Chapter 9
Engineered Split Reporter Systems for Molecular Imaging of Protein–Protein Interactions in Living Subjects

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9.1 Significance of Protein–Protein Interactions

Proteins perform cellular functions primarily as components of complexes. We now appreciate that the cell is not a simple aqueous solution, but instead a dense gel of interacting proteins forming the basis of phenomena at almost every level of cell function, in the structure of sub-cellular organelles, the transport machinery across biological membranes, packaging of chromatin, the network of sub-membrane filaments, muscle contraction, signal transduction, and regulation of gene expression, to name a few [1, 2]. Other interacting protein complexes work as components of cellular machines, such as ribosomes that read genetic information and synthesize proteins. Indeed, a frequent theme pervading biological investigation is that the great majority of proteins generally operate as constituents of complexes that contain other macromolecules, and together, carry out specific biological functions and that networks of such interactions (interactomes) connect multiple, different cellular processes [3].

Protein–protein interactions (PPIs) have been the object of intense research for many years because of their importance in development and disease. Many human diseases can be traced to aberrant PPIs either through the loss of an essential interaction or through the formation of an abnormal protein complex at an

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inappropriate time or location involving endogenous proteins, proteins from pathogens, or both [4].

A meticulous characterization of PPIs is necessary for a thorough understanding of cell function. This characterization includes, but is by no means limited to, the determination of the three-dimensional structures of these molecules [5]. Examples of the structures of a few protein interaction motifs involved in cell signaling provide an idea of the beauty and diversity of protein structure. In addition to structural considerations, the dynamic and energetic properties of these systems reveal the exquisite subtlety involved in biological specificity and control [5]. Noninvasive molecular imaging of PPIs in living subjects offers another dimension for investigating and characterizing these all important intracellular events.

9.2 Methods to Study Protein–Protein Interactions

It is useful to generate many different classes of information about proteins [6]. For any given protein, these classes of knowledge would include the following: (1) structure and sequence, (2) evolutionary history and conservation pattern, (3) expression profile, (4) intracellular localization, (5) forms of post-translational regulation to which a protein is subject, and (6) the other cellular proteins with which the protein associates. All the first five points together contribute to the determination of the sixth, and determination of the profile of PPIs is an extremely important step toward the ultimate goal of identifying the functional significance of the activity of any given protein in a cell [6].

Techniques to provide classes of information regarding PPIs fall into three categories [6]: first, there are techniques to identify every possible interacting set of proteins for a given protein of interest. Current research aims to isolate and structurally characterize all the proteins that exist in the cell. Importantly, PPIs are now considered to be so vital to cellular function that one of the first experiments performed on a protein may be a search for its interaction partners [2]. As of May 2013, 42,737 out of 83,860 proteins in the Protein Data Bank were of known protein–protein complexes; this being up from 26,069 out of 49,279 proteins in October 2008, from a variety organisms, of assemblies involving two or more protein chains. Just how many complexes exist in a particular proteome is not easy to deduce because of the different component types (e.g., proteins, nucleic acids, nucleotides, and metal ions) and the varying life spans of the protein complexes (e.g., transient PPIs, such as those involved in signaling, and stable interactions, such as in the ribosome). Until recently, the most comprehensive information about PPIs was available for the yeast proteome, consisting of approximately 6,200 proteins [7, 8]. In yeast, there are about nine protein partners per protein, although not necessarily all direct or interacting at the same time. The human proteome may have an order of magnitude more complexes than the yeast cell [7, 9].

The second set of techniques is used in circumstances where interacting proteins have been defined, and the goal is to detail the biological function and impact

of their interactions, i.e., to establish physiological significance [6]. In this case, it is essential to be able to study the interaction under conditions that correspond as closely as possible to the endogenous situation. In its current role, noninvasive strategies for molecular imaging of PPIs in living subjects fit within this second category of techniques, and the advantages of these approaches are discussed below. Finally, there are techniques to devise high-throughput methodologies to identify agents that modulate a known and well-characterized interaction in desirable ways [10].

