Multiple biological processes are regulated by complicated interaction networks formed by protein-protein or protein-RNA interactions. Nuclear bodies (NBs) are a class of membrane-less subnuclear structures, acting as reaction sites, storage and modification sites, or transcription regulating sites involved in signaling transduction. Biochemical and fluorescence-based methods are widely used to study protein-protein interactions, but false-positive results are a major issue, especially for some fluorescence-based methods. Moreover, these methods fail to be applied to study the formation of NBs, which were characterized by a popular bacterial Lac operator and/or repressor (LacO/LacI) system in mammalian cells. Methods investigating assembly of plant NBs are not available. We have recently developed a nucleolar marker protein nucleolin2 (Nuc2)-based method named Nucleolus-tethering System (NoTS) and showed its application in interaction assay among nucleoplasmic proteins and initiation of plant specific NBs, photobodies. In this extraview, we will compare NoTS with the traditional methods and discuss the assembly mechanisms of NBs, in addition to advantages, limitations, and perspectives about the application of NoTS.

NoTS Assay for Protein-Protein Interactions

Many cellular events take place step by step by dynamically assembled interaction networks, thus detecting protein-protein interactions is of importance to understand different biological processes in vivo.1,2 Traditional methods, such as yeast two-hybrid, in vitro pull-down, and co-immunoprecipitation (Co-IP), are widely used to study protein-protein interactions.3,4 However, yeast two-hybrid may miss some real interactions because posttranslational modifications of some cellular factors might be absent in yeast.5 The represented results revealed by in vitro pull-down may be not the real ones compared with the in vivo situations.2,6 Co-IP only demonstrates the two interacting proteins are in the same complex in vivo.7 Moreover, these methods fail to display the subcellular localizations of the interacting proteins.

Development of fluorescent proteins and microscopy-based methods such as Fluorescence Resonance Energy Transfer (FRET) and Bimolecular Fluorescence Complementation (BiFC) allow protein complexes to be visualized directly in living cells in their normal environments.2,8-10 The two methods monitor protein-protein interactions in vivo without antibody staining, but there exist some limitations for them. For FRET, spectral cross-talk, along with the auto-fluorescence of samples may produce “noise” signals that increase the acceptor emission falsely. Some cellular conditions unrelated to protein interactions may also produce interference on the fluorescence intensity or lifetime. For example, the high expression of proteins may cause non-specific energy transfer.
by increased random collision because of over accumulation of these proteins.\textsuperscript{7,8} Attaching large chromophore proteins at either N-terminal or C-terminal or internal site may also preclude protein interactions.\textsuperscript{3} As for BiFC, fluorescent fragments intrinsically associate with each other under some conditions, not related to the real protein-protein interactions, which may limit its applications. Additionally, the high concentration of the fused proteins in a small subcellular volume may increase false positive rate in BiFC experiments.\textsuperscript{2,9,10} Therefore, a strict negative control such as a mutated protein in its protein-interacting domain is normally necessary; however, this kind of negative control is often difficult to obtain. Conformation of the fusion protein may also affect the distance between two fluorescent fragments, disturbing the result of BiFC.

