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Biochemical and Biophysical Research Communications 367 (2008) 47–53
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Split mCherry as a new red bimolecular fluorescence complementation system for visualizing protein–protein interactions in living cells

Jin-Yu Fan [a,b], Zong-Qiang Cui [a], Hong-Ping Wei [a], Zhi-Ping Zhang [a], Ya-Feng Zhou [a], Yun-Peng Wang [a], Xian-En Zhang [a,*]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, 44#, Xiao Hong Shan, Wuhan 430071, China[1]
[b] Key Laboratory of Biomedical Photonics of Ministry of Education—Hubei Bioinformatics and Molecular Imaging Key Laboratory, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430074, China[1]

Received 7 December 2007
Available online 26 December 2007

Abstract

Bimolecular fluorescence complementation (BiFC) is a recently developed technique for detection of protein–protein interactions in living cells. In this study, a new red BiFC system was developed by splitting mCherry, a mutant monomeric red fluorescent protein, into two fragments between amino acids 159–160 and was verified using a pair of interacting proteins, SV40 large T antigen (LTag), and human p53 protein. By combined use of the mCherry-based red BiFC system with a Venus-based yellow BiFC system, the interaction between LTag and p53 as well as the interaction between sp100 and promyelocytic leukemia protein (PML), were detected simultaneously in Vero cells. The brilliant redness, short maturation time, and the long excitation and emission wavelengths (587/610 nm) of mCherry make the new BiFC system an excellent candidate for analyzing protein–protein interactions in living cells and for studying multiple protein–protein interactions when coupled with other BiFC systems.

Keywords: Bimolecular fluorescence complementation; BiFC; mCherry; Protein–protein interactions; Red fluorescent protein

Monitoring protein–protein interactions and macromolecular complex formation in living cells is extremely useful for understanding the dynamics and mechanism of biological processes. Currently, several methods are available for studying protein interactions in living cells, such as the yeast two-hybrid system [1,2], fluorescence resonance energy transfer (FRET) [3–5], and protein fragment complementation [6–11]. The recently developed bimolecular fluorescence complementation (BiFC) [12–14] assay has been proven to be a versatile and simple non-invasive technology for studying protein interactions in living cells [15–17].

The BiFC assay relies on the reconstruction of a fluorescent protein from its two non-fluorescent fragments when they are brought together and complement each other because of the association or interaction between proteins fused to each fragment [12] (Fig. 1). This technique can be used for detection of protein–protein interactions and subcellular localizations as well as multiple protein interactions due to the distinguishable spectra of various split fluorescent proteins [13]. So far, blue, cyan, green, yellow, and red BiFC systems have been developed, allowing multiple or flexible choices for studying protein–protein interactions in living cells [12,13,18,19]. Among these BiFC systems, the red one was developed most recently, using a red fluorescent protein mRFP1-Q66T that has excitation and emission wavelengths of 549 and 570 nm, respectively. To acquire a longer wavelength spectrum BiFC system, we

* Corresponding author. Fax: +86 27 87199492.
E-mail address: x.zhang@wh.iov.cn (X.-E. Zhang).
[1] Affiliated institutions a and b contributed equally to this paper.

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.12.101

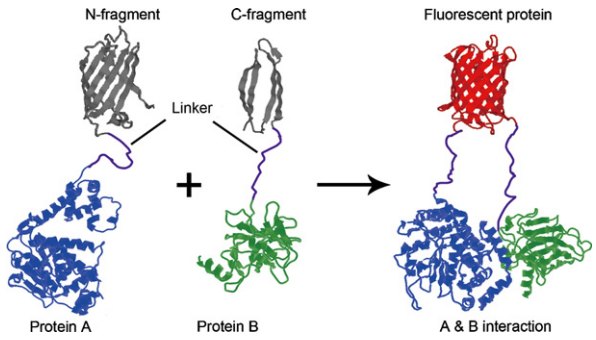

Fig. 1. Principle of BiFC system. One pair of interacting proteins, A and B, is fused to the N- and C-terminal fragments of a fluorescent protein, respectively. Each fusion protein of A–N and B–C does not emit fluorescence under excitation. When A and B interact or associate, the two fragments are brought together to facilitate reconstruction of the fluorescent protein, reproducing emission of fluorescence under excitation.

chose a second generation mRFP1 variant, mCherry, as a candidate fluorescent protein to develop a new BiFC system. Compared with the other fluorescent proteins reported so far, the mCherry protein has the longest excitation and emission wavelengths (587/610 nm, respectively) with comparable brightness and the shortest maturation time [19–21]. Moreover, mCherry is less influenced than mRFP1 by the presence of an N-terminal or C-terminal fusion protein [20]. For these features, the mCherry-based BiFC is expected to be a better red BiFC system.

