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Minireview

Protein complementation assays: Approaches for the in vivo analysis of protein interactions

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ABSTRACT

The in vivo identification and characterization of protein–protein interactions (PPIs) are essential to understand cellular events in living organisms. In this review, we focus on protein complementation assays (PCAs) that have been developed to detect in vivo protein interactions as well as their modulation or spatial and temporal changes. The uses of PCAs are increasing, spanning different areas such as the study of biochemical networks, screening for protein inhibitors and determination of drug effects. Emphasis is given to approaches that rely on signals of spectroscopic nature (i.e. fluorescence or luminescence), the ones that are more directly related to bioimaging.

1. Introduction

The protein sequence universe is expanding at vertiginous speed as a result of decoding the genomes of many divergent organisms. One of the follow-ups of whole-genome sequencing projects consists in deciphering how this myriad of proteins interacts with each other. Proteins display high connectivity in the cell. In other words, proteins never act alone; on the contrary, they associate with other proteins to form stable or transient multiprotein complexes that execute a defined function. Importantly, aberrant protein interactions are related to many diseases and therefore, they have become important targets for the development of new chemical drugs. Overall, there is a requirement of techniques that allow studying protein binding as well as their specific inhibition in vivo because the cellular environment highly affects the establishment of these interactions.

Protein-fragment complementation assays (PCAs) might fulfil the above-mentioned needs because they are particularly well suited to detect protein interactions in the cell. They are based in the fusion of the hypothetical binding partners to two rationally designed fragments of a reporter protein [1]. The interaction between bait and prey proteins brings the split reporter fragments close enough to enable their non-covalent and specific reassembly followed by the recovery of its native structure and activity.

Different types of proteins have been used as protein reporters: dihydrofolate reductase (DHFR) [2], β-lactamase [3], TEV protease [4], green fluorescent protein (GFP) or its variants [5], luciferase [6], etc. Accordingly, the signal readout can be colour, cell survival or fluorescence, among others. PCAs were originally developed with DHFR, but the availability of fluorescent or luminescent proteins has significantly extended their applicability. DHFR enables the use of two different signals to report on protein binding: cell survival (using methotrexate, a DHFR inhibitor) or fluorescence (in the presence of a fluorescent substrate) [6]. On the other hand, if fluorescent [7] or luminescent proteins [8–10] are used, the signal is of spectroscopic nature. Particularly, when the reporter proteins are fluorescent proteins (FPs), we speak about bimolecular fluorescence complementation (BIFC).

Up to now, PCAs have been used in the study of in vivo protein–protein interactions (PPIs) at different levels: from the study of specific bindings in vivo to the screening of novel PPIs in different organisms, ranging from bacteria to animals or plants. Moreover, it has been demonstrated that BIFC is a very sensitive method that can be applied to measure spatial and temporal changes in protein complexes in response to drugs that activate or inhibit particular

Abbreviations: BIFC, bimolecular fluorescence complementation; PCA, protein-fragment complementation assay; GFP, green fluorescent protein; FP, fluorescent protein; DHFR, dihydrofolate reductase; PPI, protein–protein interaction; RET, resonance energy transfer; BIFC-RET, BIFC-based resonance energy transfer; FC, flow cytometry; AP/MS, affinity purification followed by mass spectrometry; Y2H, yeast two-hybrid

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cellular pathways as well as to identify compounds that interfere in protein–protein binding [11].

2. Fundamentals

The PCAs are based on the formation of a bimolecular complex when two non-active fragments of a reporter protein are brought together due to an interaction between bait and prey (where both are fused to the split domains of the reporter) (Fig. 1). The process starts with the interaction of the bait and prey proteins (complex 1). Importantly, this binding occurs in competition with alternative endogenous interaction partners present in the cell (complex 2). The interaction brings the two split fragments in proximity enabling their non-covalent reassembly, folding and recovery of protein reporter function.

The folding and activity of the protein reporter depends completely on the interaction between prey and bait. What's more, in the case of BIFC, the fluorescence signal qualitatively correlates with the interaction strength [12]. Thus, mutations that affect the binding interface cause a decrease in fluorescence emission. Therefore, BIFC can be used to map interaction surfaces in protein complexes.

