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Novel Bimolecular Fluorescence Complementation (BiFC) assay for in vivo visualization of the protein-protein interactions and cellular protein complex localizations

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Abstract

Background

The study of protein-protein interactions (PPIs) is critical for understanding cellular processes within biological systems. The bimolecular fluorescence complementation (BiFC) assay provides a convenient approach for visualization of protein-protein interactions in living cells. Though a series of fluorescence proteins have been introduced into BiFC system, new fluorescent proteins need to be brought in this assay.

Results

Here, we applied TagBFP2 into BiFC for the first time to verify the interaction between two proteins, and EYFP was set as a positive control. Both fluorescent proteins performed well in our study. Compared to EYFP, the BiFC system based on TagBFP2 showed higher signal-to-noise ratio, which help us distinguish the signal of PPIs from noise.

Conclusion

With the joining of TagBFP2, we have another color option when we conduct BiFC assay. Especially, the green or yellow channel was occupied by a fluorescent secondary antibody in immunofluorescence application, or an interest protein tagged with a fluorescent protein. BiFC assay presented here is quite easy-to-follow, reliable, and reproducible, which can be completed within 1 week without using costly instruments and technologies that demand a high skill set.

Introduction

Protein-protein interactions (PPIs) are a vastly complicated network of reactions in intercellular and extracellular environments, which play essential roles in many cellular functions, including gene expression and regulation, and signal transduction [1]. Studying these PPIs would help us understand fundamental cellular regulations and develop novel therapeutic targets. Several methods have been developed to detect PPIs, such as canonical yeast two-hybrid assay, *in vitro* pull-down assay, and Co-immunoprecipitation assay (Co-IP) [2]. More recently, a variety of methods for surveying PPIs have emerged, of which fluorescence-based techniques, such as fluorescence resonance energy transfer (FRET) and Bimolecular fluorescence complementation (BiFC) for screening of PPIs in living cells, have attracted attention. Compared with BiFC, FRET has a lower signal-to-noise ratio, requires complicated optical setups, calculation methods and structural information to design FRET constructs. On the contrary, the BiFC assay offers a high signal-to-noise ratio and is easy to perform, which makes it the best choice to investigate PPIs in living cells.

The working principle of BiFC assay is based on the structural complementation between two non-fluorescent N- and C-terminal fragments of green fluorescent proteins (GFP) and its variants. Each of the above fragments was fused to a protein of interest respectively. If the two fused proteins do interact, fragments of fluorescent protein would be taken into such a proximity that they can reconstitute an intact fluorescent protein by autocatalysis. Besides, it also describes the spatial localization of interaction at the subcellular level, i.e., where the functional contact between two proteins takes place in a cell. Since the BiFC assay was first performed successfully by Regan and colleagues with truncated fragments of GFP in *Escherichia coli* [3], there are 15 fluorescent proteins that had been introduced in BiFC assay (Table 1).

Table 1
Fluorescent proteins used in previous work

Fluorescent proteins	Excitation Peak (nm)	Emission Peak (nm)	Cell type or organism in the first use	Additional mutation	References
EBFP	382	448	Mammalian (COS-1)	None	[4],[5]
Cerulean	439	479	Mammalian (COS-1)	None	[6]
ECFP	452	478	Mammalian (COS-1)	None	[4]
EGFP	488	512	Bacteria (*E. coli*)	None	[3],[4]
GFP-S65T	489	510	Plant (Onion epidermis)	V163A	[7],[8]
frGFP	485	510	Bacteria (*E. coli*)	None	[9]
sfGFP	503	518	Mammalian (Hela)	None	[10]
Dronpa	503	518	Mammalian (HEK293)	None	[11],[12]
EYFP	514/515	527	Mammalian (COS-1)	None	[4],[6]
Venus	515	528	Mammalian (COS-1)	None	[6]
Citrine	516	529	Mammalian (COS-1)	None	[6]
mRFP	549	570	Plant (Tobacco BY2 and Onion epidermis)	Q66T	[13]
DsRed monomer	556	556	Plant (Onion epidermis)	None	[14]
mCherry	587	610	Mammalian (Vero)	None	[15],[16]
mKate	587	621	Mammalian (COS-7)	S158A	[17]

In our study, we used EYFP fluorescence complementation system, which was first built by CD Hu et al [4] in 2002, and a novel blue fluorescence complementation system based on TagBFP2. By the way, the basic region-leucine zipper (bZIP) family was used to verify the above two systems in mammalian cells. Collectively, this approach can be used in any standard lab to get high-quality images for BiFC assay in 1 week without using advanced instruments and technologies that demand a high skill set.

