acids of Venus). By combining single- and multi-color BiFC and co-localization analyses with proper markers, the authors showed that Ypt1 interacts with Trs85, forming a few fluorescent puncta, one of which co-localizes with the Ypt1-Atg11 BiFC complex in the PAS, which appears as one dot per cell [54].

Another study used multicolor BiFC analysis to determine the association preferences of β and γ subunits of G protein in human embryonic kidney (HEK) 293 cells [55]. Using β and γ subunits fused to the C-terminal fragment of CFP and alternative interaction partners fused to the N-terminal fragment of either YFP or Cerulean, two fluorescent complexes were visualized simultaneously in HEK293 cells, and the fluorescence intensity of the BiFC complexes was then compared to determine the association preferences of β and γ subunits

[55]. Multicolor BiFC analysis was also used in plant cells to show the interaction between a protein kinase and its binding partners. The protein kinase CIPK24, which is involved in calcium-mediated signal transduction, forms complexes with the calcium sensors CBL1 and CBL10 simultaneously in distinct subcellular localizations in plant cells. Multicolor BiFC also showed that CIPK1 forms distinct complexes with CBL1 and CBL9 at the plasma membrane [56].

Despite its feasibility for the detection of multiple protein interactions in the same cell, multicolor BiFC assay has some limitations. It is possible that different fluorescent protein fragments fused to alternative partners can affect BiFC complex formation differently [53]. Thus it is important to choose appropriate fluorescent protein fragments that do not have significant difference in their ability to reconstitute. It should also be noted that, because BiFC complex formation is generally irreversible, multicolor BiFC assay cannot represent binding affinity of alternative partners in equilibrium. Nevertheless, multicolor BiFC analysis can be cost-effective and timesaving because two interactions are examined simultaneously. Cerulean- and Venus-based BiFC systems have been also used in combination with an RFP-based BiFC system for multicolor BiFC assays (see below).

Red fluorescent protein (RFP) variants

After successive application of YFP and CFP for BiFC assays, the red fluorescent protein DsRed [57] became an object of attention because its application would extend the wavelength range for BiFC assays. However, DsRed could not be used immediately because of its strong tendency to oligomerize [58]. An effort to reduce the oligomerization of DsRed resulted in the development of a monomeric RFP designated mRFP1 [59]. However, mRFP1 was still not appropriate for BiFC assay because of its weak fluorescence intensity and low photostability. Further mutagenesis of mRFP1 generated mRFP1-Q66T with improved fluorescence intensity [60]. This mRFP1-Q66T was applied for a BiFC assay in tobacco protoplasts by using humanized Renilla GFP (hrGFP), which forms homodimers [61], fused to mRFP1-Q66T fragments [60]. The mRFP1-Q66T-based BiFC assay was sensitive enough to catch weak and transient PPIs, such as dimerization of the GFP variant Emerald. Interestingly, fluorescence complementation of mRFP1-Q66T shows a distinct increase in alkaline conditions [60].

Another RFP-based BiFC system has been developed by splitting the mutant monomeric RFP mCherry with excitation and emission wavelengths at 587/610 nm [62]. The split mCherry fragments, MN159 and MC160, could visualize dimerization of EGFP or interaction between SV40 large T antigen (LTag) and human p53 protein in Vero cells [63]. Through the co-expression of LTag-MN159, p53-MC160, sp100-VN172, and promyelocytic leukemia protein (PML)-VC173, the interaction between LTag and p53 as well as the interaction between sp100 and PML were detected simultaneously in Vero cells. This study demonstrates that mCherry-based BiFC system can be used to study multiple PPIs when coupled with other fluorescent protein-based BiFC systems.

Far-red and near-infrared fluorescent proteins

Fluorescent proteins with emission spectra in the far-red wavelengths are important for imaging deep tissues in animals. The application of RFP-based BiFC systems with mRFP1 variants, mRFP1-Q66T and mCherry, had been limited because they are functional only at relatively low temperatures ($\leq 30^{\circ}\text{C}$) [60, 64]. mKate, a monomeric form of Katushka (a far-red fluorescent protein) with fast maturation and high photostability [65], was chosen as a starting point to develop an RFP-based BiFC system that can be applied at higher temperatures. The fragments of mKate fused to bJun and bFos exhibited high BiFC efficiency in COS-7 cells without pre-incubation at sub-physiological conditions [66]. However, mKate itself suffers from a relatively low level of brightness (~25% of Venus). Remarkably, S158A mutation increased the brightness of mKate by 2-fold, and this mKate-S158A variant was named as mLumin. The newly improved far-red fluorescent protein mLumin with an emission maximum of 621 nm enables a BiFC analysis at 37°C and thus in living mammalian cells [66]. When the N- and C-terminal fragments of mLumin were fused to EGFR and STAT4 and co-expressed in COS-7 cells, EGFR-STAT4 interaction was successfully visualized. Furthermore, when combined with Cerulean- and Venus-based BiFC systems, mLumin-based BiFC system allowed for simultaneous visualization of three pairs of protein–protein interactions in the same cell [66].

