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VISUALIZATION OF MOLECULAR INTERACTIONS USING BIMOLECULAR FLUORESCENCE COMPLEMENTATION ANALYSIS: CHARACTERISTICS OF PROTEIN FRAGMENT COMPLEMENTATION

Tom K. Kerppola

Howard Hughes Medical Institute and Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109-0650, Tel: (734) 764-3553, FAX: (734) 615-3397

Tom K. Kerppola: kerppola@umich.edu

Summary

Investigation of the molecular processes that sustain life must include studies of these processes in the normal cellular environment. The bimolecular fluorescence complementation (BiFC) assay provides an approach for the visualization of protein interactions and modifications in living cells. This assay is based on the facilitated association of complementary fragments of a fluorescent protein that are fused to interaction partners. Complex formation by the interaction partners tethers the fluorescent protein fragments in proximity to each other, which can facilitate their association. The BiFC assay enables sensitive visualization of protein complexes with high spatial resolution. The temporal resolution of BiFC analysis is limited by the time required for fluorophore formation as well as the stabilization of complexes by association of the fluorescent protein fragments.

Many modifications and enhancements of the BiFC assay have been developed. The multicolor BiFC assay enables simultaneous visualization of multiple protein complexes in the same cell and can be used to investigate the competition among mutually exclusive interaction partners for complex formation in cells. The ubiquitin-mediated fluorescence complementation (UbFC) assay enables visualization of covalent ubiquitin family peptide conjugation to substrate proteins in cells. The BiFC assay can also be used to visualize protein binding to specific chromatin domains as well as other molecular scaffolds in cells. BiFC analysis therefore provides a powerful approach for the visualization of a variety of processes that affect molecular proximity in living cells.

Introduction

Most of the chemistry of life takes place inside the confines of cell membranes. The molecular components that govern cell functions are organized in numerous subcellular compartments and domains. Studies of the physical and chemical processes that underlie cell functions have been conducted, for the most part, in cell extracts and in reconstituted reactions. It is essential to establish if the mechanisms identified under these *in vitro* conditions faithfully reflect the processes inside living cells. Methods for the investigation of molecular processes in living cells are therefore of great significance.

Molecular interactions are a fundamental mechanism for the transfer and integration of information in cells. Macromolecular interactions in particular constitute the regulatory network that coordinates cellular functions. Numerous methods have therefore been devised to detect and to characterize molecular interactions. Many of these methods such as co-precipitation and reconstitution of complexes using purified components require removal of the interaction partners from their normal cellular environment. The major advantage of these

and other biochemical methods is that it is possible to characterize an interaction in great detail, including analysis of the structure of the complex at atomic resolution using X-ray crystallography or NMR. Other methods, such as identification of suppressor mutations and two-hybrid assays enable detection of combinations of genes that have non-additive functional consequences, but they do not distinguish whether these consequences are produced through direct or indirect interactions of the gene products. The major advantage of these and other genetic methods is that it is possible to investigate an interaction in the normal cellular environment, including the physiological context of multicellular organisms.

Methods that enable both direct detection of interactions and characterization of the interactions in the normal cellular environment are based on various biophysical principles and the use of proteins in which the interaction partners are linked to tags whose localization or proximity to each other can be detected. The classical proximity-based assay is Förster/fluorescence resonance energy transfer (FRET) analysis^{1–4}. This assay, which has been widely used as a tool to study the architectures of macromolecular complexes in vitro, has also been applied to the study of macromolecular interactions in cells. A major advantage of this assay is the ability to detect changes in interactions virtually instantaneously. However, the relatively low sensitivity of this assay to interactions where only a small proportion of the interaction partners associate with each other at any one time often requires overexpression of the interaction partners in cells to produce a higher proportion of complexes than normal. Detection of interactions using FRET also requires the fluorophores of the two interaction partners to be in relatively close proximity (<10 nm apart) in orientations that permit dipole coupling.

An assay closely related to FRET is bioluminescence resonance energy transfer (BRET)⁵. It is potentially more sensitive than FRET because of the ability to detect very low photon flux in the absence of excitation. However, instruments have not been developed that would enable detection of the subcellular localization of BRET signal at better than approximately 10 μ m resolution. Fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation (FCCS) are based on measurement of the mobilities of single or multiple labelled molecules². The FCS approach can estimate the molecular size of a complex based on its diffusion and FCCS can evaluate whether two fluorophores have statistically correlated mobilities. Several related approaches that are based on correlation of fluorescence in images acquired using confocal microscopy are also based on measurement of the mobilities of single or multiple fluorescent molecules.

Detection of macromolecule interactions using protein fragment association

Molecular proximity can also be measured by tagging interaction partners with fragments of proteins that individually have no function, but that can form a complex with a detectable function when they are brought in proximity to each other. Fragments of many proteins have been identified that can associate with each other to form an active complex. The efficiency of association for some protein fragments can be enhanced by fusing the fragments to interaction partners. The interaction of the partners increases the local concentrations of the fragments, facilitating their association. The activity of the complex formed by the protein fragments can be used to detect the interaction.

Fragments of many different proteins have been used to detect protein interactions. The principle was originally demonstrated using fragments of ubiquitin⁶. Subsequently, fragments of β -galactosidase, dihydrofolate reductase, autofluorescent proteins, β -lactamase and several luciferases have been used^{7–14}.

