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An *in vivo* imaging-based assay for detecting protein interactions over a wide range of binding affinities

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ABSTRACT

Identifying and characterizing protein interactions are fundamental steps toward understanding and modeling biological networks. Methods that detect protein interactions in intact cells rather than buffered solutions are likely more relevant to natural systems since molecular crowding events in the cytosol can influence the diffusion and reactivity of individual proteins. One *in vivo*, imaging-based method relies on the colocalization of two proteins of interest fused to DivIVA, a cell division protein from *Bacillus subtilis,* and green fluorescent protein (GFP). We have modified this imaging-based assay to facilitate rapid cloning by constructing new vectors encoding N- and C-terminal DivIVA or GFP molecular tag fusions based on site-specific recombination technology. The sensitivity of the assay was defined using a well-characterized protein interaction system involving the eukaryotic nuclear import receptor subunit, Importin α (Impα), and variant nuclear localization signals (NLS) representing a range of binding affinities. These data demonstrate that the modified colocalization assay is sensitive enough to detect protein interactions with K_d values that span over four orders of magnitude (1 nM to 15 μM). Lastly, this assay was used to confirm numerous protein interactions identified from mass spectrometry-based analyses of affinity isolates as part of an interactome mapping project in *Rhodopseudomonas palustris*.

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Characterizing protein interactions within cellular networks is essential for gaining insight into biological functions, mechanisms of regulation, and ultimately for understanding the cell as a complex system. The ability to predict, model, and manipulate biological responses to genetic and environmental signals is dependent on a thorough understanding of the interactions within and between cellular networks. Both *in vitro* and *in vivo* methods have been used extensively to detect and characterize protein–protein interactions and each method offers different advantages and disadvantages with respect to throughput, ease of use, sensitivity, and accuracy (reviewed in [1–3]). In addition, recent technological advances have made large-scale, high-throughput interactome mapping studies both feasible and cost-efficient [1,4,5].

One method used for interactome studies involves affinity-tagged protein purification coupled with mass spectrometry identification [4,6]. This method was used to identify protein complexes in several model organisms, including *Saccharomyces cerevisiae* and *Escherichia coli* [7–11]. These studies identified numerous molecular complexes and provided insights into the physiological role of many proteins of unknown function based on their association with proteins of known function. This method, however, tends to be biased toward identification of high-affinity interactions and interactions with slow dissociation kinetics [4]. More recently, this methodology has also been applied to characterize protein interaction networks in less tractable microbes, including *Rhodopseudomonas palustris* and *Shewanella oneidensis* [12].

These *in vitro* approaches to studying protein interactions necessarily involve cell lysis and dilution of cellular components which can have significant effects on protein interactions. Within the cell, proteins do not interact in dilute buffered solutions, but rather in a highly crowded macromolecular environment. There is growing recognition of the importance that crowding events in the cytosol play in the diffusion and reactivity of individual protein molecules [13–15]. Thus, assays that detect and characterize protein interactions in intact cells may be more relevant to natural systems. For example, the yeast two-hybrid assay is commonly used to detect interactions in the molecular context of a living cell [16,17]. Numerous large-scale interactome studies have been carried out in eukaryotes and prokaryotes using the yeast two-hybrid

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assay (reviewed in [17–19]). Advantages of this assay include the ability to identify stable and transient interactions and map interaction domains. However, large-scale yeast two-hybrid screens are often challenged by high false positive and false negative rates [20]. More recently, variations of the two-hybrid assay that involve detection of interactions by protein fragment complementation have been described [3,21].

Advances in fluorescence imaging technologies have also resulted in new methods for detecting protein interactions *in vivo*. For example, interactions between two proteins that are less than 100 Å apart can be imaged and assessed by detecting fluorescence resonance energy transfer (FRET)[1] between donor and acceptor fluorophores fused to the proteins of interest [22,23]. Bioluminescence resonance energy transfer (BRET) is similar to FRET, but the donor fluorophore is replaced by a luciferase, thereby reducing extraneous fluorescence and photobleaching [24–26]. Finally, fluorescent protein fragment reassembly assays can identify protein–protein interactions based on the reconstitution of a fluorescent protein, such as GFP or YFP, by nonfluorescent fragments fused to interacting proteins [27–30].

Ding and colleagues have described an imaging-based bacterial protein interaction screen that allows for rapid identification of protein interactions *in vivo* [31]. This technique relies on the localization properties of DivIVA, a cell division protein from *Bacillus subtilis* [32]. DivIVA localizes to the cell poles and provides a general mechanism to target a protein to a discrete spatial location within a live cell. Although *E. coli* and other gram-negative bacteria lack a DivIVA homolog, its localization pattern is maintained when expressed in these cells [32]. In this assay, a protein of interest is fused to DivIVA and its potential binding partner is fused to green fluorescent protein (GFP). Following coexpression of both fusion proteins in *E. coli*, a positive protein interaction is detected if the GFP-fusion protein is recruited to the cell pole due to its interaction with its binding partner anchored to the cell pole via DivIVA. This assay has several advantages, including the ability to test interactions in the context of a living cell, ease of use, rapid results, and amenability to high-throughput screening. In addition, because the criteria for interpreting a positive interaction is based on simple GFP localization patterns in cells, this assay is amenable to automated image analysis. To this end, we have developed an algorithm that can be used to automatically identify positive interactions based on changes in the GFP-fusion protein localization patterns in cells before and after induction of DivIVA-fusion protein expression [33].