To develop the mCherry BiFC system, three loop regions, guided by mCherry's crystal structure (PDB Accession No. 2H5Q) [22], were selected to dissect mCherry and generate three pairs of fragments. Because of its tendency for dimerization and interactions [23,24], EGFP protein was fused to the three pairs of fragments individually to determinate which pair of the fragments could produce the BiFC signal. The applicability of the mCherry-based BiFC system was investigated using LTag and human p53 protein, which are known for their strong interaction [25–27]. As a negative control, the mutants of LTag and p53, which disrupt this interaction, were used to verify this system. Furthermore, to assess the ability of simultaneously detecting multiprotein interactions, a yellow BiFC system based on Venus was also introduced and used to detect protein interaction between sp100 and PML in the same cells in which LTag and p53 were detected by the mCherry-based red BiFC system. The results showed that the newly developed mCherry BiFC system is very useful for studying intracellular protein–protein interactions.

Materials and methods

Construction of plasmids. The mCherry proteins were split at three positions between amino acids 136/137, 159/160, and 174/175. The fragment pairs obtained were named MN136 and MC137, MN159 and MC160, MN174 and MC175, respectively, corresponding to the amino acid sequences of 1–136 and 137–237, 1–159 and 160–237, 1–174 and 175–237, respectively.

For construction of plasmids containing fusion protein EGFP-MN and EGFP-MC, the N- and C-terminal coding regions of mCherry were amplified by PCR from plasmid pRSET-B-mCherry. MN136, MN159, and MN174 shared the same forward primer: 5′-AAA *GGT ACC* ATG GTG AGC AAG GGC GAG-3′ (KpnI included). The reverse primers were 5′-TTT *GGA TTC* TTA GGA GGG GAA GTT GGT GC-3′ (for MN1), 5′-TTT *GGA TCC* TTA GTC CTC GGG GTA CATC-3′ (for MN2) and 5′-TTT *GGA TTC* TTA CTC CTT CAG CTT CAG CCT C-3′ (for MN3) (BamHI site was contained in each reverse primer). Each MN fragment was cut by KpnI and BamHI, and then inserted into the same pEGFP-C1 sites.

The forward primers for MC137, MC160, and MC175 were 5′-AAA *GCT AGC* ATG GAC GGC CCC GTA ATG CAG-3′, 5′-AAA *GCT AGC* ATG GGC GCC CTG AAG GGC GAG-3′ and 5′-AAA *GCT AGC* ATG GGC GGC CAC TAC GAC GCT G-3′, respectively (NheI site was included in each forward primer). The same reverse primer 5′-TTT *CTC AGA* ATA CTT GTA CAG CTC GTC C-3′ was used for MC137, MC160, and MC174 (XhoI contained). Each MC fragment was digested by NheI and XhoI, and then inserted into the corresponding sites of pEGFP-N1 (Fig. 2).

The plasmids of pMN and pMC were generated by deletion of EGFP from plasmids of pEGFP-MN and pEGFP-MC.

For construction of pLTag-MN159 plasmids, the full coding sequence of SV40 large T antigen (LTag) was generated by overlap PCR from its two exons, which were amplified from their genome. Primers for the first exon were 5′-CCC *AGA TCT* ATG GAT AAA GTT TTA AAC AGA GAG GAA TCT TTG-3′ (forward, BglII included) and 5′-CAT CAG TTC CAT AGG TTG GAA TCT CAG TTG CAT CCC AGA AGC-3′ (reverse). For the second exon: 5′-GGA GGC TTC TGG GAT GCA ACT GAG ATT CCA ACC TAT GGA ACT G-3′ (forward) and 5′-CCC *GAA TTC* GAG CCA CCC CCT CCT GTT TCA GGT TCA GGG GGA GGT GTG-3′ (reverse, EcoRI included). Then, the LTag cassette was inserted into BglII and SalI sites of pMN159, after being digested by the same enzymes. For construction of pp53-MC160 plasmids, the coding sequence of p53 was amplified from its cDNA by PCR and was then inserted into pMC160 after being digested by HindIII and PstI. Primers for p53 were 5′-CAC AAG CTT ATG GAA GAA CCA CAA TC-3′ (forward, HindIII included) and 5′-AAA CTG CAG GTC TGA GTC AGG CCC TTC-3′ (reverse, PstI included). The LTag mutant (mLTag) was acquired by deleting the amino acids from 585 to 616, which are involved in binding to p53. On the p53 side, mutant M246R was generated and marked as mp53, which thereby disrupted the interaction with LTag. mLTag, and mp53 were inserted into pMN159 and pMC160, respectively, by the same strategy. A linker encoding GGGGSGGGGS was inserted between MN159, MC160 and their fusion protein (Fig. 2).