The association of fluorescent proteins fragments fused to interaction partners is particularly useful because the intrinsic fluorescence of the complex enables visualization of the subcellular localization of the interaction without the use of additional exogenous reagents⁹. This

approach, designated bimolecular fluorescence complementation (BiFC) analysis is the most widely used method for the detection of protein interactions that is based on protein fragment association^{15, 16}

Identification of protein fragments for the investigation of protein interactions

Protein fragments that can be used for the investigation of protein interactions have been identified by empirical testing of many candidates. No procedure or algorithm that predicts protein fragments that function as reporters of protein interactions has been reported. Such fragments must have the potential to associate with each other when brought in proximity to each other by an interaction between proteins fused to the fragments. However, these fragments must not be able to associate with each other efficiently in the absence of a specific interaction between proteins fused to the fragments. These seemingly contradictory requirements, in combination with the limited understanding of protein folding pathways in living cells thwart the design of protein fragments that could associate with each other only when brought together by an interaction between proteins fused to the fragments. All of the protein fragments that have been used for the investigation of protein interactions correspond to segments of intact proteins. It is likely that the folding pathways of the protein fragments differ from those of the intact proteins, suggesting that the fragment sequences that are optimal for the investigation of protein interactions also differ from those of the intact proteins.

The structures of proteins that can be divided into fragments that can be used to investigate protein interactions vary widely (Fig. 1). Likewise, the nature of the interface formed when the two fragments associate with each other varies among these proteins. There is a prevalence of β sheet secondary structure in these proteins, but it is not clear if this is related to the ability of the fragments to associate with each other when they are brought together by protein interactions. Notably, only a few fragments of each protein that can associate to form a functional complex have been identified. This characteristic is therefore rare, at least among the proteins that have been investigated. None of the structures of the fragments are known either alone or upon association with each other. It is unlikely that the individual fragments fold into a structure resembling the intact protein in the absence of the complementary fragment. Most of the protein fragments are likely to be unfolded and to adopt an ensemble of rapidly interconverting conformations, although this has not been experimentally tested. Such an ensemble may be significant for the conditional association of the protein fragments since only a subset of the conformations may allow fragment association. However, since the folding pathways of all protein fragments used for studies of protein interactions are unknown, this information cannot be used to predict or to design protein fragments with enhanced performance characteristics.

Following protein fragment association, the structure of the complex is likely to be similar to that of the intact protein, although the discontinuity in the peptide backbone may influence local segment dynamics. The spectra of complexes formed by fluorescent protein fragments are indistinguishable from those of the corresponding intact fluorescent proteins^{9, 17}, suggesting that the structure and dynamics of complexes formed by fluorescent protein fragments are similar to those of the intact protein.

Spontaneous and facilitated association of protein fragments

It is likely that most protein fragments that can associate with each other do so more efficiently when their local concentrations are increased. Conversely, protein fragments whose association is facilitated by tethering them to a macromolecular complex are likely to have some propensity to associate with each other even in the absence of such tethering. The factors that affect the spontaneous versus facilitated association of protein fragments are not known. A higher efficiency of fragment association would enhance both spontaneous and facilitated complex

formation whereas a lower efficiency would impede both pathways. To be useful for the investigation of protein interactions, the fragments must associate with an efficiency that is high enough to detect when they are present in the same complex, but not so high as to associate independent of complex formation.

Interaction partners can facilitate the association of fused protein fragments by increasing either the rate of fragment association or the thermodynamic stability of the complex. Protein interactions in cells are not at equilibrium because of protein localization, regulation of interactions by chaperonins and the flux created by protein synthesis and degradation. The rate of fragment association can be enhanced if the rate of complex formation by the interaction partners is faster than the rate of association of the protein fragments when they are not fused to the interaction partners. If the rate of association of the protein fragments is faster than that of the interaction partners, then the interaction partners can increase complex formation only by enhancing the thermodynamic stability of the complex.

The rate of fragment association has not been measured directly for any of the fragments used to investigate protein interactions either alone or when fused to interaction partners. However, this rate can be estimated based on measurement of the activity or function of the complex formed by the fragments. Fragments of dihydrofolate reductase, β -lactamase, TEV protease and some luciferases can associate within minutes after association of the interaction partners in cells ^{10, 12, 14, 18}. The one-minute lag in the kinetics of BiFC complex formation by fluorescent protein fragments fused to bZIP domain interaction partners in vitro has been attributed to the rate of fragment association ⁹. Studies of the kinetics of BiFC complex formation by fluorescent protein fragments fused to FKBP and FRB following rapamycin induction did not detect a lag in the kinetics of BiFC complex formation ¹⁹, but the temporal resolution of these experiments does not exclude the possibility of a lag shorter than one minute.

The rates of association of most protein fragments are likely to be slower than those of typical interaction partners since the protein fragments have not evolved to associate with each other efficiently. It is therefore likely that protein fragments fold into many conformations that are not permissive for complex formation. The effect of the interaction partners on fragment association is affected by the positions of the fragments in the complex. Steric constraints on fragment association are expected to retard fragment association compared to the association of free fragments at the same local concentration. These constraints can be partly alleviated by the use of flexible linker peptides to connect the fragments to the interaction partners.

An interaction between the partners fused to the protein fragments is likely to increase the thermodynamic stability of the complex formed by the fusion proteins compared to complexes formed by the protein fragments alone. Whether this increase in complex stability materially affects the amount of complexes formed or not depends on the rate of dissociation of complexes formed by the protein fragments alone and on the rate of fusion protein degradation. These parameters are likely to vary among different protein fragments and between fusions to different interaction partners. Since association of most protein fragments produces a large interaction interface and is coupled to folding to form the native protein structure, it is likely that protein fragment association is energetically favourable even in the absence of interactions partners. Thermodynamic stabilization of complexes by interaction partners fused to the protein fragments has no detectable effect on the amount of complexes formed if dissociation of the fragments alone is slower than their degradation or takes longer than the time-frame of the experiment.