Several technologies, grouped together under the term "proteomics" (a term introduced in 1995 by Wasinger et al. [11]), have emerged with the common objective of studying protein function at the scale of an entire pathway, a whole cell, or even a whole organism. Proteomic analyses encompass large-scale studies of PPIs or complexes to establish comprehensive protein interactomes, the global examination of protein expression profiles and, more recently, of protein post-translational modifications [12]. Many experimental techniques, e.g., co-immunoprecipitation, bimolecular fluorescence complementation, fluorescence resonance energy transfer, label transfer, yeast two-hybrid screen, in vivo crosslinking of protein complexes using photo-reactive amino acid analogs, tandem affinity purification, chemical crosslinking, quantitative immunoprecipitation combined with knock-down, dual polarization interferometry, protein–protein docking, static light scattering, MALDI mass spectrometry, strep–protein interaction experiments, surface plasmon resonance, fluorescence correlation spectroscopy (many of these techniques are reviewed elsewhere [1, 13] have been developed and studied using intact cells and cell extracts to study PPIs and to facilitate these proteomic endeavors. Each of these analytical systems has its own merits and demerits, as reviewed previously [13, 14]. Clearly, one aim of proteomics is to identify which proteins interact. While the molecular imaging and study of individual PPIs (that have already been identified as such) might appear to fall outside the scope of proteomic endeavors, it is also important to note their complementary roles. Indeed, a prime challenge in the future is to conduct targeted studies of proteins of interest (including noninvasive molecular imaging analysis) while considering the larger context of whole organismal function and conversely to carefully validate systematic large-scale models of organismal function through individual test cases [6].

9.3 Advantages of Noninvasive Molecular Imaging of Protein–Protein Interactions

The overall modification of existing in vitro and cell culture-based experimental assays to study PPIs in living small animal models of disease is dependent on the challenging task of adapting them so that signals can be noninvasively detected from the exterior of living subjects upon the cellular or sub-cellular interaction of two proteins of interest. Only over the last decade has it been possible to develop

such methods as a result of the true explosion in availability of noninvasive small animal imaging technologies and the rapidly expanding field of molecular imaging, allowing signal detection from deep tissues within a living subject. We previously reviewed in detail the many advantages afforded by molecular imaging in living subjects (such as assessment of whole-body phenomena, repeatability, functionality, and quantification) [15]. One subset of molecular imaging techniques comprises reporter gene expression imaging. This represents an "indirect" imaging method involving multiple components, entailing the use of a pre-targeting molecule (an imaging reporter gene) that is subsequently activated upon occurrence of a specific molecular event. Following this, a molecular probe (a substrate or a ligand) specific for the activated pre-targeting molecule (an enzyme or receptor) is often needed (but not for fluorescent reporter proteins) and used to image its activation [16]. An important feature of reporter gene imaging techniques is their particular versatility, which allows them to be adapted for imaging diverse PPIs in intact living subjects, as outlined below, and as also reviewed by our group [14, 17, 18], and others [19–27].

The ability to noninvasively image PPIs has important implications for a wide variety of biological research endeavors, drug discovery, and molecular medicine. In particular, the visual representation, characterization, quantification, and timing of these biological processes in living subjects could create unprecedented opportunities to complement available in vitro or cell culture methodologies, in order: 1) to accelerate the evaluation in living subjects of novel drugs that promote or inhibit active homodimeric, heterodimeric, or multimeric protein assembly, and 2) to characterize more fully known PPIs (e.g., the reasons for, and the factors that drive their association) in the context of whole-body physiologically authentic environments [28].

9.4 Split Reporter Gene Imaging of Protein–Protein Interactions

The three general methods currently available for imaging protein–protein interactions in living subjects using reporter genes are as follows: a modified mammalian two-hybrid system, a bioluminescence resonance energy transfer (BRET) system, and split reporter protein complementation and reconstitution strategies. Table 9.1 outlines the chronological developments of molecular imaging strategies for the detection of PPIs in living animals to date.

Several areas of investigation are required to further refine the use of the modified mammalian two-hybrid system for noninvasive imaging of PPIs confined to the nucleus. As well as further quantitative and kinetic evaluations (e.g., characterizing the ability to follow interactions over time based on the half-life of the reporter protein(s) that are transactivated), studies are needed to optimize the choice of transactivator as well as the choice of promoters and levels of fusion

Table 9.1 Showing chronological developments of molecular imaging strategies for detection of protein–protein interactions in living animals

Years	Authors	Area of PPI study	Imaging assay	Reporter used	References
2002	Ray et al.	Development of new assay	Two-hybrid system	Fluc	[68]
2002	Luker et al.	Development of new assay	Two-hybrid system	TK and Fluc	[69]
2002	Paulmurugan et al.	Development of new assay	Split reporter complementation and reconstitution	Fluc	[31]
2003	Luker et al.	PPI between p53 and large T antigen of SV40 virus	Two-hybrid system	TK and Fluc	[70]
2004	Paulmurugan et al.	Rapamycin modulation of FRB and FKBP12 PPI	Split reporter complementation	Rluc	[55]
2004	Massoud et al.	Homodimeric PPIs	Split reporter complementation	Rluc	[28]
2004	Kim et al.	Protein nuclear transport	Split reporter reconstitution	Rluc	[45]
2004	Luker et al.	Development and applications of new split reporter fragments	Split reporter complementation	Fluc	[22]
2005	Paulmurugan et al.	Development of self-complementing split reporter fragments	Split reporter complementation	Fluc	[54]
2005	Paulmurugan et al.	Development of a fusion protein approach to image drug modulation of PPIs	Split reporter complementation	Rluc	[58]
2005	De et al.	Development of a new assay	BRET	Rluc (and GFP variant)	[71]
2005	Kim et al.	Detection of stress-related corticosterone level increases	Split reporter reconstitution	Rluc	[46]
2006	Kanno et al.	Detection of protein release from mitochondria to cytosol	Split reporter reconstitution	Rluc	[47]
2007	De et al.	Further developments of an assay	BRET	Rluc (and GFP variant)	[72]
2007	Zhang et al.	Detection of Akt kinase activity	Split reporter complementation	Fluc	[52]
2007	Paulmurugan et al.	Detection of mutiprotein PPIs	Split reporter complementation	Fluc and Rluc	in text
2008	Choi et al.	PPI between HIF-1 alpha and VHL	Split reporter complementation	Fluc	[50]