Designing fluorescent proteins that work for a BiFC assay in animal tissues is difficult. Light below 600 nm is absorbed by hemoglobin [67], and light above 1200 nm is absorbed by water molecules [68]. Thus, fluorescent proteins with excitation wavelength above 600 nm, even longer than those of mCherry and mLumin, are required for a BiFC assay in animal tissues. Further mutations of mKate produced Neptune, which is the first bright fluorescent protein with excitation peak reaching 600 nm. The monomeric variant of Neptune, mNeptune, which has excitation/emission wavelength of 600/650 nm [69], has been successfully used for BiFC assays in animals. When the N- and C-terminal fragments (MN and MC) of mNeptune were fused to EGFP and co-expressed in HeLa cells, the combination of EGFP-MN155 and EGFP-MC156 produced a bright red BiFC signal, demonstrating the validity of mNeptune-based BiFC system. Furthermore, mNeptune-based BiFC system could visualize the protein–protein interaction of bFos and bJun in live mice [70].

Recently, a BiFC reporter with even longer excitation and emission maxima optimal for whole animal imaging has been developed [71]. The near-infrared fluorescent protein iRFP retains two distinct domains PAS and GAF from its parental bacterial phytochrome *RpBphP2* [72]. It exhibits high fluorescence intensity and low cytotoxicity and utilizes endogenous concentrations of biliverdin chromophore to acquire fluorescence. iSplit, which exhibits increased brightness compared to the split iRFP, was derived from iRFP by introducing a polypeptide break between the PAS domain (amino acids 1-120) and GAF_m domain (amino acids 119-315) with three substitutions. When PAS and GAF_m were fused to the N terminus of FK506-rapamycin binding (FRB) domain of mTOR and the C terminus of FK506-binding protein 12 (FKBP), respectively, rapamycin-induced interaction between the PAS-FRB and FKBP-GAF_m fusion constructs was successfully visualized in HeLa cells and a living mouse model [71].

Efforts to improve BiFC reporter proteins

While various fluorescent protein fragment pairs covering a broad range of spectrum have been developed for BiFC assays, there have been continued efforts to improve the brightness of BiFC complex or to reduce background fluorescence. For example, a new pair of Venus fragments has been developed to visualize Smad signaling in *Xenopus* embryos [73]. *Xenopus* embryos have a large amount of yolk granule, which is responsible for high autofluorescence [74]. Thus, visualization of PPIs by Venus-based BiFC assay is not easy to achieve in *Xenopus* embryos. To develop a BiFC reporter with low background fluorescence, Venus fragments split between amino acids 144/145 (VN144 and VC145) or 154/155 (VN154 and VC155) were further subjected to point mutations. Among 30 different kinds of pairs, Vm9, VN154 fragment carrying the T153M mutation, showed high interaction-induced fluorescence and low background signal when complemented with VC155 in *Xenopus* embryos. This new pair of Venus fragments was used to visualize Smad2-Smad4 heteromeric interaction [73]. Another new pair of Venus fragments VN210 and VC210, which have low self-assembly tendency and high fluorescence intensity, has been developed to visualize the cofilin-actin interaction in HeLa cells [75].

In addition to Venus, other improved fluorescent proteins have been introduced for BiFC assays. Superfolder GFP (sfGFP) is a GFP variant with enhanced folding robustness and has reduced the probability of misfolding by fused proteins [76]. While sfGFP has a high risk of self-assembly, sfGFPC(m12), which carries the deletion of R215 and the substitution mutations V219A and L220A, has an improved signal with low background in a BiFC assay with sfGFPN, when each split sfGFP fragment was fused to Bcl-xL or Bak [77].

In general, monitoring BiFC signals by repeated capturing is limited due to photobleaching or low quantum yields. To overcome this constraint, a BiFC strategy has been developed using Dronpa, a genetically engineered monomeric GFP-like fluorescent protein cloned from *Pectiniidae*, a coral species [78]. Dronpa has a reversible photo-switching activity between the fluorescent and nonfluorescent states. Split Dronpa fragments, DN (amino acids 1-164) and DC (amino acids 165-224) fused to hMYH and hHus1 were successfully used for a BiFC assay in HEK293 cells [79]. Furthermore, complemented Dronpa exhibited almost identical reversible photo-switching activity as that of native Dronpa, suggesting that Dronpa-based BiFC assay can be used as a tool for tracking PPIs in live cells.

