In vivo quantification of protein–protein interactions in *Saccharomyces cerevisiae* using bimolecular fluorescence complementation assay

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**A B S T R A C T**

Most of the biological processes are carried out and regulated by dynamic networks of protein–protein interactions. In this study, we demonstrate the feasibility of the bimolecular fluorescence complementation (BiFC) assay for in vivo quantitative analysis of protein–protein interactions in *Saccharomyces cerevisiae*. We show that the BiFC assay can be used to quantify not only the amount but also the cell-to-cell variation of protein–protein interactions in *S. cerevisiae*. In addition, we show that protein sumoylation and condition-specific protein–protein interactions can be quantitatively analyzed by using the BiFC assay. Taken together, our results validate that the BiFC assay is a very effective method for quantitative analysis of protein–protein interactions in living yeast cells and has a great potential as a versatile tool for the study of protein function.

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1. Introduction

Protein–protein interactions are a fundamental mechanism for integrating cellular signals and generating the regulatory specificity of cellular processes. By interacting with different partners, proteins can perform different functions in different cells or under different conditions. Thus, identification and visualization of protein–protein interactions provide significant insight into the cellular roles of individual proteins. Because of this importance, analysis of protein–protein interactions is a central issue in protein research. To understand the nature of protein–protein interactions in the cellular context, several cell-based assays have been developed, including the yeast two-hybrid analysis (Fields and Song, 1989), the fluorescence resonance energy transfer (FRET) assay (Periasamy and Day, 1999; Pollok and Heim, 1999) and the protein fragment complementation assay (PCA) (Pelletier et al., 1999; Remy and Michnick, 1999; Ghosh et al., 2000; Wehrman et al., 2002; Paulmurugan and Gambhir, 2003).

More recently, a novel approach based on the PCA using fluorescent proteins, termed the bimolecular fluorescence complementation (BiFC) assay, has been developed (Hu et al., 2002). The BiFC assay is based on the formation of a fluorescent complex when two proteins fused to non-fluorescent fragments of a fluorescent protein interact with each other. The interaction between the fusion proteins facilitates the association between the fragments of the fluorescent protein. This approach enables visualization of the subcellular localizations of specific protein complexes in the normal cellular environment (Kerppola, 2008). Due to the advantages of clear visualization of in vivo protein–protein interactions and direct readout measurable with simple equipment, the BiFC technique has been used to identify and visualize protein–protein interactions in a wide range of species including animals (Hu and Kerppola, 2003; Hynes et al., 2004), plants (Bracha-Drori et al., 2004; Walter et al., 2004), yeasts (Blondel et al., 2005; Cole et al., 2007) and filamentous fungi (Hoff and Kuck, 2005).

Since regulation of various cellular processes involves dynamic protein–protein interactions, it is crucial to define the spatial and temporal changes in the involved protein complexes for comprehensive understanding of the regulation mechanisms. It is obvious that non-invasive cell-based methods are preferable for quantitative and dynamic analysis of protein–protein interactions. Although the yeast two-hybrid analysis and the PCA using dihydrolfate reductase have provided plentiful information about protein–protein interactions, these methods have some limitation in quantitative dynamic analysis of protein–protein interactions. In contrast, the FRET assay, the PCA using luciferase and the BiFC assay are suitable for quantitative dynamic analysis of protein–protein interactions because they allow the visualization of protein–protein interactions directly in living cells and the fluorescence or luminescence emitted from the reporter proteins is dependent on the amount of protein–protein interactions. Actually, phosphorylation of ErbB1 receptors stimulated with epidermal growth factor has been quantitatively visualized by the FRET assay (Verveer et al., 2000) and disassembly of protein kinase A...
regulatory and catalytic subunits induced by the G protein-coupled receptor has been quantified using the Renilla luciferase-based PCA (Stefan et al., 2007). Notably, Morell et al. (2007) reported that binding of proteins and short proline-rich peptides to the SH3 domain of Abelson Tyr kinase can be quantified by the BiFC assay in Escherichia coli. However, their study was performed in a non-natural environment, and there is so far no known report describing a systematic assessment of the feasibility of the BiFC assay for quantitative analysis of protein–protein interactions in a natural cellular context.