The yellow fluorescent protein Venus was obtained by a series of site mutagenesis of F46L/F64L/M153T/V163A/S175G from EYFP, as previously described [28]. VN stands for N-terminal amino acids (1–172) of Venus and VC for C-terminal of 173–238. The DNA sequences of VN and

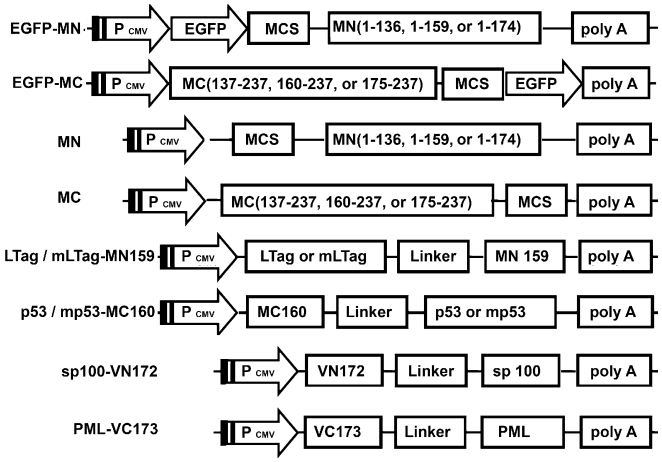

Fig. 2. Schematic view of the fusion protein constructs used in this study.

VC were cloned by PCR and replaced the EGFP of pEGFP-C1 to generate plasmids pVN-C1 and pVC-C1. The fusion proteins sp100-VN and PML-VC were generated by inserting the coding sequence of sp100 and PML to pVN-C1 and pVC-C1, respectively (Fig. 2). Primers for sp100 were 5′-AAA AGA TCT ATG GCA GGT GGG GGC GGC GAC CTG AG-3′ (forward, BglII included) and 5′-AAA GAA TTC AAA TCT TCT TTA CCT GAC CCT C-3′ (reverse, EcoRI included). Primers used for PML were 5′-AAA GTC GAC GAT GGA GCC TGC ACC CGC CCG ATC TC-3′ (forward, SalI included) and 5′-AAA GGA TCC CCT CTC CAC AAC GCG TTC CTC TGC CTC-3′ (reverse, BamHI included).

All the sequences were verified by DNA sequencing.

Cell culture and transfection. Vero cells were maintained in a humidified incubator at 37 °C with 5% CO_2 and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were plated in 35-mm tissue culture dishes at 70–80% confluence one day before transfection. The cells were transfected with plasmids using lipofectamine 2000 reagent (Invitrogen; United States) according to the manufacturer's instructions. Transfected cells were incubated either at 37 °C (5% CO_2) overnight (8–12 h) followed by 1–2 h at 4 °C or at 25 °C overnight (5% CO_2) before