Limited resolution of a BiFC analysis is greatly improved by combining BiFC with photoactivated localization microscopy (PALM) [80, 81], which has allowed visualization of PPIs inside cells with nanometer spatial resolution and single molecule sensitivity. Fluorescent proteins used in BiFC-PALM should be photoswitchable. For example, the photoactivatable fluorescent protein PAmCherry1, which was developed from mCherry by introducing 10 substitutions (E26V/A58T/K69N/L84F/N99K/S148L/I165V/Q167P/L169V/I203R) [82], can be used as a BiFC probe when split between amino acid residues 159 and 160 (RN and RC) [83]. The single molecule images of reconstituted PAmCherry1 fragments obtained by activation with 405 nm light exhibit comparable brightness and signal to background ratio with the original PAmCherry1. Importantly, the reconstitution of PAmCherry1 is highly efficient at the physiological temperature 37°C in contrast to many

other split fluorescent proteins including mCherry, which needs to be reconstituted at 25°C or 4°C. As demonstrated with PAmCherry1, BiFC-PALM allows for nanoscale-resolved visualization of PPIs under physiological conditions.

LARGE-SCALE APPLICATIONS OF BiFC

Since performing a BiFC assay is simple and inexpensive, it is suitable for large-scale screens for PPIs. Various vector systems for tagging fluorescent protein fragments to proteins of interest have been developed to date, and large-scale screens using BiFC assays have been performed in diverse species from yeast to mammalian cells, as summarized in Table 2. Below, we discuss some of these studies in yeast, plants, and mammalian cells. Many of these studies include cell-based high throughput screening by BiFC assays with either fluorescence microscopy or flow cytometry. As highlighted in some examples below, it is desirable to undertake complementary approaches in mapping interactomes to overcome limitations of each approach.

Yeast

The facile genetic methods of yeast afford an opportunity to study PPIs efficiently in this model organism. In particular, the capability of replacing wild-type genes on the chromosomes with modified copies of the genes is advantageous for BiFC assays because fusion proteins can be expressed at their endogenous levels and because there is no competition between tagged and untagged endogenous proteins. The yeast system also minimizes other complications such as non-specific PPIs due to overexpression and allows easy assessment of the functionality of N- or C-terminal fusion proteins. Another major limitation of a BiFC assay is that BiFC complex formation is irreversible *in vitro* and often *in vivo* [29], as discussed earlier. This irreversibility of BiFC assays may be due to overexpression of fluorescent protein fragments fused to the interacting proteins. Indeed, when each fusion protein has been expressed from its native promoter at the chromosomal locus in budding yeast, formation of bimolecular fluorescent complex *in vivo* was found to be reversible. For example, Pho2 and Pho4 form a BiFC complex when cells are in medium lacking phosphate, but the BiFC signal disappears in cells transferred to high phosphate-containing medium [30]. The Ras-like GTPase Rsr1 forms a BiFC complex of the homodimer transiently in the cell cycle, whereas its GDP-locked mutant protein forms a persistent BiFC complex [31]. The reversibility of BiFC complex formation has also been observed in interactions between Cdc42 and Rdi1 in yeast [32] and between phospholipase C β_2 and C δ_1 in HEK293 cells [33]. Further investigation is, however, necessary to clarify whether these data indeed reflect reversibility of the BiFC complex formation *in vivo* or rapid turnover of the BiFC complex. Nevertheless, expression of fluorescent protein fragments from the native promoter of each interacting partner on the chromosome is likely to minimize false positive interactions and the potential problem of irreversible BiFC complex formation.

Several studies in budding yeast have successfully used BiFC assays with fusion proteins expressed from their native promoters at the chromosomal loci or on a low copy number plasmid [18, 30, 31, 54, 84-91]. Although critical controls have been lacking in some previous studies, the PPIs have been often validated with other independent assays in these

studies. In an effort to apply BiFC assays for a genome-wide screen, a series of plasmids that allow expression of fluorescent protein fragment fusion constructs from their native promoters were first developed in *S. cerevisiae* [30]. These sets of plasmids allow one-step, PCR-mediated modification of genes, as originally described by Longtine et al. [92]. Plasmids for C-terminal tagging allow the expression of the N-terminal (VN) or C-terminal fragment (VC) of Venus fused to a protein of interest from its native promoter, whereas plasmids for N-terminal tagging allow the expression of these fusion proteins from the heterologous promoters of different strength. This BiFC vector system has been successfully used to validate several known interacting proteins including Pho2 and Pho4 [30].

The major hurdle of a large-scale BiFC screen has been the construction of a fusion library to express each ORF fused to an appropriate fluorescent protein fragment. To facilitate the application of BiFC assays to the genome-wide analysis of PPIs, Huh and colleagues constructed a *S. cerevisiae* fusion library, in which each endogenous gene was tagged with VN at the C terminus [90] by switching the C-terminal TAP tag of each strain of the TAP fusion library [93] with VN. This VN fusion library contains 5,911 VN-tagged strains and thus covers 95% of all ORFs annotated in the *Saccharomyces* genome database (as of April 2001; <http://www.yeastgenome.org>). This VN library was then used in a genome-wide search for proteins that interact with small ubiquitin-related modifier (SUMO) proteins in yeast [90]. The attachment of a SUMO molecule to a target protein has been shown to be important for a variety of biological processes [94]. Determination of SUMO-protein interactions would thus provide insights into the function of SUMO. All strains in the VN fusion library were mated with a strain containing VC-tagged SUMO (Smt3 in *S. cerevisiae*), and the resulting diploid strains were analyzed for the presence of a BiFC signal (Figure 2). Out of the 5,911 yeast strains screened with VC-Smt3, 367 VN strains were identified to have positive BiFC signals. Therefore, this study adds 224 proteins to the SUMO interactome, which previously had 781 interactions including 143 confirmed in this study. The identification of new 224 potential SUMO interactions suggests the power of genome-wide BiFC screens, although not all of these interactions have been validated (see below).