Several methods were developed to visualize protein-protein associations in vivo based on the relocalizations of interacting proteins. The different cytolocalization assay (DCLA) allows observing the cytoplasmic protein-protein interactions in vivo by visualizing relocalizations of preys to the cytoplasmic cell membranes because of the interactions between the bait and preys. The bait is immobilized to the cell membrane by fusing with a specific localization signal, membrane tether localization signal (MTLS).\textsuperscript{11} An orthoreovirus protein μNS-derived method is used to monitor protein-protein associations inside cells based on co-localization of preys and the bait in the large cytoplasmic aggregates formed by the bait-μNS fusion.\textsuperscript{12} Additionally, a fluorescent two-hybrid assay (E2H) successfully reveals the protein-protein interactions in living cells in real-time according to the relocalizations of preys by the fluorescent protein (FP) and the \textit{lac} repressor (LacI) fused bait, based on the specific binding of LacI to the \textit{lac} operator (LacO) repeats at a special genomic position.\textsuperscript{13} However, the former two methods are normally suitable for detecting the association between cytoplasmic proteins, and have not been applied in plants. As for the LacO/LacI system, the generation of 256 repeats of LacO sequence in the \textit{Agrobacterium}-competent binary vector for plant transformation is not easy as the backbone of a binary vector is normally big. Besides, it is difficult to detect if the introduced genes are integrated in plant genome or not in transient assay and it will take a long time to generate transgenic plants containing several genes. The NoTS is applied to visualize protein-protein interactions in the nucleus based on relocation of preys to the nucleolus in plant cells. It can be easily performed in transient assay and costs less time to get the experimental results. In NoTS, we use Nuc2, a nucleolar marker of the nucleolus, as the tethering protein to immobilize a protein of interest (X) to the nucleolus.\textsuperscript{14-16} We generated a triple fusion protein consistent of the yellow fluorescent protein (YFP), the Nuc2, and a protein of interest (X). This fusion showed a diffuse signal throughout the nucleolus and/or nuclear body-like structures at the periphery of the nucleolus. If another protein (Y) tagged by cyan fluorescent protein (Y-CFP) interacts with X, relocation of Y to the nucleolus can be monitored, which leads to co-localization of YFP and CFP signals (Fig. 1, the top panel). If there is no interaction between X and Y, Y will maintain its original distribution pattern and separated signals of FPs can be detected in vivo (Fig. 1, the middle panel). NoTS reveals protein-protein interactions by visualizing the relocalizations of interacting proteins to the nucleolus recruited by a protein of interest fused with Nuc2, a nucleolar marker of the nucleolus, which is conserved in eukaryotes.\textsuperscript{17,18} Through the nucleolar tethering of Nuc2 and recruitment of Nuc2 fusions, the interacting proteins are relocated to the nucleolus and diluted in the large volume of nucleolus, avoiding local high concentrations of these proteins at the interacting sites which easily cause false-positive results, as the cases for BiFC and FRET analysis.

NoTS is based on exogenously overexpressed Nuc2 fusion protein, however, comparing the expression levels of 18S and 25S rRNAs in wild type (Col-0) and transgenic line overexpressing Nuc2-COP1-YFP in background of \textit{cop1} mutant revealed that exogenous expression of Nuc2 is not likely to be problematic for NoTS assay.\textsuperscript{19} The relocation of a large protein such as the plant microRNA (miRNA) processing enzyme DICER-LIKE 1 (DCL1), with a molecular weight of about 214 kDa, by its interacting protein HYL1 fused by Nuc2 (Nuc2-HYL1) demonstrated the high recruitment efficiency of the interacting proteins by Nuc2 fusion proteins in NoTS.\textsuperscript{19,20} Successful recruitments of cryptochromes (cry1, cry2), UVR8, CONSTANS (CO) and LONG HYPOCOTYL IN FAR-RED 1 (HFR1) to the nucleolus by Nuc2-COP1 suggested direct interactions between COP1 and them, consistent with previous reports.\textsuperscript{19,21,22} It is possible that the relocations revealed by NoTS may be resulted from Nuc2 or its-interacting proteins, so controls are of importance to exclude this probability. The failure to detect the co-localization between COP1-interacting proteins and Nuc2 or a WD40 repeat domain-deleted COP1 (Nuc2-COP1\textit{ΔWD40}) clearly indicated that the interaction results revealed by NoTS are credible.\textsuperscript{19}

\textbf{NoTS Assay for the Assembly of Nuclear Bodies}

In addition to the application on protein-protein interactions, NoTS can be also used for studying the assembly of NBs. NBs are special subnuclear domains containing many proteins or RNAs to form complicated interaction networks, exerting multiple functions by free exchange of components in NBs with the surrounding nucleoplasm.\textsuperscript{23-27} Many NBs exist both in animals and plants, including the nucleolus, Cajal bodies (CBs), polycomb group (PcG) body and nuclear speckles.\textsuperscript{28-31} NBs such as paraspeckles, promyelocytic leukemia body (PML body) and 53BP1 nuclear body were only characterized in mammalian cells.\textsuperscript{30,34} Some plant-specific NBs are also characterized, such as nuclear dicing bodies (D-bodies), photobodies, cyclophilin-containing bodies, and AKIPI1-concentrated bodies.\textsuperscript{35-37} NBs may be reaction sites, promoting cellular processes by concentrating proteins or RNA required, such as the nucleolus and CBs.\textsuperscript{17,25,26,30} NBs may also act as hubs
recruiting gene loci for transcription regulation like PcG bodies in *Drosophila*. NBs can also serve as storage or modification sites where phosphorylation or sumoylation takes place, such as nuclear speckles and PcG bodies. In plant NBs, D-bodies might be the sites for maturation of miRNAs. Photobodies may be reaction, degradation, or storage sites during light signaling transduction.

