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TECHNIQUES FOR MOLECULAR ANALYSIS

Biochemical approaches for discovering protein–protein interactions

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Summary

Protein–protein interactions or protein complexes are integral in nearly all cellular processes, ranging from metabolism to structure. Elucidating both individual protein associations and complex protein interaction networks, while challenging, is an essential goal of functional genomics. For example, discovering interacting partners for a 'protein of unknown function' can provide insight into actual function far beyond what is possible with sequence-based predictions, and provide a platform for future research. Synthetic genetic approaches such as two-hybrid screening often reveal a perplexing array of potential interacting partners for any given target protein. It is now known, however, that this type of anonymous screening approach can yield high levels of false-positive results, and therefore putative interactors must be confirmed by independent methods. *In vitro* biochemical strategies for identifying interacting proteins are varied and time-honored, some being as old as the field of protein chemistry itself. Herein we discuss five biochemical approaches for isolating and characterizing protein–protein interactions *in vitro*: co-immunoprecipitation, blue native gel electrophoresis, *in vitro* binding assays, protein cross-linking, and rate-zonal centrifugation. A perspective is provided for each method, and where appropriate specific, trial-tested methods are included.

Keywords: co-immunoprecipitation, electrophoresis, protein binding, proteomics, mass spectrometry, sedimentation.

Introduction

A key step in the advance from genomics through functional genomics towards systems biology is the definition of protein interactions in living cells (Gandhi *et al.*, 2006). Proteins have direct physical interactions at several different levels, including; the multi-subunit enzymic nanomachines (Mitra and Frank, 2006; Mooney *et al.*, 2002; Pool, 2005), cohort and client protein binding by molecular chaperones (Chapman *et al.*, 2006; Hawle *et al.*, 2006), client protein binding by protein kinases (Chevalier and Walker, 2005; Rohila *et al.*, 2006), and multiple protein interactions in metabolic networks (Dhar-Chowdhury *et al.*, 2005; Lyubarev and Kurganov, 1989).

Scientists working in the field of bioinformatics have already developed several algorithms for predicting protein interactions (Craig and Liao, 2007; Guimaraes *et al.*, 2006; Pitre *et al.*, 2006). Refinement of these methods and the development of additional methods are ongoing; however, it is essential that any predictions are tested experimentally before being considered further.

Many synthetic genetic (Dove and Hochschild, 2004; Magliery and Regan, 2006; Parrish *et al.*, 2006; Piehler, 2005), biochemical (Kuroda *et al.*, 2006; Rohila *et al.*, 2006; Tang *et al.*, 2006), and cell biological methods (Bhat *et al.*, 2006; Demarco *et al.*, 2006; Sibarita, 2005) have been used to

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detect protein interactions *in vivo*. In contrast, here we focus specifically on methods useful for *in vitro* analyses of protein interactions.

Detecting protein interactions

Co-immunoprecipitation

Co-immunoprecipitation (co-IP) is a key technique for the analysis of protein–protein interactions, including interactions of subunits within a protein complex. The co-IP of proteins from cellular fractions is the most convincing evidence that two or more proteins physically interact *in vivo* (Monti *et al.*, 2005; Phee *et al.*, 2006; Ren *et al.*, 2003). Typically an antibody specific for one protein is incubated with a clarified homogenate or protein mixture to form an immune complex with the subject protein (antigen). A subject protein might interact with one or more other proteins in a complex. After binding to the antibodies, the entire complex can be isolated from the mixture using immobilized protein A or protein G (protein A/G).

Protocol for co-IP of the subunits of the mitochondrial pyruvate dehydrogenase complex

Chemical cross-linking antibodies to protein A/G beads For improved utility, it is desirable to chemically cross-link antibodies with protein A/G beads. The amount of antibodies and protein A/G used will depend on variables such as the avidity of antibody binding, but a useful starting point is to use 100–200 μg of antibody with 400 μl protein A/G agarose (50% slurry). The antibody and protein A/G bead mixture can then be incubated with 10 mM dimethyl pimelimidate and 10 mM dimethyl suberimidate in 20 ml phosphate-buffered saline (PBS), pH 8.5, for 1 h at 25°C. Reaction should be stopped by adding 10 ml of 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)–HCl, pH 8.8. The beads can then be passivated overnight at 4°C with 10 ml of 2 mg ml^{-1} BSA in 100 mM TRIS–HCl, pH 8.8. After washing with three bed volumes of PBS the cross-linked antibody beads are ready for use.

Disruption of purified pea seedling mitochondria. Purified pea seedling mitochondria (Fang *et al.*, 1987) were diluted with PBS to 5 mg protein ml^{-1}, then disrupted using a Kinematica Polytron homogenizer (Brinkman Instruments, http://www.brinkmann.com/). The broken mitochondria were centrifuged for 1 h at 4°C in a Beckman Coulter Optima MAX-E ultracentrifuge using a TL-100.2 rotor (109 000 ***g*** at r_{max}).

Immunoprecipitation. Supernatants were incubated with a 100 μl bed volume of monoclonal anti-E1α antibodies (Luethy *et al.*, 1995) cross-linked to POROS 20 protein G

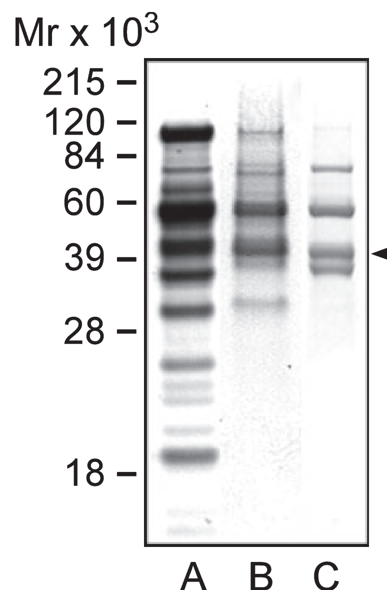

Figure 1. Immunoprecipitation of the mitochondrial pyruvate dehydrogenase complex with antibodies against the E1α subunit (Luethy *et al.*, 1995).
Lane A, total soluble matrix proteins from purified pea (*Pisum sativum* L.) seedling mitochondria (Fang *et al.*, 1987). Lane B, mitochondria were lysed, clarified, stored at −20°C for 1 month, thawed, then incubated with anti-E1α antibodies. Lane C, proteins from freshly lysed mitochondria that co-immunoprecipitated along with the E1α subunit. The position of authentic E1α is indicated with an arrowhead.