A crucial requirement for the method is that the dissected fragments should not associate spontaneously in the absence of the binding proteins because this would render the method useless due to the presence of false positives. Studying the self-assembly of FPs, it has been concluded that, if they are expressed at high levels, in certain cases, the fragments can self-associate with each other regardless of the PPI [13]. This tendency depends on the fragments, the proteins fused to them and the cellular environment. Thus, it is really important to perform appropriate controls in PCAs to ensure the specificity of the detected signal. Also, to avoid self-association, it is advisable to express the protein fusions at low levels, close to those of the endogenous counterparts, if possible.

3. Strategy design

The particular design of the PCA experiment might strongly determine its results. Different aspects have to be taken into account:

3.1. Protein reporter

The selection of the protein reporter will depend on the goal of the study and the bait protein. The Table 1 summarizes the different enzymes that have been used. Particularly, DHFR has been widely used in experiments involving library selections, whereas FPs or luminescent proteins are more suitable for studying a specific protein interaction: location, dynamics, inhibition or surface mapping. The simplest reporters are FPs because the readout is directly provided by the protein fluorophore and there is no need of any substrate (as in the case of luminescent proteins or DHFR). One important feature to consider when using FPs as reporters is that their reassembly is usually irreversible [12,14,15]. However, several studies suggest that the bimolecular complex can be partially reversible [16–18]. Nevertheless, in those cases, it has not been demonstrated convincingly that the signal fading is linked to a dissociation of the fusion proteins. Besides, during in vivo experiments, other possibilities such as proteosomal degradation cannot be excluded [19]. On the other hand, one cannot disregard that BIFC provide a stable fluorescence signal that is especially useful when working with weak or transient PPIs.

3.2. Protein reporter fragments

The fragmentation pattern for the chosen protein reporter is usually well defined [5]. Each protein requires specific breaking points that allow the non-covalent protein reconstitution while minimizing the spontaneous folding.

