Live-cell assay for simultaneous monitoring of expression and interaction of proteins

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Protein-protein interactions play a fundamental role in all cellular functions. Thus, elucidating protein interactions has emerged as a new, exciting, and important challenge in the postgenomic era.

Bimolecular fluorescence complementation (BiFC) is a powerful methodology for visualization of protein-protein interactions in living cells (1; reviewed in Reference 2). In BiFC, proteins under study are tagged either with the N-terminal (YN) or the C-terminal (YC) fragment of the yellow fluorescent protein (YFP). Protein interactions bring the YN and YC fragments together, resulting in a yellow fluorescent complex. Current BiFC assays visualize protein-protein interactions, but do not detect individual proteins under study. Consequently, expression of proteins must be evaluated with additional methods, increasing the total time and cost of interaction analysis.

To overcome this problem, we explored the feasibility of co-visualizing the expression of proteins under study in the BiFC assay by tagging them with a second, constitutively fluorescent domain.

To this end, we designed plasmids for eukaryotic expression of proteins containing either the cyan fluorescent protein (CFP) fused to YN (amino acids 1–154 of YFP) (Figure 1A) or the monomeric red fluorescent protein 1 (mRFP1) (3) fused to YC (amino acids 155–238 of YFP) (Figure 1B). Flexible linkers separate both domains in each fusion protein. Both plasmids, called C-YN and R-YC, respectively, contained unique restriction sites for insertion of additional coding sequences at the 5′ end of the artificial gene. Cells expressing both fusion proteins should display red and blue fluorescence, whereas interactions between fusion proteins should be indicated by yellow fluorescence (Figure 1, C and D).

Cotransfection of C-YN and R-YC plasmid constructs into HeLa cells, resulted in cells displaying both CFP and mRFP fluorescent signals, which were evenly distributed throughout the cells (Figure 1E). In contrast, the YFP signal was either very weak or not detectable in the same cells, indicating a very low degree of unspecific interaction (also observed with the classical BiFC assay). We did not observe significant signal crosstalk between signals (see the supplementary materials and Supplementary Figure S1 available online at www.BioTechniques.com).

The human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). The HIV Rev protein is a key regulator of virus replication. Rev is a small protein essential for the activation of