beads (Applied Biosystems, http://www.appliedbiosystems.com/) for 2 h at 25°C. The beads were collected by centrifugation, then washed three times with 10 bed volumes of PBS. Bound proteins were eluted by two 150 μl applications of 200 mM Gly, pH 2.5. The pH of the combined elutes was adjusted to neutrality with 30 μl of 1 M TRIS–HCl, pH 9.0. The immunoprecipitated samples were analyzed by SDS-PAGE (Figure 1), or subjected to solution trypsin digestion followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS).

While the antibodies used are very specific for the pyruvate dehydrogenase complex (PDC) E1α subunit, the Coomassie Brilliant Blue (CBB)-stained gel has four prominent bands (Figure 1c). These are E1α plus the E1β, E2, and E3 subunits of PDC, which were anticipated because these four subunits are known to be associated in a very large geometrically defined structure (Mooney *et al.*, 2002). There are additionally some minor bands which have been identified as known mitochondrial matrix proteins. The possibility that these proteins are associated with the PDC in a *bona fide* metabolon-type macro-complex is being evaluated.

The main advantages of using co-IP to study protein complexes are the specificity, relative ease, and compatibility with most methods of downstream analysis (SDS-PAGE, immunoblotting, MS) (Table 1). Also, after eluting the bound

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Table 1 Relative merits of the described methods for characterizing protein interactions

Method	Advantages	Disadvantages
Co-immunoprecipitation	Considered highly specific. Relatively simple. Compatible with most methods of downstream analysis. Reagents can be reused	Occasional difficulties in obtaining antibodies of high specificity and avidity. Low capacity
Blue Native PAGE	Inexpensive. Requires no specialized equipment. Compatible with most methods of downstream analysis	Works best with abundant membrane proteins. Relatively low resolution. Sensitive to low-molecular-weight solutes
In vitro binding	Relatively simple. Compatible with most methods of downstream analysis	A significant amount of recombinant DNA manipulation is necessary. Not suitable if a 'third party' mediates the interactions.
Chemical cross-linking	High capacity. Stabilizes specific interactions	Complicates downstream analysis. Potential for chemical interference Stabilizes non-specific interactions
Rate-zonal sedimentation	Non-destructive. Compatible with most methods of downstream analysis. Highly reproducible	Time intensive. Requires specialized instruments

proteins, the cross-linked antibody beads can be re-equilibrated and reused. The main disadvantage is that there are occasionally difficulties in obtaining 'good' antibodies, i.e. those of high specificity and avidity. If the target protein is cytoplasmic, co-IP can be done starting with frozen plant materials, and if the target is organellar, co-IP can be done starting with frozen isolated organelles. But regardless of the method and duration of storage, co-IP does not seem to work as well with frozen cytosolic fractions (100 000 ***g*** supernatant) or soluble fractions prepared from isolated organelles and then frozen (Figure 1b). The reason for the decrease in quality while frozen is unknown, but probably includes both proteolysis and aggregation/non-specific binding.

Two-dimensional Blue Native/SDS-PAGE

In Blue Native PAGE (BN-PAGE) proteins are solubilized using mild non-ionic detergents such as Triton X-100, digitonin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dodecylmaltoside, etc., in place of sodium dodecyl sulfate (SDS), the strong ionic detergent used to solubilize and denature proteins for separation by SDS-PAGE. Lists of the detergents successfully used in BN-PAGE can be found in Eubel *et al.* (2005) and Krause (2006). After isolation, samples are incubated with the anionic dye CBB G250 immediately before analysis. The dye binds to the surface of all proteins, primarily to Arg residues (Compton and Jones, 1985). This binding of a large number of negatively charged dye molecules to proteins facilitates migration of multi-protein complexes in a first dimension, and the tendency for protein aggregation is considerably reduced. Typically BN-PAGE is conducted using polyacrylamide gels, and the entire repertoire of concentrations/pore sizes and linear or gradient formats can be used. If the protein complexes are particularly large (>100 kDa), it is additionally possible to use agarose in place of acrylamide (Henderson *et al.*, 2000).

While one-dimensional (1D) BN-PAGE can be used directly to study protein interactions, the method is much more powerful when combined with another separation method in a two-dimensional (2D) format. Second-dimension separations can be achieved by a second round of BN-PAGE, denaturing electrophoresis (Figure 2) or isoelectric focusing. If further protein separations are necessary, there is the potential for using an assortment of three-dimensional (3D) methods (Ciambella *et al.*, 2005; Eubel *et al.*, 2005).

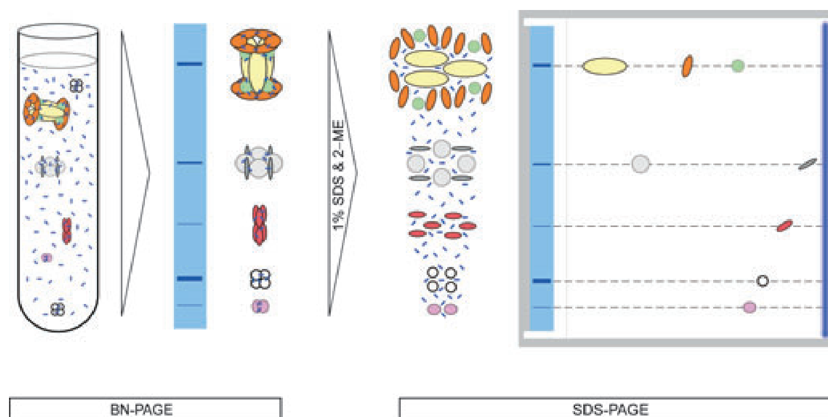

Figure 2. Two-dimensional separation of protein complexes using BNP in the first direction and SDS-PAGE in the second dimension.
Samples are incubated with Coomassie Brilliant Blue G-250 to stabilize protein complexes, then separated by native PAGE. The bands corresponding to each protein complex are excised, incubated with SDS plus a reducing agent to dissociate the complexes, and the individual subunits separated by SDS-PAGE in the second dimension.

