# Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis

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The specificity of biological regulatory mechanisms relies on selective interactions between different proteins in different cell types and in response to different extracellular signals. We describe a bimolecular fluorescence complementation (BiFC) approach for the simultaneous visualization of multiple protein interactions in the same cell. This approach is based on complementation between fragments of fluorescent proteins with different spectral characteristics. We have identified 12 bimolecular fluorescent complexes that correspond to 7 different spectral classes. Bimolecular complex formation between fragments of different fluorescent proteins did not differentially affect the dimerization efficiency of the bZIP domains of Fos and Jun or the subcellular sites of interactions between these domains. Multicolor BiFC enables visualization of interactions between different proteins in the same cell and comparison of the efficiencies of complex formation with alternative interaction partners.



Networks of protein interactions mediate cellular responses to environmental stimuli and direct the execution of developmental programs. Each protein typically has a large number of alternative interaction partners, and the selectivity of these interactions determines the developmental potential of the cell and its responses to extracellular stimuli. We recently described an approach for the visualization of protein interactions in living cells designated BiFC analysis[1]. The BiFC approach is based on the formation of a fluorescent complex by fragments of the enhanced yellow fluorescent protein (YFP) brought together by the association of two interaction partners fused to the fragments. This approach enables visualization of the subcellular sites of protein interactions under conditions that closely reflect the normal physiological environment.

Molecular engineering of the green fluorescent protein (GFP) has produced several variants with altered spectral characteristics[2]. These variants allow simultaneous visualization of the distributions of multiple proteins in living cells. Moreover, fluorescence resonance energy transfer between different variants enables investigation of interactions between individual pairs of proteins in living cells[3,4]. Thus far, it has not been possible to visualize multiple interactions in the same cell.

Selected fragments of many proteins can associate to produce functional bimolecular complexes. Such bimolecular complementation provides a convenient approach for detection of protein interactions in cells if the protein fragments can associate only when they are brought together by interaction partners fused to the fragments[1,5–9]. The unique characteristic of the BiFC approach is that the bright intrinsic fluorescence of the bimolecular complex allows direct visualization of the complex in living mammalian cells[1]. Moreover, the large number of GFP variants with distinct spectral characteristics provided the potential for parallel analysis of multiple protein interactions in the same cell. In the present study, we have realized the promise of multicolor BiFC analysis by characterizing 12 bimolecular fluorescent complexes, and we have used these complexes to compare the dimerization selectivity and subcellular sites of interactions among basic region leucine zipper (bZIP) family proteins.

## Results

The spectral characteristics of bimolecular fluorescent complexes formed by fragments of YFP are virtually identical to those of intact YFP[1]. By extension, we reasoned that fragments of other GFP variants might support bimolecular fluorescent complex formation, and that such complexes might have distinct spectral characteristics. To identify such complexes, we investigated fluorescence complementation by using the corresponding fragments of the enhanced GFP and cyan fluorescent protein (CFP) fused to the bZIP domains of Fos and Jun (bFos and bJun) (Fig. 1A). Each pair of fusion proteins was expressed in mammalian cells and the cells were examined by fluorescence microscopy (Fig. 1B–D). No complementation was detected when fragments of GFP (GN155 and GC155) fused to bJun or bFos, respectively were expressed in mammalian cells. However, fragments of CFP (CN155 and CC155) exhibited fluorescence complementation when fused to bJun or bFos, respectively (Fig. 1D). All of the fusion proteins were expressed at comparable levels as determined by western analysis (Supplementary Fig. 1 online).

To examine the selectivity of bimolecular complex formation, we tested fluorescence complementation between all nine combinations of fragments (Supplementary Fig. 2 online). YN155 exhibited fluorescence complementation with YC155 and CC155, whereas CN155

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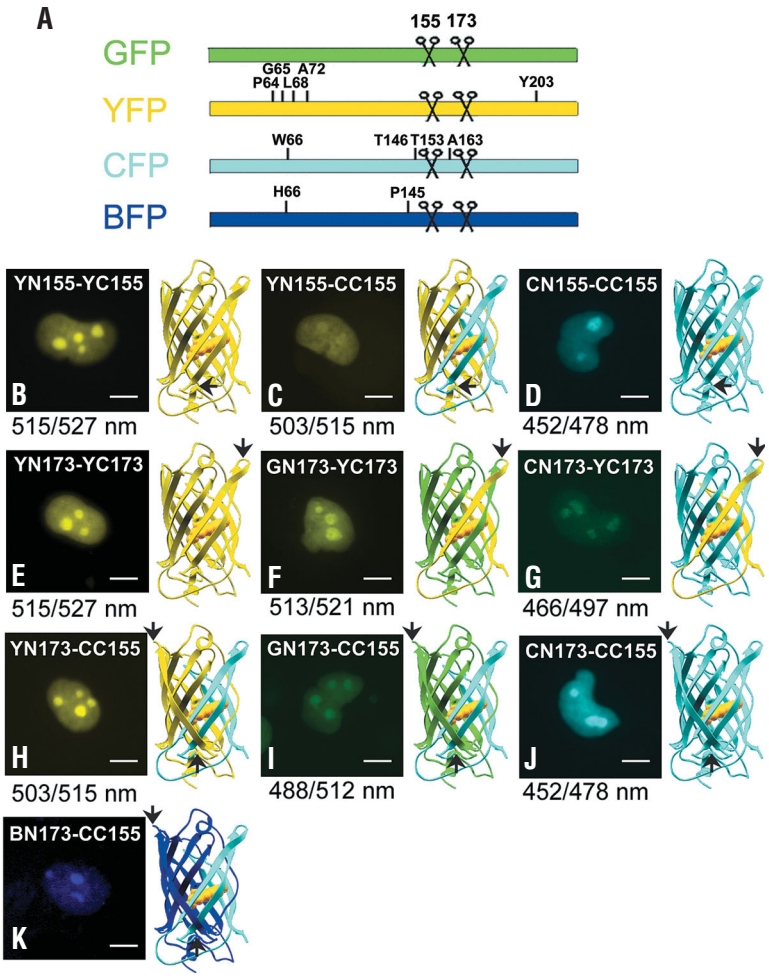