In this paper, we report modification of the colocalization assay to facilitate rapid cloning and high-throughput applications and determine the range of binding affinities that can be detected using this assay. Further, we compare the ability to detect protein interactions using the colocalization assay with the benchmark yeast two-hybrid assay. To allow rapid cloning, we constructed new vectors encoding N- and C-terminal DivIVA or GFP molecular tag fusions based on site-specific recombination technology [34]. Importantly, recombination-based reactions for cloning and plasmid preparation of this vector set can be fully automated. Once the expression vectors are successfully transformed into *E. coli* and the cells are grown in liquid culture, assay results can be obtained in 60 min. The sensitivity of the assay was defined using a well-characterized protein interaction system involving the eukaryotic nuclear import receptor subunit, Importin α (Impα) [35], and variant nuclear localization signals (NLS) representing a range of binding affinities [36–38]. Using these interaction pairs, we demonstrate that the modified colocalization assay is sensitive enough to detect protein interactions with K_d values that span over four orders of magnitude (1 nM to 15 μM). Finally, we utilized this assay to confirm numerous protein interactions identified in a large-scale protein interaction screen in *R. palustris*. *R. palustris* is a metabolically diverse, purple nonsulfur phototrophic bacterium that has the ability to grow on a wide range of carbon substrates, including aromatic acids derived from lignin. In addition, it has the ability to fix nitrogen gas into ammonia with hydrogen produced as a by-product of this reaction. This pilot study demonstrated the suitability and utility of the colocalization assay for both high-throughput and directed protein interaction studies.

Materials and methods

Bacterial growth and media

Escherichia coli strains DH5α and BL21(DE3) (Invitrogen) were grown in Luria-Bertani (LB) medium [39], which was supplemented with 50 μg/ml kanamycin, 15 μg/ml chloramphenicol, or 50 μg/ml ampicillin as needed. *E. coli* cultures were grown at 37 °C unless otherwise noted. Plasmids pNGFP, pCGFP, pNDIV, and pCDIV were transformed and propagated in chemically competent *E. coli* DB3.1 (Invitrogen) due to the presence of the *ccdB* gene in each destination vector. All other plasmids lacking the *ccdB* gene were transformed and propagated in chemically competent *E. coli* DH5α. For protein expression, plasmids were transformed into *E. coli* BL21(DE3) cells. L-Arabinose was added to a final concentration of 0.2% to LB medium to induce expression of DivIVA-fusion proteins.

DNA recombination procedures

BP and LR clonase recombination reactions were performed according to the manufacturer's instructions (Invitrogen). Purification of PCR products or plasmid DNA was performed using Qiagen DNA purification kits (Hilden, Germany). PCR were performed using a GeneAmp thermocycler (Perkin Elmer, Walthum, MA) with Vent polymerase (New England Biolabs, Ipswich, MA). All oligonucleotides used as primers for this study were purchased from Integrated DNA Technologies (Coralville, IA) and reaction protocols were optimized according to the oligonucleotide specification.

Construction of GFP destination vectors

The N-terminal GFP destination vector pNGFP was derived from pACYC184. The DEST17 cassette (nt 1–1979) was PCR-amplified from pDEST17 (Invitrogen) as a HindIII–HindIII fragment using the primers 5′-cccaagcttagatctcgatcccgcg-3′ and 5′-ccgaagcttggatatccggatatagttcc-3′. The resulting fragment included the T7 promoter, 6×His tag, V5 epitope, Cm^R gene, and ccdB gene flanked by attR1/attR2 recombination sites. This DEST17 fragment was then cloned into the HindIII site in pACYC184. The Tet^R gene was unintentionally destroyed in the pACYC184 vector as a result of this cloning strategy. Enhanced GFP (S65T) [40] was PCR-amplified from pFA6a vector [41] in two steps. First, the internal NdeI site was destroyed by amplifying the 5′ end of the gene using the primers 5′-ggaattccatatggtcagtaaaggagaagaacttttcactgg-3′ and 5′-gccgtttcatgtgatctgggtatcttg-3′. The resulting 200-bp PCR product was used as a primer along with 5′-ggaattccatatgtttgtatagttcatccatgccatgtg-3′ to amplify the full-length GFP gene flanked by NdeI sites and lacking a stop codon. This fragment was then cloned in frame into the pACYC184-DEST17 plasmid using the unique NdeI site in DEST17 to produce pNGFP. The C-terminal GFP destination vector pCGFP was also derived from pACYC184. The DEST42

[1] *Abbreviations used:* BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; Impα, Importin α; LB, Luria-Bertani; NLS, nuclear localization signals; SC, synthetic complete.