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An occasionally used variant of BN-PAGE is Clear Native PAGE (CN-PAGE). In essence, the variant simply omits CBB which prevents the charge-shift caused by the anionic dye. The resolution of CN-PAGE is lower than BN-PAGE, but the method is useful when CBB interferes with activity/function analysis of the gels (Wittig *et al.*, 2007).

Protocol for Blue Native PAGE

Membrane or soluble proteins? While BN-PAGE is suitable for separation of either soluble or cytoplasmic protein complexes, it has more typically been used for membrane protein complexes (e.g. Aivaliotis *et al.*, 2007). If membrane protein complexes are being analyzed, the first step is to solubilize the complexes by treatment with a mild detergent (Eubel *et al.*, 2005; Krause, 2006). Either purified organelles or membranes isolated from them should be incubated for 30 to 60 min at 4°C in solubilization buffer [20 mM *N*-TRIS(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–KOH, pH 8.0, containing 150 mM NaCl, and detergent] with gentle mixing on a rotary shaker. Initial analyses must be conducted using varying sample to detergent ratios. Non-solubilized proteins and membrane fragments can be removed by sedimentation at 100 000***g*** for 1 h at 4°C.

Blue Native PAGE has been particularly useful for analysis of interactions among abundant membrane protein such as members of the mitochondrial respiratory chain (Eubel *et al.*, 2005; Heinemeyer *et al.*, 2007; Krause, 2006), but can also be used for analysis of soluble protein complexes (Henderson *et al.*, 2000; Singh *et al.*, 2005; Swamy *et al.*, 2006; Wang *et al.*, 2007). Analysis of membrane proteins by BN-PAGE seems unaffected by the salts present in sample preparation buffers or low-molecular-weight (MW) compounds present in the input samples, but these compounds are problematic for the analysis of complexes of soluble proteins. A method for removal of low-MW contaminants during sample preparation has been described by Camacho-Carvajal *et al.* (2004), and improves the resolution of soluble protein complexes.

Blue Native PAGE

After isolation, samples should be adjusted to pH 7.5 and incubated at a 1:1 ratio with the anionic dye CBB G250 immediately before electrophoresis (Schagger and von Jagow, 1991). Dissociated dye, which separates from proteins during electrophoresis, can be replenished by including CBB in the cathode buffer. The running pH for BN-PAGE is 7.5, which is in the physiological range for most protein complexes *in vivo*. When separations are completed, BN gels can be destained with 10% (v/v) acetic acid, then any of the typical methods used to detect proteins be used, including silver staining, fluorescent dyes, and immunoblotting.

The main advantages of using BN-PAGE for the study of protein complexes are that it is inexpensive, no specialized equipment is necessary, and the method is compatible with most methods of downstream analysis (e.g. immunoblotting, MS). The main disadvantages are those typical of any type of native PAGE, i.e. lack of fine resolution, protein smearing because of salts, etc. in the isolation buffers (Table 1), and complex dissociation during separation.

In vitro *binding*

More than 70 protein interaction domains have been described (http://pawsonlab.mshri.on.ca/index.php?option=com_content&task=view&id=30&Itemid=63), and there is a plethora of predictive algorithms that can be used to analyze a sequence for the presence of these domains, including Ferre and King (2006). A relatively straightforward, albeit somewhat limited, strategy for the analysis of potential binding partners involves engineering the subject protein (or a domain derived therefrom) so that it can be easily retrieved from an *in vitro* incubation reaction. While many affinity tags can be considered, those commonly used include; His tags, glutathione *S*-transferase (GST), the maltose-binding protein (MBP), the CaM-binding peptide (CBP), and small epitope tags such as myc. Some affinity tags, such as GST, dimerize in solution, and this potential should be considered during experimental design. In some instances, it might be desirable to engineer a specific protease site between the subject protein and the affinity tag, so that the tag could be removed prior to downstream analysis or the subject protein can be cleaved rather than eluted from the affinity matrix.

The engineered protein (or domain) can be incubated *in vitro* with the appropriate cellular fraction, which depends upon the subcellular localization of the subject protein, retrieved from the reaction mixture using the affinity tag, washed, eluted, and the potential binding partners analyzed by protease digestion plus MS. An electrophoretic separation step could be included before the proteomic analyses, but this is not essential for identification of the interaction partners.

For illustrative purposes, an example of this strategy will be briefly described. The WW domain is a scaffold for the recognition and binding of Pro-rich proteins (Macias *et al.*, 2002). The full-length subject protein, or the derived WW domain, can be fused to GST, and this chimera incubated with a clarified supernatant derived from the appropriate subcellular fraction. The chimera can then be retrieved from the incubation mixture with glutathione beads and the interacting proteins identified by MS (Ingham *et al.*, 2005). This strategy is currently being used (W. Antoine and JAM, unpublished) to identify client proteins of the plant-unique SLT molecular chaperone (Antoine *et al.*, 2005) (Figure 3).