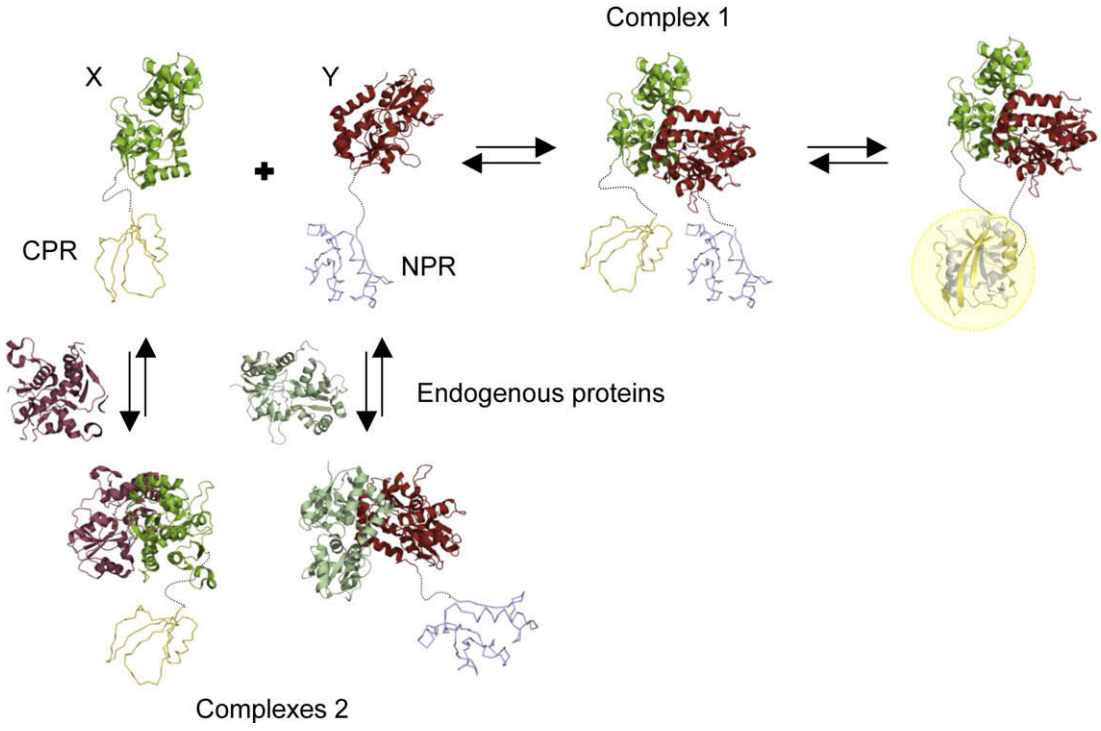

Fig. 1. Pathway for protein complementation assays (PCAs) complex formation. In PCAs each interaction partner (bait, X and prey, Y) is fused to a fragment of the reporter protein (N-terminal fragment, NRP and C-terminal fragment, CPR) and both fusions are co-expressed in the cell. The binding between X and Y (complex 1) brings the two split domains of the reporter together enabling its reassembly and recovery of its activity. It has to be taken into account that this process occurs in competition with the alternative endogenous interaction partners present in the cell (complex 2).

Table 1
Proteins used in the protein-fragment complementation assays.

Reporter protein	Readout	Application			Organism				References
		Detection of PPIs (protein–protein interactions)	Localization of PPIs	Dynamics	Lysate	Bacterial cells	Mammalian cells	Living animals	
β-lactamase	Fluorescence, absorbance	✓	×	Limited	✓	✓	✓	×	[50]
β-galactosidase	Fluorescence, absorbance	✓	×	×	✓	✓	✓	×	[51]
Dihydrofolate reductase (DHFR)	Fluorescence, cell survival	✓	✓	×	×	×	✓	×	[32,34]
Fluorescent proteins (FPs)	Fluorescence	✓	✓	Limited	✓	✓	✓	×	[52]
Ubiquitin	Reporter-gene activation	✓	×	×	×	×	✓	✓	[53]
Luminescent proteins	Bioluminscence	✓	Limited	✓	✓	✓	✓	✓	[54]
TEV protease	Reporter-gene activation	✓	×	×	×	×	✓	×	[4]

3.3. Linker

Usually the linker is a sequence of serines and glycines that provides polarity and flexibility to the protein fusion. The standard length is around 10 amino acids but it could be shorter if the bait/prey are short peptides to avoid entropic penalty upon binding [12].

3.4. Topology of the protein fusions

Fusions constructs should be designed in order to minimize steric constraints in the reconstitution of the protein reporter. Generally, it is not possible to foresee the more optimal arrangement and it should be empirically tested.

3.5. Expression system

Protein fusions need to be expressed at levels that allow detection of the regained function. However, it should be noted that high expression levels might result in non-native interactions, complex miss-localization or spontaneous reassembly of the protein reporter fragments. Therefore, the use of endogenous promoters is highly advisable.

3.6. Controls

It is important to design negative controls to ensure the specificity of the detected signal. One possibility is to substitute one of the partners with a non-binding polypeptide. Another one is to introduce mutations that reduce or eliminate the interaction, giving rise to a specific decrease or abolishment of the signal. Also, the swapping of the reporter fragments may be used as a control. In this case, no changes in the readout should be observed.

4. Detection and characterization of specific PPIs

In general, in order to study a specific protein interaction, fluorescent or luminescent proteins are chosen as reporters because they enable an easy imaging of bindings in vivo.

4.1. Split fluorescent proteins (BIFC assays)

FPs have been used to study binary interactions among a wide range of proteins in many organisms (Table 2). In the first pioneering works, strong binders were used as a proof of principle (i.e. leucine zippers interactions). But the ability of this system to detect weak interactions (around micromolar range) has also been confirmed [12,20]. This property is biologically relevant because the majority of cellular interactions are weak. Moreover, it has been shown that the fluorescence signal correlates with the interaction strength, allowing its application to map PPI surfaces by introducing site-directed or random mutations and analyzing the resulting changes in fluorescence [12].

BIFC assay also enables the visualization of multiple protein interactions [21]. The multicolour BIFC exploits the ability of fragments from different FPs to reassemble forming a protein with new and different spectral characteristics. This fact enables to simultaneously visualize multiple protein complexes in a single cell [5,22] and thus, to investigate the competition between mutually exclusive interaction partners as well as to compare their intracellular distributions [23] (Fig. 2).

