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An Overview of Current Methods to Confirm Protein-Protein Interactions



Kenji Miura^{1,2,*}

¹Department of Developmental Anatomy and Regenerative Biology, National Defense Medical College, Tokorozawa, Saitama, Japan; ²Central Research Laboratory, National Defense Medical College, Tokorozawa, Saitama, Japan

Abstract: Background: The research field of protein-protein interactions is interdisciplinary and specialized field that spans all aspects of biology, physics and chemistry. Therefore, in order to discuss the protein-protein interaction in detail and rigorously, it is desirable to integrate knowledge and methods of many related fields including boundary areas such as biochemistry, biophysics and physical chemistry in addition to biology, physics and chemistry.

Objective: The purpose of this review is to overview current methods to confirm protein-protein interactions. Furthermore, I discuss future prospects of methodology based on current status.

Results: It is often necessary to integrate, combine and validate multiple results from various methods to understand protein-protein interactions in detail.

Conclusion: It might be desirable for the addition of tags, labeling, and immobilization to solid phases to be unnecessary, and to obtain information on affinity, kinetics, and structure *via* the analytical method for protein-protein interactions. Therefore, I argue that novel methods based on principles that have already been sufficiently studied in physics or chemistry, but insufficiently applied to the life sciences, should be established to further develop the study of protein-protein interactions.

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1. INTRODUCTION

Proteins are synthesized based on base sequences of genes, but genes and proteins do not have a one-to-one correspondence. There are far more kinds of proteins than the number of genes. This is because splicing, processing, modification, and the formation of protein complexes all increase protein diversity.

Proteins can function as independent molecules or interact with other proteins to form functional complexes [1, 2]. The formation of protein complexes increases the kinds of functional units present and leads to greater protein diversity. The potential combinations of proteins that form complexes are enormous. However, information on what kinds of protein complexes can be physiologically formed cannot be directly ascertained from genomic information.

Therefore, it is necessary to investigate experimentally what combinations actually form protein complexes. Furthermore, it is also important to examine details, such as affinity and kinetic properties of protein-protein interactions, to understand the complicated biological processes in which they participate.

However, it might be relatively difficult to understand how to research protein-protein interactions. This is because protein interaction studies have the following characteristics. First, the research subject is proteins that constitute organisms. Second, protein interaction occurs based on the physical properties of individual proteins. Third, in order to analyze protein-protein interactions, it is necessary to use techniques based on established principles in physics or chemistry. The first characteristic is included in the category of biology, but the second and third characteristics are almost in the category of physics or chemistry. That is to say, the research field of protein-protein interactions is interdisciplinary and specialized field that spans all aspects of biology, physics and chemistry. Therefore, in order to discuss the protein-protein interaction in detail and rigorously, it is desirable to integrate knowledge and methods of many related fields including boundary areas such as biochemistry, biophysics and physical chemistry in addition to biology, physics and chemistry. It might be not easy to understand such broad and interdisciplinary approach in an integrated manner.

Under such situations, introducing many research methods in parallel and briefly seems to be meaningful for understanding and developing protein interaction researches. However, in this case, it is basically necessary to clarify what kind of principle each method is based on. Because, when looking over the science with a long-term span, it is certain that "analysis method based on principles and phe-

*Address correspondence to this author at the Department of Developmental Anatomy and Regenerative Biology, National Defense Medical College, Tokorozawa, Saitama, Japan; Tel: +81 4 2995 1754; E-mail: kmiura@ndmc.ac.jp

nomena in physics or chemistry" brings "accumulation of knowledge in science (*i.e.* progress of science)". It is also obvious that progress in fields requiring equipment based on sophisticated principles such as protein interaction research is dependent on the discovery and utilization of principles and phenomena in physics and chemistry. From the standpoint of these ideas and paradigms, in this paper, it is outlined principles that respective methods used for analysis of protein-protein interactions are based on rather than their overall aspects.

2. CURRENT METHODS TO CONFIRM PROTEIN-PROTEIN INTERACTIONS

Multiple methods are utilized to confirm protein-protein interactions (*i.e.*, the formation of protein complexes). Major methods and their characteristics are summarized in Table 1.