Previous studies have used TAP-MS or yeast two-hybrid assays to map the SUMO interactome. These approaches may have been unsuccessful in identifying the new interactions uncovered in this screen by BiFC assays because of the difficulty in detecting transient or weak protein interactions. The other methods used in previous studies often do not examine PPIs in their natural context. However, this BiFC-based screen also failed to identify many previously known SUMO interactions, and this may be due to topological constraints. This relays the importance of testing VN fusions at both the C and N terminus. In addition, inserting the linkers that tether VN and VC to the interaction partners may facilitate a BiFC-based screen by providing sufficient flexibility to enable VN and VC to associate with each other.

To verify positive BiFC signals, the localization of the BiFC complexes of SUMO interacting proteins was compared to the localization of the corresponding GFP fusions. Surprisingly, subcellular localizations of 26 BiFC complexes were different from those of the corresponding GFP fusions in yeast [90]. If only a small fraction of the candidate protein

is sumoylated *in vivo*, the GFP fusion localization pattern may represent the site where its unsumoylated form is present. In this case, the discrepancy between the localizations of a BiFC complex and a GFP fusion protein may indicate the different compartments or sites at which sumoylated versus unsumoylated forms function. However, further validation would be critical to ensure that these localization patterns of the bimolecular fluorescent complex are physiologically relevant. Some positive candidates identified by this BiFC screen were confirmed by IP using anti-Smt3 antibodies. A functional analysis of the SUMO-proteins was also performed by a strategy using BiFC to uncover which proteins in the VN library were substrates for desumoylation by SUMO-specific proteases, Ulp1 or Ulp2. To this end, 280 VN-tagged strains were mated with strains overexpressing Ulp1 or Ulp2, and the BiFC signal was analyzed and compared to a control strain (that did not have Ulp1 or Ulp2 overexpression) to classify the potential SUMO targets as Ulp1 or Ulp2 substrates [90].

BiFC assays were recently applied in another large-scale study to map the ATP-binding cassette (ABC) transporter interactome in budding yeast [18]. ABC transporters are transmembrane proteins that are highly conserved from prokaryotes to humans, and mutations in the ABC transporter genes are associated with numerous human diseases [95]. The ABC transporter-mediated active efflux of a broad range of xenobiotics is also believed to be one of the major causes of multidrug resistance and chemotherapeutic failure in cancer therapy [96]. This study first used membrane yeast two-hybrid (MYTH) assays to screen for proteins interacting with 19 ABC transporters, and identified 285 interactions among 209 proteins. A subset of these PPIs was further verified by Co-IP and BiFC assays using the split Venus-fusions of the candidates expressed from their chromosomal loci in yeast [18]. The results of this screen uncovered diverse and novel interactions of ABC transporters, including those involved in biological processes that have not been previously appreciated. Prior studies had shown that half-transporters such as Pxa1 and Pxa2 associate together to form full-size, functional ABC transporters [97], while the functional significance of interactions between full size ABC transporters remained elusive. This study revealed six novel interactions between full-length ABC transporters, including a relationship between Snq2 and Pdr18. Snq2 activity decreased in the absence of Pdr18. While a change in *SNQ2* transcript was not detected by quantitative real-time PCR, its protein level was lower in a *pdr18* mutant, indicating that Pdr18 affects Snq2 activity at the protein level [18].

Several ABC transporters were also found to interact with proteins involved in zinc homeostasis, and BiFC was used to determine the localization of these interactions to reveal functional significance [18]. The ABC transport protein Pdr10 was found to associate with Zrc1, a vacuolar zinc uptake transporter, which is responsible for zinc storage and detoxification [98]. The BiFC signal for the Pdr10-Zrc1 interaction was observed on the plasma membrane, while a weak BiFC signal was sometimes observed on the vacuolar membrane. It remains unclear whether the signals on the vacuolar membrane reflect all functional Pdr10-Zrc1 complex or if a subset is false-positive due to irreversible BiFC complex formation. While BiFC could not be used for a zinc transporter such as Zrt1 whose N and C termini exist in the extracellular matrix [99], co-IP verified the interactions between Zrt1 and the ABC transporters Pdr5, Pdr15, and Pdr18. This study thus provides important

insight into the function of ABC transporters by using powerful methods in proteomics including MYTH and BiFC assays.