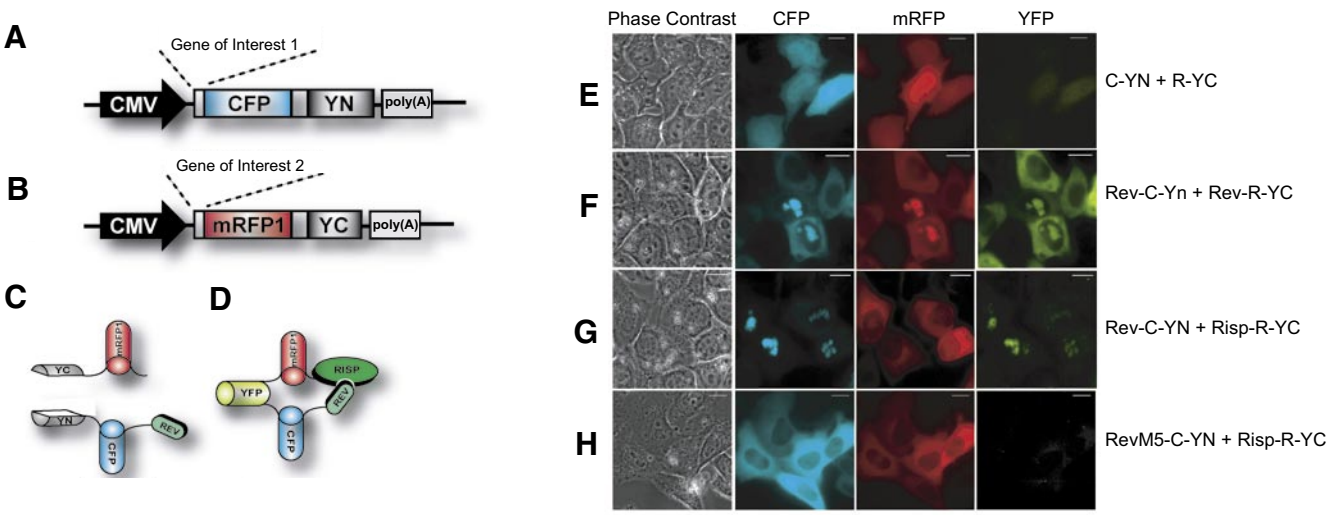

Figure 1. Principle and application of the extended bimolecular fluorescence complementation (exBiFC) assay. Panels A and B show exBiFC expression cassettes and panels C and D show exBiFC fusion proteins. Panels E–H show cells co-expressing red and blue fluorescent exBiFC proteins either (E) lacking interaction domains (C-YN + R-YC) or containing the following potential interaction domains: (F) wild-type Rev (Rev-C-YN + Rev-R-YC), (G) wild-type Rev and Risp (Rev-C-YN + Risp-R-YC), and (H) mutant RevM5 and Risp (Rev-M5-C-YN + Risp-R-YC). Significant yellow fluorescent protein (YFP) signals are only visible in panels F and G, confirming interaction of Rev with itself and with Risp. Widefield fluorescence microscopic images were acquired with an Axiovert 200 M microscope controlled by AxioVision 4.5 software (both from Carl Zeiss, Goettingen, Germany). For all images, constant exposure times and display normalizations were applied. Filters: CFP (436/20 FT 455 BP480/40), mRFP1 (530–585 FT 600 LP 615), and YFP (500/20 FT 515 BP535/30). Exposure times: CFP 50 ms, mRFP1 100 ms, and YFP 500 ms. Scale bars, 10 µm. CPF, cyan fluorescent protein; mRFP, monomeric red fluorescent protein.

HIV-1 late gene expression. Elucidation of Rev-protein interactions in cells is essential to understanding the molecular mechanisms of HIV replication and has an enormous potential for the development of novel anti-HIV strategies (4). Known cellular Rev interactors include the Rev cofactor CRM1/Exportin1 (5), a nuclear export receptor, and the recently discovered Risp factor, a modulator of Rev activity (6). In addition, Rev interacts with itself. As proof-of-concept for our assay, we inserted sequences encoding HIV Rev (wild-type and various mutants), Risp, and CRM1/Exportin1 into the C-YN and R-YC vectors and performed pairwise cotransfections to test the expression and interaction behaviors of the resulting fusion proteins.

HeLa cells transfected with Rev-C-YN and Rev-R-YC displayed distinct blue, red, and yellow fluorescent signals, confirming expression of individual Rev-containing fusion proteins and Rev-Rev interaction in the same cells (Figure 1F). Rev-Risp interaction was also verified in this assay (Figure 1G). Cells co-expressing Risp and RevM5 fusion proteins did not display a yellow signal (Figure 1H), despite of strong colocalization of mRFP1 and CFP fluorescence in cytoplasm. This confirms previous results from a yeast two-hybrid assay in which Risp failed to interact with the RevM5 mutant (6).

Interaction strengths were determined by quantitative image analysis and by flow cytometry (Figure 2, supplementary materials, and Supplementary Figure S2). Cells expressing proteins without specific interaction domains showed only very low YFP background fluorescence. Self-interaction of Rev was much stronger than interaction of wild-type Rev with a truncated Rev mutant [Rev(54–116)] lacking the multimerization domain of Rev (Figure 2, A and B). Similarly, wild-type Rev interacted much more strongly with CRM1/Exportin1 than a Rev mutant with a defective nuclear export signal [Rev NES$(A)_4$] (7) (Figure 2A). These results confirm that the extended BiFC (exBiFC) assay can be used to quantify interaction strengths in individual cells as well as in cell populations.