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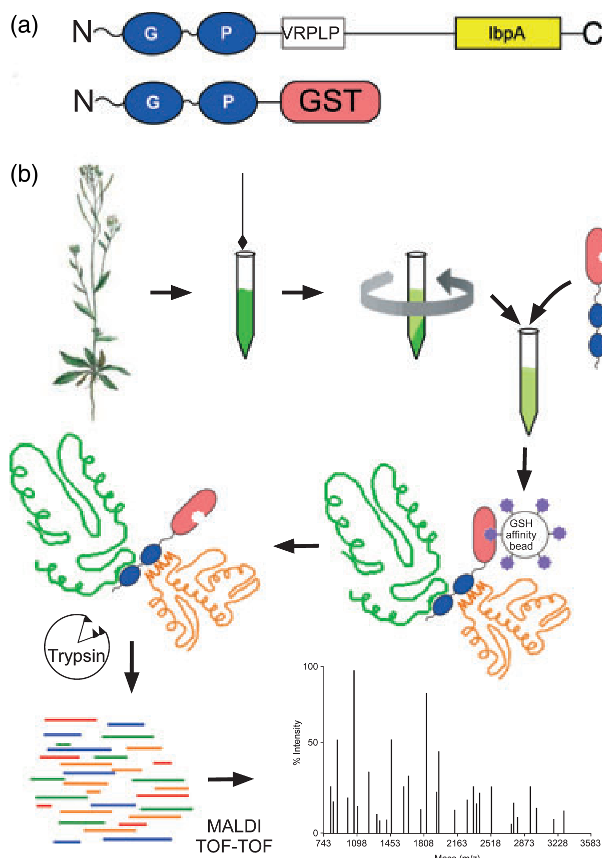

Figure 3. Use of the *in vitro* binding strategy to identify potential client proteins of the plant-unique SLT molecular chaperone (Antoine *et al*., 2005).
(a) Linear model of the *A. thaliana* SLT1A protein. G, a Gly-rich domain; P, a Pro-rich domain; VRPLP, related to the viral response protein-like protein; IbpA, related to the prokaryotic low-molecular-weight heat shock protein; GST, glutathione *S*-transferase. It was hypothesized that the G and P domains are responsible for client protein binding, and a translational fusion between these domains and GST was constructed.
(b) Summarizing the use of the *in vitro* binding strategy to isolate binding proteins which are subsequently identified by tryptic digestion plus matrix-assisted laser desorption/ionization time-of-flight MS analysis.

Protocol for in vitro *binding*

Preparation of the fusion construct. The GST sequence from a pGEX vector (GE Healthcare, http://www.gehealthcare.com/) was engineered by using the PCR to add a unique restriction site to the 3′-end of the GST coding region. Similarly, the *Arabidopsis thaliana* SLT1A sequence was engineered to add the same unique restriction site to the 5′-end at the desired position (the segment including the Pro- and Gly-rich sequences). The two PCR products were digested with the selected restriction enzyme, individually gel-purified, and then ligated yielding the translational chimera GST-SLT1AΔC (Figure 3) that was used to transform *Escherichia coli.* The transformed bacteria were induced to produce the chimeric protein, then lysed and the protein purified by affinity chromatography using glutathione–Sepharose plus anion-exchange fast protein liquid chromatography (FPLC).

Preparation of the plant sample. The SLT molecular chaperone proteins are thought to be located in the cytoplasm, so a cytoplasm-enriched fraction (cytosol) was prepared from 2-week-old *A. thaliana* seedlings essentially following the protocol described by Riedell and Miernyk (1988). The homogenizing medium should contain buffer, an osmotically active solute such as sucrose or sorbitol to help maintain membrane integrity, and protease inhibitors, reducing agents, ions, co-factors, etc., as required. The composition of the homogenizing medium must be determined empirically.

After tissue disruption, the homogenate can be centrifuged at increasing speeds for increasing times up to 100 000 ***g*** for 1 h. The 100 000 ***g*** supernatant is the cytosol (and the pellet is the microsomal fraction). Both homogenization and centrifugation should be conducted at 4°C to minimize proteolysis and loss of protein function by unfolding, etc. The cytosol fraction can be used directly for *in vitro* binding assays, or can first be dialyzed to remove low-MW compounds and to standardize the conditions for protein binding.

In vitro *incubation and isolation of interacting proteins.* The purified recombinant fusion protein can be pre-bound to glutathione–Sepharose beads. While not essential, there are instances where it is desirable to chemically cross-link the recombinant fusion protein to the glutathione beads. This could be accomplished using the method described in the co-IP section. In a typical experiment (Figure 3), 1 ml of cytosol can be incubated with 100 μg of the chimeric protein pre-coupled to glutathione–Sepharose beads and allowed to incubate for 6 h at 4°C. Incubation should include some sort of gentle mixing so that the beads do not settle to the bottom of the tube. The specific conditions of incubation will need to be determined empirically.

After incubation, the beads should be harvested by either centrifugation in a micro-centrifuge or filtration, then washed four times with incubation buffer containing 1% non-ionic detergent (Nonidet P-40, Tween 20, etc.) plus protease inhibitors. If the fusion protein has been cross-linked to the beads, then specifically interacting proteins can be released by boiling in SDS-PAGE sample buffer. The interacting proteins can be separated by electrophoresis, then excised from the gel, digested, and analyzed by MS.

The main advantage of the *in vitro* binding method is that it is relatively simple and straightforward. The main disadvantages are that a significant amount of recombinant DNA manipulation/recombinant protein purification is necessary, and the method is not suitable in situations where protein interactions are mediated by or require other factors (third-party interactions) or post-translational modifications, or

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require a specific non-native protein conformation for binding.

Protein cross-linking

Although individual proteins, and occasionally even protein complexes, can be purified to homogeneity *in vitro* (a proud achievement for any protein biochemist), it is without question that the frequently harsh conditions necessary to isolate and purify a target protein are markedly different from those that exist *in vivo*. Upon cell breakage, proteins are diluted and exposed to a plethora of small molecules which destabilize native interactions. These conditions, while useful or even necessary for protein purification, interfere with the detection and analysis of protein interactions. In many instances, interactions are transient and/or characterized by high dissociation constants. The use of chemical cross-linking agents is an excellent way to stabilize *in vivo* protein interactions. Protein cross-linking is a venerable technique for discovering protein–protein interactions as evidenced by the hundreds of publications employing the use of these reagents, dating back to the 1960s (Spirin *et al.*, 1965). A schematic model for cross-linking cohort proteins that interact transiently is presented in Figure 4. In this example, a hetero-bifunctional, UV-activated, cleavable cross-linker is employed.