In our previous study, we reported that the BiFC assay can be used to reliably analyze in vivo protein–protein interactions in Saccharomyces cerevisiae (Sung and Huh, 2007). Here, we demonstrate that the BiFC assay can be used to quantify not only the amount but also the cell-to-cell variation of protein–protein interactions in S. cerevisiae. Additionally, we show that protein sumoylation and condition-specific protein–protein interactions can be quantitatively analyzed by using the BiFC assay. To our knowledge, this is the first report on in vivo quantitative analysis of protein–protein interactions. We also report an in vivo kinetic analysis of the bimolecular fluorescence complex formation between the fusion proteins. Our results validate that the BiFC assay is a very effective method for quantitative analysis of dynamic protein–protein interactions in living yeast cells. This study will contribute to expanding the applicability of the BiFC assay in protein researches and stimulate quantitative analysis of several issues of protein–protein interactions under various conditions.

2. Materials and methods

2.1. Yeast strains and growth media

The yeast strains used in this study are listed in Supplementary Table S1. All S. cerevisiae strains were derived from BY4741 (MATα hisΔ1 leu2Δ0 met15Δ0 ura3Δ0) and BY4742 (MATα hisΔ1 leu2Δ0 lys2Δ0 ura3Δ0). Yeast cells were grown at 30°C in YPD or appropriate synthetic complete dropout media (Sherman, 2002). For ectopic overexpression of Ulp1 from the inducible GAL1 promoter, cells were grown in synthetic medium with raffinose or galactose as a carbon source. Low fluorescence medium was prepared by dissolving all needed components as previously described (Sheff and Thorn, 2004).

2.2. Plasmid construction

To construct N-terminal tagging vectors that allow modulation of expression of the fused proteins, the promoter regions of HXK2 and RPL7B were amplified by PCR and ligated into BglII/PacI-digested N-terminal tagging vectors for the BiFC assay (Sung and Huh, 2007). Construction of the N-terminal tagging vector with the CET1 promoter has been previously described (Sung and Huh, 2007). To construct N-terminal tagging vectors that induce stochastic expression of the fused proteins, the promoter regions of PCM2 and YHR087W were amplified by PCR and ligated into BglII/PacI-digested N-terminal tagging vectors for the BiFC assay (Sung and Huh, 2007). For ectopic expression of Ulp1, a PCR product of ~1900 bp obtained using BY4741 genomic DNA as a template, a forward primer ULP1 + 1(SpeI) and a reverse primer ULP1 + 1866R[Xhol] was digested with SpeI and Xhol, and ligated into SpeI/Xhol-digested p415GAL plasmid (Mumberg et al., 1994), thus generating p415GAL-Ulp1 vector. The primers used in plasmid construction are listed in Supplementary Table S2.

2.3. Microscopic analysis

Yeast cells grown to mid-logarithmic phase in synthetic complete dropout medium were microscopically analyzed in 96-well glass bottom microplates (Whatman) pre-treated with concanavalin A (Sigma) to ensure cell adhesion. Microscopic analysis was performed on a Zeiss Axiovert 200 M inverted microscope with a Plan-NeoFluar 100×/1.30 NA oil-immersion objective lens. Fluorescence images for the BiFC assay were taken using a standard fluorescein isothiocyanate (FITC) filter set (excitation band pass filter, 450–490 nm; beam splitter, 510 nm; emission band pass filter, 515–565 nm).

2.4. Flow cytometry analysis

Flow cytometry analysis was performed using a FACSCanto cytometer (BD Biosciences). To measure fluorescence intensity, yeast cells grown to mid-logarithmic phase in synthetic complete dropout medium were collected and resuspended in phosphate-buffered saline. Cells were excited with a 488-nm, air-cooled, solid state argon ion laser. FITC fluorescence was detected through a 530-nm band pass filter. To exclude the cellular debris and the aggregated cells, approximately 50% of the cell population was gated.

2.5. Western blot analysis

The cell extracts were prepared using a previously described method (Sung et al., 2008). SDS-PAGE and Western blot analysis were performed by using standard methods with HRP-conjugated anti-GFP antibody (Rockland) and anti-hexokinase antibody (United States Biological).