(continued)

Table 9.1 (continued)

Years	Authors	Area of PPI study	Imaging assay	Reporter used	References
2008	Chan et al.	Detection of HSP90 inhibitors	Split reporter complementation	Rluc	[59]
2008	Zhang et al.	Enhanced detection of Akt kinase activity	Split reporter complementation	Fluc	[53]
2008	Luker et al.	Activation of the chemokine receptor CXCR4	Split reporter complementation	Fluc	[73]
2008	Pichler et al.	Development of a universal transgenic reporter mouse for PPI detection	Two-hybrid system	Fluc	[74]
2009	De et al.	Further developments of an assay	BRET	Rluc (and GFP variant)	[75]
2009	Chan et al.	Detection of protein phosphorylation mediated by protein kinases	Split reporter complementation	Fluc	[51]
2010	Pan et al.	Monitoring of rapamycin pharmacodynamics	Two-hybrid system	Fluc	[76]
2010	Massoud et al.	Development of a PET-based split reporter	Split reporter complementation	TK	[65]
2011	Dragulescu-Andrasi et al.	Detection of deep seated PPIs	BRET	Rluc	[77]

Fluc Firefly luciferase
Rluc Renilla luciferase
TK Herpes simplex virus type-1 thymidine kinase
GFP Green fluorescent protein
FRB FK506-binding protein (FKBP12) rapamycin-binding domain
FKBP12 FK506-binding protein
PPI Protein–protein interaction
BRET bioluminescence resonance energy transfer
HIF-1alpha hypoxia-inducible factor-1alpha
VHL von Hippel-Lindau tumor suppressor
HSP90 Heat shock protein 90
Akt Enzymes that are members of the serine/threonine-specific protein kinase family. Akt was originally identified as the oncogene in the transforming retrovirus, AKT8

proteins. This strategy will not be discussed further in this chapter; and a separate chapter dedicated to BRET imaging can be found elsewhere in this book.

In certain circumstances, functional proteins can assemble from one or more polypeptide fragments, with the occurrence and efficiency of assembly commandeered into a strategy to measure real-time PPIs. Indeed, synthetically separated fragments of some enzymes can reconstitute functionally active protein particularly if the interaction is helped along by fusion of the enzyme fragments to

strongly interacting moieties. Thus, in the "split protein" strategy, a single reporter protein/enzyme is cleaved into N-terminal and C-terminal segments; each segment is fused to one of two interacting proteins (X and Y). Physical interactions between the two proteins X and Y reconstitutes the functional reporter protein leading to signal generation that can be measured. This split protein strategy can work either through protein-fragment complementation assays (PCA), or intein-mediated reconstitution assays. In the former, noncovalent assembly of the reporter protein occurs, and in the latter case, reconstitution of the full reporter protein occurs through covalent bonding. To date, several reporter proteins (e.g., β-lactamase, β-galactosidase, ubiquitin, dihydrofolate reductase, firefly luciferase [Fluc], Renilla luciferase [Rluc], Gaussia luciferase [GLuc], Click beetle luciferase [CBLuc], green fluorescent protein) have been adapted for split protein strategies by finding various split sites for each reporter protein [29–32]. If a full-length reporter can be imaged in living subjects, and this reporter can be appropriately split, then the split reporter assay could possibly be used to noninvasively image PPIs. The appropriate split point should lead to two fragments that do not have significant affinity for each other and yet when brought together (through interaction of the two proteins being studied for their mutual affinity) lead to detectable signal.