BiFC and its recent variants (2) is a well-established methodology for detection and quantification of protein complexes that has been applied to plant (8) and mammalian cells (9–11). In this study we extended BiFC technology by generating tools for co-visualization of not only protein-protein interactions, but also expression and localization of individual interaction partners in a single assay. Previously, this required use of fluorescence resonance energy transfer (FRET), which is a very powerful tool for analysis of transient interactions with rapidly disassociating partners (reviewed in Reference 12). In BiFC, protein interactions are stabilized by formation of the fluorescent complex, resulting in a more robust signal than in FRET, which is particularly useful for detection of weak interactions. Furthermore, the generation of the interaction signal in BiFC appears to be less distance-dependent than in FRET, suggesting that BiFC/exBiFC has the potential to detect proteins associated in large complexes (2). It is noteworthy that, to some extent, FRET between CFP, YFP, and mRFP could also occur when an exBiFC complex has formed (13; reviewed in Reference 14). We observed that the degree of FRET between CFP and YFP and between CFP and mRFP is negligible compared with the generated exBiFC signal (see supplementary materials and

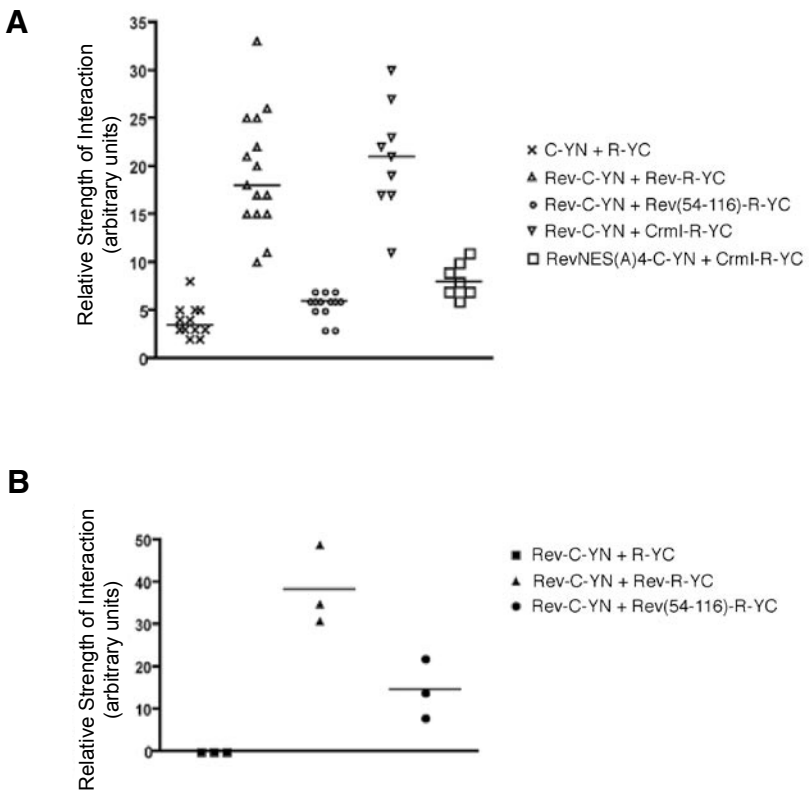

Figure 2. Quantitative analysis of protein-protein interactions in the extended bimolecular fluorescence complementation (exBiFC) assay. Relative strengths of interactions are shown on the y-axis. Symbols represent values of a single analysis, and horizontal lines represent median values. (A) Analysis of single cells by fluorescence microscopy. The intensity of the yellow fluorescent protein (YFP) signal was normalized to the sum of red fluorescent protein (RFP) and cyan fluorescent protein (CFP) signal intensities and multiplied by 100 (arbitrary units). Values obtained for fluorescent proteins lacking specific interaction domains (C-YN + R-YC) represent unspecific YFP background fluorescence. Rev-Rev interaction (Rev-C-YN + Rev-R-YC) is stronger than interaction of Rev with a multimerization-deficient truncated Rev mutant [Rev-C-YN + Rev(54–116)-R-YC]. Similarly, wild-type Rev interacts much more strongly with CRM1/Exportin1 (Rev-C-YN + CRM-1-R-YC) than the nuclear export deficient Rev mutant [Rev NES$(A)_4$-C-YN + CRM-1-R-YC]. (B) Analysis of cell populations by flow cytometry. Cells were harvested 48 h after transfections and analyzed with a FACSAria™ cell sorter (BD Biosciences, Heidelberg, Germany). A healthy, homogenous cell population was determined by a gate and drawn in the forward scatter/side scatter (FSC/SSC) dot blot; subsequently only this population was analyzed. The median intensity of YFP (ex: 488; em: 530/30) fluorescence was determined in CFP (ex: 407; em: 480/30) and monomeric red fluorescent protein 1 (mRFP1) (ex: 543; em: 610/20) double-fluorescent cell populations (arbitrary units) and corrected for background YFP signal of the control pair Rev-C-YN and R-YC. In agreement with panel A, Rev-Rev self-interaction is stronger than interaction of Rev with Rev mutant (54–116).