Similar approaches were undertaken to map the interactome of Sho1, an integral membrane protein involved in the high-osmolarity glycerol (HOG) mitogen-activated protein kinase pathway in budding yeast. This recent study used BiFC and co-IP assays to validate PPIs that were first identified by MYTH assays [91].

Plants

Many vector systems have been developed for BiFC assays in plants. The sequences for the N- and the C-terminal parts of EYFP, which were split between amino acid residues 174 and 175, were introduced into a set of plasmids (pSATN). These plasmids allow versatile and simple cloning of genes and assembly of several expression cassettes in a single binary vector for simultaneous expression of multiple genes [100]. To obtain high expression levels in a wide range of plant species and tissues, fusion proteins were expressed under the control of the constitutive tandem cauliflower mosaic virus (CaMV) 35S promoter, the tobacco etch virus (TEV) translation leader, and the CaMV 35S poly(A) terminator. These vector series allow detection of PPIs in various cellular compartments, including the nucleus, plasmodesmata, and chloroplasts of different plant species and cell types. The pSATN series of vectors have been further modified for multicolor BiFC assay [101] using the bait protein tagged with the C-terminal portion of CFP (amino acids 155-238) and prey proteins tagged with the N-terminal portions (amino acids 1-173) of either Venus or Cerulean.

A high-throughput screen has been performed using BiFC and YTH assays to map PPIs between core cell cycle proteins of *A. thaliana* [102]. The BiFC vectors were created with the MultiSite Gateway technology that combined the cell cycle ORFs and the GFP fragments downstream of the constitutive CaMV 35S promoter. Each core cell cycle protein was C-terminally tagged with either the N- or C-terminal fragment of GFP. The constructs were transiently co-expressed in leaf epidermal cells of tobacco (*Nicotiana benthamiana*) by *A. tumefaciens*-mediated leaf infiltration. BiFC assays were applied to test a total of 917 pairwise interactions for 58 cell cycle regulatory proteins and identified 341 (37%) interacting pairs [102]. In another BiFC-based screen, an *Arabidopsis* cDNA library of colonies representing $\sim 2 \times 10^5$ cDNAs was screened for PPIs by sequential screening of subsets of cDNAs in *Arabidopsis* leaf or tobacco (*N. tabacum*) Bright Yellow-2 protoplasts [103]. This screen identified single cDNA clones encoding proteins that interact with bait proteins, VirE2 and VirD2. This cDNA library was also used to confirm VirE2-interacting proteins in orchid (*Phalaenopsis amabilis*) flowers [103], demonstrating that this technology can be applied to several plant species.

To facilitate large-scale PPI investigations, a set of 12 GATEWAY-compatible BiFC vectors have been created to efficiently permit the combination of candidate protein pairs with every possible N- or C-terminal fragment of SCFP3A (Super Cyan Fluorescent Protein 3A) [104] or Venus [105]. This vector set enables the investigation of dual complex formation by single-color BiFC as well as the performance of multicolor BiFC for simultaneous detection of multiple protein interactions in a single cell. These vectors have been used to establish the interaction of the proteins Cnx6 and Cnx7 within the

molybdopterin synthase complex as well as the simultaneous formation of homomeric Cnx6-Cnx6 and heteromeric Cnx6-Cnx7 complexes within the same cell [105].

A genome-wide study in *Arabidopsis* used BiFC assays in combination with flow cytometry [106]. In this screen, an *Arabidopsis* cDNA library was recombined into pE-SPYCE (split YFP C-terminal fragment expression) plasmid to generate a random prey YC-cDNA library. This library was screened with a YN-bait fusion partner in protoplasted *Arabidopsis* plant cells. Positive BiFC signals were sorted by flow cytometry, and the candidates were confirmed by further BiFC assays and FRET-FLIM (Fluorescence-lifetime imaging microscopy) assays. This strategy led to the discovery of four CPK3 (calcium-dependent protein kinase 3)-interacting proteins including At2g39050, a ricin B-related lectin domain containing protein [106].

BiFC assays were recently used across species to determine essential PPIs involved in wheat defense response [107]. PPIs have been difficult to study in wheat because of the lack of the complete genome sequence. The protein interactome involved in defense response has been studied in rice, whose complete genome sequence is available. The rice proteins were examined in combination with wheat proteins by BiFC assays to determine novel regulators of defense response as well as to evaluate the conservation of these PPIs [107]. Extensive conservation was observed between rice and wheat protein interactors involved in defense response, indicating that the rice interactome data can be used to predict wheat defense protein interactions. These insights into wheat defense response will provide essential information for creating resistant wheat plants that can be used for agriculture. Overall, this work exemplifies the use of BiFC for testing any protein interactions, even between proteins from different species.