Choosing a cross-linker. Protein cross-linking reagents are varied but can be defined by a minimum of two reactive groups and classified according to reactivity (primary amines, sulfhydryls), water solubility and membrane permeability, cleavability, and arm length between the two reactive groups (Kluger and Alagic, 2004). An extensive array of protein cross-linking reagents is available from Pierce Chemicals (http://www.piercenet.com/) who also provide an on-line interactive cross-linker selection guide. The availability of diverse cross-linking agents presents some interesting possibilities for discovering and characterizing protein interactions.

Variable spacer arm-length agents, including the reactive polyethylene glycol derivatives which have spacer lengths ranging from 18 to 53 Å, could be useful for systematically probing spatial interactions among partners in protein complexes (Veronese and Pasut, 2005). Label transfer cross-linkers allow bait:prey type analysis using high-sensitivity detection with labels such as biotin or ^{125}I (Lam *et al.*, 2002). The sensitivity of this approach and the ability to capture the bait:prey complex using a photosensitive aryl-azide group offer tight control over the activation conditions for probing protein–protein interactions.

When using any cross-linking agent one must be aware of the chemical reactivity, and avoid using interfering buffers (e.g. TRIS and Gly are incompatible with *N*-hydroxysuccin-

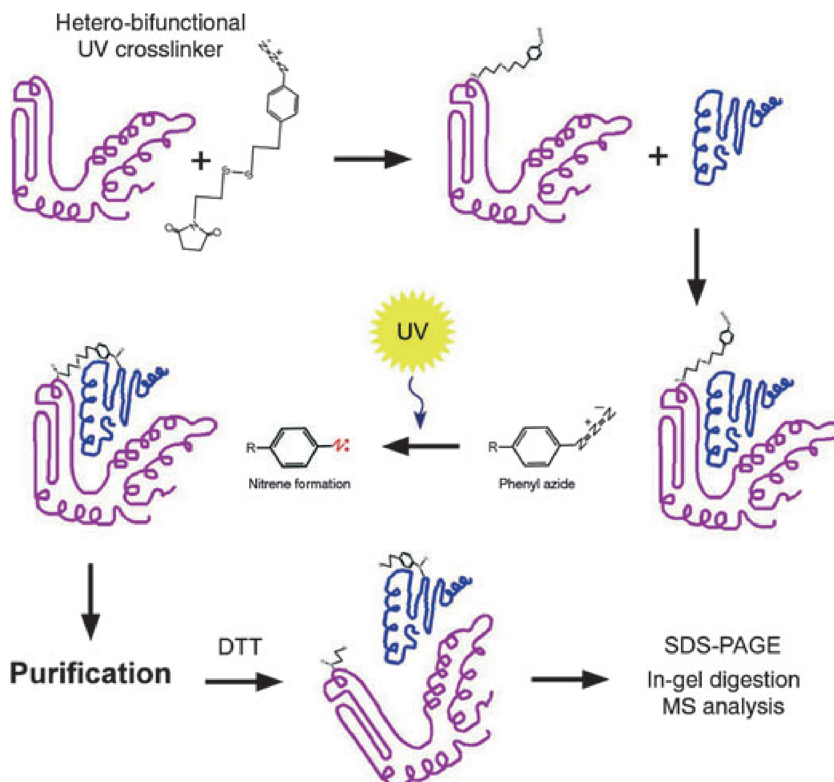

Figure 4. A schematic model for cross-linking cohort proteins that interact transiently, using a hetero-bifunctional, UV-activated, cleavable cross-linker.
In the first step, the maleimide function of the cross-linker reacts with a –SH group exposed on the surface of protein 1. Protein 1 then binds to and forms a complex with protein 2. Upon UV irradiation, the phenyl-azide function of the cross-linker is converted to a highly reactive nitrene which reacts with a primary amine group on the surface of protein 2. The cross-linking stabilizes the previously labile complex so that it can be purified. After purification, the sulfhydryl reducing reagent DTT is added, which cleaves the disulfide bond of the cross-linker and reduces both Cys. The two proteins are then separated by SDS-PAGE, and identified by MS analysis of the tryptic peptides.

imidyl- and formaldehyde-reactive cross-linkers) or peptide protease inhibitors in the reactions. Downstream MS-based identification of proteins must also be considered when deciding which type of cross-linker to use. For example, Lys-reactive agents interfere with trypsin digestion, which cleaves on the C-terminal side of Lys and Arg residues. All cross-linkers carry a 'molecular footprint' that must be accounted for when making MS-based protein assignments (Soderblom and Goshe, 2006). The identification of the chemically modified regions of the protein and the amino acid residues that specifically contain the modification remains challenging. Most strategies involve SDS-PAGE to separate cross-linked from non-cross-linked proteins, with subsequent in-gel tryptic digestion. The peptides extracted from the gel are a complex mixture that complicates identification by MS. Peptide identification using tandem MS is difficult because the fragmentation spectrum contains product ions originating from both peptides involved in the cross-link which precludes sequential amino acid assignments for each peptide based on *de novo* sequencing or database-searching algorithms. Even with more advanced MS instrumentation, the identification of the cross-linked species is dependent upon protein size and complexity. These problems are being addressed with the synthesis, utilization, and analysis of cross-linkers specifically designed for proteomic analyses (Sinz, 2003; Soderblom and Goshe, 2006).

Formaldehyde cross-linking. A simple, inexpensive, and commonly used protein cross-linker is formaldehyde, which reacts primarily with Lys residues. Several properties of formaldehyde make it a useful reagent for characterizing protein–protein interactions *in vivo*. It is membrane permeable which allows rapid fixation and consequently inactivation of many cellular proteases. Some enzymes are immediately inactivated upon addition of formaldehyde to growing cells (Hall and Struhl, 2002). Formaldehyde is also considered to be a 'zero-length' cross-linker (although the actual arm distance is 2 Å), so only close-proximity associations are characterized, minimizing non-specific protein interactions. The reversibility of formaldehyde cross-links would seem to circumvent missed trypsin cleavage after Lys residues (Vasilescu *et al.*, 2004). Formaldehyde cross-links can be reversed by boiling in SDS-PAGE sample buffer (Hall and Struhl, 2002). Formaldehyde can be used from a 37% concentrated liquid stock (Hall and Struhl, 2002) or paraformaldehyde powder (Vasilescu *et al.*, 2004), at concentrations ranging from 0.1–1.0% depending upon protein or cell density. After quenching with 1 to 2 M Gly, *in vivo* formaldehyde cross-linked samples can be used directly with co-IP. Formaldehyde is volatile, and in order to avoid cross-linking proteins in the skin and nasal passage it is important to wear gloves and work in a chemical fume hood.