Analysis of molecular proximity

The association of protein fragments can be facilitated by the elevated local concentration produced by confining the fragments to a limited space. The facilitated association therefore

does not require direct contact between proteins fused to the fragments. Assembly of the fusion proteins in a macromolecular complex can facilitate protein fragment association even if there is no direct contact between the proteins fused to the fragments. The presence of proteins within the same macromolecular complex generally indicates that they participate in the same biological process. Thus, the ability to detect protein assembly within a macromolecular complex in the absence of direct contact is a valuable characteristic of assays based on protein fragment association.

The ability to detect protein fragment association when they are tethered to the same complexes can be used to detect other macromolecules in cells. Thus, if two fusions to protein fragments bind to a macromolecule or macromolecular complex, the presence of the macromolecule or complex can be detected in cells. The fusions can include protein domains selected to recognize specific endogenous molecules. Thus protein fragment association could in principle be used to detect endogenous proteins in living cells.

Molecular proximity can be modulated by both non-covalent and covalent interactions. Covalent linkage of fusion proteins can facilitate association of protein fragments when the fragments are fused to proteins that become covalently linked or when the fusion proteins interact with proteins connected by covalent bonding. The effects of covalent bonds on molecular proximity have been used to visualize ubiquitin family peptide conjugation to substrate proteins²⁰ as well as polypeptide ligation by intein ligases²¹.

Co-localization within a small spatial domain can in principle also facilitate protein fragment association. Assuming unconstrained fragment mobilities and ignoring excluded volume effects, the efficiency of protein fragment association is predicted to decrease with the cube of the radius of the space that is available for fragment diffusion. This relationship can provide a rough estimate of the relative efficiencies of association of fragments that are tethered to the same molecular complex and fragments that are confined to a space with a radius ten times larger than the molecular complex. Although the latter fragments would co-localize by confocal microscopy, their rate of association would be three orders of magnitude lower than that of the fragments tethered to the complex. In practice, the rate of fragment association is affected by steric constraints that are only partially alleviated by flexible linker peptides and dynamic motions of the fusion proteins. Nevertheless, the association of many protein fragments is inefficient when they are not tethered by covalent or non-covalent interactions, even if the fragments co-localize at the resolution of light microscopy.

Bimolecular fluorescence complementation analysis

The bimolecular fluorescence complementation (BiFC) assay is based on the observation that the association of fluorescent protein fragments can be facilitated by an interaction between proteins fused to the fragments⁹ (Fig. 2). The BiFC assay enables visualization of protein interactions at high spatial resolution without the need for other exogenous agents. A wide range of structurally divergent protein complexes in a variety of different organisms and cell types have been visualized using the BiFC assay^{15, 22}. In principle, the BiFC assay can be used to visualize interactions between any proteins that can be fused to fluorescent protein fragments. The assay can be used to detect interactions in any subcellular compartment in any aerobically growing organism or cell that can be genetically modified to express the fusion proteins.

A palette of BiFC complexes

A great advantage of bimolecular fluorescence complementation compared to complementation by other protein fragments is the intrinsic fluorescence of the complex. This enables detection of protein interactions without the use of other exogenous reagents.

Moreover, multiple protein interactions can be visualized in the same cell using multicolor BiFC analysis (Fig. 3) ¹⁷. In this approach alternative interaction partners are fused to fluorescent protein fragments that produce BiFC complexes with distinct spectra. Many spectrally distinct BiFC complexes have been identified ^{17, 23}. In addition, some fluorescent protein fragments can associate with several different complementary fragments to produce BiFC complexes with distinct spectra ¹⁷ (Table I). These fragments enable visualization of the competition for complex formation among alternative interaction partners ²⁴. The relative efficiencies of BiFC complex formation by different combinations of fluorescent protein fragments do not reflect the equilibrium among alternative interactions (see below). However, these efficiencies reflect the relative efficiencies of complex formation among alternative interaction partners at the time of fusion protein synthesis.

Design of fusion proteins for BiFC analysis

The most important objective in the design of fusion proteins for use in BiFC analysis is to reproduce as closely as possible the characteristics of the endogenous proteins. The fluorescent protein fragments can be fused to either end of each protein. Exchange of the fragments between the two fusions produces complexes that are sterically non-equivalent. Thus, eight different combinations of fusions can be tested (Fig. 4). The linker sequences used to join the fluorescent protein fragments to the interaction partners can also affect the functions of the fusion proteins and BiFC complex formation.

Two different positions in YFP and other GFP family members can be used to divide the proteins in two fragments whose association can be facilitated by protein interactions ¹⁷. The positions correspond to amino acid residues 155 and 173 in YFP (Table I). Fluorescent protein fragments that contain overlapping sequences encompassing these fragments can also be used. No major differences in the characteristics of BiFC complexes formed by the two different types of fragments have been reported. The large number of fusion protein configurations that can be used for BiFC analysis facilitates the identification of fusion proteins that can form BiFC complexes and reproduce the characteristics of the endogenous proteins.

To reproduce the properties of endogenous proteins it is critical not to express the fusion proteins at levels higher than their endogenous counterparts. Overexpression can cause promiscuous interactions, mis-localization and alter protein functions. Ideally, the functions of the fusion proteins should be tested by replacing the endogenous proteins with the fusions. When this is not feasible, the fusion proteins should be tested under conditions that reflect those experienced by their endogenous counterparts as closely as possible.

