Video Article

Protein Membrane Overlay Assay: A Protocol to Test Interaction Between Soluble and Insoluble Proteins *in vitro*

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Abstract

Validating interactions between different proteins is vital for investigation of their biological functions on the molecular level. There are several methods, both *in vitro* and *in vivo*, to evaluate protein binding, and at least two methods that complement the shortcomings of each other should be conducted to obtain reliable insights.

For an *in vivo* assay, the bimolecular fluorescence complementation (BiFC) assay represents the most popular and least invasive approach that enables to detect protein-protein interaction within living cells, as well as identify the intracellular localization of the interacting proteins [1,2]. In this assay, non-fluorescent N- and C-terminal halves of GFP or its variants are fused to tested proteins, and when the two fusion proteins are brought together due to the tested proteins' interactions, the fluorescent signal is reconstituted[3-6]. Because its signal is readily detectable by epifluorescence or confocal microscopy, BiFC has emerged as a powerful tool of choice among cell biologists for studying about protein-protein interactions in living cells [3]. This assay, however, can sometimes produce false positive results. For example, the fluorescent signal can be reconstituted by two GFP fragments arranged as far as 7 nm from each other due to close packing in a small subcellular compartment, rather that due to specific interactions[7].

Due to these limitations, the results obtained from live cell imaging technologies should be confirmed by an independent approach based on a different principle for detecting protein interactions. Co-immunoprecipitation (Co-IP) or glutathione transferase (GST) pull-down assays represent such alternative methods that are commonly used to analyze protein-protein interactions *in vitro*. However, iIn these assays, however, the tested proteins must be readily soluble in the buffer that supportsused for the binding reaction. Therefore, specific interactions involving an insoluble protein cannot be assessed by these techniques.

Here, we illustrate the protocol for the protein membrane overlay binding assay, which circumvents this difficulty. In this technique, interaction between soluble and insoluble proteins can be reliably tested because one of the proteins is immobilized on a membrane matrix. This method, in combination with *in vivo* experiments, such as BiFC, provides a reliable approach to investigate and characterize interactions faithfully between soluble and insoluble proteins. In this article, binding between Tobacco mosaic virus (TMV) movement protein (MP), which exerts multiple functions during viral cell-to-cell transport[8-14], and a recently identified plant cellular interactor, tobacco ankyrin repeat-containing protein (ANK) [15], is demonstrated using this technique.

Video Link

The video component of this article can be found at https://www.jove.com/video/2961/

Protocol

1. Expression and extraction of the proteins

1. Differentially tag the proteins to be tested for their detection. Label the protein to be immobilized on the membrane (ProIM) with a tag of a larger size (e.g., GST), and the unfused tag can be used as an immobilized negative control (ProIMnc). Label the protein to be used as a soluble probe (ProSOL) with either a large or a small tag. Fuse the same tag to an appropriate negative control soluble protein (ProSOLnc) and include in the interaction assay to confirm the specificity of the detected binding.
2. Choose the protein expression system to express tagged proteins, i.e., *E. coli*, baculovirus, etc., depending on the requirement for potential post translational modifications and yields of the proteins.
3. Extract ProIM and $ProIM_{nc}$ from the organism of choice (step 1.2) using SDS-PAGE loading buffer (20% glycerol, 4% SDS, 20 mM Tris-HCl, pH6.8) containing proteinase inhibitor cocktail. Leave the cell suspension at room temperature for 15 min, add 0.5% of 2-mercaptoethanol and boil for 5 min.
4. Extract ProSOL and $ProSOL_{nc}$ from the organism of choice (step 1.2) using a standard protocols [16]. These proteins should be readily soluble in the binding buffer (140 mM NaCl, 10 mM, Tris-HCl pH 7.4, 2 mM EDTA, 2 mM DTT, 1 % BSA, 0.1% Tween 20) used in the step 4.1.

5. Determine the concentration of the recombinant proteins using a standard method, such as Bio-Rad protein assay kit. such as Bio-Rad protein assay kit. If crude extract, rather than purified protein, is used for the assay, estimate the concentration of the protein of interest in this extract by scanning densitometry of the corresponding band on an SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R-250 and using known concentrations of BSA as reference.

