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Bimolecular fluorescent complementation (BiFC) assay for visualization and characterization of protein –protein interactions in living cells

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Abstract

Finding a trustworthy method to recognize protein-protein interactions is going to be a big task in the future. Bimolecular fluorescent complementation (BiFC) assay enables direct visualization of protein interactions in living cells. The BiFC assay is based on the discoveries that two non-fluorescent fragments of a fluorescent protein interact and form a fluorescent complex. Protein function is often mediated by formation of stable or transient complexes. The BiFC assay involves splitting of yellow fluorescent protein into two non-overlapping N-terminal (YN) and C-terminal (YC) fragments. Each fragment was cloned in a frame to a gene of interest, enabling expression of fusion proteins. No fluorescence was detected following co-expression free non-fused YN and YC or non-interacting protein pairs. Reconstitution of a fluorescing yellow fluorescent protein (YFP) chromospheres occurred only when the inquest proteins interacted. It can be used in visualizing the interaction of a phytoplasma effector with its proteinaceous host partner in *Nicotiana benthamiana* mesophyll protoplasts. The BiFC assay has been used for the visualization of interactions between many types of proteins in different sub cellular locations and in different cell types and organisms. This assay growing popularity is a result of its simplicity, usability, and capacity to conduct experiments. It is technically straight forward and can be performed using a regular fluorescence microscope and standard molecular biology and cell culture reagents.

Key words: Bimolecular fluorescent complementation, Green fluorescent protein, protein-protein interaction, sub-cellular interaction, yellow fluorescent protein

Introduction

Bimolecular fluorescence complementation also known as (BiFC) is a technology typically used to validate protein interactions. It is based on the association of fluorescent protein fragments that are attached to components of the same macromolecular complex. Protein-protein interactions are essential to almost all biological processes. Protein-protein interactions can be studied *in vivo* using blue, green,

and yellow bimolecular fluorescence complementation (BiFC) systems based on GFP and its variations. The connection of fluorescent protein fragments with elements of a single macromolecular complex serves as the foundation for this theory (Ghosh et al 2000). In order to express the proteins that are thought to interact, complementary unfolded segments of a fluorescent reporter protein are fused to them. The reporter protein can reorganize in its natural three-dimensional structure and release its fluorescent signal when these proteins interact, bringing the fluorescent fragments close by. Using an inverted fluorescence microscope that can image fluorescence in cells, this fluorescent signal can be found and placed inside the cell. Additionally, the strength of the connection is inversely correlated with the intensity of the fluorescence released, with higher fluorescence levels indicating close or direct interactions and lower fluorescence levels indicating interaction within a complex. Therefore, one may determine both the location and interaction partners of proteins of interest by visualizing and analysing the intensity and distribution of fluorescence in these cells. Studies of protein interactions have provided fundamental insights into the regulation of cellular functions. In *E. coli* cells, Ghosh et al. in 2000 created a technique that made it possible to reassemble a green fluorescent protein (GFP) using an anti-parallel leucine zipper. By separating GFP into C- and N-terminal GFP fragments, this was accomplished. This study was also the first to describe an *in vivo* method known as the bimolecular fluorescence complementation (BiFC) assay, which aims to shed light on the structural underpinnings of protein complex formation by detecting fluorescence produced by the assembly of fluorescent reporter protein fragments attached to interacting proteins.

History

First discovered in subtilisin-cleaved bovine pancreatic ribonuclease, biochemical complementation was further broadened utilising mutant -galactosidase that allowed cells to grow on lactose. Later, it was discovered that many proteins possess the capacity to spontaneously coalesce into functional complexes. Additionally, it was discovered that protein fragments possess the capacity to coalesce as a result of the spontaneous functional complex assembly of interaction partners to which they are fused.