Material And Methods

RNA extraction and Reverse-transcription

Complementary DNA (cDNA) was obtained from HEK 293T cells. Briefly, the total RNA was extracted from HEK293T with RNA isolator Total RNA Extraction Reagent (R401, Vazyme, Nanjing, China) and estimated by Nanodrop (Thermo Fisher Scientific, Massachusetts, U.S.). The total RNA was then reverse transcribed into cDNA using a reverse transcription kit (Cat. R312-01, Vazyme, Nanjing, China) according to the manufacturer's instructions.

Fragments and linearized vector for clone

The non-fluorescent fragments and Jun and Fos of fused proteins were obtained by PCR. And the templates were pRP[Exp]-Puro-CMV-EYFP and pLV-Puro-EF1A-TagBFP2 (Vector Builder) for fragments and Human total cDNA for Jun and Fos. The primers we used in this study was list in Table S1 (See in supplemental materials). The vector used for cloning in our study is pHY009 (purchased from Hanyin biotech, Shanghai). This vector plasmid was digested with *XhoI* (FD0694, Thermo Fisher Scientific, Massachusetts, U.S.) and *EcoRI* (FD0274, Thermo Fisher Scientific, Massachusetts, U.S.). The fragments and linearized vector obtained above were purified with FastPure Gel DNA Extraction Mini Kit (DC301, Vazyme, Nanjing, China). Then, we cloned the Fragments to the vectors by ClonExpress MultiS One Step Cloning Kit (C113, Vazyme, Nanjing, China).

Construction of Plasmid Expression Vector

The following eukaryotic expression vectors were used in this study. (i) Plasmids encoding fusion proteins of Jun (bJun) jointed with YN (BN), and Fos (bFos) jointed with YC (BC), wherein YN (BN) represent the N-terminal fragments of EYFP (TagBFP2), and YC (BC) represent C-terminal fragments of EYFP (TagBFP2), respectively. (ii) Plasmids encoding fusion proteins of YC (BC) jointed with FosΔbZIP, wherein, FosΔbZIP represent that the bZIP region of Fos was deleted, so that Jun could not interact with FosΔbZIP. The components of above fusion proteins were connected with an oligopeptide linker (2x GGGGS) (iii) Plasmids encoding fusion protein of U2AF2 connected with EGFP.

Cell culture and transfection

Human cervical cancer cell line (Hela) was purchased from National Collection of Authenticated Cell Cultures and maintained in a humidified incubator at 37 ℃ with 5% CO_2 and grow in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Bovine Serum, 100U/mL penicillin, and 100mg/mL streptomycin. The day before transfection, cells were seeded on 6-well dishes which were pre-paved with cell glass slides for imaging with Laser Scan Confocal Microscope. The Hela was allowed to grow to 80–90% confluence.

Transfections was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The plasmids pair for transfection was list as follows: pHY009-Jun-YN/pHY009-YC-Fos, pHY009-bJun-YN/pHY009-YC-bFos, pHY009-Jun-BN/pHY009-Fos-BC/pHY009-U2AF2-EGFP, and pHY009-

Jun-YN/pHY009-YC-FosΔbZIP, pHY009-Jun-BN/pHY009-BC-FosΔbZIP, wherein pHY009-U2AF2-EGFP was nuclear marker and pHY009-YC-FosΔbZIP, pHY009-BC-FosΔbZIP were false positive control group. The ratio for transfection were 1μg: 1μg for plasmids pair and 1μg: 1μg: 1μg for plasmids triplet.

Mild hypothermia incubation for Bimolecular fluorescence maturation

After transfection, allow the cells grow for 24 hours. Incubate the cell dishes at room temperature or an incubator set as 25–30 ℃ for 2–4 h. Because EYFP and TagBFP2 are sensitive to 37℃ so that they cannot form the intact fluorescent proteins. In this period, we checked the fluorescence with fluorescence microscope occasionally until it met our expectation. Mounting the cell slides with antifade mounting medium and seal the edges of slides with transparent nail enamel. All the samples should store at humid and dark room.