**Figure 1.** Visualization of complementation between fragments of different fluorescent proteins fused to bFos and bJun. (A) Diagram of amino acid substitutions among enhanced green fluorescent protein variants and the positions where they were fragmented (155 and 173). (B–K) Fluorescence images of COS-1 cells transfected with plasmids expressing the protein fragments indicated in each panel fused to the bZIP domains of Fos and Jun. The C-terminal fragments were fused to bFos and the N-terminal fragments were fused to bJun. Structural models of the bimolecular fluorescent complexes shown to the right of each image are based on the X-ray crystal structure of full-length GFP[10]. The positions of fragmentation are indicated by arrows in the structures. The position and structure of the duplicated β-strand shown in H–J is unknown. The excitation/emission maxima of each complex are shown below the images. The bar represents 10 μm in all images.

exhibited fluorescence complementation only with CC155 when fused to bFos and bJun (Fig. 1B–D). The fluorescence spectrum of cells expressing YN155 and CC155 fusions was distinct from those of cells expressing either YN155 and YC155 or CN155 and CC155. GN155 and GC155 did not exhibit detectable fluorescence complementation with any of the other fragments. YC155 and CC155 differ from GC155 by single amino acid residues whereas YN155 and CN155 differ from GN155 by four and three amino acid residues respectively (Fig. 1A). These amino acid substitutions determined the selectivity of complementation among these fragments.

We used a genetic screen in *Escherichia coli*[1] to identify a second pair of YFP fragments (YN173 and YC173) that exhibit complementation when fused to bFos and bJun. We examined fluorescence complementation between these fragments and the corresponding fragments of GFP, CFP, and the enhanced blue fluorescent protein (BFP) fused to bFos and bJun. The sequences of the C-terminal fragments of GFP, CFP, and BFP are identical (Fig. 1A), and thus only YC173 and GC173 were tested. YC173 exhibited fluorescence complementation with YN173, GN173, and CN173 when fused to bFos and bJun (Fig. 1E–G), whereas GC173 did not exhibit detectable fluorescence complementation with any of the fragments tested. The two positions of fragmentation (155 and 173) enabled complementation to take place between distinct combinations of fluorescent protein fragments (Supplementary Fig. 2 online). Thus, a small number of amino acid substitutions can influence the positions at which proteins must be fragmented to support bimolecular complementation.

GFP is a β-barrel structure containing 11 strands surrounding a central α helix[10]. The two sites of fragmentation in YFP that support BiFC are separated by one strand of the β-barrel that surrounds the fluorophore[10]. We examined fluorescence complementation between all 24 combinations of fragments truncated at these positions fused to bFos and bJun (Supplementary Fig. 2 online). None of the N-terminal fragments truncated at residue 155 exhibited fluorescence complementation with C-terminal fragments truncated at residue 173. In contrast, all of the N-terminal fragments truncated at residue 173 (YN173, GN173 and CN173) formed bimolecular fluorescent complexes with CC155 and YC155 (Fig. 1H–J and data not shown). The corresponding N-terminal fragment of BFP (BN173) also exhibited fluorescence complementation with CC155 (Fig. 1K). Duplication of the segment separating the points of truncation therefore facilitated bimolecular fluorescence complementation. This duplication had no detectable effect on the spectra of the bimolecular complexes (Fig. 2). To establish whether complementation between fragments of different fluorescent proteins fused to bFos and bJun required the leucine zipper dimerization interface, we examined complementation by bFos fusions in which the carboxy-terminal half of the leucine zipper was deleted (bFosΔZIPYC155 and bFosΔZIPCC155). We compared the complementation efficiencies of the wild-type and mutant fusions with bJun fused to fragments of different fluorescent proteins (Fig. 3, Supplementary Table 1 online). Mutation of the leucine zipper resulted in a more than tenfold reduction in the efficiencies of fluorescence complementation between all fragments of fluorescent proteins tested. Cells expressing fragments of different fluorescent proteins fused to wild-type bFos and bJun produced different intensities of fluorescence emissions at different times after transfection. Cells expressing the same fragments, but with a deletion in the leucine zipper of Fos, produced either undetectable or lower fluorescence emissions at all times after transfection (Supplementary Table 1 online). Similar results were obtained when the fluorescence emissions of the cell populations were measured using fluorescence spectroscopy (data not shown). The mutant proteins were expressed at the same levels and were localized to the same subnuclear sites as the wild-type proteins. Thus, efficient fluorescence complementation between all fragments of fluorescent proteins examined required a specific interaction interface.