Three assembly models have been put forward to explain the formation of NBs in mammalian cells. The first is a stochastic assembly model in which individual component interacts stochastically to build up NBs in an equal and random order. Different components in CBs like coilin, SMN complex, or special ribonucleoproteins (RNPs) can initiate de novo formation of CBs which share similar morphology, composition and dynamics to the endogenous CBs, suggesting a self-organized model for the assembly of CBs. The second is an ordered assembly model, emphasizing the sequential assembly fashion. The third one is a seeding assembly model proposing a seed function during the initiation of NBs. A protein or RNA can be the seed during the initiation of NBs. A protein of interest (X) is fused to Nuc2 and YFP to make Nuc2-X-YFP, which is tethered to nucleolus. The interacting protein (Y) of X is fused to CFP. If Y interacts with X, Y-CFP will be relocated to the position of Nuc2-X-YFP and the co-localization signals of YFP and CFP can be detected in the nucleolus, which is labeled by Nuc2-mCherry (the top panel). If Y does not interact with X, Y-CFP will maintain its original position and separated signals of YFP and CFP will be observed in the nucleus (the middle panel). For nuclear body initiation assay, a component of NBs (X) is fused with Nuc2 and YFP to make Nuc2-X-YFP and displays body-like structures at the periphery of nucleolus, which contain other components of the NBs (Y-CFP), suggesting X has initiation ability for the assembly of NBs (the bottom panel).

![Figure 1. Schematic outline of NoTS assay for nuclear protein-protein interactions and initiation of nuclear bodies.](image)

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nucleoplasmic proteins or components of NBs based on immobilization of the bait by fusing with Nuc2 in plants.\(^{19}\) It is quite easy to operate in transient assay in plants and costs less time to display experimental results. As nucleolus is a conserved organelle, it is of great interest to extend the application of NoTS to other cell lines or organisms to detect the nuclear protein-protein interactions.\(^{14,17}\) NoTS does not easily produce a false-positive result, which is a major issue for BiFC and FRET, as when assessing a protein-protein interaction in the nucleolus or at the periphery foci of the nucleolus, full or partial co-localization signal of FPs can be observed throughout the nucleolus in NoTS, avoiding a local high concentration of the proteins by diluting them in the large volume of the nucleolus. However, false-negative result might be a problem for NoTS. The failed recruitment of photoreceptors PHYTOCHROME A (phyA) and PHYTOCHROME B (phyB) to nucleolus by Nuc2-COP1 showed in NoTS indicated indirect or weak interactions may be not suitable for NoTS assay.\(^{19,48,49}\) Thus, the interaction affinity between two proteins is a key factor for application of NoTS, very weak protein-protein interactions may be out of the sensitivity of NoTS. It is a good choice to conjunct NoTS with other fluorescence-based methods like BiFC and FRET. NoTS was now only used to detect the interactions among nucleoplasmic proteins, extending its application to analyze the interactions among cytoplasmic proteins fused with nuclear localization signals will be also of great interest. In this case, it should point out that this may produce unexpected off-target effects in some situations. If the interaction of cytoplasmic proteins depends on modifications by other cytoplasmic proteins, it may miss the interaction result in NoTS. For the assembly of NBs, NoTS is easier to be generated by transient co-expression in plants compared with the bacterial LacO/LacI system. Besides, tethering a protein of interest to the nucleolus by Nuc2 can be easily achieved and monitoring the recruitment and localization of these components to the de novo formed bodies by fluorescent proteins is more direct and simple than antibody staining. Previously, the RNA fused by LacI and MS2 stem loop is tethered to LacO repeats through specific binding of LacO/lacI and MS2 coat/MS2 stem loop and visualized by the fluorescence in site hybridization (FIHS).\(^{44,46,47}\) A λN22 RNA stem-loop binding system is also proposed for visualization of RNA in vivo in plant cells.\(^{50}\) NoTS may be modified to test the function of RNAs in the formation of NBs by using MS2 coat protein/MS2 stem loop or similar systems. This modification may be also helpful for NoTS to test the RNA-protein interactions. In plants, D-bodies are related to maturation of miRNAs; nuclear speckles are involved in splicing of messenger RNAs (mRNAs). It is of interest to explore the assembly of D-bodies or plant nuclear speckles and reveal the potential functions of RNAs in these NBs.\(^{36,51}\) As NoTS revealed the assembly of photobodies follows a self-organization model, further tests are needed to address whether this kind of assembly model is also applied to other NBs in plants.\(^{19}\)