Fluorescence microscopy

Fluorescence microscopy was carried out with a fluorescence microscope or confocal laser scanning microscope. The max excitation wavelength/max emission wavelength of EYFP and TagBFP2 were 513 nm/527 nm and 399 nm/454 nm, respectively. If your microscope has no special optical filter for EYFP, the channel of FITC can be a substitute. And the channel of DAPI can be used to observe TagBFP2 and DAPI.

Results

Principle of the BiFC assay

The working principle of BiFC assay is based on the structural complementation between two non-fluorescent N- and C-terminal fragments of fluorescent proteins and its variants. Each of the above fragments was fused to a protein of interest respectively. If the two fused proteins do interact, fragments of fluorescent protein would be taken into such proximity that they can reconstitute an intact fluorescent protein by autocatalysis (Fig. 1A). Therefore, fluorescence can be seen in living cells when these fluorescent fragments fused proteins interact with each other (Fig. 1B).

Experimental design

In our study, we used EYFP (Enhanced yellow fluorescent protein) fluorescence complementation system, which was first built by CD Hu et al [4] in 2002, and a novel blue fluorescence complementation system based on TagBFP2 (Tag blue fluorescent protein) (Fig. 2A). The EYFP was split at 155A/156D [4] or 173E/174D [18], and generate two pairs of non-fluorescent fragment, named YN155/YC156, YN173/YC174 correspondingly. Based on above fragments, it developed two sets of BiFC system. Protein A was fused to YN155 (YN173), and protein B, which could interact with protein A, was jointed to YC156 (YC174). When protein A interact with protein B, the two non-fluorescent fragments were brought in

proximity. And then, they would assemble into an intact fluorescent protein via self-catalysis and emitted fluorescence. Both BiFC systems perform well in previous study. According to the stereoscopic structure of fluorescent proteins, we speculated that splitting joints between the β-sheet of the barrel-like fluorescent protein would scarcely impact on their natural structure and physiology. Therefore, we truncated TagBFP2 at 151D/156G, 166G/167G, 191G/192V, all of which were located at the connective loops. Non-fluorescent fragments pairs generated before named BN151/BC152, BN166/BC167, BN191/BC192, which underlay our following BiFC assay.

To construct the EYFP-BiFC and TagBFP2-BiFC systems, the N-terminal sequence encoding EYFP or TagBFP2, YN/BN, was fused to the N-terminal of Jun or bJun (the bZIP region of Jun). The C-terminal sequence encoding EYFP or TagBFP2, YC/BC was fused to the C-terminal of Fos or bFos (the bZIP of Fos). The two fused fragments were connected by a linker amino acid sequence 2x GGGGS. Finally, these fused sequences were inserted into the vector-PHY009 (Fig. 2B).

EYFP fluoresce mediated by interplay between Jun and Fos

We co-transfected Hela with fusion protein pHY009-Jun-YN (pHY009-bJunYN) and pHY009-YC-Fos (pHY009-YC-bFos) and imaged the cells with fluorescence microscope. Our results show that yellow fluorescence was co-localized with blue nucleus (stained with DAPI) (Fig. 3A). These yellow light spots emitted by EYFP indicated the interaction between Jun and Fos, whose nuclear localization coincide with their normal physiology. On the contrary, when we deleted the bZIP domain of Fos and generated the fusion proteins YC-FosΔbZIP. Subsequently, we co-transfected Hela with Jun-YN and -YC-FosΔbZIP. As expected, there was no significant yellow fluorescent signal in these groups (Fig. 3B). Above results revealed that BiFC assay was competent to verify whether two proteins exist interaction or not.

TagBFP2 was introduced into BiFC assay

According to methods of EYFP BiFC system, we cleaved TagBFP2 at three sites and obtained three pairs of non-fluorescent fragments, which has been elaborated above. Identically, we fused these fragments to Jun and Fos respectively and transfected Hela with them.