In a study to uncover the *Arabidopsis* G-protein interactome, BiFC assays were used to validate yeast two-hybrid interactions between G-protein regulators and effector proteins [108]. Randomly selected 78 interactions (and 28 negative controls) of G-protein regulators and effector proteins were examined by BiFC assays using bait and prey constructs expressed at low levels to reduce false positives, as high protein expression can lead to false positives due to non-specific binding and irreversible bimolecular fluorescent complexes. This BiFC assay resulted in a high validation rate with confirmation of 74 out of 78 potential interactions, indicating that BiFC analysis in conjunction with yeast two-hybrid is a robust method of detecting protein-protein interactions. Analysis of the BiFC signal showed diverse subcellular localizations of interactions, likely reflecting functional differences. In addition, many novel interactions were uncovered, suggesting a possible role for G-proteins in previously unrecognized processes such as cell wall biogenesis and morphogenesis in *Arabidopsis*.

Mammalian cells

In one of the early large-scale screens by BiFC, split GFP-based BiFC system was combined with fluorescence-activated cell sorting (FACS) to identify proteins interacting with protein kinase B (PKB)/Akt. PKB fused to the C-GFP was used as a bait to screen a human brain cDNA library that expressed prey N-GFP fusion proteins in COS-1 cells. This screen identified hFt1, a novel PKB binding protein [40]. Additional analyses showed that hFt1

protein interacts directly with PKB and that it is likely to function as a regulator of PKB activity that controls apoptotic signals [40].

A high-throughput BiFC-based screen was performed to identify regulators of telomere signaling in human cells [109]. This protein complementation array screen combined BiFC and flow cytometry analyses to detect low-affinity or transient interactions of six core telomeric proteins TRF1, TRF2, POT1, RAP1, TIN2 and TPP1. Each telomeric protein was tagged with the N-terminal fragment of Venus (VN) at either N or C terminus. The retroviral array library of the VC fusion prey contained 11,880 ORFs tagged with VC at either N or C terminus, which were generated by Gateway cloning of 12,212 ORFs from hORFeome [110]. The cell lines expressing each VN-tagged telomeric protein bait were then infected with the retroviral array library of the VC-tagged prey to test four combinations of fusions for each protein pair. Analyses of these BiFC signals validated that the cell mixing and BiFC strategies had worked successfully to detect positive PPIs of the core telomeric proteins. Variations in bait or prey protein expression level did not affect the ability to detect a BiFC signal, suggesting the robustness of their assay [109]. BiFC might thus have stabilized transient interactions of the telomeric proteins, leading to the efficient detection of the PPIs. However, one caveat is that transient versus stable PPIs might be difficult to distinguish because formation of a bimolecular fluorescent complex may be irreversible.

Significant in this study of the telomere interactome is the development of an automated data analysis program called 'CytoArray' used to analyze the large amount of flow cytometry data [109]. Data processing by this custom designed analysis program involves four major steps: defining positive regions in flow cytometry plots, calculating weighted positive ratios (WPR), determining statistically significant cutoff values, and removing common contaminants. Calculating WPR rather than positive percentages (PP: positive cell number/total live cell number) improves the sensitivity of detection of BiFC signals because signal intensity is not considered in PP. CytoArray also helped to eliminate false positives based on a bait-specific threshold. Subsequent GST-pull down assays confirmed 72% of BiFC results, verifying the effectiveness of this BiFC-based screen. This screen identified over 300 proteins that interact with the six core telomeric proteins, and greater than 80% of these PPIs had been previously unknown [109]. As expected, most proteins identified were found to be involved in processes related to the major roles of the six core telomeric proteins. However, some proteins including protein kinases and E3 ubiquitin ligases are novel interactors of telomeric proteins. This study is the first to use live human cells to perform a genome-wide search of PPIs by BiFC assays, and it further extends the application of BiFC-based genome-wide studies.

Recent development of adenoviral BiFC vectors allows efficient BiFC-based analysis in mammalian cells. Adenovirus is a convenient gene delivery tool because it can transfect various types of cells *in vivo* and *in vitro*, and the expression of transgene is quite consistent [111]. However, adenoviral vectors had not been amenable for a large-scale study because of the difficulty in recombination with a target gene. A simple and robust system for constructing recombinant adenoviruses, termed AdHTS (adenovirus high-throughput system), has been developed using Gateway *in vitro* site-specific recombination technology and terminal protein (TP)-coupled adenoviral vectors [112]. AdHTS enables generation of

multiple recombinant adenoviruses in 96-well plates simultaneously without the need for additional cloning or recombination in bacteria or mammalian cells. Adenoviral BiFC vectors have been generated based on AdHTS and have been used to monitor G protein-coupled receptor (GPCR) activation in human cells by an adenovirus-based β -arrestin BiFC assay [113]. Human GPCR cDNAs were cloned into adenoviral BiFC vectors to generate the cDNAs for expression of the GPCR tagged with either VN or VC at the C termini and the subsequent β -arrestin BiFC assay. This study found that 33 GPCRs belonging to various classes exhibit markedly increased BiFC signals in intracellular pits and vesicles upon stimulation with the appropriate agonists, suggesting that this assay represents a rapid and universal method for monitoring GPCR activation [113].

