

## A DELICATE TOUCH

The notion of a cell membrane serving to contain a large mixture of proteins randomly moving about in a cytosol may once have been the grade school introduction to cellular biology. However, when one considers the nature and coordination of cellular activities, it becomes evident that random collisions between proteins could not result in the spatial and temporal reaction and information-carrying cascades now known to occur in cells. Proteins must interact in very specific ways in order to coordinate nearly all cellular processes including DNA replication and transcription, RNA splicing and translation, protein modification and secretion, cell cycle control and apoptosis as well as signal transduction and gene expression. Therefore, a disruption in the interaction of proteins is likely to contribute to the onset of disease and is the reason so much research effort is focused on understanding the nuances and implications of such interactions. **By Alfred Doig**

**P**rotein-protein interactions (PPIs) occurring on the exterior of the cell membrane provide signals as to the cell's external environment. Within the cell membrane such signals are propagated by other specialized PPIs that serve to deliver the message to one or more of the compartmentalized cell structures, such as the nucleus or mitochondria, which might result in, for example, a change in gene expression or ATP production.

PPIs are very diverse but all protein interactions occur in a highly specific manner determined by structural and physiochemical properties of the interacting proteins. At the molecular level PPIs can be characterized by their binding strength (permanent or transient), specificity (specific or nonspecific), the location of interacting segments (within one or more polypeptide chains), and the degree of similarity between interacting protein subunits.

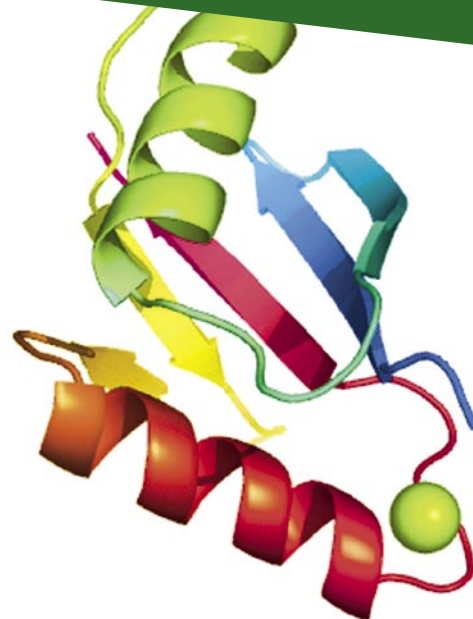
### Experimental Approaches

PPIs are affected by a number of variables including protein concentrations—as determined by protein synthesis and degradation—and the location of the interacting protein participants within the cell. In recent years, innovations in software, reagents, and instrumentation as well as improvements to experimental protocols have given a clearer understanding of the biological roles of many PPIs.

Methods available to study PPIs extend from very qualitative approaches to highly quantitative measurements. The methods of choice are determined by the nature of the experimental study and can range from the desire to discover new PPIs to determining the dissociation kinetics between well-established interacting pairs. There exist a number of *in vivo* and *in vitro* methods used to identify and characterize PPIs. The techniques are based on a variety of biological, biophysical, or physiochemical measurements and some lend themselves to high throughput format development.

### Biological Methods

If a yes/no answer regarding the significance of a suspected two-protein interaction is required, a synthetic lethality approach may be appropriate. This method involves the construction of cells containing two mutations, one in each of a suspected PPI pair. Neither of the mutations alone results in loss of cell viability, but if both should occur in the same cell, death results. Another established method used in PPI research is the two-hybrid approach, first demonstrated using yeast strains and subsequently adapted for use with mammalian and human cell lines. The yeast two-hybrid methodology utilizes a nutrient-dependent yeast strain into which separate bait and prey plasmids are introduced. One plasmid produces a known protein (the bait) with a fused DNA-binding domain (BD) fragment while the other plasmid produces a protein product in which an activation domain (AD) fragment is fused onto **continued »**



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## Protein-Protein Interactions

the so-called prey protein. The prey protein can be either a single known protein or a library of known or unknown proteins. If the bait and prey proteins bind, transcription of a reporter gene takes place, indicating the formation of a PPI.

This “bait and prey” approach has been incorporated into a variety of commercial reagent formats. **BD Biosciences** markets the BD Matchmaker Two-Hybrid System 3, an enhanced GAL4 two-hybrid system. The product uses yeast strain AH109, in which four reporter genes are integrated in the host genome. A similar system, DUAL-membrane from **Dualsystems Biotech**, is specifically designed to detect interactions involving integral membrane proteins with other such proteins, membrane-associated proteins, or soluble proteins. A human cell-based “bait-prey” PPI assay system, known as GRIP, is produced by **Biolmage** (part of Thermo Fisher Scientific). The GRIP technology is based on the translocation of human cAMP phosphodiesterase PDE4A4 and provides a high throughput method to screen for inhibitors of protein interactions.