Multicolor BiFC allows comparison of the efficiencies of complex formation between alternative interaction partners, provided the fragments do not influence the selectivity of protein interactions. To compare the effects of fragments of different fluorescent proteins on dimerization efficiency, we examined the competition between proteins in which bJun was fused to fragments of different fluorescent

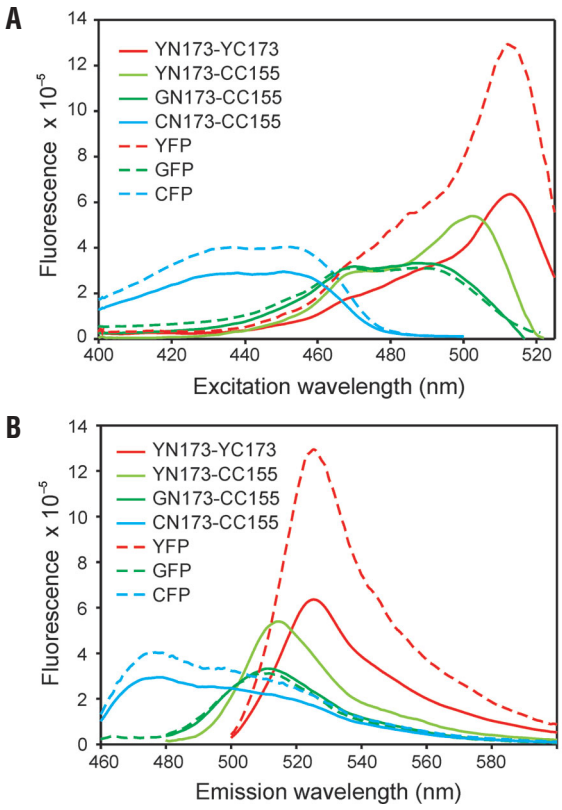


**Figure 2.** Excitation and emission spectra of cells expressing fragments of different fluorescent proteins fused to bFos and bJun. Solid lines correspond to bimolecular fluorescent complexes and dashed lines to intact fluorescent proteins. (A) The excitation spectra were collected by measuring emissions at 535 nm through a 530 nm long-pass filter, and were normalized by the ratio between the emission maximum of each complex and the emission intensity at 535 nm. (B) The emission spectra of YN173-YC173 and YFP were collected through a 500 nm long-pass filter using excitation at 480 nm and the other emission spectra were collected through a 450 nm long-pass filter using excitation at 430 nm; the spectra were normalized on the basis of the ratio between the excitation maximum of each complex and the excitation efficiencies at 480 nm and 430 nm, respectively. The cells were transfected with 0.5 μg of the plasmids encoding the fluorescent protein fragments fused to bFos and bJun or with 0.05 μg of the plasmids encoding the full-length proteins.

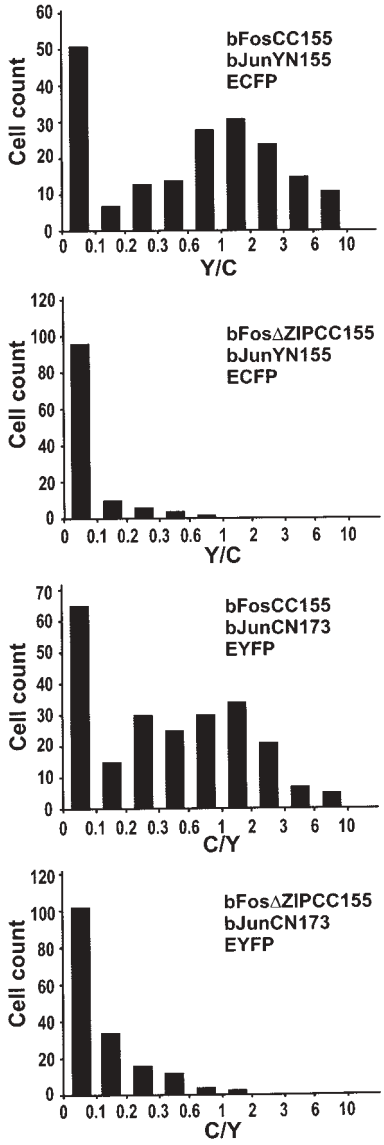


**Figure 3.** Effects of a deletion in the leucine zipper on the efficiencies of complementation between fragments of different fluorescent proteins fused to the bZIP domains of Fos and Jun. The efficiencies of fluorescence complementation were determined in individual cells by measuring the ratio between the fluorescence emissions of the bimolecular fluorescent complex and that of a coexpressed intact fluorescent protein. Plasmids encoding the proteins indicated in each panel were transfected into cells (0.25 μg of each fusion protein and 0.025 μg of the ECFP or EYFP internal control) and the ratio of fluorescence emissions produced by the bimolecular complex and the internal control (Y/C or C/Y) was measured. Note the logarithmic scaling of the categories in the histograms.

proteins for dimerization with bFosYC173 *in vitro* (Fig. 4). The ratio of bimolecular fluorescent complexes formed by the alternative interaction partners was calculated by fitting the spectrum of the mixture to the weighted sum of the spectra of the two complexes. This ratio showed a perfect correspondence with the ratio of bJun fusion proteins that was added to each reaction. Thus, the bZIP domains of Fos and Jun have identical dimerization efficiencies when fused to fragments of different fluorescent proteins, suggesting that the fusions do not have differential effects on interactions between bFos and bJun *in vitro*.