In *E. coli* cells, Ghosh et al. in 2000 created a technique that made it possible to reassemble a green fluorescent protein (GFP) using an anti-parallel leucine zipper. By separating GFP into C- and N-terminal GFP fragments, this was accomplished. The heterodimerization of the anti-parallel leucine zipper produced a GFP protein that could be seen since each leucine zipper had a GFP fragment linked

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to it via a linker. The fact that the fluorescent signal was successful demonstrated that the various GFP peptide fragments could rejoin properly and achieve tertiary folding. Therefore, it was proposed that this method may be utilized to analyze the interaction of protein-protein pairs whose N-C termini are adjacent to one another.

Multicolor BiFC

Hu et al. (2002) have added multicolor BiFC as an extension of their original BiFC system, demonstrating the feasibility of PCAs between the complementing segments of YFP and CFP as well as between YFP and GFP fragments. The resultant complexes of GFP- YFP and CFP-YFP have distinct, resolvable emission spectra (Hu and Kerppola, 2003). When examining the complex formation of a specific protein with various interaction partners, multicolor BiFC experiments are helpful. Preferable interactions have been predicted thanks to careful examination of fluorescence intensity.

The Principle of BiFC

The principle is based on splitting of green or yellow fluorescent protein to form a functional fluorophore. It takes interaction between the proteins or peptides fused to each of the split YFP/GFP/cyan fluorescent protein (CFP) molecules for the association of the molecule to take place (fig 1). The split fluorophore fragments may interact with these fused proteins/peptides to create a fluorescent protein that shares the same spectral characteristics as the unsplit YFP. If the proteins that are fused to the split fluorophore fragments do not interact, reconstitution of the YFP/GFP/CFP usually does not take place and no fluorescence is detected.

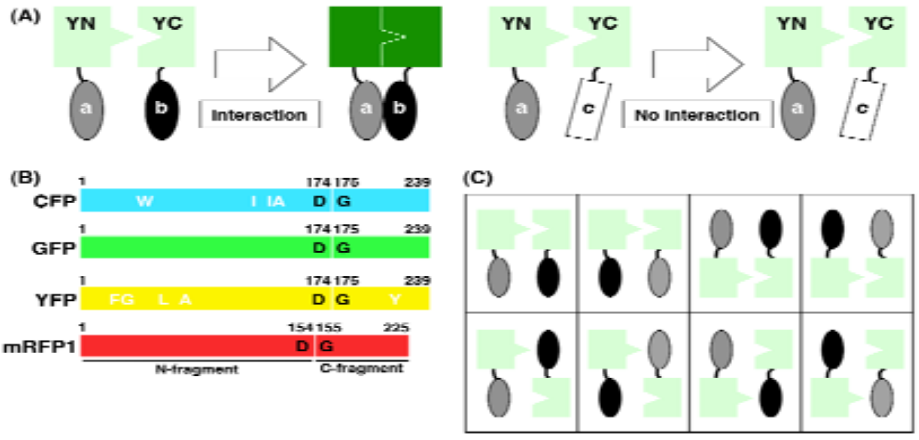

Fig 1. Principle of BiFC assay (Hu & Kerppola, 2003; Waadt et al., 2008)

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When tested proteins or peptides (a, b, and c) to which they are fused interact, nonfluorescent fragments of a fluorescent protein (YN and YC) are brought together. The two proteins' interaction results in the reconstruction of a fluorescent signal. (B) Diagram showing the sites where the proteins CFP, GFP, YFP, and mRFP1 were fragmented and the amino acid alterations between them. The CFP, GFP, and YFP in our system were split between residues 174 and 175 and mRFP1, which contains an amino acid substitution of the 66th glutamine to aspartic acid, despite the fact that there are alternative positions to split a fluorescent protein into two fragments (Hu & Kerppola, 2003; Waadt et al., 2008). The amino acid threonine was divided between positions 154 and 155. CFP and YFP's amino acid composition