We utilized U2AF2 as the nuclear localization protein as TagBFP2 is blue fluorescent [19]. Green fluorescence was co-localized with blue nucleus, as seen in Fig. 4A. These blue light dots emitted by TagBFP2 further demonstrated the relationship between Jun and Fos. Similarly, when we removed the bZIP domain of Fos and created the YC-FosΔbZIP fusion proteins. Following that, we co-transfected Hela with Jun-YN and -YC-FosΔbZIP. There was no substantial blue fluorescence signal in these groups, as expected (Fig. 4B). The BiFC-TagBFP2 assay was also capable of evaluating whether two proteins interact in living cells, according to the findings.

Discussion

Revealing the formation, distribution, and dynamics of PPIs in situ in living cells can deepen our understanding of how cells coordinate their multiple components to fulfill their activities. Here we improved the method of BiFC to detect and visual PPIs in living cells. Compared with other methods, BiFC is superior in the following aspects. First, BiFC does not require structural information of interacting proteins, the addition of dye or fluorophore staining, etc. Merely the complementation of two fluorophore segments (N-terminal and C-terminal) will produce the fluorescence and reveal the information about the interaction of the proteins [20]. In addition to the detection of PPIs, it also describes the spatial localization of interactions at the subcellular level, i.e., where the functional contact between two proteins takes place in a cell. Third, BiFC does not rely on energy transfer between fluorophores as seen in FRET and BRET assays but it can be detected by employing conventional fluorescence microscopy. And fourth, the intensity of the fluorescence signal is proportional to the strength of protein-protein interactions.

In our study, we introduced TagBFP2 into BiFC assay for the first time. Based on stereochemical structure of fluorescent proteins, we speculated that splitting at the disorder loop region would scarcely impact on their native structure and physiology. Hence, we tried above-mentioned three sites of TagBFP2 in this study. The results proved our hypothesis. As for other fluorescent proteins, it is necessary to conduct more experiments to estimate our conjecture. Between the interest protein and non-fluorescent fragment, we designed a flexible linker (2x GGGGS), which would avoid interplay between the two components. Compared with BiFC based on EYFP, TagBFP2 exhibited the higher signal-to-noise ratio, which help us distinguish the PPIs induced fluorescence from the noise signal.

Despite the advantages of BiFC assay, it could not be ignored that the reconstitution of BiFC complexes is irreversible [4]. In some cases, this property offers a great advantage to detect low affinity and transient PPIs by facilitating the readout of complex formation, which is not readily achieved by other methods [21]. Nevertheless, the persistent complexes hinder us to study the dynamics of signaling networks. In other words, BiFC assay cannot be used for function analysis. Furthermore, fluorescence would arise occasionally even though the fused protein pair did not interact with each other, which led to the background signal in the BiFC assay. Further research on the folding and dynamics of bimolecular complex formed by the two non-fluorescent fragments could help us solve these problems. At present, it is strongly recommended to introduce an appropriate negative control, such as one of the partners being deleted or mutated in the interacting region, to exclude the false-positive results in this assay.

The BiFC assay has become one of the most common approaches for visualizing PPIs in living cells. It is proved in this article that the BiFC assay is a reliable tool for detection of PPIs with appropriate negative controls. Mutational engineering of full-length GFP family members has produced proteins with an astounding range of photophysical and photochemical characteristics. Various fluorescence proteins are adapted for new purpose thanks to their own characteristics. Recently, the BiFC assay was introduced into experiments on animals for visualizing PPIs [22]. For extending the applications of the BiFC assay, more fluorescent proteins should be taken into research to evaluate their relative merits.

Abbreviations

BiFC	Bimolecular Fluorescence Complementation
PPIs	Protein-protein interactions
Co-IP	Co-immunoprecipitation
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent proteins
bZIP	basic region-leucine zipper
cDNA	Complementary DNA
DMEM	Dulbecco's modified Eagle's medium
EYFP	Enhanced Yellow Fluorescent Protein
TagBFP2	Tag blue fluorescent protein

Declarations

Acknowledgements

Not applicable

Authors' contributions

Y.L. designed the structure of the manuscript. Z.S. and X.G. contributed to the written sections to the manuscript. W.Z. contributed to the figures and figure legends. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest

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Figures

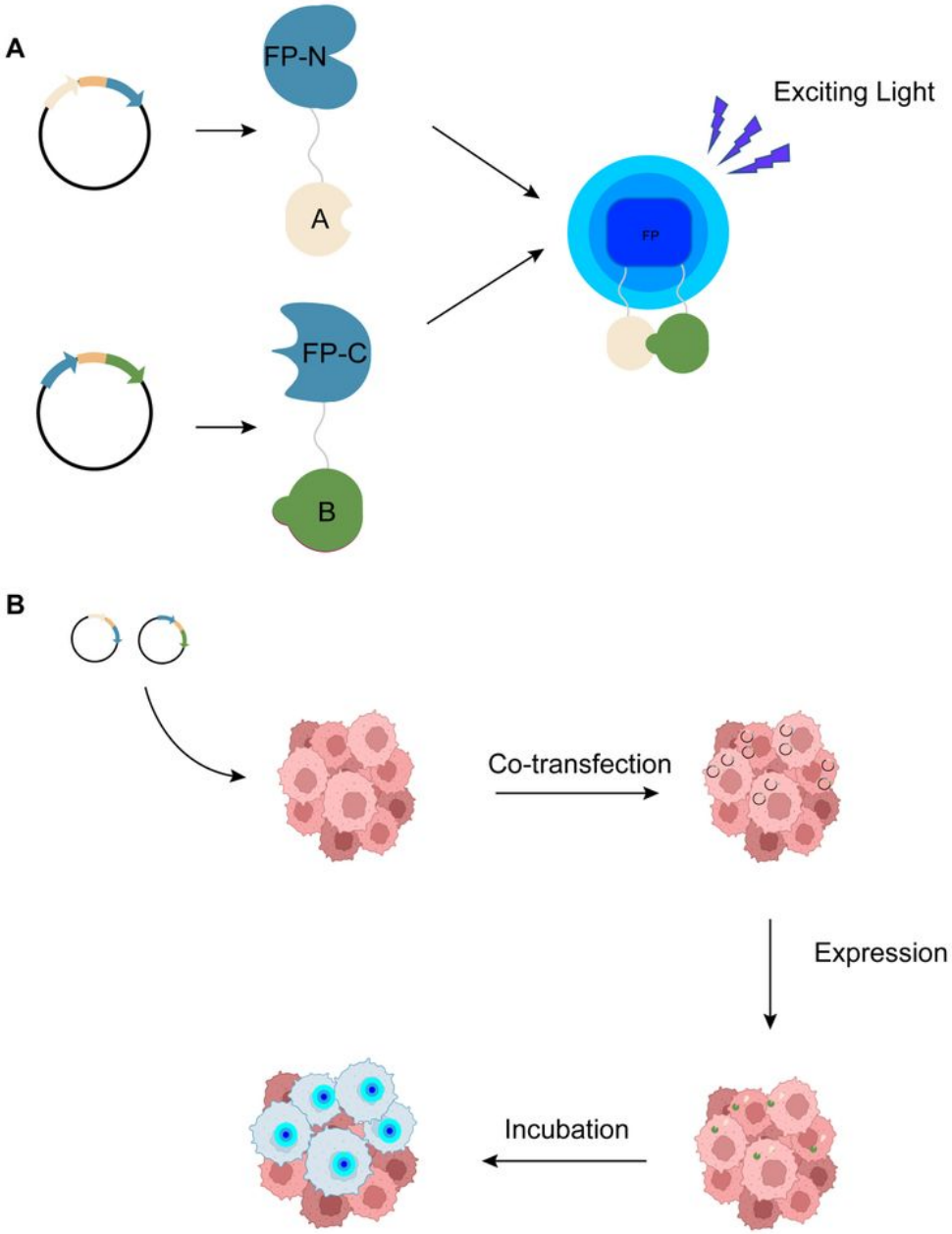

Figure 1

Graphic abstract of BiFC and workflow of BiFC assay in living cell. (A) The bimolecular fluorescent fragments fusion proteins were expressed by the corresponding plasmids pair. When they interact with each other, the fragments will be brought in proximity, form an intact fluorescent protein and fluoresce. **(B)** The workflow of BiFC assay in living cell. We co-transfected Hela with the BiFC plasmids pair. After

expression of these plasmids, incubate the cells at room temperature or in mild hypothermia incubator for maturation of fluorescent protein.