Kinetics of BiFC complex fluorescence

The fluorescence intensities of BiFC complexes do not match the kinetics of interactions between the corresponding endogenous proteins for several reasons. First, there is a delay between the time of fusion protein interaction and the time when the complex becomes fluorescent. This delay is due to the time required both for fluorescent protein fragment association and for the chemical reactions that produce the fluorophore ^{9, 15}. Nevertheless, BiFC complex fluorescence can often be detected within minutes after fusion protein interaction. In studies of the time-course of BiFC complex formation by YFP fragments fused to FKBP and FRB, fluorescence was detected within 10 minutes following rapamycin induction and continued to increase for at least 8 hours ¹⁹. Rapid detection of signal does not indicate a short half-time of fluorescent BiFC complex formation, but is due to stochastic association of a small fraction of the fluorescent protein fragments followed by fluorophore maturation. Detection of this weak signal is possible because of the high sensitivity of fluorescence detection and the minimal background fluorescence of most cells.

Fluorescent protein fragments chemically conjugated to oligonucleotides can form BiFC complexes more than two orders of magnitude faster than fluorescent protein fragments fused to interacting proteins²⁵. The reasons for this difference are unclear, but may be related to chemical synthesis of the oligonucleotide conjugates versus biological synthesis of the fusion proteins. In general, BiFC analysis enables rapid (within tens of minutes) detection of complex formation, but does not allow instantaneous visualization of complexes or analysis of the kinetics of complex formation in real time.

BiFC complex stabilization by fluorescent protein fragment association

The dynamics of BiFC complexes differ from those of endogenous protein interactions because of stabilization of BiFC complexes by association of the fluorescent protein fragments^{9, 19}. BiFC complexes formed by fragments of YFP fused to leucine zipper dimerization partners have a half-life of more than 24 hours *in vitro*⁹. BiFC complexes containing the fluorescent protein fragments alone are stable ($t_{1/2} > 1$ hour) in the presence of up to 5 M urea²⁶. In living cells, BiFC complexes formed by fluorescent protein fragments fused to FKBP and FRB are stable for at least 8 hours in the presence of the competitive inhibitor FK506¹⁹. The stabilization of BiFC complexes by fluorescent protein fragment association is not surprising considering the extensive interaction interface that forms upon association of the two fragments.

The interaction interface between the fluorescent protein fragments comprises four new β strand interfaces. Each interface contains between 6 and 9 hydrogen bonds between the peptide backbones of the two fragments. These hydrogen bonding networks are very stable since the protons mediating these hydrogen bonds have binding energies of 6–11 kcal mol⁻¹ based on deuterium exchange studies of intact fluorescent proteins²⁷. It is likely that the fluorescent protein fragments are at least partially unfolded prior to their association since neither of the fragments forms a contiguous β sheet in the intact fluorescent protein. Thus, dissociation of BiFC complexes requires breaking more than 30 hydrogen bonds and solvating the hydrophobic interior of the β barrel.

Despite their great predicted stability, several cases where the fluorescence of BiFC complexes is reduced under conditions predicted to cause dissociation of the interaction partners fused to the fragments have been reported. The fluorescence intensity of BiFC complexes formed by YFP fragments fused to phospholipase C β dimers is reduced by addition of a competitor protein to membrane preparations or by acetylcholine treatment of living cells²⁸. Likewise, the fluorescence intensities of cells that contain BiFC complexes formed by YFP fragments fused to actin are reduced in the presence of latrunculin that can inhibit actin polymerization²⁹. In some of these cases, it is difficult to exclude the possibility that the decrease in fluorescence intensity is caused by a change in the environment of the BiFC complexes that causes a change in fluorescence intensity or by degradation of the fusion proteins. The fluorescence intensity of BiFC complexes formed by fluorescent protein fragments conjugated to oligonucleotides also decreases rapidly upon addition of an oligonucleotide competitor or 2 mM MgCl₂ to the reaction²⁵. It is formally possible that the fluorescence intensity of BiFC complexes is affected by the changes in conditions. Consequently, it is essential to consider potential effects of stabilization of protein complexes by fluorescent protein fragment association when interpreting results of BiFC analysis. The effects of such stabilization on cellular functions are likely to vary depending on the proteins investigated and could become more severe over time. It is therefore advantageous to visualize interactions between fusion proteins as soon as possible after complex formation.

Specificity of BiFC complex formation

The association of fluorescent protein fragments is presumably energetically favorable since it can stabilize complexes formed by specific interaction partners. It is therefore possible that

fragment association can also stabilize complexes formed by proteins that do not normally interact with each other. To determine if BiFC complex formation reflects a specific interaction between the putative interaction partners, it is essential to determine the effects of mutations that eliminate the interaction on BiFC complex fluorescence. Ideally, mutations that eliminate the interaction but have no other effects on the characteristics of the fusion proteins should be tested. In particular, it is essential to establish that mutations do not affect the levels of fusion protein expression or their localization. If such mutations produce a significant change in the efficiency of BiFC complex formation, it is likely that a specific interaction between the proteins fused to the fluorescent protein fragments facilitates their association.

Since association of the fluorescent protein fragments is energetically favourable, it may seem surprising that they do not associate with each other independent of fusions to interaction partners. The explanation to this conundrum is that the low rate of autonomous fluorescent protein fragment association generally produces very low levels of fluorescent complexes. The efficiency of BiFC complex formation is determined by the rate of fragment association and not by the thermodynamic stability of the complex. Since the rate of fragment association is determined by the proportion of the fusion proteins that form complexes with each other, BiFC complex formation reflects the proportion of the fragments that are present in the same complex. This proportion is reduced if the fusion proteins favour interactions with other cellular proteins.