Development of BiFC technique

Sites within the GFP molecule that permit insertions without changing fluorescence properties have been discovered through mutational investigations. These results led to the discovery that coupled to interacting anti-parallel coiled-coil peptides, broken GFP fragments could be reassembled in vitro and in Escherichia coli (Ghosh et al., 2000). The same study demonstrated that when expressed in E. coli, each GFP fragment is insoluble on its own, but the reassembled GFP protein complex is soluble and stable and has a low dissociation coefficient. A chimeric protein known as Pericam was created when a peptide spacer was inserted into GFP and to which calmodulin (CaM) and a CaM target peptide M13 were fused at its N- and C-terminal ends, respectively. When Ca21 binds to the CaM moiety, which in turn interacts with the M13 peptide, the GFP fluorescence in the Peri-cam is quenched. Pericam was utilised to track dynamic shifts in Ca21 levels in several HeLa and cardiomyocyte cellular compartments (Nagai et al., 2001; Robert et al., 2001).

Requirements for performing BiFC

The simplicity of the BiFC test and the ability to conduct studies with either a standard epifluorescence microscope outfitted with the necessary filter sets and a CCD camera or with a CLSM are among its main advantages. The signals are usually strong enough to overcome background fluorescence, especially when using split YFP. However, in weak fluorescence situations, certain filter sets, such the Ziess Pinkel Set 40, can be utilised to distinguish between actual YFP fluorescence and autofluorescence (for more detail see Bracha-Drori et al., 2004). The determination of fluorescence spectra is made possible by the spectrum scanning capabilities of some CLSMs. A fluorescence spectrophotometer can also be used to check whether the sample emits the necessary spectrum. Finally, it is important to examine the fluorescence that results from an unintentional interaction between a

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candidate protein fused to either YN or YC and the corresponding YN or YC only, or preferably with a mutant noninteracting protein.