Another in vivo method for visualizing PPIs involves use of the fluorescence resonance energy transfer (FRET) approach. In this technique, two different fluorescent molecules (fluorophores)—the donor and acceptor—are genetically fused to the two proteins of interest. Regular fluorescence occurs when the protein-bound fluorophores emit energy at the emission frequency. When the two labeled proteins interact and are stimulated by light energy at the excitation frequency for the donor fluorophore, some of this energy transfers to the acceptor, which then re-emits the light at its own emission wavelength. The result is that the donor partner in the PPI emits less light energy, while the acceptor emits more. FRET equipped microscopes and fluorescence-based cell sorter systems are used in conjunction with these FRET reagents to quantify the PPIs.

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## Isolating Protein Pairs

In studies where the isolation of proteins involved in a PPI is desired, “pull down” assays can be used. **Pierce Biotechnology** (now a part of Thermo Fisher Scientific) offers its ProFound PPI pull down kits for this application. The product is based on either GST- or His-tagged fusion proteins. The tagged fusion protein is used as the “bait” protein and the pull-down process is based on a bead-based, affinity purification technique. The captured PPIs are eluted for analysis by Western blot.

For recovering interacting proteins from mammalian cells, **Stratagene**, an **Agilent Technologies** company, produces its InterPlay Mammalian TAP System. The method is based on expression of a protein of interest fused to two affinity tags: a streptavidin binding peptide (SBP) and a calmodulin binding peptide (CBP). “A two-step tandem affinity purification protocol yields exceptionally pure and intact interacting proteins through gentle elution and elimination of a protease digestion step,” says Benjamin Pricer, the product manager for functional biology at Stratagene. “The isolated proteins,” he continues, “can be identified using Western blotting or mass spectrometry.”

## Array Screening

Protein arrays are another, more recent addition to the PPI toolbox. As in the two-hybrid approach, a “bait and prey” strategy is used. The methods involve incubating an array of surface anchored proteins with cell supernatant, washing the assay surface, and analyzing for PPIs. A variety of anchoring methods and formats is used including glass slides, polymeric beads, and chromatographic media. **Hypromatrix** supplies a general purpose PPI screening array called the AntibodyArray, which comprises membrane tethered antibodies against hundreds of well-studied proteins. The antigenic protein binds to the antibody, thus capturing any PPIs between the antigen protein and its interacting partners. Similarly, **Invitrogen's** ProtoArray Human Protein Microarray contains approximately 8,000 unique human proteins, selected from multiple gene families and arrayed in duplicate on a 1 inch x 3 inch nitrocellulose-coated glass slide. To provide maximum flexibility, these microarrays are compatible with fluorescent, chemiluminescent, radioisotopic, and other detection methods.

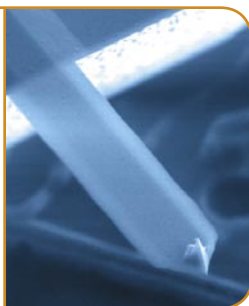
Specialty arrays for PPI screening are also available and include **Sigma-Aldrich's** Panoram Human Cancer v1 Protein Array used to screen for interactions with 130 fully functional cancer proteins. Also, **Panomics' TF Protein Array** is useful for determining how a particular protein interacts with the 140 known transcription factor proteins spotted on this array.

## Instrumentation for Bound Proteins

Over the past 15 years, specific instrumentation has evolved to measure the physiochemical properties of known interacting proteins. These instruments are based on crystal resonance, surface plasmon energy measurements, microcalorimetry, scanning tunneling microscopy, and total internal reflection fluorescence.

The principle of crystal resonance has been applied by **Attana** to develop a quartz crystal microbalance (QCM) technology to obtain label-free, real-time measurements of PPI kinetics, affinity, and specificity. QCM uses a piezoelectric effect to oscillate a crystal at its resonance frequency. This frequency shifts when molecules are added to or removed from the surface of the crystal. By attaching a specific bait protein to the biotin treated crystal surface, **continued »**

“The AFM instrument employs a sensitive probe, the deflection of which is a measure of sample surface topography.”



an interaction with an introduced prey protein can be detected due to the resulting change in protein mass.