Supplementary Figure S3). However, occurrence of FRET between YFP and mRFP reduces signal intensity of YFP fluorescence. This effect will not yield false positive results, but can lead to an underestimation of YFP signal strength (see supplementary materials and Supplementary Figure S3). The extent of FRET between YFP and mRFP can be determined by acceptor photobleaching of mRFP.

The classical BiFC assay measures interaction efficiencies by quantifying the intensity of the signal of the fluorescent complex, without taking into account the expression levels of the individual fusion proteins. In this respect, the exBiFC assay represents a major advance, because it allows easy identification of cells expressing both fusion proteins and the normalization of the interaction signal to expression levels of both fusion proteins in these cells. We were able to monitor complex formation in living cells over extended time periods (data not shown), suggesting that the exBiFC methodology will provide unprecedented insights into protein-protein interaction properties. Nevertheless, it is possible that in cases where these features of the exBiFC assay are not wanted or needed, the classical BiFC assay may also be suitable. Indeed BiFC utilizes a shorter tag at the protein of interest, and this may be advantageous for interactions sensitive to steric hindrance.

It is well-known that adding tags, such as fluorescent proteins, to proteins of interest has the potential to affect subcellular localization behavior of proteins. Also the intracellular localization of the YFP signal does not necessarily indicate the location where the interaction took place, but rather reflects an equilibrium state of the exBiFC complex. These aspects have to be taken into consideration before experiments are planned and obtained data are interpreted.

As folding and chromophore maturation of YFP (and other fluorescent proteins) is sensitive to higher temperatures, a short preincubation at temperatures lower than 37°C may be required before BiFC analysis (see supplementary materials). This problem could be circumvented by adapting the YFP-based exBiFC assay to other fluorescent proteins (e.g., Venus or Citrine) (15). Additionally, recent and ongoing advances in the availability of fluorescent proteins (16) with distinct and improved spectral and biochemical properties should allow generation of a variety of new exBiFC constructs for multicolor analysis.

Several future applications can be envisioned for the exBiFC assay. Importantly, it should prove useful in identifying small molecule inhibitors of a particular protein-protein interaction and therefore may be an important tool in developing novel therapies. The exBiFC approach may also be adapted to screening of plasmid cDNA libraries for specific interactors. This would be greatly facilitated by constitutive expression of at least one exBiFC fusion protein as bait. Indeed, we were successful in generating HeLa cell lines stably expressing Rev-R-YC and Risp-R-YC, respectively, indicating that the R-YC plasmid can be used to generate stable cell lines.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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