3. Results and discussion

3.1. Quantification of the change in the amount of protein–protein interactions

To examine the feasibility of the BiFC assay for quantification of protein–protein interactions, we chose to analyze Cet1, an essential RNA 5′-triphosphatase known to form a homodimer (Lima et al., 1999). It has been shown that homodimerization of Cet1 can be reliably visualized by the BiFC assay if the N-terminal fragment (amino acids 1–172; VN) and C-terminal fragment of Venus (amino acids 155–238; VC) are tagged at the N-terminus of Cet1 (Sung and Huh, 2007). To modulate the expression level of VN- or VC-tagged Cet1, we constructed the N-terminal tagging vectors that allow expression of the fusion protein under the CET1, HXK2 or RPL7B promoter. Using the resulting vectors, we tagged the N-terminus of Cet1 with VN in BY4741 cells, thus generating HY0255, HY0819 and HY0821 cells that express VN-tagged Cet1 under the CET1, HXK2 and RPL7B promoters, respectively. Similarly, VC was tagged at the N-terminus of Cet1 in BY4742 cells, generating HY0256, HY0820 and HY0822 cells that express VC-tagged Cet1 under the CET1, HXK2 and RPL7B promoters, respectively. The resulting cells were mated with each other, generating nine diploid strains co-expressing VN- and VC-tagged Cet1 under different combinations of three promoters.

When analyzed by Western blot, HY0838 and HY0839 cells expressing VC-Cet1 under the control of HXK2 and RPL7B promoter, respectively, showed higher expression of VC-Cet1 than HY0837 cells expressing VC-Cet1 under its own CET1 promoter (Fig. 1A). This observation demonstrates that the expression level of Cet1 can be successfully modulated by switching the promoter. When analyzed by fluorescence microscopy, all HY0837, HY0838 and HY0839 cells showed clear BiFC signals in the nucleus (Fig. 1B, left panel). Remarkably, however, the brightness of BiFC signals was different among cells and seemed to be proportional to the expression level of VC-Cet1. Among nine diploid cells, HY0827 cells that express
Both VN-Cet1 and VC-Cet1 under the CET1 promoter showed the lowest BiFC signal intensity (FITC value of 117), and HY0839 cells that express both VN-Cet1 and VC-Cet1 under the RPL7B promoter showed the highest BiFC signal intensity (FITC value of 220) (Fig. 1C), correlating well with the strength of the promoters. Together, these results show that modulation of the expression level of Cet1 can
induce the change in the amount of Cet1–Cet1 interaction, which can be rapidly and precisely quantified using the BiFC assay coupled with flow cytometry.