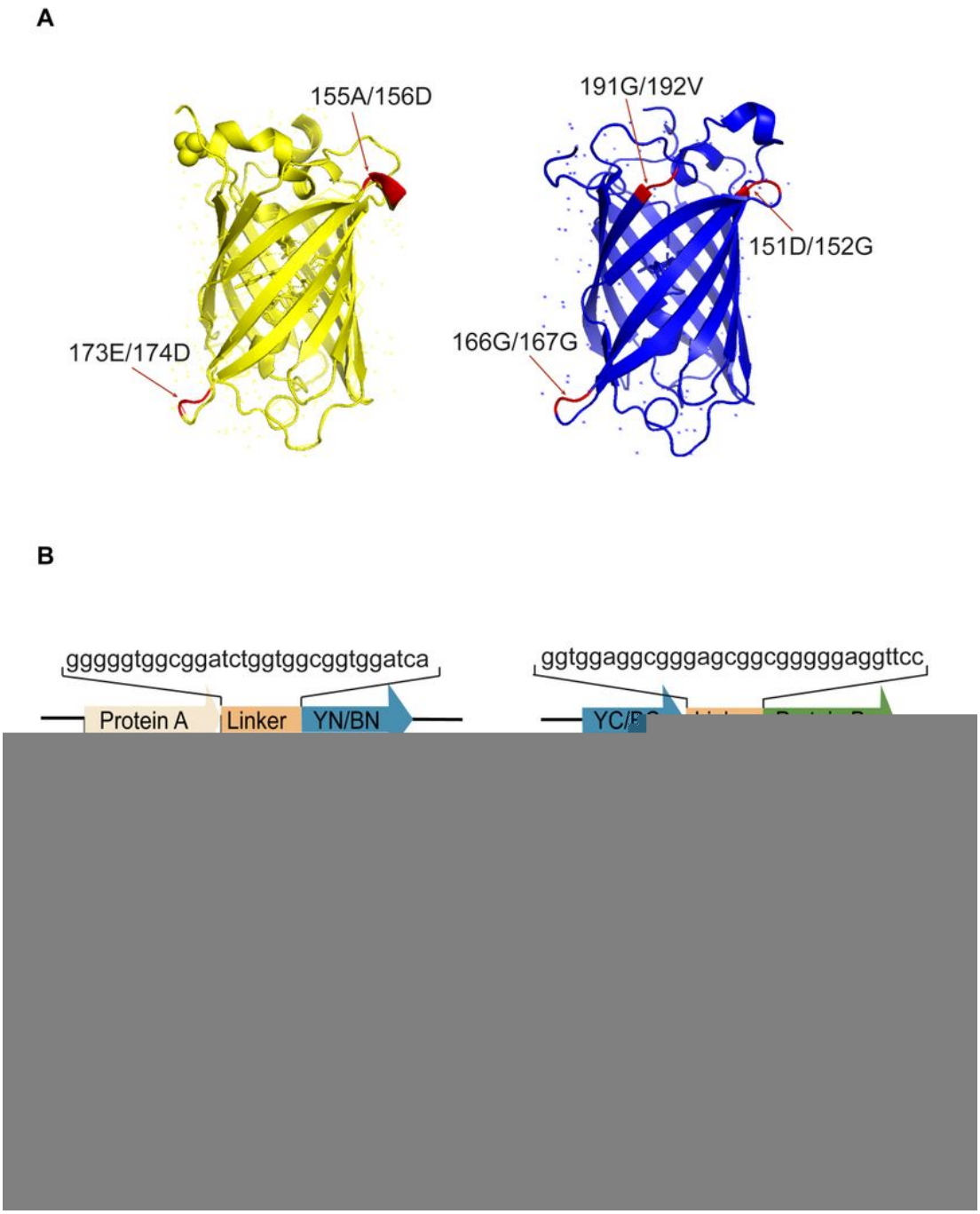

Figure 2

stereochemical structure of EYFP and TagBFP2 and schematic diagram of BiFC plasmids. (A) Stereochemical structure of EYFP and TagBFP2, and the split sites (arrows) in the loop regions of the β-

barrel structure of EYFP and TagBFP2 are indicated. **(B)** Protein A was fused to the YN/BN with linker while Protein B was fused to the YC/BC, both of which were linked by an oligopeptide linker (2x GGGGS).