The main advantage of using chemical cross-linking is that covalent bonds are formed between partner proteins, greatly stabilizing their association (Table 1). Unfortunately, both legitimate and artifactual associations are stabilized, the latter becoming especially problematic when cross-linkers with long spacer arms are employed. This requires a greater effort to be made toward independent validation of putative interactions. Additionally, even when cleavable cross-linkers are used the downstream analysis is convoluted by the residual molecular footprint (Soderblom and Goshe, 2006).

Rate-zonal centrifugation

Protein complexes can reach truly amazing sizes. For example, the purified mitochondrial PDC, a multimeric assembly comprising at least six different proteins, was estimated to be approximately 10 MDa (Thelen *et al.*, 1998). The core of this complex was shown to be a 2.7-MDa homomeric 60-mer of 55 kDa dihydrolipoamide acetyltransferase subunits, based upon a combination of size-exclusion chromatography, rate-zonal sedimentation, and negative-staining transmission electron microscopy (Thelen *et al.*, 1999). The massive size of this complex enabled purification of either the complete complex or the core structure from isolated maize mitochondria or *E. coli*, by a simple combination of 400 000 ***g*** centrifugation or ultrafiltration, respectively, to concentrate the protein sample, followed by rate-zonal sedimentation in linear glycerol gradients.

Other large plant protein complexes have been resolved or purified by rate-zonal centrifugation, including 60S ribonucleoprotein particles (Hanano *et al.*, 1996), the 26S proteasome (Malik *et al.*, 2004), and the mitochondrial respiratory chain supercomplexes (Dudkina *et al.*, 2005), as well as relatively smaller protein assemblies such as Photosystem I and II complexes (Huber *et al.*, 2004; Swiatek *et al.*, 2001) and DNA primase (Garcia-Maya and Buck, 1998).

Rate-zonal centrifugation separates protein complexes based upon size and shape. Glycerol and sucrose are the most commonly used media. Large-volume (>20 ml) linear gradients are generally prepared using a gradient maker and peristaltic pump whereas small-volume gradients for sucrose or glycerol require sequential layering of decreasing stock concentrations followed by passive diffusion for a fixed period of time depending upon the size of the gradient (Basi and Rebois, 1997). Gradient linearity should be verified by refractometry. Gradient concentration range, centrifugal force, and duration of centrifugation are the variable parameters in rate-zonal centrifugation, and can generally be obtained from the literature or empirically determined. Unlike isopycnic centrifugation, 'too much' rate-zonal centrifugation will ultimately result in the sample collecting at the bottom of the tube. For analysis of the mitochondrial PDC (Thelen *et al.*, 1998), the sample was applied on top of a

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10–50% linear glycerol gradient and centrifuged for 16 h at 25 000 ***g*** in a SW28 swinging bucket rotor. Under these conditions, the native complex sedimented to approximately 30% glycerol, indicating that for smaller protein complexes a lower percentage of glycerol, slower speed, or shorter duration will be necessary.

Protocol for density gradient isolation of protein complexes

Forming the gradients. The two main approaches suitable for forming linear density gradients are active (gradient maker) and passive (diffusion of a multi-step gradient). It is important to verify linearity by refractometry, and simple hand-held refractometers, available from scientific instrument, food science, or home-brewing sources, are sufficient for this purpose. It is important to properly match the volume of the gradient with the volume and amount of protein being analyzed. A common mistake is sample overloading. As a rule, the volume of the analyte should be minimized. With respect to protein amount, we have successfully resolved >5 mg of total protein on a 40 ml gradient.

Linear gradient formation using a gradient maker. A gradient maker is a dual-chamber mixing device. One should first determine the final volume size of a typical gradient that will be poured and select a gradient maker that can minimally accommodate twice the volume, i.e. for 40 ml gradients select a gradient maker with a total volume of at least 100 ml.

Prepare the 'light' and 'heavy' solutions separately. Sucrose and glycerol are commonly used, although synthetic iodinated-sugars (e.g. Metrizamide, Nycodenz) have advantages in terms of density and viscosity. The concentration of the two limit-solutions depends upon the size of the complex to be purified, but in general 10% and 50% are good starting points. Solutions must also contain buffers, protease inhibitors, and any stabilizing agents or detergents (for membrane complexes) to ensure the integrity and solubility of the complex.

Attach clear plastic tubing to the outlet (light chamber) of the gradient maker, and connect to a peristaltic pump fitted to a long metal needle placed near the bottom of the centrifuge tube. Center the outlet chamber of the gradient maker over a magnetic stirrer. Close the stopcock valve connecting the two sides of the gradient maker. Add the heavy solution (the one with the higher percentage of glycerol) to the non-outlet side of the gradient maker. This volume should be half of the final gradient volume! To purge the air from the stopcock valve (and prevent 'burping' of light solution into the heavy chamber), slowly open this valve until it is filled with the heavy solution. Fill the outlet chamber with an equal volume of the light solution. Gently add stir bars of equal size to each of the chambers. Although mixing will take place only in the outlet chamber, the volume displacement of the stir bar should be equal in both chambers.

Turn the stirrer motor on to a setting that achieves maximal stirring without the stir bar bumping. Open the stopcock valve and slowly increase the pump speed until the desired flow rate is achieved, which is generally no faster than 1–2 ml min^{-1} to allow adequate mixing. When the centrifuge tubes are filled it is important to stop the flow before air bubbles pass through the tubing and destroy the gradient. Then carefully remove the metal needle from the centrifuge tube. A linear gradient has now been formed, and the sample can be layered on top in preparation for centrifugation.