2. Immobilization of ProIM and ProIMnc on the membrane

1. Resolve two sets of extracts, each containing 1 µg of ProIM and $ProIM_{nc}$ on an SDS-polyacrylamide gel according to the standard protocol [16].
2. After electrophoresis, place the transfer the gel to in 100 ml of transfer buffer (60 mM Glycine, 10 mM Tris, 0.0006% SDS, 20% MeOH) and incubate with gentle agitation for 20 min at room temperature.
3. Electrotransfer the proteins contained in the gel to a nitrocellulose membrane according to the standard protocol [16].

3. Re-folding of membrane-bound proteins

1. After electrotransfer, incubate the membrane for 15 min in 15 ml of buffer A (30 mM Tris-HCl pH7.4, 0.05% Tween 20) with gentle agitation to remove residual SDS.
2. After draining carefully, transfer the membrane to 25 ml of denaturation buffer (7 M guanidine hydrochloride, 2 mM EDTA, 50 mM DTT, 50 mM Tris-HCl pH 8.3). and incubate for 2 hours at room temperature with gentle agitation. (Note: The nitrocellulose membrane becomes opaque when incubated in the denaturation buffer). and incubate for 2 hours at room temperature with gentle agitation.
3. Transfer the membrane to 25 ml of TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) and incubate with gentle agitation for 5 min (Note: During this step, the membrane regains its original white color).
4. Transfer the membrane to 25 ml of binding buffer (see step 1.2) and incubate at 4°C with gentle agitation for overnight.

4. Probing the ProIM by ProSOL

1. Cut the membrane into two strips, both containing ProIM and $ProIM_{nc}$.
2. Make the ProSOL and ProSOLnc hybridization solutions by diluting the preparation with 1-10 µg of either ProSOL or ProSOLnc in 10 ml of fresh binding buffer. Transfer each membrane into the ProSOL or ProSOLnc hybridization solution and incubate for 1.5 h at room temperature with gentle agitation.
3. Remove the membranes from the hybridization solutions and rinse three times for 15 min each in TBS.

5. Visualizing protein-protein interaction by immunoblotting

1. Block the membrane with 2.5% skim milk in TBST (10 mM Tris-HCl, 140 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h at room temperature. Dilute the primary antibody (anti-strepII polyclonal rabbit antibody) in 0.5% skim milk in TBST at the concentration recommended by the manufacturer.
2. Place the blocked membranes in the antibody solution, and incubated for 1 h at room temperature or for overnight at 4°C with gentle agitation.
3. Rinse the membranes in 20 ml TBST for 15 min, and twice for 5 min at room temperature with gentle agitation.
4. Dilute the secondary antibody (anti-rabbit IgG antibody) conjugated with horse radish peroxidase (HRP) in 0.5% skim milk in TBST at the concentration recommended by the manufacturer. Place the membranes in the secondary antibody solution and incubate for 1 h at room temperature with gentle agitation.
5. Rinse the membranes in 20 ml TBST for 15 min, and twice for 5 min at room temperature with gentle agitation. After the final rinse in TBS, visualize the protein-protein interaction using a HRP chemiluminescence substrate (for example, Millipore Immobilon western chemiluminescent HRP substrate).
6. Probe the same membranes with the primary antibody for the tag fused to ProIM, followed by the appropriate secondary antibody as described in steps 5.1 to 5.4. Visualize ProIM and ProIMnc as described in step 5.5 to validate the identity of the bands obtained in the protein membrane overlay assay (step 5.5). In most cases, stripping is not necessary, because the signal obtained by this step is much stronger than residual signal obtained from step 5.5.