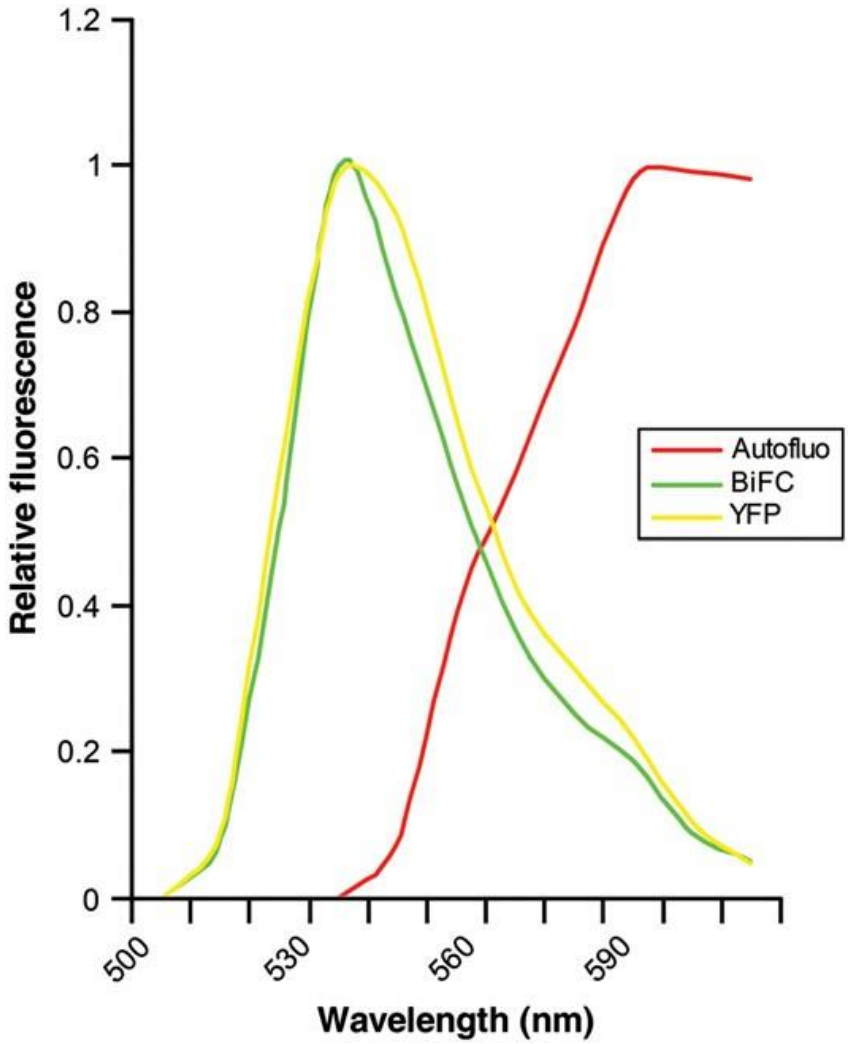

Fig. 2; Bracha-Drori et al., 2004

Advantages of BiFC

BiFC has a number of noteworthy benefits.

(1) There is no complicated specialized equipment needed for the assay, which is straightforward.

(2) Because a fluorescing YFP would only emerge after interactions between proteins fused to broken fragments, there is either no background signal or a weak one.

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(3) BiFC makes it possible to determine the sub cellular localization of interacting protein complexes as well as how different interacting partners influence the complex's sub cellular localization.

(4) The stability of the reconstituted YFP complexes plays a key role in the sensitivity of the BiFC test, which allows for the identification of weak and transient interactions (Hu et al., 2002).

However, the assay has a number of flaws that must be considered. (1) Real-time detection of dynamic changes in protein-protein interactions is hampered by the slow maturation period of the reconstituted GFP/YFP/CFP (Ghosh et al., 2000; Hu et al., 2002; Kerppola, 2006a, 2006b).

Successful BiFC and multicolor BiFC experiments have been conducted using fragments generated from Venus and Cerulean, a modified form of CFP (Shyu et al., 2006). Analysis of the kinetics of protein-protein dissociation is hampered by the stability of the reassembled YFP complexes (Hu et al., 2002). When the expression levels of the split YFP fragments are high, this can result in the detection of nonspecific interactions. Chimeric fusion proteins may have distinct molecular characteristics than native proteins.

Disadvantages of BiFC

The following factors need to be carefully taken into account with BiFC systems. The stability of the reconstituted YFP/GFP/CFP complexes and the slow maturation times of fluorescent dyes other than Venus may compromise the results, despite the fact that BiFC-based systems have been used successfully for monitoring dynamic changes in Ca21 concentration (Nagai et al., 2001; Robert et al., 2001). However, the majority of BiFC tests in plants use transient expression methods, in which transformed tissues are often examined after a few or even 24 to 48 h. This gives proteins adequate time to mature. Expression levels should be kept low to alleviate the issue of nonspecific interactions. It is crucial to use negative controls in the form of tested protein point mutants that do not interact. It is essential to monitor expression levels of the relevant proteins with antibodies to make sure that a lack of fluorescence is not caused by low expression (Fig. 3; Bracha-Drori et al., 2004; Walter et al., 2004). Thus, if the fluorescent signal can be quantified and normalized to the levels of protein expression, it might be very helpful.

By analyzing expression patterns, colocalization assays, and, if applicable, genetic analyses and determining protein-protein interactions in plants using an independent approach, the validity of the BiFC results should be confirmed. The important message is that employing the appropriate controls and calibration is crucial, just like with any other experimental apparatus.

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Conclusion

Many cellular activities are governed by dynamic networks of protein-protein interactions, which also determine how well cells can react to external cues. Nonetheless, the study of protein complex synthesis in vivo in plant cells has continued using techniques like fluorescence resonance energy transfer. experimentally challenging, time-consuming, and requiring high-end technical tools. In this article, we describe how a bimolecular fluorescence complementation (BiFC) approach was used to visualise protein-protein interactions in plant cells. This method depends on two nonfluorescent fragments of the yellow fluorescent protein coming together to produce a fluorescent complex when interacting proteins are fused to these fragments.

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