Linear gradient formation by passive diffusion. Prepare a minimum of three solutions that span the desired range in equal increments (e.g. for a broad gradient such as 10–50% prepare solutions with concentrations of 10%, 20%, 30%, 40%, and 50% glycerol or sucrose). Buffer, protease inhibitors, and any essential stabilizing or solubilizing agents must also be included in each of the solutions. Pipette an equal volume of each solution into the ultracentrifuge tube, beginning with the solution of highest density and proceeding with the solution with the next highest density. Try to minimize mixing at the interface of the layers. When finished, the four interfaces separating the five solutions should be clearly visible.

Allow the gradients to form by passive diffusion by incubating at 4°C overnight or 6–8 h at 25°C (suggested times are based upon gradient volumes of 8 ml). The length of incubation required varies with incubation temperature, gradient volume, and the number of solutions employed, and must be determined empirically. This can be easily achieved by initially setting up a series of tubes, fractionating them at desired time increments, and checking for linearity by determining the refractive indices.

When layering the solutions, it is best to pipette along the side of the tubes in a slow, controlled manner. Rapid pipetting will cause mixing and ultimately a non-linear gradient. To minimize evaporation, be sure to cover the centrifuge tubes during the passive incubation step. To reduce the incubation time required for linear gradient formation, one can tightly seal the centrifuge tubes containing the stepped solutions using a rubber stopper and slowly turn the tubes or rack (having secured the tubes on their side) along the horizontal axis. When incubation is complete, slowly bring tubes back to the vertical orientation.

Ultracentrifugation of protein samples. Once the gradients have been formed, samples can be carefully layered on top for centrifugation. It is important to carefully balance the centrifuge tubes, and to place them into the rotor buckets without disturbing the sample/gradient interface. After

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completing centrifugation, allow the rotor to decelerate with the brake turned off.

The appropriate speed and temperature for centrifugation can be determined in preliminary experiments. It is handy, although certainly not essential, to have the multi-protein complex migrate approximately half-way into the gradient. The viscosity of a solute (sucrose, glycerol, etc.) changes with temperature, so both speed (***g***-force) and temperature can be manipulated. In any case, the stability/lability of complexes must be considered, and conditions modified in order to maintain association of transient or highly labile complexes.

When designing conditions for centrifugation, it is important to calculate ***g***-force at a position approximating that of the protein complex. For calculations, the distance to the mid-point of the gradient should be used rather than the top (r_{min}) or bottom (r_{max}) of the gradient. A useful concept is that of '***g***-minutes'. A good starting point for isolation of a multi-protein complex is centrifugation at 82 000 ***g*** for 10 h. This is 49 200 000 ***g***-minutes. The equivalent separation could be achieved by centrifugation at 200 000 ***g*** for 4 h 6 min, or by sitting on the bench at 1 ***g*** for 34 d 3 h 50 min!

Gradient fractionation. There are several options for gradient fractionation, depending upon resource availability. Gradients can be fractionated from the bottom by either pumping or gravity flow, or from the top by pumping. The simplest method is to puncture the bottom of the tube with an 18-gauge syringe needle, and fractionate the gradient drop-wise. Using this method, drop counting (e.g. 20 drops/fraction) yields more uniform fractions than time-controlled fraction collecting. This method requires the use of thin-walled tubes that can be punctured, but which obviously cannot be reused.

Fractions can be pumped from the bottom of the tubes by what is essentially a reversal of gradient formation. Reconstruct the metal needle/peristaltic pump configuration used during formation, being careful not to disrupt the gradient. Connect the outlet tubing from the pump to a fraction collector set for timed advance. Drop advance will yield fractions of unequal size due to the changes in solute concentration. Turn on the pump to a predetermined speed which results in fractions of approximately 0.5 ml.

In the third method, a dense but not viscous liquid such as Maxidens (Accurate Scientific Corp., http://www.accurate-chemical.com/) is pumped to the bottom of the tube and the gradient fractionated by upward displacement. This method requires a tube sealed at the top with a syringe point connected by plastic tubing to a fraction collector.

The main advantages of using rate-zonal sedimentation to study protein complexes are that it is non-destructive, compatible with all downstream methods of analysis, and it is a highly reproducible method capable of preparative sample loadings ($\sim$6 mg 40 ml^{-1} gradient). The main disadvantage is the time (forming the gradients, centrifugation, fractionation) and instrumentation (gradient maker, peristaltic pump, ultracentrifuge and rotor, and fraction collector) requirements.

Identifying interacting partners

A downstream protocol to routinely identify putative interactors is essential for the often iterative analyses required for protein–protein characterization. With the arrival of proteomics we are confident that biochemistry-based analysis of protein association can proceed unimpeded and will no doubt experience a resurgence. Proteomics is a catch-phrase to describe the integration of high-resolution MS with search-and-match algorithms to discover the identity of a protein based upon proteolytic peptide mass alone (peptide mass fingerprinting) or peptide mass plus fragmentation (tandem MS) information (for recent review see Ahn *et al.*, 2007).

Conveniently, proteins of interest can be identified directly from SDS gels by excising the stained band and performing in-gel digestion, typically with sequencing grade trypsin (Shevchenko *et al.*, 1996). The bands on SDS gels are frequently a composite of multiple proteins with similar migration. For example, tandem MS analysis of 39 bands from a high-resolution SDS gel of a chloroplast subfraction of macromolecular structures yielded on average eight proteins per band, clearly demonstrating that SDS-PAGE by itself is insufficient to resolve protein mixtures of high to medium complexity (Phinney and Thelen, 2005). Thus if quantification is important, SDS-PAGE is only sufficient for low-complexity protein samples (fewer than 10 proteins). However, if the identity of interacting proteins is the only goal, SDS-PAGE coupled with tandem MS, particularly liquid chromatography-tandem MS (LC-MS/MS), is a facile approach suitable for any of the aforementioned strategies.

Gel-free approaches for protein identification require only the incubation of a protein sample, free in solution or bound to affinity matrices, with trypsin followed by reverse phase or multi-dimensional (strong cation exchange preceding reversed phase) LC-MS/MS analysis. Although gel-based or gel-free methods are both routinely used for protein identification, the two methods are complementary and can improve proteome coverage when employed in parallel (Katavic *et al.*, 2006).