6. Representative Results:

The ANK-MP interaction was observed by BiFC in tobacco epidermal cells (Figure 1A). Because MP is a highly insoluble protein when expressed in bacteria or in plants, the protein membrane overlay assay was adopted to validate this interaction *in vitro* (Figure 1B). Protein extracts containing 1 µg of GST-MP (ProIM) or unfused GST ($ProIM_{nc}$) were resolved by SDS-polyacrylamide gel electrophoresis, followed by electrotransfer to a nitrocellulose membrane. When these ProIMs were probed with soluble ANK-strepII (ProSOL), GST-MP, but not unfused GST, exhibited binding (Figure 1B, lanes 1, 2, compare to lanes 5, 6). Moreover, when the same set of ProIMs were probed with an unrelated ProSOLnc, i.e., *Arabidopsis cytoplasmic* NADH kinase tagged strepII (NADH3-strepII), no binding was not observed, further demonstrating the specificity of the ANK-MP interaction (Figure 1B, lanes 3, 4).

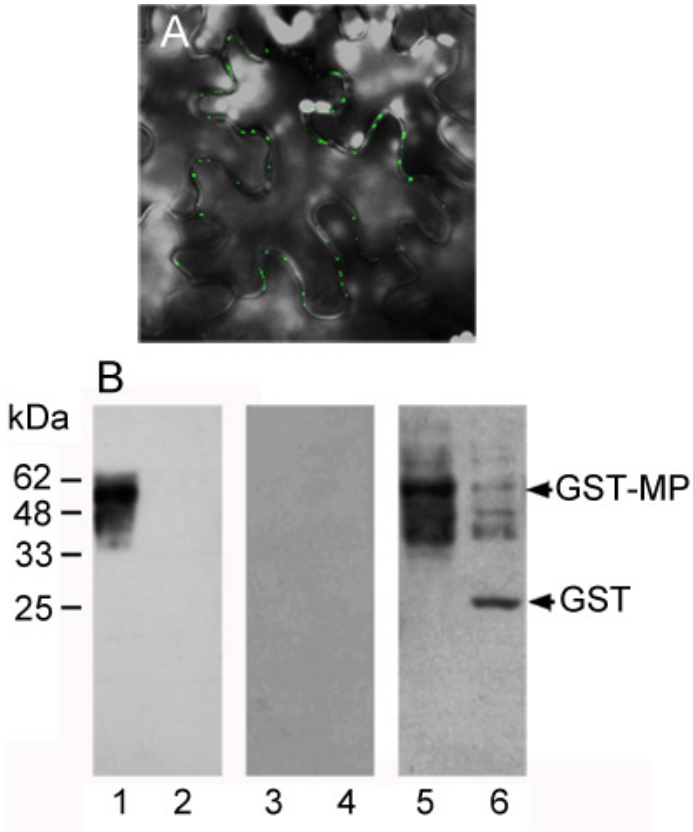

Figure 1. Specific binding of tobacco ANK to TMV MP *in vivo* and *in vitro*. (A) ANK-MP interaction in living tobacco epidermal cells as detected by BiFC. Strong YFP signal was reconstructed when MP and ANK, fused to C-terminal and N-terminal halves of YFP, respectively, were coexpressed in tobacco epidermis following microbombardment of their encoding genes. This BiFC signal accumulated in puncta at the cell periphery, which are diagnostic of plasmodesmata [15]. (B) ANK-MP interaction *in vitro* as detected by protein membrane overlay assay. Protein extracts containing 1 µg of GST-MP (ProIM) or unfused GST (ProIMnc) were resolved on a 15% SDS-polyacrylamide gel, followed by electrotransfer onto a nitrocellulose membrane. The GST-MPProIM and GSTProIMnc were incubated with 0.5 µg/ml of ANK-strepII (ProSOL), and ANK binding was detected by probing the membrane with anti-strepII rabbit polyclonal antibody, followed by anti-rabbit IgG+M secondary antibody conjugated to HRP (lanes 1 and 2). Neither GST-MPProIM nor GSTProIMnc interacted with an unrelated protein, *Arabidopsis* cytoplasmic NADH kinase, fused to the strepII tag (ProSOLnc, lanes 3 and 4). The identity of the band observed in this assay was confirmed by probing the membrane with anti-GST antibody (lanes 5 and 6). When the membrane was treated with denaturation buffer without being washed with buffer A, the binding of the GST-MP to ANK is lost, while unidentified proteins contained in the GST-MP and GST contining protein extracts reacted with ANK-strepII, demonstrating the importance of the step 3.1 before the denaturation process (lanes 7 and 8).