On the other hand, BIFC-based resonance energy transfer (BIFC-RET) has been recently created to image and identify ternary protein complexes [24]. There are two versions of this assay. First, a fluorophore reconstituted by BIFC can be an acceptor for RET (resonance energy transfer) from a bioluminescent protein in a process of bioluminescence-RET. Alternatively, the reassembled FP can act as either acceptor/donor for RET from/to another FP (leading to fluorescence-RET). In these assays, together with the two proteins fused to the FP fragments, a third partner is linked to a fluorescent/luminescent protein. The binding between the two fist proteins reconstitutes the FP, which can act as a donor/acceptor to the other reporter if the ternary complex is formed (Fig. 2).

Furthermore, BIFC can be used to detect the presence of compounds that perturb protein binding, being able to distinguish inhibitors with dissimilar potency [25]. One key issue in this application is the practical irreversibility of the FP reassembly. As a result, the addition of competitors upon reconstitution does no effect the fluorescence emission preventing the detection of interaction

Table 2
Examples of protein interactions visualized using bimolecular fluorescence complementation (BIFC) in different organisms.

Proteins	Organism
Adenosine and dopamine receptors [55], spliceosomal proteins [56], nucleosome binding proteins [57]	Mammalian cells culture
Chaperones [25], SH3 domains [12], leucine zippers [14]	Bacteria (*Escherichia coli*)
Stomatin-like proteins (SPL) related to locomotion [58], proteins related to DNA repair [59]	Nematode (*Caenorhabditis elegans*)
Growth factors that regulate embryonic cell differentiation [60]	Amphibian (*Xenopus laevis*)
Proteins related to MicroRNA biogenesis [61], membrane proteins [62], transcription factors related to leaf senescence [63]	Plants (*Arabidopsis thaliana*, *Nicotiana benthamiana*)
Signaling proteins [16], cytoskeletal proteins [64], proteins involved in meiotic silencing [65]	Fungi (*Saccharomyces cerevisiae*, *Neurospora crassa*)