The spectral differences between bimolecular complexes formed by fragments of different fluorescent proteins enable comparison of the subcellular sites of interactions between different proteins in the same cell, provided the fragments do not differentially affect complex localization. To compare the subcellular locations of bFos-bJun heterodimers fused to fragments of different fluorescent proteins, we coexpressed bJunCN173 and bJunYN173 with bFosYC173 in COS-1 cells and imaged the cells using filters optimized for the detection of CN173-YC173 and YN173-YC173 complexes, respectively (Fig. 5A). Both complexes were localized preferentially to the nucleoli, and showed perfect colocalization. Similar results were obtained when bJunCN173 and bJunYN173 were coexpressed with bFosYC155 or with bFosCC155. Although the efficiency of nucleolar localization of the complexes varied between different cells in the population, the efficiencies of nucleolar localization of complexes containing fragments of different fluorescent proteins were identical in individual cells. Thus, fragments of different fluorescent proteins did not have differential effects on the subcellular sites of interactions between bFos and bJun.

To visualize the subcellular sites of interactions between different proteins in the same cell, we coexpressed bJunCN173 and JunYN155 with bFosYC155. bJunCN173-bFosYC155 was localized

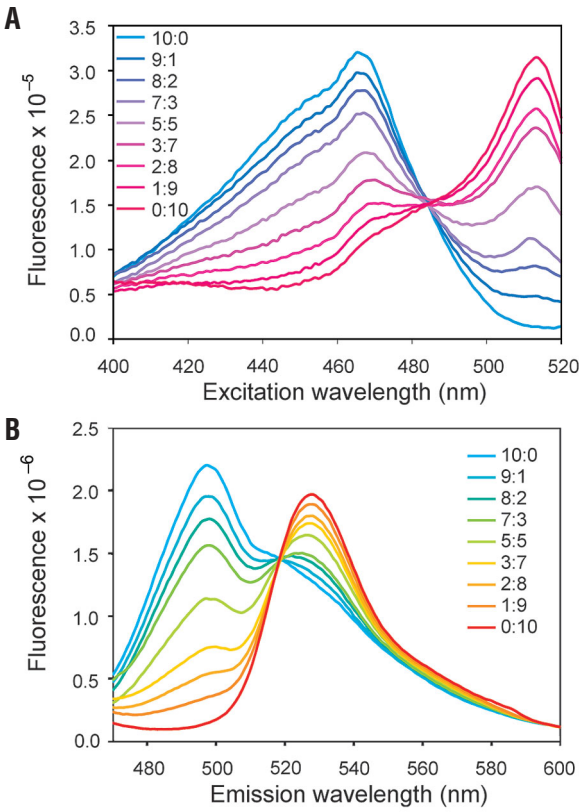


**Figure 4.** Comparison of the efficiencies of bimolecular complex formation by fragments of different fluorescent proteins fused to bFos and bJun. We examined the competition between different ratios of bJun fused to fragments of different fluorescent proteins for a limiting concentration of bFos fused to the complementary fragment. (A) The indicated ratios of GN173 and CN173 fused to bJun were mixed with a fixed concentration of YC173 fused to bFos. The excitation spectra were collected after 12 h by measuring emissions at 530 nm. (B) The indicated ratios of YN173 and CN173 fused to bJun were mixed with a fixed concentration of YC173 fused to bFos. The emission spectra were collected after 12 h during excitation at 450 nm.