2.1. Pull Down Assay

Pull down is essentially small-scale affinity purification [3-5]. A protein of interest is expressed with a tag (*e.g.*, GST) and then purified with an appropriate matrix (*e.g.*, GSH agarose) that specifically binds to the tag. At this time, the protein is immobilized on the matrix. Then, the protein with matrix is incubated with a solution containing candidate interactor protein(s) such as cell lysates or purified protein solutions. If a candidate interactor protein binds with the protein of interest and forms a complex, it is possible to purify this complex to collect the matrix. The protein complex is then subjected to SDS-PAGE. The electrophoresed and separated protein can be identified by western blotting, mass spectroscopy, or protein sequencing.

2.2. Two-Hybrid Assay

This is a method for binding of two kinds of proteins in living cells, such as yeast cells [3, 6]. Plasmid DNA encoding the protein of interest (the bait) fused with the DNA-binding domain of GAL4 is transfected and expressed in cells. The bait then binds to genomic DNA of the host cell *via* the DNA-binding domain of GAL4. Then plasmid DNA encoding a candidate interactor protein (the prey) fused with the DNA-activation domain of GAL4 is also transfected into the cells. When the prey binds with the bait, proteins that are essential for survival and proliferation of the cells encoded by reporter genes locating downstream of GAL4 responsive promoter are transcribed. Therefore, when the prey binds with the bait, the cell proliferates, and when they do not bind, the cell does not proliferate on the appropriate selective medium. The protein bound with bait can be identified based on the sequence of the DNA fused with DNA-activation domain of GAL4 in cells of a colony formed when transfected cells are sparsely seeded on a plate.

2.3. Gel Filtration Chromatography

Gel filtration is a technique to separate molecules dissolved in a solution based on differences in mobility owing to their respective molecular weights [7]. In short, gel filtration is a kind of molecular sieve.

A solution containing purified protein of interest and a solution containing purified candidate interactor proteins are

mixed. Then the mixture is applied to gel filtration and the proteins are separated. If these two proteins form a complex, a peak is detected at the retention time corresponding to the molecular weight that totals each protein. It is possible to purify the protein complex by fractionating the fraction of this peak.

2.4. Isothermal Titration Calorimetry (ITC)

ITC is a method to assay whether there is an interaction between proteins by precise analysis of the thermodynamic profile [8, 9]. An interaction that occurs between protein molecules is accompanied by a change of heat balance (*i.e.*, exothermic or endothermic change). Given this, when a solution of candidate interactor protein is titrated to a solution containing the protein of interest under the condition that both solutions are isothermal with each other, it is possible to determine the presence or absence of an interaction between these proteins by very precise monitoring of the heat balance.

2.5. Förster Resonance Energy Transfer (FRET)

Förster resonance energy transfer is a phenomenon by which energy moves directly from a donor fluorescent dye to an acceptor fluorescent dye by electronic resonance without passing through an electromagnetic wave (fluorescence from the donor) when one (donor) of two neighboring fluorescent dyes (donor and acceptor) is excited. This phenomenon was discovered by Theodor Förster in 1946. In the life sciences, fluorescent proteins such as green fluorescent proteins are generally used as chromophores [10].

Two proteins, one fused with the donor fluorescent chromophore and the other with the acceptor fluorescent chromophore, are expressed and subjected to a binding assay. When the two proteins do not bind, the donor fluoresces when it is excited but the acceptor does not. If the two proteins bind, the donor and acceptor are inevitably in close proximity. When the donor is excited in this state, the fluorescence from the donor is bypassed at a certain efficiency, and the fluorescence is emitted from the acceptor by electronic resonance that directly transfers energy from donor to acceptor. In short, when FRET occurs, the intensity of fluorescence of the donor decreases, whereas that of the acceptor increases. Based on this phenomenon, it is possible to evaluate the binding of proteins by analyzing the fluorescence from the donor and acceptor.

2.6. Luminescent Oxygen Channeling Assay (LOCI)

LOCI is a method to examine protein-protein interactions by mixing two kinds of proteins immobilized on appropriate beads for the donor and the acceptor respectively and irradiating with light [11-14]. This method is also called AlphaScreen.

Two protein solutions, one with protein bound to phthalocyanine and the other to an olefinic compound, are mixed. When phthalocyanine is irradiated with 680 nm light only for a moment, a singlet oxygen is generated and spreads over a very short range because of its very short half-life. Just after irradiation at 680 nm is stopped, emitted light at 612 nm is detected. If the two proteins bind, 612 nm light is

emitted as a result of the reaction of singlet oxygens with the olefinic compound. On the other hand, if the two proteins do not bind, 612 nm light is not emitted, because singlet oxygens do not reach the olefinic compound.