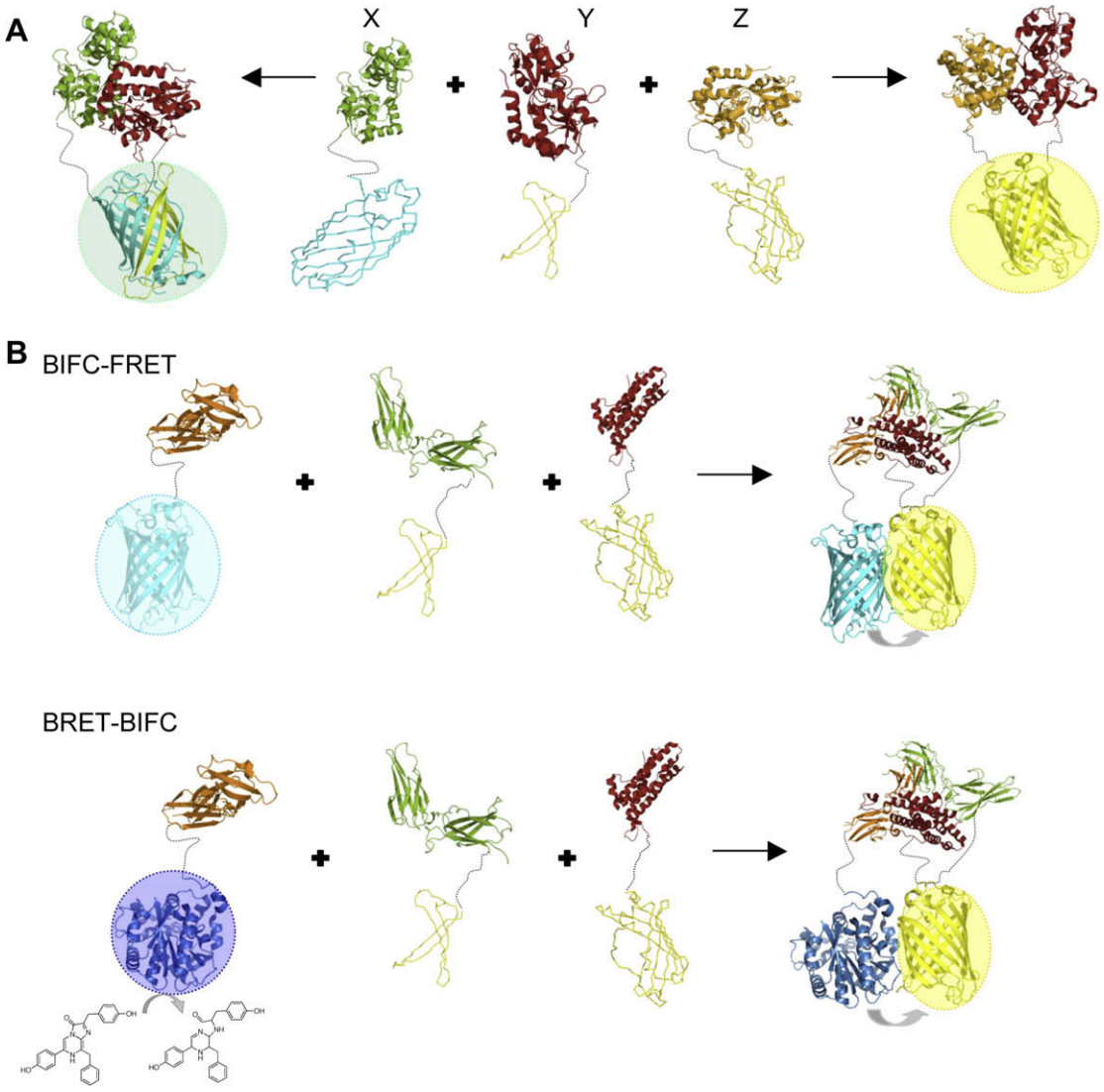

Fig. 2. Bimolecular fluorescence complementation (BIFC) application to study specific protein–protein interactions (PPIs). (A) Principle of the multicolour BIFC assay. Proteins X and Z are fused to the N-terminal fragments of two different FPs (fluorescent proteins) (cyan and yellow, respectively); and protein Y (a common binder of proteins X and Z) is fused to the C-terminal fragment of the yellow FP. The complex between X and Y can be distinguished from complex Y–Z due to the differences between the fluorescence spectra of the reassembled FPs. (B) Assays for the visualization of ternary complexes using BIFC-based resonance energy transfer (BIFC-RET). One interaction partner is linked to a FP (Cyan FP) and each of the other two partners, to a fragment of another FP (in this case, Yellow FP). If these last two proteins interact, the yellow FP reassembles and recovers its 3D structure and activity. Then, if the ternary complex is formed, a fluorescence-RET process occurs, where the cyan FP acts as a donor and the yellow FP, as acceptor. In the BRET-BIFC assay, one interaction partner is fused to a luminescent protein (*Renilla* luciferase, Rluc) whereas the other two are linked to fragments of the yellow FP. Under normal conditions Rluc oxidizes coelenterazine to colenteramide emitting light. If the three proteins interact forming a multiprotein complex, the reassembled yellow FP can act as receptor of the light emitted by Rluc in a bioluminescent-RET phenomenon.

antagonists. Therefore, in this case, the modulator needs to be already present in the cell before the expression of the binding partners. In fact, this circumstance turns to be helpful in the screening of biologically relevant inhibitors: a pre-incubation step avoids the selection of high affinity compounds that cannot penetrate or be stable enough in the cell.

4.2. Split luminescent proteins

Renilla and firefly luciferases have become the most widely used luminescent reporters. A common feature of this type of enzymes is that they need to process a substrate to generate bioluminescence. Thus, in contrast to BIFC, this compound has to be added in the system in order to obtain a signal.

One of the main advantages of using luminescent proteins is their reversible reassembly due to the presence of structurally independent sub-domains in the protein. Accordingly, the addition of an inhibitor leads to the disruption of the bimolecular complex with the concomitant signal disappearance. Therefore, PCAs with bioluminescent proteins as reporters are specially suited for the study of PPI dynamics [26,27]. The choice between fluorescent or bioluminescent proteins as reporters will depend on the objective of the study and the characteristics of the target interaction.

5. PCAs applied to high-throughput requirements

Perhaps, one of the most exciting properties of PCAs is that they can be easily implemented for the high-throughput a analysis of PPIs in living cells, becoming a very useful tool for proteomic and system biology studies, as we discuss in the next sections.

5.1. Screening of interactions

PCAs can be used as screening tool to identify potential interaction partners of a specific protein [28]. The bait protein fused to one of the reporter fragments is screened against a cDNA library fused to the complementary portion (Fig. 3). One recent development to create the library of endogenous proteins is the use of a retroviral mutagen vector that efficiently activates and tags host genes. This approach applied in BIFC has been used to successfully identify novel substrates and regulators of the serine/threonine protein kinase PKB/Akt [29].