The principle of the PCA strategy for detecting PPIs was first demonstrated by Pelletier et al. using the enzyme dihydrofolate reductase (DHFR) [33], following inspiration from a 1994 paper by Johnsson and Varshavsky [34] describing what they called the "ubiquitin split protein sensor." In all PCAs, splitting a specific reporter protein into two distinct fragments abolishes its function. Bringing the two fragments back together in a controlled manner then restores functional activity [35]. Selected fragments of many proteins can associate to produce functional bimolecular complexes [36]; the PCA system can therefore be generalized for a number of enzymes for the detection of PPIs, examples including DHFR, glycinamide ribonucleotide (GAR) transformylase, aminoglycoside, and hygromycin B phosphotransferases, all reviewed by Michnick et al. [35], *Escherichia coli* TEM-1 β-lactamase [29, 37], green fluorescent protein and its variants [36], and the molecular imaging reporters Fluc [31] and Rluc [30, 38]. A review of this subject was also published recently by Shekhawat and Gosh [39].

9.4.1 Intein-mediated Reconstitution Assays

These assays are based on the restoration of the full reporter protein through covalent bonding. Inteins have been defined as protein sequences embedded in-frame within a precursor protein sequence and excised during a maturation process termed protein splicing [40]. Protein splicing is a post-translational event involving precise excision of the intein sequence and concomitant ligation of the flanking sequences (N- and C-exteins) by a normal peptide bond [41]. Inteins are intervening DNA sequences that are not present in the mature gene product as a result of splicing at the protein level instead of at the RNA level. In 1998, it was

discovered that the gene for the catalytic α subunit of the replicative DNA polymerase III from *Synechocystis* sp. PCC6803 (*Ssp*) is encoded in two segments *dnaE-n* and *dnaE-c* [42]. Inteins represent a potentially powerful means of protein manipulation, because two peptide bonds are broken, and a new peptide bond is formed during the protein splicing process. Protein splicing is an exceedingly complex self-catalyzed process that requires neither cofactors nor auxiliary enzymes. It requires no source of metabolic energy and therefore involves bond rearrangements rather than bond cleavage followed by re-synthesis. The elucidation of the reaction steps involved in protein splicing has made it possible to modulate the reactions by mutations and to design proteins that can undergo highly specific self-cleavage and protein ligation reactions. An intein can be viewed as an enzyme whose substrate is the adjacent amino acid residues in the two exteins to which it is linked.

Ozawa et al. [43] initially demonstrated that Fluc can be split between amino acid positions 437 and 438 and used with inteins (DnaE) in a reconstitution strategy to detect insulin-induced interaction of phosphorylated insulin receptor substrate 1 (IRS-1) and its target N-terminal SH2 domain of phosphoinositide-3 kinase (PI-3 K) in a cell culture assay. Upon interaction of the two proteins, the two DnaE fragments are brought close enough to fold together and initiate splicing and linking of the two Fluc halves with a peptide bond. The *Fluc* gene has to be rationally dissected so that each half of Fluc is inactive. After ligating the Fluc fragments together, the resultant mature Fluc recovers its bioluminescence activity [44].

We subsequently reasoned that it may be possible to split Fluc and use split reporter complementation *without* inteins. We therefore studied PCA and intein-mediated reconstitution of Fluc fragments and found that a complementation strategy was as sensitive as the intein-mediated reconstitution strategy under the conditions tested [31]. Thus, we demonstrated for the first time the feasibility of imaging PPIs using split reporters in small living animals. We studied a PCA based on split Fluc (cleaved into two fragments nFluc: residues 1–437; and cFluc: residues 438–550), using the interaction of Id and MyoD as test proteins [31].

Subsequently, Kim et al. [45] developed a genetically encoded bioluminescence indicator for monitoring and imaging the nuclear trafficking of target proteins in vitro and in vivo. The principle is based on reconstitution of split fragments of Rluc by protein splicing with a DnaE intein. A target cytosolic protein fused to the N-terminal half of Rluc is expressed in mammalian cells. If the protein translocates into the nucleus, the Rluc moiety meets the C-terminal half of Rluc, and full-length Rluc is reconstituted by protein splicing. They demonstrated quantitative cell-based in vitro sensing and in vivo imaging of ligand-induced translocation of androgen receptor, which allowed high-throughput screening of exogenous and endogenous agonists and antagonists of this receptor.

The same authors used a similar approach to noninvasive molecular imaging of physical and emotional stress by developing a method for detecting physiological increases in the endogenous corticosterone caused by exo- and endogenous stress in living animals [46]. They constructed a pair of genetically encoded indicators composed of cDNAs of glucocorticoid receptor (GR), split Rluc, and a DnaE

intein. The GR fused with C-terminal halves of Rluc and DnaE was localized in the cytosol, whereas a fusion protein of N-terminal halves of Rluc and DnaE was localized in the nucleus. If corticosterone induces GR translocation into the nucleus, the C-terminal Rluc meets the N-terminal one in the nucleus, and full-length Rluc is reconstituted by protein splicing with DnaE. Cell-based methods provided a quantitative bioluminescence assay of the extent of GR translocation into the nucleus. The authors further demonstrated that the indicator enabled noninvasive imaging in mice subjected to two different types of imposed stress: a forced swimming and metabolic perturbation caused by 2-deoxy-D-glucose. This stress indicator should be valuable for screening pharmacological compounds and in studying mechanisms of physiological stress.