3.2. Quantification of the stochasticity in protein–protein interactions

It has long been appreciated that expression of some proteins are subject to stochastic variation. The variation of protein expression in isogenic populations is dominated by the stochastic production/ destruction of mRNAs (McAdams and Arkin, 1997; Elowitz et al., 2002). Recently, an elegant study revealed a remarkable structure to biological noise and suggested that the protein noise levels have been selected to reflect the costs and potential benefits of this variation (Newman et al., 2006). Since the amount of protein–protein interactions depends on the expression levels of the involved proteins as shown above, it is plausible that the noise in protein expression may bring about the cell-to-cell variation of protein–protein interactions. To test this possibility, we constructed the N-terminal tagging vectors that allow expression of the fusion protein under the promoters of PGM2 and YHR087W, which show noisy expression with high values of coefficient of variation (CV: (standard deviation/mean) × 100(%)) (Newman et al., 2006). To confirm whether switching the promoter of non-noisy gene to that of PGM2 or YHR087W could induce stochastic expression, we first generated HY0802, HY0805 and HY0806 cells that express green fluorescent protein (GFP)-tagged Cet1 under the CET1, PGM2 and YHR087W promoters, respectively. When analyzed by fluorescence microscopy, all HY0802, HY0805 and HY0806 cells showed GFP signal in the nucleus but the cell-to-cell variation of GFP signal intensity was different from one another. While the signal intensity of HY0802 cells was homogeneous from cell to cell, that of HY0805 and HY0806 cells was variable and stochastic from cell to cell (data not shown). In addition, when analyzed by flow cytometry, the CV values of HY0805 and HY0806 were significantly higher than that of HY0802 cells (data not shown). Stochastic expression of Cet1 was also maintained in diploid HY0812, HY0815 and HY0816 cells, which were generated by mating HY0802, HY0805 and HY0806 cells, respectively, with BY4742 cells (Fig. 2A). While the CV value of HY0812 cells was 41.1, those of HY0815 and HY0816 cells significantly increased to 52.2 and 59.7, respectively. These observations demonstrate that expression of Cet1 can be induced to be stochastic by switching the promoter of CET1 to that of noisy PGM2 or YHR087W.
For quantitative analysis of the stochasticity in protein–protein interactions using the BiFC assay, we first tagged the 5′ end of CET1 gene with VC under the control of the CET1, PG12 or YHR0027/YHR007W promoter. The resulting HY0256, HY0824 and HY0826 cells were mated with HY0821 cells expressing N-terminally VN-tagged Cet1 under the RPL7B promoter, generating HY0837, HY0840 and HY0841 cells, respectively. When analyzed by fluorescence microscopy, all cells showed the BiFC signal in the nucleus. However, there was a clear difference in the cell-to-cell variation of the BiFC signal intensity among cells. While the BiFC signal intensity of HY0837 cells was rather homogeneous, that of HY0840 and HY0841 cells was quite variable from cell to cell (Fig. 2B, left panel). This cell-to-cell variation of the BiFC signal intensity could be quantified by flow cytometry. The CV values of HY0837, HY0840 and HY0841 cells measured by flow cytometry were 28.9, 46.1 and 42.1, respectively (Fig. 2B, right panel). Interestingly, although the expression levels of VC-Cet1 from noisy promoters (FITC values of 71 and 2003) were similar to each other (data not shown), the averaged BiFC signal intensity of HY0840 and HY0841 cells expressing VC-Cet1 from noisy promoters (FITC values of 71 and 72, respectively) was much lower than that of HY0837 cells (FITC value of 145). This observation indicates that the extent of protein–protein interaction represented by the BiFC signal intensity depends not only on the level but also on the stochasticity of expression of interacting proteins. Taken together, our results demonstrate that stochastic expression of interacting proteins induced by noisy promoters leads to the cell-to-cell variation of protein–protein interactions in isogenic populations and this variation of protein–protein interactions can be quantified by flow cytometry functioning with single-cell resolution.

3.3. Visualization and quantification of protein sumoylation

Small ubiquitin-related modifier (SUMO) proteins are covalently attached to specific lysine residues of their target proteins and modulate the activity of the target proteins involved in a wide variety of cellular pathways, including protein transport, transcriptional regulation, DNA repair and cell cycle progression (Seeler and Dejean, 2003; Zhao, 2007). Like other post-translational modifications, sumoylation process is reversible. Many proteins undergo cycles of sumoylation and SUMO deconjugation; sometimes these occur only at certain times (for example, at a certain stage of the cell cycle) and places (such as the nucleus) (Meulmeester and Melchior, 2008). Thus, quantitative analysis of the extent of sumoylation as well as subcellular localization analysis of sumoylation would be very useful in understanding the function of sumoylation.

A previous study has shown that ubiquitinated or sumoylated proteins can be detected and visualized in animal cells by a modified approach of the BiFC assay (Fang and Kerppola, 2004). Prior to quantitative analysis of sumoylation, we first tested whether the BiFC assay could be used to detect and visualize sumoylated proteins in S. cerevisiae. To test this, we chose a Cu–Zn superoxide dismutase, Sod1, which is a well-characterized sumoylated protein involved in oxygen radical detoxification (Hannich et al., 2005; Wykoff and O’Shea, 2005). Using the tagging plasmids for the BiFC analysis, we tagged the 3′ end of SOD1 gene with VN in BY4741 cells and the 5′ end of SMT3 gene encoding SUMO with VC in BY4742 cells, generating HY0852 and HY0839 cells, respectively. These cells were mated and the resulting HY0853 cells co-expressing Sod1-VN and VC-Smt3 were analyzed by fluorescence microscopy. As expected, HY0853 cells showed a clear BiFC signal in the nucleus and cytoplasm, demonstrating that sumoylated proteins can be visualized by the BiFC assay in S. cerevisiae (data not shown; see Fig. 3A). Interestingly, although Sod1 is known to be located primarily in the cytoplasm (Crapo et al., 1992; Huh et al., 2003), the BiFC signal for Sod1 sumoylation was detected mainly in the nucleus. This observation suggests that a sumoylated form of Sod1 is localized to the nucleus and sumoylation may be needed for its targeting to or retention in the nucleus.