The dominant role of kinetics rather than thermodynamics in BiFC complex formation may seem counterintuitive, especially since BiFC complexes in cells can accumulate over several days. There are many reasons the association of fluorescent protein fragments does not reach equilibrium. In vitro studies of BiFC complex formation have demonstrated that many fluorescent protein fragment fusions undergo irreversible misfolding when they do not associate with complementary fluorescent protein fragments⁹. The efficiency of BiFC complex formation under these conditions is determined by the competition between BiFC complex formation and mis-folding, each of which is irreversible. A specific interaction between proteins fused to the fluorescent protein fragments can shift the balance in favour of BiFC complex formation. Thus, the specificity of BiFC complex formation in vitro depends on the folding characteristics of the fluorescent protein fragments.

Studies in cells have identified another mechanism that contributes to the specificity of BiFC complex formation. Cells that express fluorescent protein fragments fused to proteins that do not interact with each other often have lower fluorescence intensities than cells that express the fluorescent protein fragments alone. Thus, fusions to non-interaction partners have a negative effect on BiFC complex formation, presumably through interactions with endogenous proteins that reduce BiFC complex formation either by spatially separating the fusion proteins or by sterically interfering with fluorescent protein fragment association. The efficacy of this negative effect depends on the characteristics of the fluorescent protein fragments. Fragments of Venus that fold rapidly form BiFC complexes more efficiently than fragments of YFP that fold slowly¹⁹. Thus, fragments of Venus can exhibit a higher efficiency of BiFC complex formation than fragments of YFP even when the fragments are fused to proteins that do not interact with each other (Table I). Misfolding of the fluorescent protein fragments does not appear to limit BiFC complex formation in cells since fusion proteins synthesized prior to the induction of complex formation can form BiFC complexes with the same efficiency as newly synthesized proteins¹⁹. Competition by interactions with endogenous proteins is likely to be the major mechanism that determines the specificity of BiFC complex formation in living cells.

The low efficiency of association of the fluorescent protein fragments is reflected by the low fluorescence intensities of cells that produce BiFC complexes relative to the fluorescence intensities of cells that produce the corresponding intact fluorescent proteins. The lower

fluorescence intensities are unlikely to be due to a difference in intrinsic fluorescence intensity (absorbance or quantum yield) between BiFC complexes and the corresponding intact fluorescent proteins since BiFC complexes formed *in vitro* have a fluorescence intensity comparable to that of the same concentration of the corresponding intact fluorescent protein⁹. The fluorescence intensities of cells that produce BiFC complexes are typically between 0.1% and 10% of the fluorescence intensity of cells that produce the corresponding intact fluorescent protein. This difference is due, at least in part, to competition for complex formation by endogenous proteins. Thus, even for specific interaction partners, typically only a minority of the fluorescent protein fragments form BiFC complexes.

Choice of fluorescent protein fragments

Many, but not all combinations of GFP-family fluorescent proteins fragments truncated at residue 155 or 173 produce BiFC complexes in mammalian cells⁹. The wide selection of fluorescent protein fragments provides a choice among a variety of fluorescent protein fragments that produce BiFC complexes with varying degrees of brightness as well as other characteristics. The importance of low levels of fusion protein expression, the advantages of visualizing BiFC complexes soon after their formation and the small proportion of fluorescent protein fragments that form BiFC complexes put a great premium on the brightness of BiFC complexes. Consequently, many attempts have been made to identify fluorescent protein fragments that produce higher intensities of BiFC complex fluorescence^{30–32}. Venus is the brightest fluorescent protein that has been identified³³, and fragments of Venus produce the highest intensity of BiFC complex fluorescence³¹.

Studies of Venus fragments fused to different interaction partners have revealed differences in the specificity of BiFC complex formation^{19, 31, 32}. In many cases, mutations in the interaction partners have reduced BiFC complex formation by Venus fragment fusions, corroborating the specificity of BiFC complex formation by these fragments^{31, 34, 35}. In a direct comparison of BiFC complex formation by fragments of Venus and fragments of YFP fused to wild type and mutant bZIP domains, the fragments of Venus exhibited a higher specificity of BiFC complex formation than the YFP fragments³¹. Conversely, expression of the Venus fragments alone in *Xenopus* embryos produced a higher fluorescence intensity than expression of the YFP fragments³². Analysis of the inducible interaction between FKBP and FRB using fusions to different fluorescent protein fragments revealed a higher background of spontaneous association and reduced inducibility of BiFC complex formation by the N-terminal fragment of Venus than by the corresponding YFP fragment¹⁹ (Table I). The higher intensity of BiFC complex formation by Venus fragments is therefore compromised by the higher background of spontaneous fragment association at least under some circumstances.

Analysis of the contributions of individual amino acid residues to the difference between YFP and Venus fragment association indicated that substitution of any one of the amino acid residues in Venus by the corresponding residue in YFP eliminated the high level of autonomous fragment association in *Xenopus* oocytes, but not in HEK293T cells³². The Venus fragments containing the M153T substitution fused to Smad2 and Smad4 exhibited conditional BiFC complex formation in *Xenopus* oocytes³². Likewise, the N-terminal Venus fragment containing the M153T substitution fused to FKBP and several complementary C-terminal fragments fused to FRB exhibited inducibility comparable to that of the YFP fragments. However, cells that expressed these fusions had an order of magnitude higher intensities of both spontaneous and inducible BiFC fluorescence¹⁹. The Venus fragment containing the M153T substitution therefore represents a compromise between the needs for high fluorescence intensity and for low autonomous BiFC complex formation (Table I). However, this chimeric fragment exhibits constitutive BiFC complex formation and low inducibility at lower

temperature (30 °C) when fused to FKBP. Thus, each combination of fluorescent protein fragments has a distinct set of advantages and disadvantages as BiFC analysis probes.