Kanno et al. [47] also developed a genetically encoded bioluminescence indicator for monitoring the release of proteins from the mitochondria in living cells. The principle of this method is based on reconstitution of split Rluc fragments by protein splicing with a DnaE intein. A target mitochondrial protein connected with an N-terminal fragment of Rluc and an N-terminal fragment of DnaE is expressed in mammalian cells. If the target protein is released from the mitochondria toward the cytosol upon stimulation with a specific chemical, the N-terminal Rluc meets the C-terminal Rluc connected with C-terminal DnaE in the cytosol, and thereby, the full-length Rluc is reconstituted by protein splicing. The extent of release of the target fusion protein was evaluated by measuring activities of the reconstituted Rluc. To test the feasibility of this method, the authors monitored the release of Smac/DIABLO protein from mitochondria during apoptosis in living cells and mice. Their method allowed high-throughput screening of an apoptosis-inducing reagent, staurosporine, and imaging of the Smac/DIABLO release in cells and in living mice. This rapid analysis may be used for screening and assaying chemicals that would increase or inhibit the release of mitochondrial proteins in living cells and animals.

The split-intein system generally suffers from slow kinetic response rates posing problems for quantitative interrogation of reversible biochemical reactions, drug-induced protein associations, or shifts in equilibrium states of interacting proteins. The truly important aspects of studying PPIs in living cells or animals lie in the ability to do so in real time. The inevitable delay in the ability to detect an interaction using the split-intein strategy can be attributed to the time required for the splicing reaction. While this may not be a factor for slow reactions occurring over long time frames, numerous drugs, chemicals, and natural ligands exert their effects in seconds to minutes. The system also exhibits a high false-positive rate on account of the split-intein fragments being so small that at times it is believed they act merely as simple linker proteins, thus limiting the quantification of protein interactions.

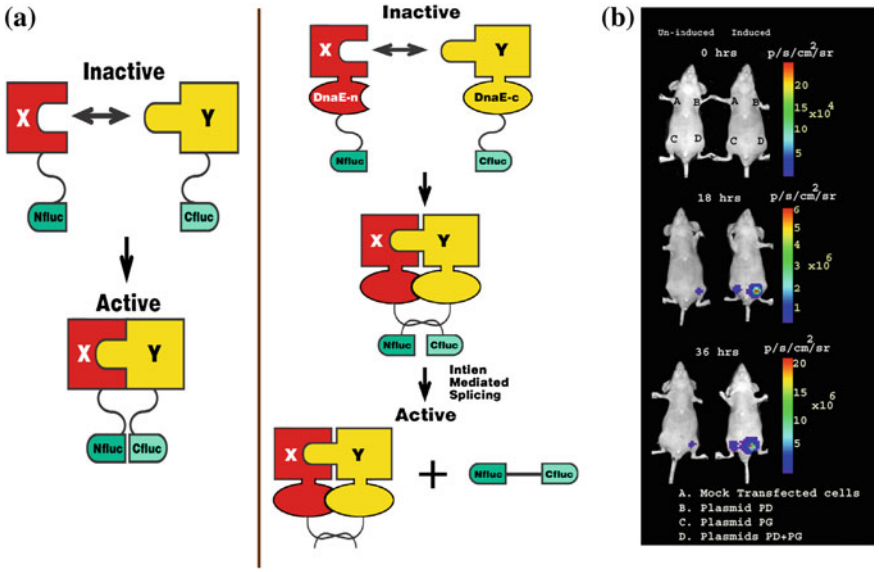

Fig. 9.1 Schematic diagram of the split reporter–based complementation strategy used to optically image PPIs in living mice. The N-terminal half of firefly luciferase is attached to protein X through a short peptide linker FFAGYC, and the C-terminal half firefly luciferase is connected to protein Y through the peptide linker CLKS. Interaction of proteins X and Y recovers Fluc activity through protein complementation (Fig. 9.1a). In vivo optical cooled CCD camera imaging of mice carrying transiently cotransfected 293T cells for the induction of the complementation-based split-luciferase system. All images shown are the visible light image superimposed on the optical CCD bioluminescence image with a scale in p/sec/cm^2/sr. Mice were imaged in a supine position after IP injection of D-Luciferin. (Fig. 9.1b). A set of nude mice was repetitively imaged after subcutaneous implantation of 293T cells transiently cotransfected with various plasmids as described in Paulmurugan et al. [31]. One group of mice was induced with TNF-α, and the other group was not induced. The images were one representative mouse from each group immediately after implanting cells (0 h), and 18 and 36 h after TNF-α induction. The induced mouse showed higher Fluc signal at site D (where interacting proteins result in reporter protein complementation) when compared with the mouse not receiving TNF-α. The Fluc signal significantly increases after receiving TNF-α. Reproduced with permission from Paulmurugan et al. [31]