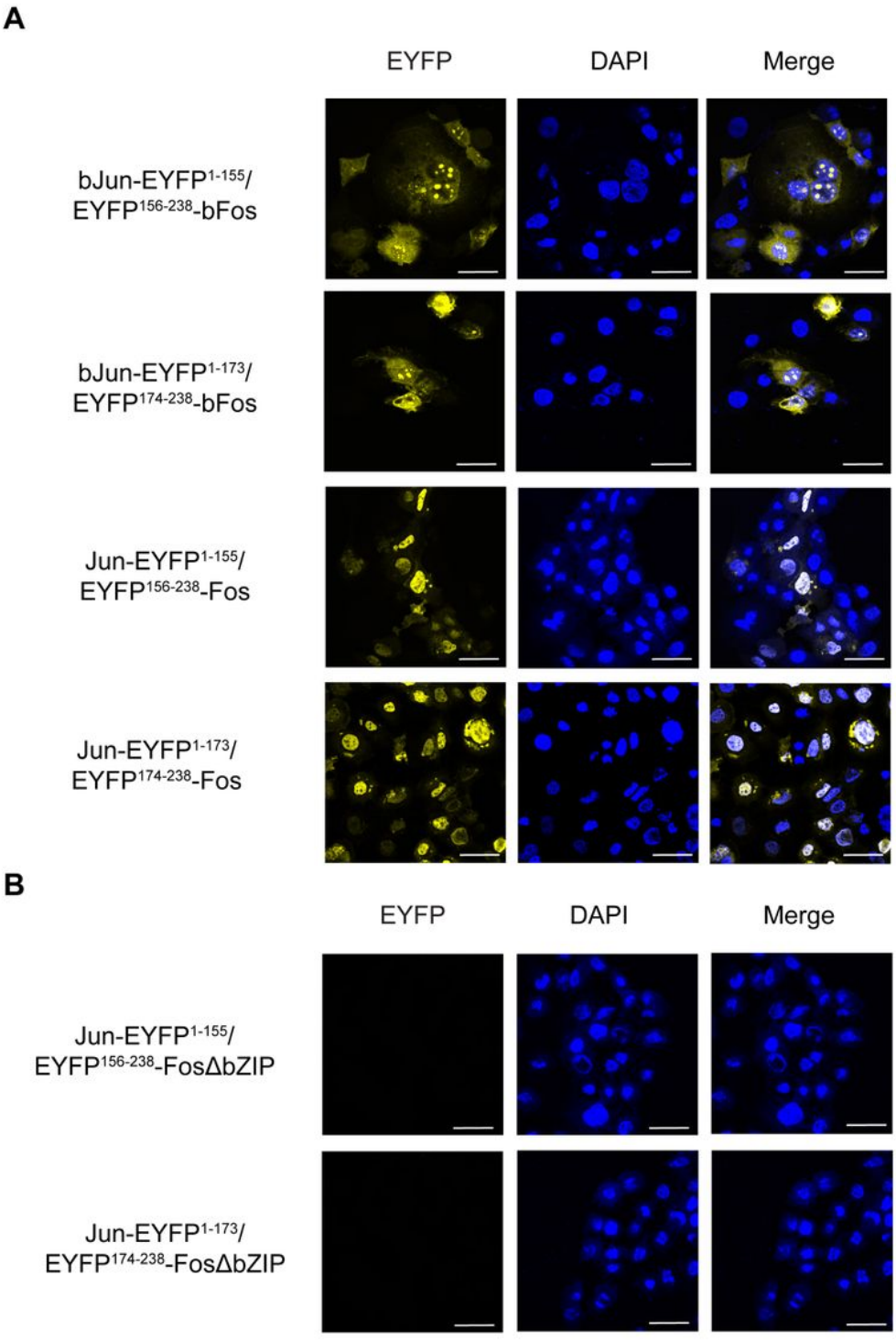

Figure 3

EYFP fluoresce mediated by interplay between Jun and Fos. (A) BiFC assay monitoring interaction between Jun/Fos and bJun/bFos (core domains for their interaction) in Hela. Bright yellow spots in Hela

nucleus were observed. The Jun-Fos and bJun-bFos interactions occurs in the nucleus, as evidenced by colocalization with the DNA stain DAPI. (**B)** Jun and FosΔbZIP cannot interact with each other and no fluorescence emerged. The scale bar in all images is 40 μm.