It is notable that the emission wavelength is shorter than the excitation wavelength, and the excitation light is not on when the emitted light is detected. These factors therefore reduce background emission and improve sensitivity.

2.7. Reflectometric Interference Spectroscopy (RIFS)

RIFS is a method for analyzing protein-protein interactions based on the analysis of interference of white light reflected by multiple surfaces, such as an internal reference layer near the tip of a sensor chip and the surface of a protein layer immobilized on the tip [15, 16]. This method is also called BioLayer Interferometry (BLI).

The protein of interest is immobilized on the tip of the surface of the glass sensor. Then white light is irradiated onto the immobilized protein *via* epi-illumination, which is to say, from the opposite side of the surface of the sensor (also opposite from the protein), and the spectrum of the reflected wave is precisely recorded. This reflected wave is an interference (or synthetic) wave of the light reflected at the internal reference layer near the sensor distal surface and the substance (protein) that is bound to the distal surface.

Sequentially, this sensor is soaked into the buffer where a candidate interactor protein is dissolved. If the candidate interactor protein binds to the protein immobilized on the tip surface, the density (*i.e.*, refractive index) of the layer including these proteins increases. Because the velocity of light is slightly lower in material in which the refractive index is higher, the velocity of the light in a layer in which proteins form complexes becomes slightly lower, and the reflected wave reaches the sensor with a slight delay. On the other hand, arrival times to the sensor of waves reflected at the internal reference layer are constant in the presence or absence of protein complex formation. Therefore, the interference spectrum of reflected light modulates with the formation of protein complexes on the tip surface. In this way, it is possible to examine the binding of proteins by comparing and analyzing the interference spectra of reflected waves. In general, the density of the tip surface calculated from the detected interference spectrum is converted to a sensorgram, a graph describing values versus time.

2.8. Surface Plasmon Resonance Spectroscopy (SPR)

Protein-protein interactions are confirmable using the method to detect the change of conditions causing surface plasmon resonance when another protein binds to a protein that was previously immobilized on a metal surface sensor [17].

Free electrons existing in the metal film collectively vibrate as a longitudinal wave when the prism that is attached to the metal film is irradiated with light. This phenomenon is called a surface plasmon. When p-polarized light in a transverse wave is irradiated at an angle larger than the critical angle, the surface plasmon is resonant with the vibration of the irradiated light. This phenomenon is called surface plasmon resonance. When the surface plasmon resonance is ex-

cited, the light of the resonated wavelength is absorbed on the metal surface to propagate a near-field (evanescent wave), and the near-field transmits in the metal surface. On the other hand, light of a wavelength that is not absorbed is totally reflected. Therefore, there is a dark line in the spectrum of the reflected wave corresponding to a wavelength absorbed in the metal surface when surface plasmon resonance is excited. Alternatively, the shift of the incidence angle causing SPR depends on what is added to the metal surface, such as an increase of protein density. Therefore, it is possible to detect protein-protein interactions by measuring the change of conditions causing SPR, such as the shift of the angle of reflection at which the dark line is detected. The incidence angle causing SPR (*i.e.*, the angle to cause the dark line) is called resonance angle.

One side of the gold film surface sensor contacts with a buffer, and the protein is immobilized on this surface. Then, p-polarized light is irradiated on the surface opposite that where the protein is immobilized at angles around those causing SPR. The light irradiated with an incidence angle causing SPR corresponding to the density of protein immobilized on the gold film is absorbed as a near-field (evanescent wave) by the SPR, but the light at other incidence angles is totally reflected. Next, the surface on which the protein is immobilized is brought into contact with the solution containing a candidate interactor protein, and the reflected light is detected in the same way. If the candidate interactor protein binds to the protein immobilized on the surface, the incidence angle causing SPR (resonance angle) shifts accompanying the increase in density of the proteins bound to the surface. Therefore, it is possible to examine the binding of proteins by comparing the resonance angle. In general, the density of the tip surface calculated from the reflected wave is converted to a sensorgram describing values versus time.

2.9. Circular Dichroism Spectroscopy (CD)

When proteins form a complex, it is often accompanied by a conformational change of each protein. In such cases, it is possible to detect protein-protein interactions by structural analysis.

Circular dichroism spectroscopy is an analytical method for assessing the chirality of molecules in solution from the difference between the absorbance of left and right circularly polarized light. It is possible to evaluate the secondary structure of proteins by this method [18].