For quantitative analysis of sumoylation, we tried to modulate the level of Sod1 sumoylation. To do this, we analyzed Sod1 sumoylation under overexpression of Ulp1, a SUMO-specific protease, which deconjugates SUMO from the substrate proteins (Li and Hochstrasser, 1999). Since overexpression of Ulp1 is toxic and lethal to yeast cells, we controlled expression of Ulp1 under the inducible GAL1 promoter. HY0853 cells were transformed with p415GAL-ULP1 plasmid, and the resulting HY0854 cells were analyzed by fluorescence microscopy. Remarkably, the BiFC signal for Sod1 sumoylation disappeared from the nucleus upon galactose induction of Ulp1 (Fig. 3A), indicating that Sod1 sumoylation in the nucleus is reduced by overexpression of Ulp1. We measured the change in Sod1 sumoylation by counting the number of cells showing the BiFC signal in the nucleus. After 2 h of galactose induction, the BiFC signal for Sod1 sumoylation disappeared from the nucleus in more than 80% of cells (Fig. 3B). To examine the possibility that the disappearance of the BiFC signal from the nucleus might be due to the change in level or subcellular localization of Sod1 and Smt3 proteins, we first checked the expression level of Sod1 and Smt3 proteins under galactose induction condition. The expression level of Sod1 and Smt3 was not altered under this condition (Fig. 3A). In addition, GFP-tagged Sod1 and Smt3 did not show any difference in their subcellular localization pattern under galactose induction condition (Fig. 5B). Taken together, these results indicate that disappearance of the BiFC signal for Sod1 sumoylation from the nucleus is due to deconjugation of Smt3 from Sod1 by a SUMO-specific protease Ulp1. Furthermore, this example of Sod1 sumoylation shows that the BiFC assay can be used for quantitative analysis of the extent of sumoylation as well as subcellular localization analysis of sumoylation.

3.4. Quantification of condition-specific protein–protein interactions

To test whether the BiFC assay could be used to quantify protein–protein interactions induced under specific conditions, we analyzed the interaction between Pho2 and Pho4, both of which are transcription factors involved in phosphate metabolism. When cells are grown in medium containing a high concentration of phosphate, Pho4 is fully phosphorylated and localized to the cytoplasm. In response to phosphate limitation, Pho4 is dephosphorylated and transported into the nucleus, where it binds cooperatively with Pho2 and activates transcription of phosphate-responsive genes (Lenburg and O’Shea, 1996). We have previously shown the occurrence of Pho2–Pho4 interaction in the nucleus under phosphate limitation condition using the BiFC technique (Sung and Huh, 2007). A recent study reported that differential phosphorylation of Pho4 by Pho80–Pho85 produces phosphorylated forms of Pho4 that differ in their ability to activate transcription, contributing to multiple outputs (Springer et al., 2003). We hypothesized that differential phosphorylation of Pho4 under different concentrations of phosphate might lead to differential interaction between Pho2 and Pho4 proteins. To test this hypothesis, we measured the extent of interaction between Pho2 and Pho4 in media containing different concentrations of phosphate (0, 7, 70, 700, and 7000 M) using the BiFC method. After incubation for 4 h in medium containing 0 or 7 M phosphate, HY0200 cells co-expressing C-terminally VN-tagged Pho2 and VC-tagged Pho4 showed the BiFC signal in the nucleus (Fig. 4A). However, the BiFC signal for Pho2–Pho4 interaction was not detected in HY0200 cells incubated in medium containing 70, 700 or 7000 M phosphate. This result is consistent with the previous finding that Pho4 is dephosphorylated, transported into the nucleus and interacts with Pho2 only under phosphate limitation condition (Lenburg and O’Shea, 1996).