9.4.2 Protein-Fragment Complementation Assays Using Split Firefly Luciferase

The most commonly used bioluminescence reporter gene for research purposes has been the luciferase from the North American Firefly (*Photinus pyralis*; *Fluc*). Fluc (61 kDa) catalyzes the transformation of its substrate D-Luciferin into oxyluciferin in a process dependent on ATP, Mg^{++}, and O_2, leading to self-emission of light from green to yellow wavelengths (560–610 nm, peak emission at 562 nm). In 2002, we demonstrated for the first time the feasibility of imaging PPIs using split

reporters in small living animals [31]. We studied a PCA based on split Fluc (cleaved into two fragments, nFluc: residues 1–437; and cFluc: residues 438–550), using the interaction of Id and MyoD as test proteins (Fig. 9.1).

Subsequently, Luker et al. [48] described a systematic truncation library yielding alternative complementary N- and C-fragments of Fluc (nFluc: residues 2-416; and cFluc: residues 398-550). These fragments were used to monitor rapamycin-mediated interaction of rapamycin-binding proteins FRB and FKBP12. We similarly used the Fluc fragments previously tested with Id and MyoD to study rapamycin-mediated interactions and found the complementation to be too weak for optical imaging in living animals using the CCD camera (unpublished data).

Further studies on reporter complementation assays for imaging of PPIs in living subjects led us to use a combinatorial strategy to identify a novel split site for Fluc with improved imaging characteristics over previously published split sites [49]. A combination of fragments with greater absolute signal and near-zero background signal was achieved by screening 115 different combinations. The identified fragments were further characterized by using five different interacting protein partners and an intramolecular folding strategy (see below). Cell culture studies and imaging in living mice were performed to validate the new split sites. In addition, the signal generated by the newly identified combination of fragments (nFluc 398/cFluc 394) was compared with different split-luciferase fragments then in use for studying PPIs and was shown to be markedly superior with a lower self-complementation signal and equal or higher post-interaction absolute signal. This study also identified many different combinations of nonoverlapping and overlapping Fluc fragments that can be used for studying different cellular events such as sub-cellular localization of proteins, cell–cell fusion, and evaluating cell delivery vehicles, in addition to PPIs, both in cells and small living animals.

The developed split Fluc system was used to study the crucial role of tumor hypoxia in tumorigenesis [50]. Under hypoxia, hypoxia-inducible factor 1 alpha (HIF-1 alpha) regulates activation of genes promoting malignant progression. Under normoxia, HIF-1 alpha is hydroxylated on prolines 402 and 564 and is targeted for ubiquitin-mediated degradation by interacting with the von Hippel-Lindau protein complex (pVHL). We developed a novel method of studying the interaction between HIF-1 alpha and pVHL using the split Fluc complementation-based bioluminescence system in which HIF-1 alpha and pVHL are fused to amino-terminal and carboxy-terminal fragments of the luciferase, respectively. We demonstrated that hydroxylation-dependent interaction between the HIF-1 alpha and pVHL leads to complementation of the two luciferase fragments, resulting in bioluminescence in vitro and in vivo. Complementation-based bioluminescence is diminished when mutant pVHLs with decreased affinity for binding HIF-1 alpha are used. This method represents a new approach for studying interaction of proteins involved in the regulation of protein degradation.

In another application, protein phosphorylation mediated by protein kinases was studied using a genetically encoded, generalizable split Fluc-assisted complementation system [51]. This was developed for noninvasive monitoring of phosphorylation events and efficacies of kinase inhibitors in cell culture and in

small living subjects by optical bioluminescence imaging. The serine/threonine kinase Akt mediates mitogenic and anti-apoptotic responses that result from activation of multiple signaling cascades. It is considered a key determinant of tumor aggressiveness and is a major target for anticancer drug development. An Akt sensor (AST) was constructed to monitor Akt phosphorylation and the effect of different PI-3 K and Akt inhibitors. Specificity of AST was determined using a nonphosphorylable mutant sensor containing an alanine substitution (ASA). It was found that the PI-3 K inhibitor LY294002 and Akt kinase inhibitor perifosine led to temporal- and dose-dependent increases in complemented Fluc activities in 293T human kidney cancer cells stably expressing AST (293T/AST) but not in 293T/ASA cells. Inhibition of endogenous Akt phosphorylation and kinase activities by perifosine also correlated with increase in complemented Fluc activities in 293T/AST cells but not in 293T/ASA cells. Treatment of nude mice bearing 293T/AST xenografts with perifosine led to a twofold increase in complemented Fluc activities compared with that of 293T/ASA xenografts. Our system was used to screen a small chemical library for novel modulators of Akt kinase activity. It is foreseen that this generalizable approach for noninvasive monitoring of phosphorylation events will accelerate the discovery and validation of novel kinase inhibitors and modulators of phosphorylation events.