Since aberrant PPIs contribute to the pathology of several diseases and viral protein interactions are often important for their function, PPIs have been major targets for drug development [114]. Cell-based BiFC assays have been successfully applied to high-throughput screenings for small molecule inhibitors of PPIs in mammalian cells. Dimerization of human immunodeficiency virus (HIV)-1 proteins, such as Nef and viral protein R (Vpr), are essential for viral pathogenesis and AIDS progression [115]. To develop anti-retroviral drug leads, BiFC assays have been performed to screen a library of small molecules for HIV viral protein inhibitors that interfere with dimerization of these viral proteins [116, 117]. In another recent study, BiFC technology was used to screen anti-influenza A virus drug candidates that could reduce autophagy induction by affecting Beclin-Bcl2 heterodimer dissociation [118]. As discussed earlier, to facilitate anticancer drug discovery, mKG-based BiFC system was used to screen for PPI inhibitors in a natural product library [44]. These studies demonstrate that BiFC assays have great potential for high-throughput screening in drug discovery. BiFC assays may also provide a deeper understanding of the mechanisms of action of potential drugs by uncovering where and when they have an effect in the cell.

CONCLUDING REMARKS

The development of BiFC has allowed efficient detection and visualization of PPIs *in vivo*. Recent application of BiFC in large-scale studies has demonstrated its potential for uncovering protein interactomes in live cells. In particular, cDNA libraries in plants and mammalian cells as well as the VN fusion library in budding yeast have allowed BiFC assays to be more widely applied in high-throughput studies. As in many other large-scale screening methods, individual candidates identified by a BiFC-based screen need to be carefully validated for their interactions by using proper controls and other complementary methods. With a variety of fluorescent proteins already designed for use with this assay, other combinatorial methods and further development of programs to handle large-scale data resulting from BiFC assays will provide versatility to BiFC-based high-throughput studies. While the greatest capability of BiFC lies in the detection of subcellular localization of a complex, one caveat is that BiFC complexes often become stabilized after association of the fluorescent protein fragments, and thus the dynamics of BiFC complexes might be different from those of the endogenous protein-protein complexes [25]. Although the irreversibility often limits its use for monitoring temporal changes in protein interactions in real time, this irreversible nature of BiFC system facilitates identification of weak and transient

interactions. Future development of a reversible BiFC system will greatly increase the flexibility of its application. To minimize any potential problem of non-physiological protein interactions or subcellular localization due to overexpression, fusion proteins should be expressed at their endogenous levels whenever possible. Quantification of fluorescence signals of BiFC complexes would also be more relevant under the physiological condition.

A critical factor for further applications of this simple, yet powerful screening method is the development of additional cDNA or genome-wide prey libraries in diverse cell types. As in the case of any fluorescent-based techniques, development of additional fluorophores with higher quantum yield and variants with enhanced signal-to-noise ratio will further improve this technology. In addition, BiFC analysis combined with recent advances in fluorescence microscopy will provide new insights into the molecular mechanisms of protein functions in live cells with high spatiotemporal resolution. As PPIs underlie many cellular functions including numerous signaling pathways and PPIs are also implicated in several diseases, an efficient quantification of the BiFC signals in a cell-based assay is likely to allow further applications to the discovery of novel drugs that modulate PPIs.

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Abbreviations

BiFC	bimolecular fluorescence complementation
PPI	protein-protein interaction
GFP	green fluorescent protein
YFP	yellow fluorescent protein
RFP	red fluorescent protein
CFP	cyan fluorescent protein
IP	immunoprecipitation
ORF	open reading frame
GST	glutathione S-transferase
PCA	protein fragment complementation assay
MYTH	membrane yeast-two hybrid
FRET	fluorescence resonance energy transfer
TAP	tandem affinity purification
mKG	monomeric Kusabira-Green

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Highlights

- A BiFC analysis is used to detect protein-protein interactions in living cells.
- A BiFC assay provides a simple, sensitive method for high throughput screening.
- Recent development and applications of BiFC in proteomics research are described

Figure 1. Schematic representation of the BiFC analysis

The N-terminal and C-terminal fragments (YN and YC) of YFP are fused to two proteins of interest (A and B). The interaction between A and B allows formation of a bimolecular fluorescent complex. In contrast, b (a mutant form of B or a non-binding partner) cannot form a complex with YN-A, showing no fluorescence. The images show an example of BiFC complex formed on the vacuolar membrane of yeast cells (upper panel) and a control with a mutant that fails to form a complex. The image is reproduced from Singh et al. [85]. Copyright (2008) National Academy of Sciences, U.S.A.