Taking a slightly different tack, two companies, **GE Healthcare/Biacore Systems** and **Bio-Rad Laboratories**, market instrumentation based on surface plasmon resonance (SPR). These instruments use a gold foil detector surface and reflected light energy to generate surface electron charge density waves (plasmons). PPIs forming between surface-tethered proteins and introduced proteins on the detector surface interact with these plasmons resulting in measurable refractive index changes that are proportional to changes in surface mass. Stefan Löfås, chief scientist, Biacore Systems, GE Healthcare Bio-Sciences AB, explains that “the combination of label-free SPR detection, sophisticated microfluidics and a broad range of surface chemistries allows characterization of almost any type of biomolecular interaction at a very detailed level. The kinetic information obtained greatly facilitates the characterization of molecular mechanisms and biological processes.”

The introduction of higher throughput screening capacities for SPR instruments is a focus for Biacore and Bio-Rad. As Cathy Mainini, senior product manager, protein function division, Bio-Rad Laboratories, points out, “We have combined surface plasmon resonance technology with an innovative 6 x 6 microfluidic design that allows one to measure an array of 36 biomolecular interactions simultaneously. This parallel approach can generate a complete kinetic profile of a biomolecular interaction in a single experiment without regeneration.”

**TIRF Technologies’** instruments are based on total internal reflection fluorescence (TIRF). The process uses light propagating within a quartz crystal which, when it reaches an interface with a less dense aqueous solution, although fully reflected, generates an evanescent field that extends beyond the interface and into the aqueous solution. The fluorophores adsorbed, adhered, or bound to the surface will fluoresce while fluorophores in bulk solution will not. The sensitivity of the TIRF approach is reported to be 10,000 times higher than SPR-based instruments.

Based on the scanning tunneling microscopy—the inventors of which received half of the 1986 Nobel Prize in Physics—the atomic force microscope (AFM) is an imaging and measurement tool that provides a three-dimensional map of a sample’s surface. The AFM instrument employs a sensitive probe, the deflection of which is a measure of sample surface topography. With the capacity to observe and manipulate biological surfaces under physiological conditions, AFM can be used to explore biological structures at the single molecule level and measure mechanical ligand-receptor interactions with 3D resolution and sensitivity down in the piconewton ( $N^{-12}$ ) range. W. Travis Johnson, senior scientist at Agilent Technologies, explains,

“We are combining AFM with surface chemistry and bioconjugation chemistry in order to study individual, discrete ligand-receptor interactions far from equilibrium. This is increasing our understanding of how biological systems work at the single molecule level.”

### Instrumentation for Proteins in Solution

**MicroCal** has found a way to adapt ultrasensitive calorimetry to the study of PPIs. Utilizing isothermal titration calorimetry (ITC), the instrument measures the heat that is absorbed or generated when a biomolecular interaction occurs. Uniquely, the method does not require that the target protein be labeled or bound to a surface, allowing the proteins to be studied in solution in a native state. Ernesto Freire, Henry Walters Professor, Biology and Biophysics, Johns Hopkins University, reports his laboratory uses “ITC in all our projects involving protein-protein interactions. ITC not only provides the most accurate determination of binding affinity, but also it is the only technique that reveals the nature and magnitude of the forces involved in the binding process by being able to measure the binding enthalpy and entropy in a single experiment.”

### Bringing It All Together

Understanding of the physiological and disease associated importance of protein-protein interactions continues to expand. For example, researchers recently reported using a Biacore system to generate binding kinetics measurements to identify a site on the HIV-1 Env protein that may be a target for vaccine development. PPI research is moving forward aided by innovations in instrumentation, availability of reagents, and the generation of robust data sets.

PPI research is also benefitting from advances in knowledge software such as **Ingenuity Systems’** Pathways Analysis. Megan Laurance, senior scientist at Ingenuity Systems, points out “our software application contains a comprehensive network of protein-protein interactions and regulatory events [transcriptional effects, post-translational modifications, epigenetic events] manually curated from the scientific literature.” This enables researchers to put the particular PPI under investigation into a broader context of normal and abnormal cellular function. As Laurance puts it, this allows them to “rapidly understand their experimental system as a whole, based on what was detected at the protein interaction level.”

Emphasizing the importance of depending on multiple technologies, Michael Snyder of **Yale University** points out that in his laboratory “we use a number of methods including two-hybrid, protein arrays, affinity chromatography, and SPR to investigate the biological significance of PPIs. All of these methods have their inherent limitations; however, by integrating the resulting datasets we gain confidence in our understanding of the function and importance of specific PPIs.” These technologies are enabling exciting new research on the characterization of multiprotein interactions involving “scaffold” proteins responsible for bringing other proteins together so they can interact.

Continued advancements in the ability to measure the various parameters associated with PPIs coupled with knowledge-based software will undoubtedly provide the understanding required for systems biology modeling and serve to uncover new targets for therapeutic developments.

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