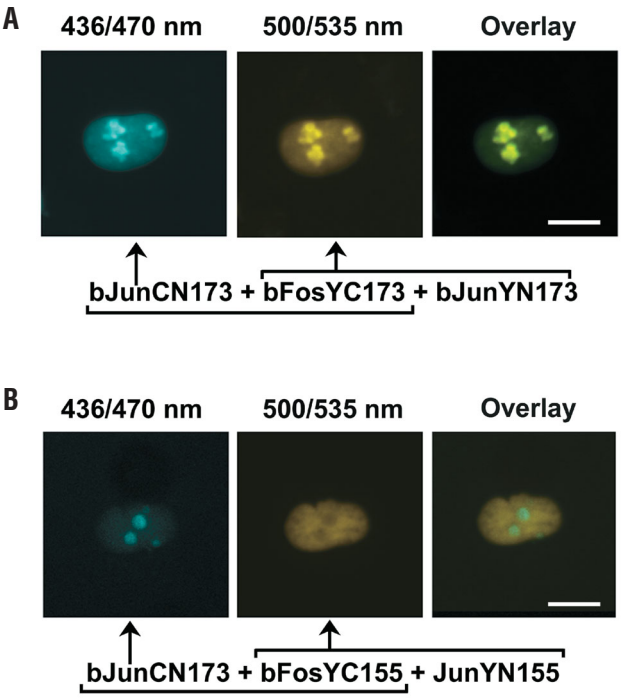


**Figure 5.** Multicolor BiFC analysis of the subcellular sites of interactions between different proteins in the same cell. (A) Colocalization of bJunCN173-bFosYC173 and bJunYN173-bFosYC173 bimolecular fluorescent complexes. (B) Differential localization of bJunCN173-bFosYC155 and JunYN155-bFosYC55 complexes. Plasmids encoding the proteins indicated below the images were cotransfected into COS-1 cells. The images show the fluorescence emissions of the same cell using a 436 nm and 470 nm 'C' filter or a 500 nm and 530 nm 'Y' filter. The bar represents 10 μm in all images.

to the nucleolus, whereas JunYN155-bFosYC155 was localized to the nucleoplasm (Fig. 5B). The two complexes showed distinct distributions in the same nucleus, indicating that regions outside the bZIP domain of Jun affected the subcellular localization of the complex. Similar results were obtained when bJunCN173 and JunYN155 were coexpressed with bFosCC155. Thus, multicolor BiFC can be used to compare the subcellular sites of interactions between different proteins in the same cell.

The bZIP domains of Fos, Jun, and ATF2 can interact with each other in all pairwise combinations *in vitro*, and activate different genes in cells[11–14]. To investigate the competition between alternative interaction partners in cells, we examined the relative efficiencies of bJun interactions with bFos and bATF2. We expressed bJunCC155 with bFosCN173 and bATF2YN155 together as well as with each separately, and compared the ratio between the fluorescence emissions of the complexes (Fig. 6A, Supplementary Table 2 online). Cells expressing the two pairs of proteins separately exhibited cyan and yellow fluorescence, respectively. Coexpression of a limiting amount of bJunCC155 with bFosCN173 and bATF2YN155 together resulted in cyan fluorescence that was comparable to that observed in cells transfected with bJunCC155 and bFosCN173, but in yellow fluorescence that was 100-fold lower than that of cells transfected with the same amounts of bJunCC155 and bATF2YN155 (Supplementary Table 2). The ratio between yellow and cyan fluorescence was therefore comparable for cells expressing all three proteins and those expressing only bJunCC155 and bFosCN173 (Fig. 6A). Similar results were obtained when bJunCC155 was expressed with bFosCN155 and bATF2YN155 (data not shown). When excess bJunCC155 was expressed with bFosCN173 and bATF2YN155, both cyan and yellow fluorescence was observed in the cells. It is therefore likely that the inhibition of complementation between bATF2YN155 and bJunCC155 in the presence of either bFosCN155 or bFosCN173 was caused by more efficient complex formation by bJunCC155 with either bFosCN155 or bFosCN173 than with bATF2YN155.

To confirm that the identity of the fragments fused to bJun, bFos, and bATF2 did not affect the preference for bimolecular fluorescent complex formation, we exchanged the fragments between bFos and bATF2 (Fig. 6B). Cells expressing a limiting amount of bJunCC155 with bFosYN155 and bATF2CN173 produced yellow fluorescence comparable to that of cells transfected with bJunCC155 and bFosYN155, but produced cyan fluorescence that was tenfold lower than that of cells transfected with the same amounts of bJunCC155 and bATF2CN173 (Supplementary Table 2). Again, the ratio between yellow and cyan fluorescence for cells expressing all three proteins was similar to that of cells expressing only bJunCC155 and bFosYN173 (Fig. 6B). The fluorescent protein fragments were fused to the same positions in bJun and bATF2 relative to the leucine zipper, and the same linker sequences were used for all fusions. The competing proteins were expressed at comparable levels (Supplementary Fig. 1 online). Consequently, it is likely that the higher efficiency of complementation between bJun and bFos fusions reflected preferential heterodimerization of bJun with bFos than with bATF2 in cells.