While protein identification by MS analysis is the most common method currently used, it is certainly not the only one. Both biochemical and histochemical methods for measuring enzyme activity can be productively employed (Hall, 2004), and when the identity of a specific protein is suspected then immunoblot analysis remains the gold standard. In some instances, protein arrays can be effectively used to identify interacting proteins (e.g. Popescu *et al.*, 2007).

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Validating results

At present there are relatively few high-quality protein-interaction data available for plants (Uhrig, 2006). Several high-throughput studies of the *E. coli* and *Saccharomyces cerevisiae* interactome have been completed, and studies of *Caenorhabditis elegans* and *Drosophila melanogaster* are at the draft-interactome stage. There is, however, a concern about the utility of data from these high-throughput analyses. Very little corroboration of interaction partners has come from the two independent studies of the yeast interactome using the two-hybrid method (Ito *et al.*, 2001; Uetz *et al.*, 2000). Similarly, there was relatively little agreement between the two independent studies of the yeast interactome based upon TAP-tag/proteomic analyses (Gavin *et al.*, 2002; Ho *et al.*, 2002). Even more disturbing, there is little agreement between the two-hybrid and TAP-tag datasets. This begs the question, how does one distinguish among *bona fide* interactions, false-positive, and false-negative interactions?

Clearly, very abundant proteins have a relatively high probability of being contaminants rather than true interaction partners (Graumann *et al.*, 2004). For example, both the large and small subunits of Rubisco consistently show up as interactors with the SLT molecular chaperone proteins (W. Antoine and J.A.M., unpublished). Because *in planta* Rubisco and SLT are separated by the plastid envelope membranes, this is probably an artifactual interaction; the result of Rubisco abundance. It has been suggested that certain thresholds can be used to predict artifactual interactions. If a yeast protein is found associated with more than 20% of other proteins analyzed then this is considered an artifactual interaction (Gavin *et al.*, 2002; Graumann *et al.*, 2004; Ho *et al.*, 2002). While this might be a reasonable strategy for whole-proteome analyses, in smaller experiments it is important to specifically validate interactions rather than relying on an arbitrary assignment.

It is not adequate to validate experimental results solely using bioinformatic analyses, and, unless a protein interaction has been well-studied and described from several experimental systems, it is not acceptable to validate interaction results solely by analogy. Regardless of the method initially used to identify potentially interacting proteins, it is essential that the interactions be independently and experimentally validated (e.g. Marchand *et al.*, 2006). All systems have the potential for generating false-positive results. A good example of the validation of results can be found in Basmaji *et al.* (2006). Proteins potentially interacting with Knr4/Smi1 were isolated and identified from yeast by TAP-tag/proteomic analyses. Comparison of TAP-tag results with two-hybrid analysis revealed multiple common interacting proteins. Co-identification of the interacting partners using independent methods greatly increases confidence that the interactions are valid *in vivo*. All of the methods described herein are suitable for both discovery and validation.

Ultimately one must consider that protein interactions are constantly plastic and potentially higher order. A legitimate interaction under one physiological or developmental condition might not be observed under different conditions (Graumann *et al.*, 2004). A noteworthy example of two quite different, but equally valid, lists of interacting proteins comes from analysis of client proteins of the *E. coli* molecular chaperone GroE. Kerner *et al.* (2005) analyzed spheroplasts that were lysed and quickly depleted of ATP in order to lock client proteins into physical association with GroEL. Eighty-five obligate client proteins, a surprisingly small number, were found. Using a very different experimental system (a temperature-sensitive GroEL mutant) and growth conditions, Chapman *et al.* (2006) found a dramatically different and much larger client protein population associated with GroE. These results emphasize both the need for uniform experimental design for high-throughput analyses and the importance of low-throughput, varied, and validated methods.

Characterizing the basis of the interactions

After interacting protein partners have been identified, perhaps the ultimate strategy for validating interactions comes from biochemical and biophysical analyses. Specific protein interactions are typically mediated by one or more well-defined sequences/motifs/domains (Ingham *et al.*, 2005; Perez-Torrado *et al.*, 2006; Spaller, 2006). In some instances, a recognition domain includes a post-translational modification such as phosphorylation (Huber *et al.*, 2002). Docking of a client protein with a recognition domain is amenable to quantitative analysis by surface plasmon resonance spectroscopy (Liu *et al.*, 2007; Phillips and Cheng, 2007), isothermal titration calorimetry (Ababou and Ladbury, 2006), electron paramagnetic resonance (EPR) spectroscopy (Crane *et al.*, 2006), or a quantitative version of the two-hybrid methods more commonly used in protein discovery (Griffith and Wolf, 2002; Suo and Miernyk, 2004). Association and/or dissociation constants can be calculated using any of these methods (e.g. Liu *et al.*, 2007, Moll *et al.* 2006). Typically, the interaction domains within the sequences of two binding proteins might be predicted using bioinformatic tools, the interaction itself quantified, the domains deleted or modified by site-directed mutagenesis, and the predictions verified by a second round of analysis.

If binding is mediated by sequence features other than one or more of the known protein interaction domains, identification of the interacting sequences can be approximated by deletion analysis or screening of combinatorial peptide libraries (Eichler, 2005; Smith and Scott, 1993). As with the known protein interaction domains, results from the

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predictive analyses can be validated by mutagenesis to define the sequence(s) mediating the protein–protein interactions.

Conclusions

Herein we have described a suite of methods useful for detecting protein–protein interactions *in vitro*. The list was not intended to be all-inclusive, but rather typifies methods that the authors have found useful in their own research. Not all of the methods will yield useful information in all studies of protein interactions. A negative result obtained with one of the methods does *not* indicate that a protein has no interacting partners *in vivo*. In some, and possibly many, instances, it will be productive to use more than one of the methods described.

It is the dawn of the age of protein interactions. Using the methods cited and described, along with your own ingenuity, we encourage you to find out which partners are associated with your own favorite protein! And then to extend this knowledge so that it additionally encompasses the questions of how and why they interact?

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