Discussion

This approach is suitable for testing protein-protein interactions between combinations of the proteins,when at least one of which the proteins is readily soluble in the binding buffer, and was successfully applied to other combination of proteins [17,18]. The iInteractions between the proteins that are both insoluble under these conditions cannot be tested by this protocol.

Also, successful refolding of ProIM is critical for the assay. Rinsing the membrane in TBS after the electrotransfer is the key step, because the residual SDS can impair the denaturation/renaturation process.

Finally, to avoid non-specific binding, the concentration of ProSOL in the binding buffer should not exceed 1 µg/ml. ProSOL which is too concentrated may exhibit non-specific binding to ProIM. Also, to block the non-specific binding of membrane immobilized proteins to ProSOL, BSA in the hybridization buffer used during step 4.2 can be substituted to skim milk.

Disclosures

No conflicts of interest declared.

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References

1. Hu, C.D., Chinenov, Y., & Kerppola, T.K. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol Cell*. **9**, 789-798 (2002).
2. Citovsky, V., *et al*. Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. *J Mol Biol*. **362**, 1120-1131 (2006).
3. Kerppola, T.K. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nat Protoc*. **1**, 1278-1286 (2006).
4. Shyu, Y.J., *et al*. Visualization of protein interactions in living Caenorhabditis elegans using bimolecular fluorescence complementation analysis. *Nat Protoc*. **3**, 588-596 (2008).
5. Citovsky, V., Gafni, Y. & Tzfira, T. Localizing protein-protein interactions by bimolecular fluorescence complementation in planta. *Methods*. **45**, 196-206 (2008).
6. Ohad, N., Shichrur, K. & Yalovsky, S. The analysis of protein-protein interactions in plants by bimolecular fluorescence complementation. *Plant Physiol*. **145**, 1090-1099 (2007).
7. Zamyatnin, A.A., Jr., *et al*. Assessment of the integral membrane protein topology in living cells. *Plant J*. **46**, 145-154 (2006).
8. Citovsky, V., Knorr, D., Schuster, G., & Zambryski, P.C. The P30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. *Cell*. **60**, 637-647 (1990).
9. Heinlein, M., Epel, B.L., Padgett, H.S., & Beachy, R.N. Interaction of tobamovirus movement proteins with the plant cytoskeleton. *Science*. **270**, 1983-1985 (1995).
10. Tomenius, K., Clapham, D., & Meshi, T. Localization by immunogold cytochemistry of the virus coded 30 K protein in plasmodesmata of leaves infected with tobacco mosaic virus. *Virology*. **160**, 363-371 (1987).
11. Ding, B., *et al*. Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants. *Plant Cell*. **4**, 915-928 (1992).
12. Meshi, T., *et al*. Function of the 30 kd protein of tobacco mosaic virus: involvement in cell-to-cell movement and dispensability for replication. *EMBO J*. **6**, 2557-2563 (1987).
13. Wolf, S., Deom, C.M., Beachy, R.N., & Lucas, W.J. Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. *Science*. **246**, 377-379 (1989).
14. Waigmann, E., Lucas, W.J., Citovsky, V., & Zambryski, P.C. Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. *Proc. Natl. Acad. Sci. USA* **91**, 1433-1437 (1994).
15. Ueki, S., Spektor, R., Natale, D.M., & Citovsky, V. ANK, a host cytoplasmic receptor for the Tobacco mosaic virus cell-to-cell movement protein, facilitates intercellular transport through plasmodesmata. *PLoS Pathog* **6**, e1001201 (2010).
16. Coligan, J.E., Dunn, B.M., Ploegh, H.L., Speicher, D.W. & Wingfield, P.T. (ed G. Tayler) (John Wiley & Sons, Inc., 2011).
17. Tzfira, T., Vaidya, M., & Citovsky, V. VIP1, an Arabidopsis protein that interacts with Agrobacterium VirE2, is involved in VirE2 nuclear import and Agrobacterium infectivity. *EMBO J*. **20**, 3596-3607 (2001).
18. Chen, M.H., Sheng, J., Hind, G., Handa, A. & Citovsky, V. Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. *EMBO J*. **19**, 913-920 (2000).