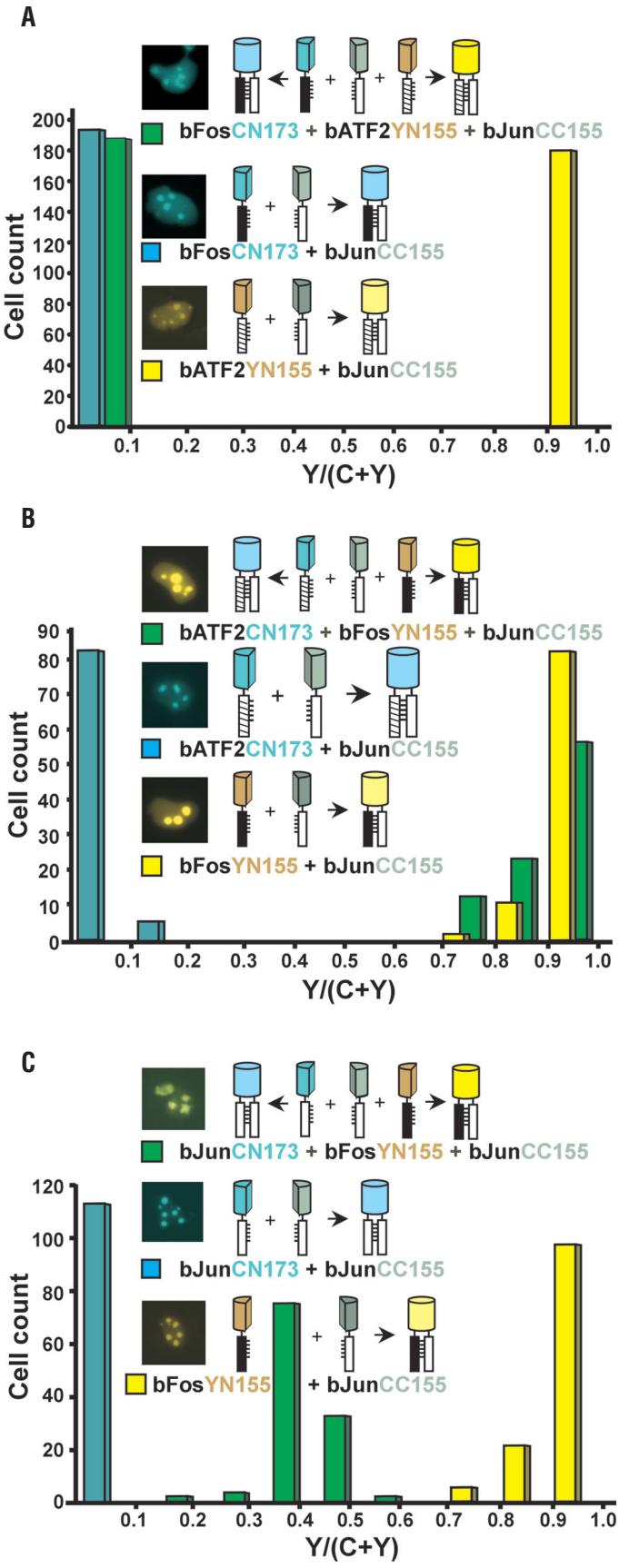


**Figure 6.** Multicolor BiFC analysis of the competition between alternative interaction partners in cells. Two combinations of interaction partners producing bimolecular complexes with different spectra were transfected into different cell populations (cyan and yellow bars) or cotransfected into the same cell population (green bars) as indicated above each graph. The ratio between the fluorescence emissions corresponding to each complex (Y/(C + Y)) was determined in individual cells in each population, and was plotted in the histograms. (A) Competition between bFosCN173 and bATF2YN155 for dimerization with bJunCC155. (B) Competition between bFosYN155 and bATF2CN173 for dimerization with bJunCC155. (C) Competition between bFosYN155 and bJunCN173 for dimerization with bJunCC155. Plasmids encoding the proteins indicated in each panel were cotransfected into cells. The plasmid encoding the shared interaction partner fused to CC155 was used at limiting concentration (0.1 μg), whereas the other plasmids were used at a higher concentration (0.5 μg). The fluorescence intensities of individual cells were quantified using a 436 nm/470 nm 'C' filter and a 500 nm/535 nm 'Y' filter and the Y/(Y + C) ratios were plotted in the histograms.

To examine the relative efficiencies of bFos interactions with bJun and bATF2 in cells, we expressed limiting bFosCC155 with bJunCN173 and bATF2YN155. The cells exhibited cyan fluorescence comparable to cells transfected with bFosCC155 and bJunCN173, but markedly lower yellow fluorescence than cells transfected with the same amounts of bFosCC155 and bATF2YN155 (data not shown). A similar inhibition of bFos-bATF2 and bJun-bATF2 heterodimer formation was observed in cells transfected using a limiting concentration of bATF2YC155 with bFosYN155 and bJunCN173 (data not shown). The higher efficiency of bFos-bJun heterodimer formation therefore did not require bimolecular complex formation because bFosYN155-bJunCN173 heterodimers could not form a bimolecular fluorescent complex. Thus, the bZIP domains of Fos and Jun favor dimerization with each other over dimerization with the bZIP domain of ATF2 in cells.

To examine the relative efficiencies of Fos-Jun heterodimerization and Jun homodimerization in cells, we expressed bJunCC155 with bFosYN155 and bJunCN173 (Fig. 6C). The cells produced on average 60% of the cyan fluorescence and 35% of the yellow fluorescence produced by the control cells. The fluorescence intensities of individual cells were normally distributed and showed a moderate ($r = 0.83$) correlation between cyan and yellow fluorescence. Thus, Fos-Jun heterodimers and Jun homodimers can coexist in cells, consistent with the similar thermodynamic stabilities of these dimers *in vitro*[15].

## Discussion

The multicolor BiFC assay provides a unique approach for the simultaneous visualization of multiple protein interactions in a living cell. Although we describe only cases where two interactions were compared in parallel, the number of interactions that can be visualized simultaneously is limited in principle only by the number of spectrally distinct bimolecular fluorescent complexes. In practice, the number of complexes that can be visualized simultaneously depends on the spectral resolution of the imaging system. In typical cases where two different complexes need to be resolved, the broad range of spectral characteristics among the bimolecular complexes identified here enables visualization of the complexes without interference from spectral overlap using standard fluorescence microscopy.