Zhang et al. [52] have also described a new reporter molecule whose bioluminescence activity within live cells and in mice can be used to measure Akt activity. Akt activity in cultured cells, and tumor xenografts was monitored quantitatively and dynamically in response to activation or inhibition of receptor tyrosine kinase, inhibition of PI-3 K, or direct inhibition of Akt. The results provided unique insights into the pharmacokinetics and pharmacodynamics of agents that modulate Akt activity, revealing the usefulness of this reporter for rapid dose and schedule optimization in the drug development process. Having constructed a genetically engineered hybrid bioluminescent Akt reporter (BAR) molecule that reports on Akt serine/threonine kinase activity (containing an Akt consensus substrate peptide, consisting of a domain that binds phosphorylated amino acid residues (FHA2) flanked by nFluc and cFluc reporter domains), the same authors subsequently described a modified version of this reporter molecule (myristoylated and palmitoylated bioluminescent Akt reporter [MyrPalm-BAR]), which is membrane bound and the bioluminescence activity of which can be used to monitor Akt activity at the cell membrane [53]. This was based on the fact that Akt is recruited to the plasma membrane upon activation. Using changes in Akt activation status with small molecule inhibitors of Akt, they demonstrated that the membrane-targeted Akt reporter was more sensitive and quantitative. In addition, inhibition of upstream signaling kinases such as epidermal growth factor receptor and phosphatidylinositol 3-kinase activity resulted in changes in Akt activity that was quantitatively monitored by bioluminescence imaging. Based on these results, the authors proposed that the membrane-associated Akt reporter may be better suited for high-throughput screening and identification of novel compounds that modulate the Akt pathway.

In a slight variation on the theme of imaging PPIs, we identified different fragments of *Fluc* based on the crystal structure of Fluc; these split reporter genes, which encode fragments distinctly different from those currently used for studying PPIs, can *self*-complement and provide Fluc enzyme activity in different cell lines in culture and in living mice [54]. The comparison of the fragment complementation associated recovery of Fluc activity with intact Fluc was estimated for different fragment combinations and ranged from 0.01 to 4 % of the full Fluc activity. Using a cooled optical charge-coupled device camera, the analysis of Fluc fragment complementation in transiently transfected subcutaneous 293T cell implants in living mice showed significant detectable enzyme activity upon injecting D-Luciferin, especially from the combinations of fragments identified (nFluc and cFluc are the N and C fragments of Fluc, respectively): nFluc (1–475)/cFluc (245–550), nFluc (1–475)/cFluc (265–550), and nFluc (1–475)/cFluc (300–550). The cFluc (265–550) fragment, upon expression with the nuclear localization signal (NLS) peptide of SV40, showed reduced enzyme activity when the cells were cotransfected with the nFluc (1–475) fragment expressed without NLS. We also proved in this study, by delivering TAT-cFluc (265–550) to cells stably expressing nFluc (1–475) and recovering signal, that the complementing fragments could be efficiently used for screening macromolecule delivery vehicles. These complementing fragments should be useful for many reporter-based assays including intracellular localization of proteins, studying cellular macromolecule delivery vehicles, studying cell–cell fusions, and also developing intracellular phosphorylation sensors based on fragment complementation.

9.4.3 Protein-Fragment Complementation Assays Using Split Renilla luciferase

The enzyme Rluc (or the synthetically mutated humanized version, hRluc), is a 311-residue, 36-kDa monomeric bioluminescence imaging reporter protein, being the smallest optical reporter protein identified to date for studying PPIs in a PCA strategy [30, 38]. This PCA strategy, using N- and C-terminal halves of split Rluc functions in both cell culture and in living animals, and has been demonstrated with several different protein partners. We used fragments generated by splitting between residues 229 and 230 to study rapamycin-induced interaction of the human proteins FRB and FKBP12 [55]. Moreover, protein interaction between IRS1 and the SH2 domain of PI3 K in the insulin-signaling pathway was located in living mammalian cells using Rluc split between residues 91 and 92 [30].