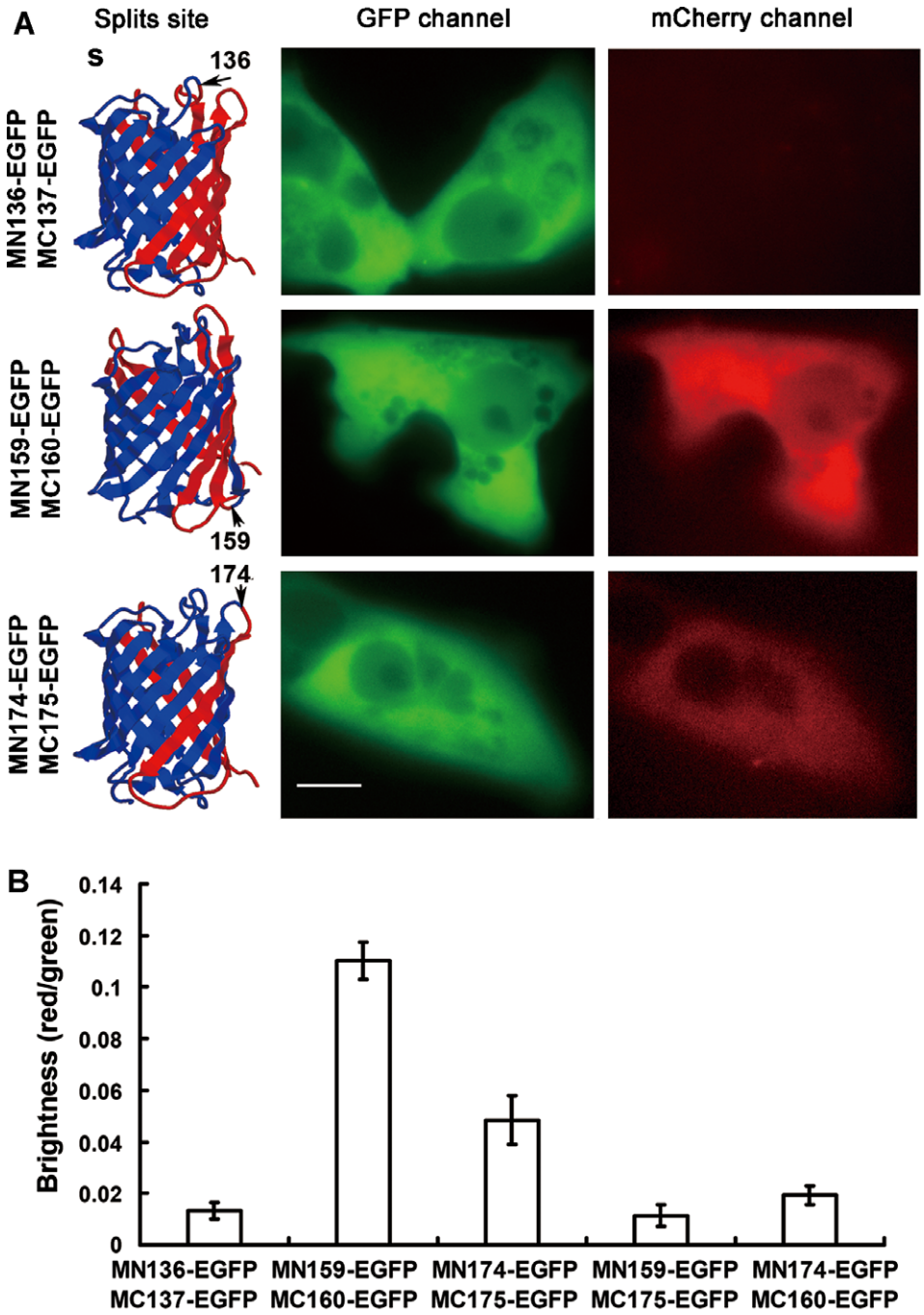

Fig. 3. Construction of the mCherry-based BiFC system. (A) Images of Vero cells after transient co-expression of various combinations of the fusion proteins. Cells cotransfected with the plasmids were incubated overnight at 37 °C, and then transferred to 4 °C for 1–2 h. The images were acquired by epifluorescent microscopy under 100× oil objective lens (scale bar, 10 μm). (B) Quantitative analysis of the mCherry and EGFP signal strength obtained upon transient expression of various construct combinations in Vero cells.

imaging. The cells were imaged by an inverted fluorescence microscopy after replacing the medium with fresh medium. The nucleus was stained with Hochest 33258.

Fluorescence microscopy, image acquisition and data analysis. Cells were imaged with an inverted wide-field fluorescent microscope (Axiovert 200, from Carl Zeiss, Germany). A filter set with 472/30 nm for excitation, 510 nm for dichroic beam splitter, and 520/35 nm for emission was used to view the green fluorescence from EGFP channel. The red fluorescence from mCherry channel was viewed using an excitation filter of 531/40 nm, a dichroic beam splitter of 600 nm, and an emission filter of 593/40 nm, while the yellow fluorescence from Venus channel was viewed using an excitation filter of 500/24 nm, a dichroic beam splitter of 515 nm, and an emission filter of 542/27 nm (All filters are from Semrock, United States). Images from the mCherry, EGFP, and Venus channels were captured using a cooled CCD camera (Model Cascade 512B, Photometrics, United States) with the same exposure times. The camera was operated at 5 MHz with 16-bit digitization and controlled by MetaMorph 6.0 software (Molecular Devices, United States).