These experiments can be followed by a second step that enables the functional validation of the detected PPIs. This is an advantage because, regardless of the employed approach, the detected bindings between bait and prey proteins are sometimes only tentative. The interaction is confirmed through the addition of an agent that perturbs the biochemical network in which the bait protein is known to be involved. Changes in PCA signal intensity or localization allow to confirm that a visualized PPI is not a false positive or an artefact.

Proteomic experiments require high-throughput approaches able to detect and analyse the generated signals in an automated and fast manner. In this sense, the coupling between BIFC and flow cytometry (FC) is a sensitive way of measuring weak PPIs; at the same time, it allows dealing with large libraries providing a fast sample rate and a qualitative evaluation of the interaction strength. Thus, BIFC–FC has found application in the screening of mutations that modulate protein interaction affinity and specificity [30].

One special feature of PCAs is that they allow a library-versus-library selection. This type of experiments is not so straightforward employing another method and it is particularly useful in protein design and to reveal structural/functional relationships. For example, it was used in bacteria to screen two libraries for leucine zipper forming peptides using DHFR as reporter [31].

On the other hand, PCAs have also been applied to a genome-wide screening in *Saccharomyces cerevisiae* using DHFR as split reporter and cell growth as signal readout [32]. Particularly, it has been employed a mutated version DHFR that it is not sensitive to the inhibitor methotrexate. When this drug is added to growth media, only the cells expressing an interaction pair will survive. Particularly, 2770 interactions have been detected identifying new binding pairs and discovering an unknown sub-space of the yeast interactome.