Fragments of different fluorescent proteins did not differentially affect bZIP protein dimerization or the localization of those dimers. These fragments therefore enable comparison of the efficiencies and subcellular locations of complex formation with alternative interaction partners in the same cell. It remains possible that these fragments have distinct effects on interactions between other proteins or the localization of those complexes. It is therefore necessary to compare the efficiencies of complex formation by proteins fused to these fragments, and to establish the effects of the fragments on complex localization, in order to use multicolor BiFC for comparison of

interactions among other proteins. In addition, because formation of the bimolecular fluorescent complex is essentially irreversible, the efficiency of fluorescence complementation represents the efficiency of the association between the interaction partners at the time of complex formation, and does not reflect subsequent shifts in the equilibrium among alternative interaction partners[1].

The systematic analysis of complementation between fragments of different fluorescent proteins provides several insights into protein complementation. Complementation between fragments of different fluorescent proteins was selective, and single amino acid substitutions had greater than 100-fold effects on the efficiency of fluorescence complementation. These same substitutions had little effect on the quantum yields of the intact proteins[2], and some were located at a distance from the fluorophore. Substitutions that resulted in different efficiencies of fluorescence complementation had only small effects on the kinetics of fluorophore maturation (data not shown). It is therefore likely that these amino acid substitutions influence the efficiency of bimolecular complex formation.

Two different positions were identified where fragmentation of fluorescent proteins enabled bimolecular complementation to take place. These positions are within loops at opposite ends of the β-barrel structure. The bFos-bJun interaction and the linker sequences used in these experiments therefore provide sufficient flexibility for the fragments to associate with their N-termini separated by a distance of either 10 or 50 Å. We have used the multicolor BiFC assay to investigate interactions among several structurally unrelated protein families. The results from these experiments confirm that the interaction partners do not need to juxtapose the fragments in a specific orientation, providing that the linkers that tether the fragments have sufficient flexibility to allow the fragments to associate with each other. Multicolor BiFC therefore provides a general approach for the analysis of complex formation among alternative interaction partners in living cells.

The β-barrel structure that surrounds the fluorophore in GFPs is composed of three segments of contiguous peptide sequence, each of which forms an anti-parallel β-sheet that together form the β-barrel[10]. Both of the truncations that allow fluorescence complementation interrupt one of these β-sheet segments. Thus, all of the fragments that allow fluorescence complementation contain an incomplete β-sheet. Fragments that were truncated at the junctions between the three β-sheet segments did not exhibit fluorescence complementation[1]. Other structurally unrelated protein fragments that exhibit complementation also contain incomplete domains that are unlikely to fold in the absence of the complementary fragments[6,7,16–18]. It is therefore likely that bimolecular complementation by many protein fragments requires at least part of the fragments to be unfolded, which may facilitate their association.

Comparison of the efficiencies of interactions between alternative interaction partners using multicolor BiFC requires that the fusion proteins meet several criteria. First, fragments of different fluorescent proteins must not differentially affect interactions between the proteins. In the case of bFos-bJun heterodimers, identical efficiencies of complex formation were observed between proteins fused to fragments of different fluorescent proteins. Second, the efficiencies of complementation between the fragments must be equivalent once they have been brought together by interactions between the alternative interaction partners. In the case of bFos-bJun and bJun-bATF2 heterodimers fused to the same fragments, the fluorescence emissions of bimolecular complexes formed separately were comparable, consistent with the structural similarity between these complexes. Finally, similar amounts of the alternative interaction partners must be expressed and be localized to their normal subcellular compartments. Comparable amounts of the bZIP domains of Fos, Jun, and ATF2 were expressed and were localized to the nucleoli. When these criteria are met, multicolor BiFC enables comparison of the efficiencies and subcellular sites of complex formation between alternative interaction partners in living cells.

## Experimental protocol

**Plasmid construction.** The sequences encoding amino acid residues 1–154, 155–238, 1–172, and 173–238 of enhanced YFP, GFP, CFP, and BFP (Clontech, Palo Alto, CA) were fused downstream of sequences encoding residues 118–210 of Fos (bFos) and residues 257–318 of Jun (bJun) using linker sequences encoding RPACKIPNDLKQKVMNH and RSIAT, respectively. The chimeric coding regions were cloned into pFLAG-CMV2 (Sigma, St. Louis, MO) and pHA-CMV (Clontech, Palo Alto, CA) vectors for expression in mammalian cells and into pDS56[11] for expression in *E. coli*. As a control, residues 179–193 in the leucine zipper of Fos were deleted to produce bFosΔZIP. Plasmids encoding JunYN155, bATF2YN155, and bATF2YC155 were described previously[1].