The CD spectra of each protein solution alone are obtained, and the ratio of alpha helices and beta sheets of each protein is estimated. Then, a CD spectrum of a mixture of both proteins is obtained, and the ratio of alpha helices and the beta sheets is again estimated. If alpha helices or beta sheets ratio of the mixed solution is different from that expected from the original two solutions, this confirms that the secondary structure changed due to forming a protein complex.

2.10. Raman Optical Activity Spectroscopy (ROA)

Scattering occurs when light is incident on a sample. Most of the scattered light has the same wavelength as the incident light; however, a small amount is different. As the light is scattered by a molecule, some of the energy of a pho-

ton is absorbed and the wavelength becomes longer, or energy from the molecule is added and the wavelength becomes shorter. This phenomenon is called Raman scattering.

A sample is irradiated with monochromatic light, and the scattered light is detected through a spectroscope. The spectrum thus obtained is called a Raman spectrum, and it reflects the vibration and structure of the irradiated molecule. In Raman optical activity spectroscopy, spectra are acquired by irradiating a sample with monochromatic left and right circularly polarized light. By analyzing these spectra, molecular structural information can be obtained [19-21]. Therefore, it might be possible to examine protein-protein interactions by comparing and analyzing the Raman spectra of single-protein solutions and their mixtures.

2.11. Small Angle X-ray Scattering (SAXS)

Small angle X-ray scattering is a method to obtain structural information of a substance by analyzing scattered X-rays within a small scattering angle when the substance is irradiated with X-rays [22, 23].

SAXS is performed separately for solutions of two proteins, and structural information is acquired. Then, SAXS is performed on a mixture of the two proteins, and structural information is again acquired. The formation of a protein complex is judged by comparing the information obtained from the independent solutions and mixed solution.

2.12. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance is a phenomenon in which a specific frequency (Larmor frequency) of an electromagnetic wave (radio wave) irradiated from outside a material is absorbed by atomic nuclei when the material is placed in a static magnetic field. The Larmor frequency slightly shifts depending on the local structure, such as local electron density around an atomic nucleus. The Larmor frequency is generally converted to a value called chemical shift for convenient description and treatment. Nuclear magnetic resonance spectroscopy is a method to analyze the structure of a molecule based on chemical shift.

A protein of interest is labeled with a stable isotope (*e.g.*, ^{13}C , ^{15}N), and an NMR spectrum of the protein solution is acquired. Then, a spectrum of a mixture of the labeled protein and candidate interactor protein is acquired. It is possible to judge structural changes and interactions by comparing these two spectra [24]. Even if a tertiary structure cannot be determined because of the difficulty of each signal exact assignment, it is still possible to examine broader spectral changes and thus acquire information on changes in tertiary structure accompanied by protein complex formation.

2.13. Cryo-Electron Microscopy (Cryo-EM)

Cryo-EM is a method to directly observe the structure of a molecule with a transmission electron microscope [25-27].

Table 1. Current methods to confirm protein-protein interactions.

Methods	Detection of Protein Binding	Screening of Interactor Protein	Purification of Protein Complex	Tag Free Analysis	Matrix (Solid Phase)-Free Analysis	Affinity Analysis (KD)	Kinetic Analysis (Kon, Koff)	Higher Structure Analysis	Index to Confirm Protein-Protein Interaction
Pull down	+	+	+	-	-	-	-	-	Band/spot by SDS-PAGE
Two-hybrid	+	+	-	-	+	-	-	-	Colony formation
Gel Filtration	+	-	+	+	+	-	-	-	Retention time
ITC	+	-	-	+	+	+	-	-	Temperature
FRET	+	-	-	-	+	+	-	-	Fluorescence
LOCI	+	-	-	-	-	+	-	-	Luminescence
RIFS	+	-	-	±	-	+	+	-	Interference spectrum
SPR	+	-	-	±	-	+	+	-	Resonance angle
CD	±	-	-	+	+	-	-	±	Absorbance of circularly polarized light
ROA	±	-	-	+	+	-	-	±	Raman scattering by circularly polarized light
SAXS	±	-	-	+	+	-	-	±	Scattered X rays
NMR	±	-	-	+	+	-	-	±	Chemical shift
Cryo-EM	+	-	-	+	+	-	-	+	Images of proteins

+: possible and suitable; ±: possible depending on conditions; -: rather unsuitable or impossible
Kon: association rate constant; Koff: dissociation rate constant; KD: dissociation constant

In addition to improvements in the performance of the electron microscope itself, this method was made possible by advances in several technologies such as electron beam detectors and image analysis methods.