To closely investigate the effect of phosphate concentration on the bimolecular fluorescence complex formation between Pho2-VN and
Pho4-VC, we examined the kinetics of appearance of the BiFC signal following incubation of HY0200 cells in media containing different concentrations of phosphate. At 0 μM phosphate, cells showing the BiFC signal began to appear after 30 min and approximately 80% of cells showed the BiFC signal after 4 h (Fig. 4B). At 7 and 70 μM phosphate, cells began to show the BiFC signal after 2 and 4 h, respectively. However, cells did not show the BiFC signal even after 6 h at extracellular phosphate concentrations greater than 700 μM phosphate. There was no significant change in the levels of Pho2 and Pho4 over this time range at different concentrations of phosphate (data not shown), indicating that the increase in the BiFC signal for Pho2–Pho4 interaction under phosphate limitation condition do not result from an increase in the levels of Pho2 and Pho4 in cells. To examine the relationship between transport of Pho4 into the nucleus and appearance of the BiFC signal for Pho2–Pho4 interaction, we also monitored the kinetics of transport of Pho4 into the nucleus in media containing different concentrations of phosphate. As expected, the rate of transport of Pho4 into the nucleus was inversely proportional to the extracellular phosphate concentrations (Fig. 4C). Notably, at 0 μM phosphate, more than 80% of cells showed nuclear accumulation of Pho4 within 1 h. This observation indicates that there is a significant delay between transport of Pho4 into the nucleus and appearance of the BiFC signal for Pho2–Pho4 interaction. This delay in appearance of the BiFC signal seems to be due to slow fluorophore maturation of the bimolecular fluorescence complex rather than slow association of Pho2-VC and Pho4-VC, as observed in the previous study (Hu et al., 2002).

Previously, we have shown that the bimolecular fluorescence complex formation between Pho2-VC and Pho4-VC is reversible; the BiFC signal for Pho2–Pho4 interaction disappears quickly. Upon transfer to medium containing a high concentration of phosphate, almost all cells lost the BiFC signal for Pho2–Pho4 interaction within 1 h (data not shown). Furthermore, there was no delay between export of Pho4 out of the nucleus and disappearance of the BiFC signal for Pho2–Pho4 interaction (data not shown). This observation indicates that the
bimolecular fluorescence complex between Pho2-VN and Pho4-VC is readily dissociated by phosphate replenishment, at a comparable rate to that of Pho4 export. Taken together, our results show that the interaction between Pho2 and Pho4 is tightly controlled depending on extracellular phosphate concentrations and the BiFC assay enables a reliable quantitative analysis of protein–protein interactions induced under specific conditions.

4. Conclusions

Protein–protein interactions are a fundamental mechanism for integrating cellular signals and generating the regulatory specificity of cellular processes. By interacting with different partners, proteins can perform different functions in different cells or under different conditions. Moreover, protein levels are dynamically changed as a result of cellular signaling events or in response to environmental perturbations. Changes in protein concentrations will affect inevitably the occurrence and extent of protein–protein interactions. Hence, quantitative analysis of dynamic protein–protein interactions is crucial to understanding the functional roles of the proteins. In this study, we have demonstrated the feasibility of the BiFC assay for quantitative analysis of protein–protein interactions. Furthermore, we have shown that, when combined with flow cytometry, the BiFC assay can be used to monitor rapidly and precisely protein–protein interactions at single-cell resolution. This feature broadens the potential of the BiFC assay as a tool for in vivo analysis of protein–protein interactions. Probably the most exciting application of the BiFC assay system would be a genome-wide high-throughput screening of protein–protein interactions. The powerful gene manipulation techniques available for yeast enable construction of a collection of strains in which each protein is expressed as a C-terminal epitope fusion from its endogenous promoter and natural chromosomal position. Thus, construction of a genome-wide collection of VN- or VC-tagged yeast strains for the BiFC assay would be possible and greatly contribute to understanding the global structural organization of the networks of dynamic protein–protein interactions in eukaryotic system.

Supplementary materials related to this article can be found online at doi:10.1016/j.mimet.2010.08.021.
References