Figure 2. Schematic diagram of the genome-wide BiFC analysis in budding yeast
 For the genome-wide BiFC analysis, each strain of the VN fusion library is mated with a $MAT\alpha$ strain expressing a protein of interest tagged with VC, thus generating a diploid collection. Each strain of the obtained diploid collection expressing both the VN fusion and the VC fusion is analyzed by fluorescence microscopy, and the images are collected and quantitatively analyzed. Adapted from Sung et al. [90].

Table 1

Major properties of available BiFC reporter proteins

<i>Fluorescent Protein</i>	<i>Excitation/Emission Maxima (nm)</i>	<i>Split Site</i>	<i>Temperature Sensitivity of Maturation</i>	<i>Interactions Tested</i>	<i>Experimental Cells or Organisms</i>	<i>Reference</i>
<i>EYFP</i>	514/527	154/155	Yes (poor maturation at 37°C)	bJun-bFos ATF2-Jun p50-p65 p50-Jun p50-Fos p65-Jun p65-Fos p65-IκBα	COS-1 NIH3T3 HeLa	[23]
<i>Venus</i>	514/529	154/155 or 172/173	No	bJun-bFos	COS-1	[51]
<i>Citrine</i>	516/528	154/155 or 172/173	No	bJun-bFos	COS-1	[51]
<i>ECFP</i>	452/478	154/155 or 172/173	Yes (poor maturation at 37°C)	bJun-bFos bATF2-bJun	COS-1	[41]
<i>Cerulean</i>	439/479	172/173	No	bJun-bFos	COS-1	[51]
<i>EGFP</i>	475/505	157/158	ND ^a	Z peptides TPR domains- Hsp90 TPR domains- Hsc70	<i>E. coli</i>	[22, 39]
<i>mRFP1-Q66T</i>	549/570	168/169	Yes (poor maturation at 37°C)	hrGFP homodimer GC3-CPC BP/KNAT1- BLH7	Tobacco protoplasts Onion epidermal cells	[60]
<i>mCherry</i>	587/610	159/160	Yes (poor maturation at 37°C)	EGFP homodimer LTag-p53	Vero cells	[64]
<i>mLumin</i>	587/621	151/152	No	bJun-bFos EGFR-STAT5A EGFR-STAT5B	COS-7	[66]
<i>mNeptune</i>	600/650	155/156	No	EGFP homodimer bJun-bFos	HeLa	[70]
<i>mKG</i>	494/506	168/169	No	PX-PB1 in p47 ^{phox}	HEK293T	[42]
<i>Dronpa</i>	503/518	164/165	No	hHus1-hRad1	HEK293	[79]
<i>iRFP</i>	690/713	120/123 (between PAS and GAF domains)	No	E-coil-K-coil FRB-FKBP	HeLa	[71]

^aND: not determined

Table 2

Summary of BiFC-based large-scale PPI studies in various cell types

Organism	Bait	Prey	Fluorescent protein fragments used	Strategy for generating BiFC constructs	Screening results	Reference
Yeast	Smt3	5,911 yeast proteins	VN and VC	PCR-mediated chromosomally tagged VN/VC	367 SUMO interactor candidates	[90]
	17 ABC transporters	Interactor candidates found by MYTH screening	VN and VC	PCR-mediated chromosomally tagged VN/VC	44 positive BiFC signals out of 79 interactions	[18]
Plant	58 core cell cycle proteins	58 core cell cycle proteins	nGFP and cGFP	Vector construction by Multi-Site Gateway technology	341 interactions out of 917 pairs	[102]
	CPK3	cDNA library of <i>A. thaliana</i>	YN and YC	Vector construction by Multi-Site Gateway technology	8 interactor candidates	[106]
	19 proteins from G-protein complexes	33 proteins from G-protein complexes	nYFP and cYFP	Vector construction by Gateway technology	74 positive BiFC signals out of 78 pairs tested	[108]
	VirE2, VirD2, CTE domain	cDNA library of <i>A. thaliana</i>	nVenus/nYFP and cYFP	cDNA expression vector construction by Gateway technology	5, 6 and 2 interactor candidates for each bait	[103]
Mammalian cells	Telomeric proteins TRF1, TRF2, POT1, RAP1, TIN2, TPP1	11,880 retroviral array library	VN and VC	Retroviral array library construction by Gateway cloning	320 interactor candidates of telomeric core proteins	[109]
	GPCRs	β -arrestin 2	VN and VC	Construction of adenoviral BiFC vectors by AdHTS	Activation signals of 33 GPCRs	[113]
	PKB	Human brain cDNA library	GFP[1] and GFP[2]	Categorization of cDNA library vectors into 4 fractions to maximize their incorporation into cells	17 novel PKB interactor candidates	[40]
<i>In vitro</i>	TGF7, PAC1, PAC3	β -catenin, PAC2, PAC3	mKG_N and mKG_C	Vector construction by Gateway technology	11 inhibitors for 3 PPIs out of 123,599 samples in the natural product library	[44]