A solution of the purified protein is frozen so as not to produce crystals, and two-dimensional images of protein molecules are directly acquired with a transmission electron microscope. With only single images, the resolution is low. Therefore, several thousand similar images are acquired and computed to reconstruct the original structure. By doing this, it is possible to determine the three-dimensional structure of a protein with high resolution. This method is called single-particle analysis. The formation of a protein complex can also be detected and the structure determined by this analysis.

CONCLUSION

At present, one of the main tasks of proteomics or biochemical target discovery might be the precise description of whole protein-protein interactions. Proteins are not uniformly distributed at the same concentrations in cells, often showing a locally concentrated distribution. Subcellular localizations are diverse depending on the kind of protein. Given this, the overall distribution and status of whole proteins represents an extremely complex aspect of living cells. Therefore, in addition to knowing which proteins combine with which to form protein complexes, a detailed understanding of their affinity and kinetics is also essential. It is thus necessary to integrate, combine and validate multiple

results from various methods (Table 1) to disclose protein-protein interactions in detail. At present, this might be the practical answer to the question as to which is the best approach to confirm protein-protein interactions in detail.

In the near future, however, the addition of tags, labeling, and immobilization to solid phases should become unnecessary, and information on affinity, kinetics, and structure should be able to be obtained through new, effective, and powerful analytical methods. Looking back on the history of methodology, many practical analytical methods that have been optimized for use in the life sciences have been based on well-established principles in physics and chemistry, yet took many decades to be utilized. From the analogy of the development of methodology, in order to further develop the study of protein-protein interactions, it will likely be necessary to discover or extract principles and phenomena in physics and chemistry that are not utilized yet for analysis. And it will be expected to accumulate cross correlated detailed information utilizing that novel analytical method (Figure 1).

LIST OF ABBREVIATIONS

CD	=	Circular Dichroism Spectroscopy
Cryo-EM	=	Cryo-Electron microscopy
FRET	=	Förster Resonance Energy Transfer
ITC	=	Isothermal Titration Calorimetry
LOCI	=	Luminescent Oxygen Channeling Assay
NMR	=	Nuclear Magnetic Resonance Spectroscopy
RIFS	=	Reflectometric Interference Spectroscopy
ROA	=	Raman Optical Activity Spectroscopy
SAXS	=	Small Angle X-ray Scattering
SPR	=	Surface Plasmon Resonance Spectroscopy

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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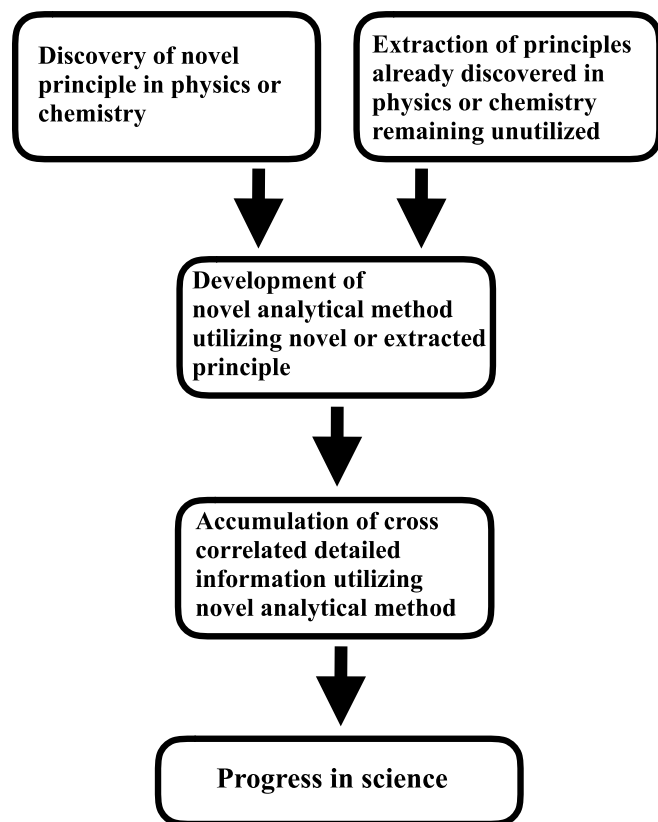


Figure 1. Stepwise flow towards progress in science.

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