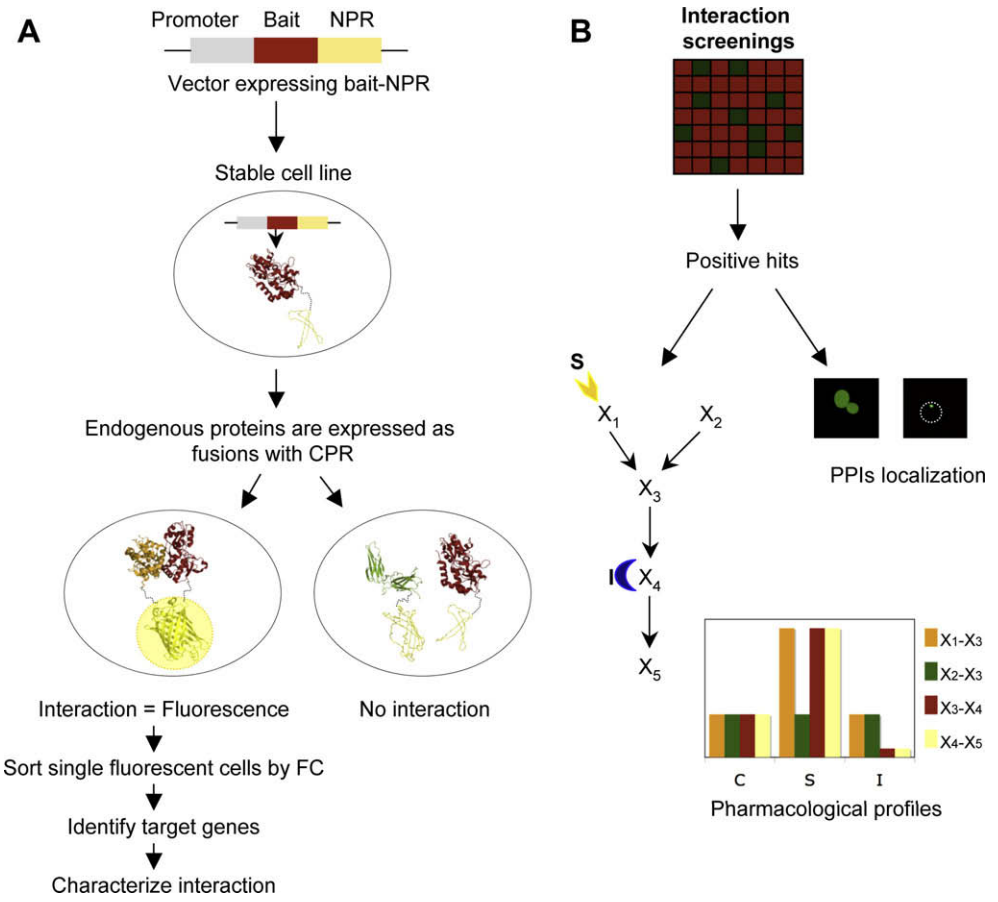

Fig. 3. High-throughput application of protein-fragment complementation assays (PCAs) to study protein interactions. (A) Procedures of a screening for interaction partners using PCAs. The bait protein is fused to a fragment of the reporter protein (in this case, the N-terminal fragment, NPR). In a host cell line, the protein fusion is stably expressed under preferably an endogenous promoter. Afterwards, employing for example a retroviral mutagen vector, the cells are induced to express also endogenous proteins linked to complementary protein fragment (CRP). Cells co-expressing an interaction pair could be isolated by FC due to their fluorescence. After a cell sorting process, the target genes can be sequenced and identified. (B) Strategy for inferring pathway organization using PCAs. First, all the interaction pairs are identified using PCAs: each protein supposed to be involved in the pathway is screened against the other ones. The results are summarized in a matrix: a red square means a negative result (no interaction) and a green one corresponds to an interaction. All the positive hits are afterwards subjected to two different analyses. First, the subcellular locations of the PPIs are imaged using microscopy. Second, the interactions are tested for responses to compounds that perturb the network. For example, the addition of a stimulator (S) will increase the PCA readout in all the interactions that compose that pathway. And an inhibitor (I) will decrease the signal in all the bindings situated downstream of its target. The data derived from these experiments enable to create a pharmacological profile of each interaction and even to deduce the pathway organization.

5.2. Studies of biochemical networks

If the bait protein is involved in cellular trafficking or in a specific metabolic route, PCAs could provide information to infer the pathway organization or, additionally, to detect unknown connections between different pathways [33]. First, a screening of PPIs between proteins supposed to be involved in a specific pathway needs to be performed. The results are then represented in a matrix that summarizes the positive (binding) and negative (non-binding) outcomes. The positives clones (i.e. cells expressing a interacting pair) are selected for further analysis. Particularly, they are treated with drugs known to target the studied pathway. The approach allows to generate a pharmacological profile that helps in the understanding of the pathway organization (Fig. 3). A specific PPI inhibitor will impinge on all the interactions situated downstream the cascade without affecting the ones upstream of its point of action. In addition, with a fluorescent readout, changes in the localization of the interaction pair upon upstream drug action can also be imaged. This strategy has been used to study a signal transduction pathway involved in the control of initiation of eukaryotic translation as well as its linkage with another pathway controlled by a kinase. Importantly, the data gave rise to a detailed representation of the pathway including both the PPIs and their response to specific antagonists [34].

5.3. Identification of drug effects

On the other way around, PCA can be used to undercover the specific effect of pharmaceutical compounds by measuring the spatial and temporal changes in protein complexes in response to drugs that act over particular pathways.

A foundational study has been reported by MacDonald et al. [35]. First, different PPI pairs were chosen as 'sentinels' of different biochemical pathways (with a pharmacological interest or involved in essential cellular functions) and fused to FP fragments. The strategy is based in the above-mentioned principle that drugs having an activity on another protein complex could also affect the downstream BIFC labelled complex due to a cascade effect. Using microscopy, changes in protein complexes were measured directly in cells grown on micro plates. The signal was extracted using different algorithms that quantify variations in intensity or localization of the fluorescence. Finally, the effect of each drug on each particular BIFC assay was subjected to hierarchical clustering. The data allow inferring the mechanism of action of novel drugs enabling a further optimization. Also, in principle, structure–function relationships could be established between conformationally unrelated chemical compounds. Overall, this strategy promises to be of great help to enhance the productivity of drug-discovery research.

5.4. Screening of PPIs inhibitors

The potentiality of PCAs in the screening of compounds that interfere with PPIs has been proved using as reporters β-galactosidase [36] or FPs [19]. If an inhibitor of the target interaction is present, the recorded signal decreases in correlation with its inhibitory potency. For example, it has been applied in the definition of the active site of a peptidic inhibitor [25] or in the identification of antagonist receptors in libraries of around 30 000 compounds [37].