For fluorescence quantitative analysis, the red and green fluorescence intensity was acquired from their original images by subtracting their background fluorescence. The background values were the average fluorescence intensities obtained from a region (50×50 pixel^2) without red signal in the mCherry channel and the corresponding region in the green fluorescence channel. The ratio of fluorescent intensity of red to green was calculated by dividing the intensity of red fluorescence by that of green fluorescence.

Results

The sites for the gene interruption were picked out based on the crystal structure of mCherry [22]. Three sites between amino acids 136–137, 159–160, and 174–175 at the top or bottom loops of the barrel were chosen to split the protein and develop the mCherry-based BiFC system. These regions contain no periodic secondary structure, and they are flexible and topologically feasible for the protein to fold (Fig. 3A).

Because EGFP has a propensity for dimerization and enhanced fluorescence, it was used in this investigation as a test model of weak interacting proteins, and served as a fluorescent tag to monitor the expression of mCherry fragments, as well as a standard for quantifying the relative red fluorescence. The coding sequences of these fragments were inserted into two kinds of plasmids pEGFP-C1 and pEGFP-N1. As a negative control, MN/MC fragments without EGFP were also expressed in Vero cells by deleting GFP from each fusion protein of MN-EGFP or MC-EGFP.

As shown in Table 1, when there was no interacting protein pair fused to MN/MC, the transient expression of either MN or MC alone or their co-expression in Vero cells did not result in red fluorescence. When the fusion proteins with EGFP were co-expressed in Vero cells, one combination of MN159-EGFP and MC160-EGFP produced strong red fluorescence, indicating a functional BiFC signal. The combination of MN174-EGFP and MC175-EGFP yielded a weak BiFC signal. Other combinations did not result in observable red fluorescence (Fig. 3A and B).

The relative BiFC efficiencies of MN and MC fragments of mCherry were calculated by dividing the intensity of red fluorescence to that of green fluorescence [19], after subtraction of the background fluorescence, when the fusion proteins of MN-EGFP and MC-EGFP were co-expressed in the cells. For each combination, the average fluorescence intensities from 30 fluorescent cells were used for the relative BiFC efficiency calculations. As shown in Fig. 2B, the BiFC efficiency of the combination of MN159 and MC160 fragments was approximately 5-fold higher than that of the combination of MN174 and MC175 fragments and 10-fold higher than other combinations. These findings demonstrated the high BiFC efficiency of the combination of MN159 and MC160 fragments of mCherry. The pair MN159/MC160 was then selected to construct the mCherry BiFC system.

To assess the validity and reliability of the mCherry-based BiFC for studying protein interactions in living cells, LTag and p53 were selected for their well-known strong interactions. When the fusion proteins LTag-MN159 and p53-MC160 were co-expressed in Vero cells, strong red fluorescence signals were detected, indicating a functional BiFC signal (Fig. 4A). As a negative control, mutant LTag (mLTag) and Mutant p53 (mp53), a pair of proteins without known interactions [27], were also used to assess the new BiFC system. The amino acids (585–616) of LTag, which are known to be involved in binding to p53, were deleted from LTag to construct the mLTag [27]. Then, mLTag was fused to MN159 to generate the fusion protein mLTag-MN159. On the other hand, mp53 and mp53-MC160 were generated by a single amino acid substitution (M246R) in p53 because the M246R mutation has been shown to disable the interaction between p53 and LTag [27]. Co-expression of mLTag-MN159 and mp53-MC160 in Vero cells did not result in red fluorescence (Fig. 4A). The mCherry-based BiFC was then demonstrated.