Analysis of the characteristics of BiFC complexes using fluorescence techniques

Fluorescence provides a variety of sophisticated methods for the investigation of molecular characteristics. BiFC analysis enables extension of these methods to the investigation of multiprotein complexes. The mobilities of protein complexes can be visualized by fluorescence recovery after photobleaching (FRAP) or fluorescence loss in photobleaching (FLIP) of BiFC complexes^{36, 37}. These approaches provide a unique strategy for comparison of the mobilities of the individual interaction partners with the mobility of the complex formed by the proteins. The mobility of the BiFC complex reflects the mobility of the interaction partners while they are associated with each other. Dissociation of the interaction partners can affect the apparent mobility of the complex in the absence of BiFC complex formation. Comparison of the mobilities of the individual subunits and the BiFC complex can provide a way to evaluate the contribution of interactions with a particular partner to the mobilities of the entire population. Fluorescence photobleaching can also be used to evaluate if a BiFC complex is associated with a cellular scaffold or if it is free to diffuse within the cell³⁷. These approaches provide powerful tools for characterization of the dynamics of protein complexes in living cells.

BiFC analysis can also be combined with other interaction assays to study multimeric protein complexes. BiFC analysis combined with fluorescence resonance energy transfer (FRET) can be used to visualize interactions between BiFC complexes and proteins fused to intact fluorescent proteins³⁸. In these studies, it is important to establish that changes in the ratio of fluorescence emissions at different wavelengths represent energy transfer rather than changes in the spectra of the individual fluorophores resulting from changes in their environments. Several strategies, including acceptor photobleaching and donor lifetime analysis can be used for this purpose. BiFC analysis can also be combined with bioluminescence resonance energy transfer (BRET) to detect interactions of the BiFC complex with proteins fused to an intact luminescent protein^{39–41}. Due to the limited spatial resolution of luminescence detection, this strategy sacrifices direct visualization of the subcellular localization of the complex. In principle, these strategies could be extended to detection of quaternary or larger complexes in cases where detection of such complexes in living cells would be scientifically useful.

Applications of BiFC analysis

BiFC analysis has been used to visualize a wide range of cellular processes. These include non-covalent interactions between proteins of many different structural classes in virtually every subcellular compartment (see sitemaker.umich.edu/~kerppola.bifc for an updated list).

These assays have been used in many different cell types as well as in several multicellular plants and animals. Moderate throughput screens for interaction partners have been conducted^{42, 43}. The influence of the levels of protein expression on BiFC complex formation and the need for controls using mutant proteins for interpretation of the results challenge high-throughput application of BiFC analysis for proteome-wide analysis of protein interactions. Nevertheless, in principle BiFC analysis can be used to visualize every protein interaction in the interactome in virtually every cell type and organism.

Visualization of ubiquitin family peptide conjugates

The linkage of ubiquitin to many substrate proteins produces conjugates that have properties distinct from the unmodified proteins. Although ubiquitination was originally described as a signal for proteasomal degradation, subsequent studies have demonstrated that ubiquitination can serve a variety of functions in protein trafficking, DNA repair and transcription regulation.

The facilitated association of fluorescent protein fragments has also been used to detect covalent protein modifications. Ubiquitin-mediated fluorescence complementation (UbFC) is based on the association of fluorescent protein fragments fused to a ubiquitin family peptide and a putative substrate protein²⁰. Conjugation of the ubiquitin family peptide to the substrate protein can bring the fluorescent protein fragments in proximity to each other and facilitate UbFC complex formation (Fig. 5). Analysis of UbFC complex formation by mutants of ubiquitin and the substrate protein can be used to identify the determinants of ubiquitin conjugation and conjugate localization⁴⁴. UbFC analysis therefore provides a unique approach for investigation of the molecular determinants for ubiquitin conjugate formation and trafficking.

Re-constitution of a unimolecular fluorescent proteins has also been used to detect intein-mediated protein splicing²¹. In studies of covalent protein modifications, it is critical to establish that the fluorescence results from covalent bond formation rather than non-covalent association of the fluorescent protein fragments. This is readily tested by examining the effects of mutations that block covalent bond formation, but do not interfere with potential non-covalent interactions between the fusion proteins. In the case of UbFC analysis, non-covalent interactions by many proteins with ubiquitin or ubiquitin binding proteins can facilitate association of the fluorescent protein fragments²⁰. However, even if noncovalent interactions can produce BiFC complexes, differences in the subcellular distributions of covalent and non-covalent complexes can illuminate the consequences of covalent bond formation for conjugate localization²⁰.

BiFC analysis can also be used to visualize nucleic acid binding in cells by using fluorescent protein fragment fusions whose association is facilitated by nucleic acid binding. This principle has been applied to the visualization of RNA binding in several studies. Several different RNA-binding motifs have been used, targeting both exogenously expressed RNAs containing recognition sequences for the RNA binding proteins^{30, 45} as well as endogenous mRNAs⁴⁶. The challenge of endogenous RNA visualization is engineering of RNA binding domains with sufficient specificity to detect a unique mRNA target. In studies of nucleic acid binding, it is also critical to establish that bimolecular complex formation in the absence of nucleic acid binding does not facilitate association of the fluorescent protein fragments. The interpretation of experiments that attempt to visualize cellular macromolecules through their ability to facilitate fluorescent protein fragment association must also consider the possibility that the fragments can remain associated as a fluorescent complex after dissociation from the macromolecule that facilitated their association.