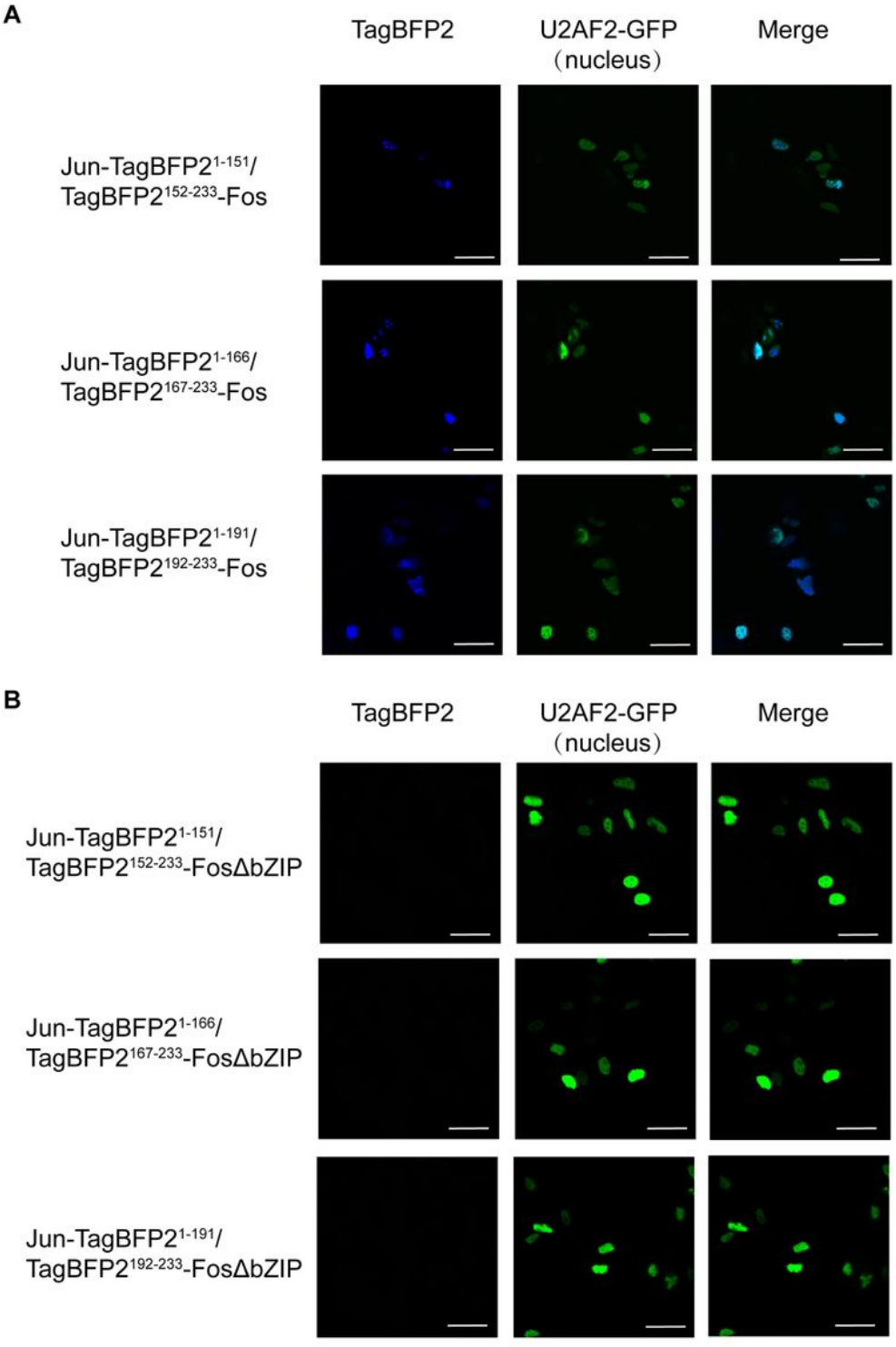

Figure 4

Fluorescence emitted by fusion proteins complex of TagBFP2 fragments and Jun/Fos. (A) BiFC assay monitoring interaction between Jun and Fos in Hela as evidenced by intense TagBFP2 signal. **(B)** No fluorescence signals were detected in the group of Jun and FosΔbZIP. The scale bar in all images is 40 μm.

Supplementary Files

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- SupplementaryMaterial.docx