**Discussion and Perspectives**

Revealing protein-protein interactions by relocations of proteins through its interacting protein is powerful. Proteins relocated to cell membrane, viral factory-like structures (FLS) and the genomic locus containing LacOs have been visualized to detect protein-protein associations or interactions in cytoplasm and nucleus.\(^{7,11,12}\) NoTS uses the nucleolus as an anchor structure to detect the interactions among nucleoplasmic proteins or components of NBs based on immobilization of the bait by fusing with Nuc2 in plants.\(^{19}\) It is quite easy to operate in transient assay in plants and costs less time to display experimental results. As nucleolus is a conserved organelle, it is of great interest to extend the application of NoTS to other cell lines or organisms to detect the nuclear protein-protein interactions.\(^{14,17}\) NoTS does not easily produce a false-positive result, which is a major issue for BiFC and FRET, as when assessing a protein-protein interaction in the nucleolus or at the periphery foci of the nucleolus, full or partial co-localization signal of FPs can be observed throughout the nucleolus in NoTS, avoiding a local high concentration of the proteins by diluting them in the large volume of the nucleolus. However, false-negative result might be a problem for NoTS. The failed recruitment of photoreceptors PHYTOCHROME A (phyA) and PHYTOCHROME B (phyB) to nucleolus by Nuc2-COP1 showed in NoTS indicated indirect or weak interactions may be not suitable for NoTS assay.\(^{19,48,49}\) Thus, the interaction affinity between two proteins is a key factor for application of NoTS, very weak protein-protein interactions may be out of the sensitivity of NoTS. It is a good choice to conjunct NoTS with other fluorescence-based methods like BiFC and FRET. NoTS was now only used to detect the interactions among nucleoplasmic proteins, extending its application to analyze the interactions among cytoplasmic proteins fused with nuclear localization signals will be also of great interest. In this case, it should point out that this may produce unexpected off-target effects in some situations. If the interaction of cytoplasmic proteins depends on modifications by other cytoplasmic proteins, it may miss the interaction result in NoTS. For the assembly of NBs, NoTS is easier to be generated by transient co-expression in plants compared with the bacterial LacO/LacI system. Besides, tethering a protein of interest to the nucleolus by Nuc2 can be easily achieved and monitoring the recruitment and localization of these components to the de novo formed bodies by fluorescent proteins is more direct and simple than antibody staining. Previously, the RNA fused by LacI and MS2 stem loop is tethered to LacO repeats through specific binding of LacO/lacI and MS2 coat/MS2 stem loop and visualized by the fluorescence in site hybridization (FIHS).\(^{44,46,47}\) A λN22 RNA stem-loop binding system is also proposed for visualization of RNA in vivo in plant cells.\(^{50}\) NoTS may be modified to test the function of RNAs in the formation of NBs by using MS2 coat protein/MS2 stem loop or similar systems. This modification may be also helpful for NoTS to test the RNA-protein interactions. In plants, D-bodies are related to maturation of miRNAs; nuclear speckles are involved in splicing of messenger RNAs (mRNAs). It is of interest to explore the assembly of D-bodies or plant nuclear speckles and reveal the potential functions of RNAs in these NBs.\(^{36,51}\) As NoTS revealed the assembly of photobodies follows a self-organization model, further tests are needed to address whether this kind of assembly model is also applied to other NBs in plants.\(^{19}\)

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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