Association of the fluorescent protein fragments requires that they can collide with each other. This association therefore depends on the steric arrangement of the fragments in the complex. BiFC analysis can in principle be used to probe the architectures of proteins and protein complexes in living cells. The architecture of the complex formed by the N-terminal domain of titin in the presence of telethonin was corroborated by BiFC analysis⁴⁷. Several homology models are also consistent with results of BiFC analysis^{48, 49}. Similarly, protein conformational changes in cells can in principle be detected using BiFC analysis. Addition of maltose to maltose binding protein fused to the two fluorescent protein fragments enhances the fluorescence intensity *in vitro*⁵⁰. Since association of the fluorescent protein fragments can stabilize the BiFC complex, this is predicted to be successful only in cases where the conformational equilibrium is sufficiently biased in favor of one conformation so as to prevent trapping of one conformer by fragment association. Fluorescent protein fragment fusions to membrane proteins have also been used to probe their topology using BiFC analysis⁵¹. In such studies, it is important to consider the potential influence of the fluorescent protein fragments on the topology of the fusion protein as well as the possibility that the fragments could associate before or during insertion into the membrane. In all cases where two fluorescent protein

fragments are fused to the same protein, it is essential to consider the fact that BiFC complex formation can be affected both by bimolecular association of two fusion proteins as well as by the conformations of the individual fusions. Taking these limitations into account, BiFC analysis is one of the few experimental approaches that provides the potential for visualization of rare conformational states in proteins expressed at low levels in living cells.

Visualization of protein association with molecular scaffolds

Many protein interactions in cells occur on scaffolds that serve as assembly platforms for protein complexes. One such scaffold is chromatin that consists of chromosomal DNA, histone octamers and associated proteins. Chromatin serves numerous roles including the regulation of gene transcription. To perform this function, chromatin facilitates the gene-specific assembly of regulatory protein complexes.

BiFC analysis can be used to visualize regulatory protein binding to specific chromatin domains by fusing one fluorescent protein fragment to the regulatory protein and the complementary fragment to one of the histones that comprise the repeating nucleosome unit of chromatin^{52, 53}. Regulatory protein binding to specific genes can bring the fluorescent protein fragments in proximity to each other, facilitating BiFC complex formation at subnuclear locations corresponding to sites of chromatin association. The binding specificities of different regulatory proteins can be compared by examining their patterns of chromatin association to establish if the proteins bind to the same or different chromatin domains in individual living cells⁵³. To compare regions of chromatin association, it can be useful to stain the cells using dyes or antibodies that mark specific subnuclear domains. These staining patterns can be used as ancillary markers to compare the regions of chromatin association by regulatory proteins between different cells. The regions of chromatin association by different regulatory proteins can also be compared by using multicolor BiFC analysis. It should also be possible to mark specific genes by fluorescence in situ hybridization (FISH). However, this has not been experimentally demonstrated and the conditions required for FISH may cause loss of at least part of the BiFC complex fluorescence.

The subnuclear distribution of BiFC complexes formed by chromatin binding proteins with histones is expected to represent the distribution of the sites of chromatin association by the regulatory protein in the nucleus. Any dissociation of regulatory proteins from chromatin is predicted to eliminate their fluorescence unless the nucleosome containing the histone fusion is also disrupted. It is important to test the potential influence of stabilization of regulatory protein association with histones on the specificity of chromatin binding. If the cell population is homogeneous, this can be evaluated by comparing the binding specificities of the BiFC complexes with those of the endogenous proteins using standard ChIP analysis. To establish if BiFC complex formation requires regulatory protein binding to chromatin, the effects of mutations in histones that prevent their assembly into nucleosomes can be examined⁵³. Strategies analogous to BiFC analysis of chromatin association should be applicable to the investigation of protein binding to other cellular scaffolds (actin, tubulin, lamin etc.). In these cases, validation of the specificities of the BiFC complexes must rely on alternative strategies based on antibodies and functional complementation.

Strategies that take advantage of BiFC complex stability

The stabilization of protein complexes by association of the fluorescent protein fragments provides many advantages in addition to enhancing the sensitivity of BiFC analysis. BiFC complexes can be purified under conditions where interactions between the corresponding proteins in the absence of BiFC complex formation are unstable. Such purified complexes can be used to identify modifications that are enriched in proteins that are associated in a BiFC complex. The isolation of BiFC stabilized complexes (iBiSC) has been used to identify

modifications in histones that are bound by specific polycomb family members⁵³. This approach is generally applicable and can be used to identify characteristics of small subpopulations of proteins that are associated with a specific interaction partner. As an *in vitro* approach, this strategy does not provide information about the subcellular localization of complexes or about the variation in the abundance of the complexes in individual cells. However, in combination with imaging studies, it can provide biochemical information about the composition of the complexes that are visualized using BiFC analysis.

Future prospects and challenges

Strategies based on BiFC analysis will undoubtedly be applied to new scientific questions. Many of these applications will require the development of new BiFC assays with enhanced performance and novel characteristics. A basic characteristic of BiFC analysis is the fluorescence intensity produced by an interaction between a particular combination of interaction partners. The demand for higher fluorescence intensity is driven by the need to detect fewer protein complexes in more challenging imaging situations. This demand is particularly keen in the use of BiFC analysis to visualize protein interactions in multicellular plants and animals. Increasing the number of BiFC complexes that are formed by either increasing the levels of expression of the fusion proteins or enhancing the efficiency of association of the fluorescent protein fragments risks producing complexes that do not reflect the properties of the endogenous proteins. It is therefore necessary to use other strategies such as enhancement of the intrinsic brightness of BiFC complexes to reach this objective. This requires optimization of fragments for the use in BiFC analysis rather than adoption of fragments optimized in the context of the intact fluorescent proteins. Given the important role of protein folding in BiFC complex formation, it is possible that the fluorescent protein fragments must be optimized separately for use in different organisms or even in different subcellular compartments. It should be possible to optimize the fragments by screening libraries of mutated fragments for increased fluorescence intensity.