Table 1
BiFC Signal[a] observed after co-expressing various combinations of the mCherry fragments in Vero cells

C-terminal fragment with or without EGFP fusion	N-terminal fragment with or without EGFP fusion					
	MN136	EGFP-MN136	MN159	EGFP-MN159	MN174	EGFP-MN174
MC137	−	na	−	na	−	na
EGFP-MC137	na	−	na	−	na	−
MC160	−	na	−	na	−	na
EGFP-MC160	na	−	na	++	na	−
MC175	−	na	−	na	−	na
EGFP-MC175	na	−	na	−	na	+

[a] Detected in mCherry channel. ++, strong fluorescence; +, weak fluorescence; −, no fluorescence; na, not applicable.

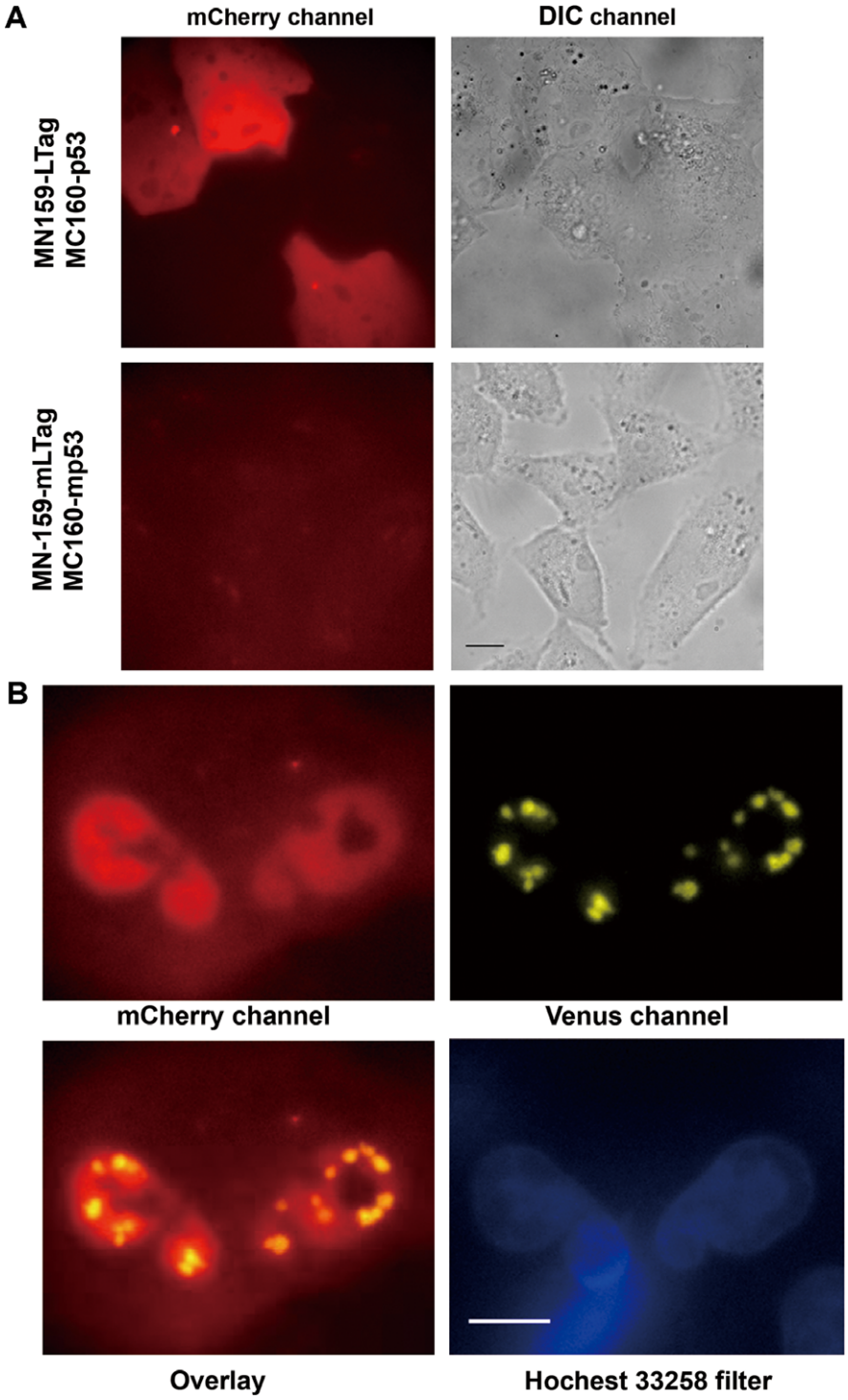

Fig. 4. Visualization of protein–protein interactions using the mCherry-based BiFC system. (A) Visualization of the interaction between LTag and p53, as well as the interaction between mLTag and mp53 (as negative control) in Vero cells, using the mCherry BiFC system based on MN159 and MC160 fragments. Left, DIC images; right, red fluorescent images. (B) Simultaneous visualization of the interaction between LTag and p53 (mCherry Channel), as well as the interaction between sp100 and PML (Venus Channel) in Vero cells after transient co-expression of MN159-LTag, MC160-P53, VN172-sp100, and VC173-PML. The cells were dyed with Hochest 33258 to indicate the position of cell nucleus. Scale bar, 10 μm.