It has to be taken into account that the use of PCAs entails the in vivo screening of biologically relevant antagonists. In the conditions of the experiment, the compounds selected as good inhibitors are the ones displaying a high affinity for its target and high cell penetrability and stability in the harsh cellular environment.

6. Advantages and disadvantages of PCAs

PCAs have some features that are advantageous in comparison with alternative and widespread techniques to detect and identify PPIs. The use of FPs or other enzymes that can provide fluorescence as signal readout enables a direct detection of PPIs. Like PCAs, fluorescence-RET experiments also use fluorescence emission as signal [38] and have been successfully coupled to FC [39]. With the fluorescence-RET technique, complex dynamics can be also analyzed due to the reversibility of the interaction between reporter proteins. But, in contrast, it has lower sensitivity: the detection of the signal can be difficult due to background fluorescence resulting from direct acceptor excitation. This problem is avoided in PCAs where the detection of enzymatic activity does not occur in the absence of the interacting partners if the experiment is properly designed [12].

Two important pros of PCAs are their applicability to high-throughput experiments and their ability to work in vivo. Therefore, the monitored PPIs are established in the endogenous background. This is of basic importance because many interactions are dependent on post-translational modifications or on the presence of additional partners. PCAs share with phage display technology [40] its high-throughput sample rate as well as the ability to distinguish between protein binders with different specificity and/or affinity. In comparison to PCAs, phage display technique has important limitations like its difficult application to weak interactions and its in vitro context.

Like PCAs, yeast two-hybrid (Y2H) permits in vivo genome-wide screening for weak PPIs [41], but the system requires the nuclear importation of the fusion proteins. In comparison, PCAs work in all the cellular compartments tested so far. In addition, Y2H assays are limited to the study of binary interactions whereas PCAs can be applied to study ternary protein complexes, the competition for the binding to a specific protein or the subcellular localization of PPIs.

Affinity purification followed by mass spectrometry (AP/MS) [42] has been widely used to address PPI networks. In this approach, interactions take place in the cell but the need for cellular disruption and stringent purifications steps might perturb native complexes and preclude the detection of weak interactions. In a recent paper, a comparative quality study of the yeast interactome datasets obtained using various methods has been performed [43]. It proposes that the Y2H (and to some extent, other methods to detect binary interactions like PCAs) and AP/MS are essential tools in the study of the interactome because the data obtained from both approaches is complementary and fundamental for a comprehensive representation of the PPI networks. In particular, it is found that interactome datasets obtained by detecting binary interactions are significantly enriched in transient interactions.

7. Perspectives

The present review exemplifies how PCAs are increasingly being adopted by the 'interactomic' community as standard methods to detect and characterize PPIs in the cell. The approach is extremely flexible and, as a consequence, PCAs applications are spreading to unsuspected areas of research such as the visualization of cellular processes involving other biomolecules, such as interactions between proteins and RNA [44,45] and likely, in a near future, to the analysis of lipids or sugars [21].

An outcome of massive proteomic studies and the deciphering of PPI networks is that both the traditional concept of one protein/one function and the assumption that drugs are always specific against a single protein target are not longer valid. The protein function is now considered in the context of systems biology

framework. It is clear that drugs can interact with more than one target, disturb other cellular functions or cause feedback reactions in the targeted pathway. The ability of PCAs to detect these undesired side reactions is expected to become very useful in the drug-discovery pipeline.

The establishment of PPIs is a dynamic phenomenon regulated by the cellular environment. Most structural biology methods only permit to capture a static view of the architecture of multiprotein complexes. In the next future it is foreseen that the structural resolution of a multiprotein complex by experimental or computational approaches would be combined with PCA experiments either to confirm the new experimental structures or the homology models [46].

Fortunately there is still much room to improve the characteristics of PCAs. Particularly, it is important to improve some properties of FPs like their kinetics of folding or chromophore maturation [47]. Another important aspect is to diminish the self-assembly tendency of the protein fragments. Also, it has to be taken into account that, for live-cell imaging in vivo, spectral variants with long-wavelength fluorescence emission have to be developed [48]. Finally, improvements in the sensitivity and spatial resolution of fluorescence imaging will help to overcome the uncertainty of the microscopy techniques [49].

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