BiFC complexes formed by fluorescent protein fragments have remarkably similar spectra to those of the intact fluorescent proteins from which the fragments were derived⁹. It is therefore likely that it will be possible to produce BiFC complexes with photophysical characteristics similar to those of other fluorescent proteins with unique properties. Such properties include photo-activation and photo-conversion. BiFC complexes with these characteristics would be particularly useful for tracking the trafficking of BiFC and UbFC complexes as well as for super-resolution imaging using PALM and related enhanced microscopy techniques. BiFC complexes with time-dependent spectral characteristics would enable determination of the properties of protein complexes at different times after complex formation. Finally, BiFC complexes whose spectra depend on their local environments could be used to probe the local microenvironments of specific protein complexes in living cells.

The ability of BiFC complexes to stabilize protein complexes could be used to investigate the functional consequences of stabilizing interactions between specific proteins. The fraction of fusion proteins that forms stable BiFC complexes is likely to vary for different interaction partners. The proportion of stable complexes is likely to be proportional to their fluorescence intensity. Thus, it is in principle possible to quantify differences in tethering efficiency by mutated versions of the interaction partners in order to determine how such differences affect biological functions.

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Figure 1.

Examples of protein fragments that can be used to study protein interactions. The fragments are shown in red and blue using models based on the X-ray crystal structures of the intact proteins. In β -galactosidase, the overlap between the fragments is shown in magenta. The discontinuity in the polypeptide backbone is shown in translucent grey. The images were generated using jmol. The images were reproduced from Nature Methods 3, 969–971⁵⁴, Copyright (2006), Nature Publishing Group.

Figure 2.

Principle of bimolecular fluorescence complementation analysis. The red and blue rectangles represent putative interaction partners (A and B). They are fused to fragments of a fluorescent protein, represented by silver half-cylinders (YN and YC). If the proteins interact with each other, they can facilitate association of the fluorescent protein fragments to produce a fluorescent complex (green cylinder YN-YC). The image shows an example of a complex formed by nuclear proteins. The image is reproduced from *Molecular and Cellular Biology* 24, 5694–5709⁵⁵, Copyright (2004) American Society of Microbiology.

Figure 3.

Principle of multicolor BiFC analysis. Two alternative interaction partners, A and B, are fused to fragments of different fluorescent protein fragments (tinted gray half-cylinders). These fusions are co-expressed in cells with a shared interaction partner, Z, fused to a complementary fragment (grey half-cylinder). Complexes formed by A and Z can be distinguished from complexes formed by B and Z based on the difference in their fluorescence spectra. The images under the diagrams show an example of the visualization of two protein complexes in the same cell, one nucleolar (cyan) and the other nucleoplasmic (yellow). The images were reproduced from *Nature Biotechnology* 21, 539¹⁷, Copyright (2003) Nature Publishing Group.

Figure 4. Multiple combinations of fusion proteins should be tested for bimolecular fluorescence complementation. Amino- and carboxyl-terminal fusions can be used to test eight distinct combinations. The labels are the same as in Fig. 2.

Figure 5.

Principle of ubiquitin-mediated fluorescence complementation (UbFC) analysis. The UbFC assay is based on the association between fluorescent protein-fragments that are brought together by covalent conjugation of ubiquitin that is fused to one fragment to a substrate that is fused to the complementary fragment. The fluorescent protein fragment must be fused to the N-terminal ends of ubiquitin-family peptides since their C-terminals ends are essential for conjugation to substrate proteins. Fusions to the N termini of ubiquitin and small ubiquitin-like modifier-1 (SUMO-1) do not interfere with the activities of the enzyme complexes that conjugate these peptides to protein substrates. It is essential to determine if the conjugates that contain the fluorescent protein fragments retain the biological functions of the unmodified conjugates. Because ubiquitin can be conjugated in different monomeric and polymeric configurations to substrates, it is important to establish that the stoichiometry and configuration of the ubiquitin conjugate are not altered by the fusions. The images on the right show multicolor UbFC analysis of ubiquitin (green) and SUMO1 (blue) conjugation in the same cell. The images were reproduced from Proceedings of the National Academy of Sciences of the United States of America 101, 14782–14787²⁰, Copyright (2006) the National Academy of Sciences.

Table 1

Advantages and disadvantages of different combinations of fluorescent protein fragments in BiFC analysis.

#	N-terminal fragment ^a	C-terminal fragment ^a	Advantages	Disadvantages	References ^b
1	YN155	YC155	Low spontaneous association	Weak fluorescence intensity	9, 19
2	YN173	YC173			17
3	CN155	CC155	Low spontaneous association, multi-color analysis	Weak fluorescence intensity	9, 19
4	YN155	CC155			
5	VN155 or VN173	YC155 or VC155 or VC173	Higher fluorescence intensity vs. #1, #2, #4, #7, #8	Higher spontaneous association vs. #1, #2, #4, #7, #8	19, 31, 32
6	VyN155	VC155	Lower spontaneous association vs. #5	None noted	32
7	VyN155	YC155	Lower spontaneous association vs. #1, #4	Lower fluorescence intensity vs. #5; Higher spontaneous association vs. #1, #4; temperature-dependent	19
8	VyN155	CC155			

^aThe first letter(s) indicate the fluorescent proteins from which each of the fragments was derived (Y: YFP; C: CFP; V: Venus; Cr: Cerulean; Vy: Venus with M153T substitution from YFP). The next letter indicates the N- or C-terminal fragment. The number indicates the position in the sequence where the fragment ends or starts.

^bThe list of references is not comprehensive.