The applicability of the mCherry BIFC system for the detection of multiple protein–protein interactions was tested coupled with a Venus-based yellow BiFC system in the same cells. According to published data [18], Venus was dissected between amino acids 172–173, generating N- and C-terminal fragments (referred as VN172 and VC173 here). These two fragments were then fused to proteins sp100 and PML, respectively. PML and sp100 are

members of PML nuclear bodies or Nuclear Dot 10 (ND 10). They can form a large protein complex with other proteins [29]. As shown in Fig. 4B, both red fluorescence and yellow fluorescence were detected in Vero cells after transient co-expression of LTag-MN159, p53-MC160, sp100-VN172, and PML-VC173. Yellow fluorescence was scattered in various sizes of dots in the nucleus, which is consistent with the location of ND10 in cells; while the red fluorescence was distributed across the whole cell. These images indicate the distribution and localization of the interactions between sp100 and PML as well as LTag and p53 in the cells, which is consistent with a previous report [29]. Therefore, the combined use of the mCherry-based and Venus-based BiFC systems could be used to simultaneously monitor interactions between two pairs of proteins.

Discussion

Features of the BiFC systems have been summarized previously. Considering all the BiFC systems developed to date, the mCherry-based system has the longest wavelength spectrum with enough brightness. These features would introduce lower background signals in fluorescence imaging with little cellular damages. Compared to the existing techniques for studying intracellular protein–protein interactions, such as the widely adopted FRET method, the BiFC systems have some advantages. First, the BiFC system is more sensitive, since the complementation of two fluorescent fragments in BiFC is stable [14]. Second, it may detect weak interactions that would occur when interacting partners are either over a distance of 7 nm apart or have low expression, which is difficult to be detected by FRET. However, this stable complementation is sometimes a disadvantage for BiFC when two interacting proteins are in a state of dynamic equilibrium or only in a transient association, which is good for the FRET technique. Finally, multiple protein interactions in living cells can be simultaneously visualized when different color BiFC systems are utilized. This has been demonstrated here through interactions of LTag and p53, and PML and sp100. In particular, the longest spectra of the mCherry-based system make it more distinguishable than other BiFC systems including the mRFP1-Q66T-based red BiFC system.

The main existing problem of all BiFC systems is their temperature dependence. Low temperatures favor the reconstitution of fluorescence upon proximity of the two fluorescent fragments, but may affect the behaviors of the target proteins. In our test, the mCherry splits produced good BiFC signals when cells were incubated at room temperature (25 °C) overnight (data not shown). However, the long duration of reconstitution (several hours) is unpractical for dynamic studies of protein–protein interactions. In such cases, FRET is recommended.

In summary, we have successfully constructed a red BiFC system by splitting mCherry at its 159–160 position. This system has the longest emission wavelength reported so far and can therefore be easily distinguished from other BiFC systems. A long emission wavelength is advantageous when imaging cells because a longer wavelength produces weaker fluorescence background. With its other advantages of a short maturation time and brilliant fluorescence, the mCherry BiFC system would find particular applications for analyzing protein–protein interactions in living cells and for studying multiple protein–protein interactions when coupled with other BiFC systems.

Acknowledgments

We are grateful to Dr. Roger Y. Tsien for providing the plasmid pRSET-B-mCherry and to Dr. Christian Klein for the cDNA of p53 and to Dr. Roger D. Everett for PML gene and to Dr. Michael D. Hebert for sp100 gene. ZQ Cui and HP Wei are supported by National Basic Research Program of China 2006CB933102 and the National Natural Science Foundation of China (20775085; 30700169). The others are supported by the Nano projects of the Chinese Academy of Sciences (kjcx2-sw-h12). Affiliations a and b tie for the first unit.

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