**Imaging and spectral analysis of fluorescence in cells.** COS-1 cells were transfected with plasmids encoding the fusion proteins indicated. Transfected cells were incubated at 37 °C for 24 h and then switched to 30 °C for 0–24 h to promote fluorophore maturation. To image the fluorescence emissions of cells expressing different combinations of fusion proteins, we used filter pairs centered at 436 nm excitation and 470 nm emission (C filter) or 500 nm excitation and 535 nm emission (Y filter) together with dichroic mirrors with transmission windows at 450–490 nm and 520–590 nm. The fluorescence intensities of individual cells were quantified using automated feature-recognition software (Complix, Granberry Township, PA). The signal in an area of the field containing no cells was used as background and subtracted from all values. There was less than 2% overlap between the signals from bimolecular fluorescent complexes formed by YN173 and CN173 with either YC155 or CC155 using these filters. Thus, no correction for crosstalk was necessary. To measure the spectra of bimolecular fluorescent complexes, the cells were washed and resuspended in PBS. All spectra were corrected for background signal produced primarily by scatter by subtracting the spectrum of untransfected cells. The level of expression of each fusion protein was quantified by western blotting using antibodies directed against the FLAG and HA epitopes (Sigma, St. Louis, MO).

**Analysis of fluorescence complementation *in vitro*.** Chimeric fusion proteins were purified from *E. coli* using nickel chelate affinity chromatography in the presence of 6 M guanidine as described[1]. The proteins indicated in each experiment were heated to 95 °C for 5 min and diluted to a final concentration of 30 μg/ml in buffer (50 mM $NaPO_4$, pH 8.0, 150 mM NaCl, 5% glycerol (vol/vol), 0.1 mg/ml BSA, and 1mM dithiothreitol) at room temperature. Excitation and emission spectra were collected after no further change in fluorescence was observed. The fluorescence intensities of the bimolecular fluorescent complexes were comparable to those observed for the intact fluorescent proteins. The spectra were corrected for scatter by subtracting the spectrum of the buffer alone.

*Note: Supplementary information is available on the Nature Biotechnology website.*

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*Competing interests statement*
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1. Hu, C.-D., Chinenov, Y. & Kerppola, T.K. Visualization of interactions among bZIP and Rel proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789–798 (2002).
2. Tsien, R.Y. The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544 (1998).
3. Miyawaki, A. *et al*. Fluorescent indicators for $Ca^{2+}$ based on green fluorescent proteins and calmodulin. *Nature* **388**, 882–887 (1997).
4. Romoser, V.A., Hinkle, P.M. & Persechini, A. Detection in living cells of $Ca^{2+}$-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators. *J. Biol. Chem.* **272**, 13270–13274 (1997).
5. Johnsson, N. & Varshavsky, A. Split ubiquitin as a sensor of protein interactions *in vivo*. *Proc. Natl. Acad. Sci. USA* **91**, 10340–10344 (1994).
6. Rossi, F., Charlton, C.A. & Blau, H.M. Monitoring protein-protein interactions in intact eukaryotic cells by β-galactosidase complementation. *Proc. Natl. Acad. Sci. USA* **94**, 8405–8410 (1997).
7. Pelletier, J.N., Campbell-Valois, F.X. & Michnick, S.W. Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. *Proc. Natl. Acad. Sci. USA* **95**, 12141–12146 (1998).
8. Ghosh, I., Hamilton, A.D. & Regan, L. Antiparallel leucine zipper-directed protein reassembly: application to the green fluorescent protein. *J. Am. Chem. Soc.* **122**, 5658–5659 (2000).
9. Galarneau, A., Primeau, M., Trudeau, L.E. & Michnick, S.W. Beta-Lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein-protein interactions. *Nat. Biotechnol.* **20**, 619–622 (2002).
10. Ormö, M. *et al*. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* **273**, 1392–1395 (1996).
11. Kerppola, T.K. & Curran, T. Selective DNA bending by a variety of bZIP proteins. *Mol. Cell. Biol.* **13**, 5479–5489 (1993).
12. Tsai, E.Y. *et al*. A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter *in vivo*. *Mol. Cell. Biol.* **20**, 6084–6094 (2000).
13. Falvo, J.V., Parekh, B.S., Lin, C.H., Fraenkel, E. & Maniatis, T. Assembly of a functional beta interferon enhanceosome is dependent on ATF-2-c-jun heterodimer orientation. *Mol. Cell. Biol.* **20**, 4814–4825 (2000).
14. Daury, L. *et al*. Opposing functions of ATF2 and Fos-like transcription factors in c-Jun-mediated myogenin expression and terminal differentiation of avian myoblasts. *Oncogene* **20**, 7998–8008 (2001).
15. O'Shea, E.K., Rutkowski, R., Stafford, W.D., Kim, P.S. & Stafford, W.F. Preferential heterodimer formation by isolated leucine zippers from fos and jun. *Science* **245**, 646–648 (1989).
16. Ullmann, A., Jacob, F. & Monod, J. On the subunit structure of wild-type versus complemented β-galactosidase of *Escherichia coli*. *J. Mol. Biol.* **32**, 1–13 (1968).
17. Kippen, A.D. & Fersht, A.R. Analysis of the mechanism of assembly of cleaved barnase from two peptide fragments and its relevance to the folding pathway of uncleaved barnase. *Biochemistry* **34**, 1464–1468 (1995).
18. Koebnik, R. *In vivo* membrane assembly of split variants of the *E. coli* outer membrane protein OmpA. *EMBO J.* **15**, 3529–3537 (1996).