Rluc is capable of generating a flash of blue light (460–490 nm, peak emission at 482 nm) upon reaction with its substrate coelenterazine. One limitation associated with the use of Rluc is its relatively rapid reaction kinetics, requiring early time-point measurements [56]. Nevertheless, this split reporter system appears highly suitable for studying PPIs in cells and in living animals owing to its optical

bioluminescence nature, and its signal that is amplifiable through an enzymatic process. Further, the complementation strategies based on Rluc fragments, with smaller fragment size than Fluc, have less hindrance with interacting protein partners, and work more efficiently with different imaging assays. Another clear advantage of using the split Rluc system when compared to the split Fluc is that the former's enzymatic reaction is ATP independent and therefore could be used in specific situations where the PPI under study itself requires ATP, e.g., the binding of Hsp90 to the co-chaperone protein p23. Unfortunately, several coelenterazines have been found to be substrates for the efflux transporter *MDR1* P-glycoprotein, including coelenterazine f, h, and hcp [57]. This raises some general concern for the indiscriminant use of coelenterazine and Rluc reporters in live cell assays and noninvasive whole-animal imaging. The photon output of the reporter can be impacted by changes in P-glycoprotein transport activity that alter substrate availability within the cells, thereby introducing signal artifacts not related to the biological process under investigation, that is, PPIs. Furthermore, coelenterazine cannot be used in experimentation involving transport across the blood–brain barrier because brain capillaries are rich in outwardly directed P-glycoprotein, effectively excluding coelenterazine from the central nervous system.

To date, the split Rluc system has been used in several molecular imaging applications, including PPIs, small molecule-induced PPIs, small molecule-mediated inhibition of PPIs, and protein homodimerization. More recently, we developed a novel fusion protein approach for studying rapamycin-mediated interaction of fused FRB and FKBP12 with either split hRluc or split enhanced GFP, to achieve a system with greater sensitivity for detecting lower levels of drug-mediated PPIs in vivo [58]. These applications are described more fully below.

Networks of protein interactions mediate cellular responses to environmental stimuli and direct the execution of many different cellular functional pathways. Small molecules synthesized within cells or recruited from the external environment mediate many protein interactions. The study of small molecule-mediated interactions of proteins is important to understand abnormal signal transduction pathways in cancer and in drug development and validation. In one study, we used split hRluc protein-fragment-assisted complementation to evaluate heterodimerization of the human proteins FRB and FKBP12 mediated by the small molecule rapamycin [55] (Fig. 9.2). The concentration of rapamycin required for efficient dimerization and that of its competitive binder ascomycin required for dimerization inhibition were studied in cell lines. The system was dually modulated in cell culture at the transcription level, by controlling nuclear factor kappaB promoter/enhancer elements using tumor necrosis factor alpha, and at the interaction level, by controlling the concentration of the dimerizer rapamycin. The rapamycin-mediated dimerization of FRB and FKBP12 also was studied in living mice by locating, quantifying, and timing the hRluc complementation-based bioluminescence imaging signal using a cooled charge-coupled device camera. It was found that this split reporter system can be used to efficiently screen small molecule drugs that modulate PPIs and also to assess drugs in living animals. Both are

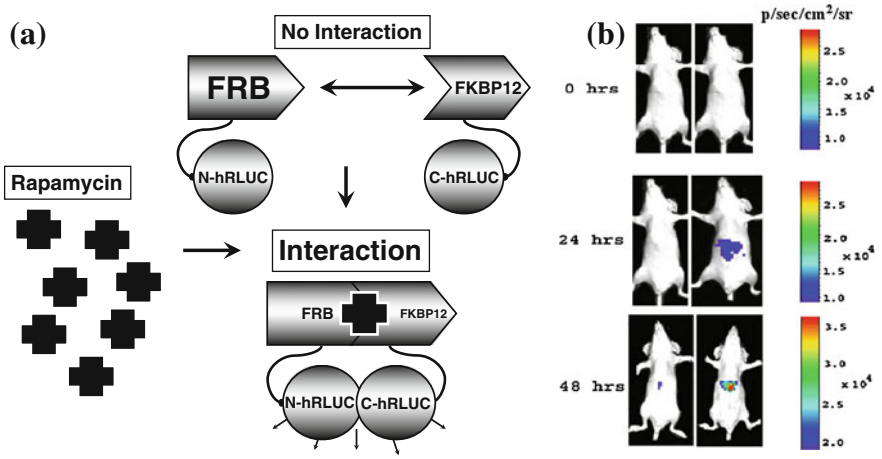

Fig. 9.2 Schematic diagram of rapamycin-mediated synthetic Renilla luciferase (hRluc) protein-fragment-assisted complementation strategy. In this strategy (Fig. 9.2a) N-terminal and C-terminal portions of hRluc fragments are attached to proteins FRB and FKBP12, respectively, through a short peptide linker GGGGSGGGGS. The N- and C-portions of hRluc fragments are closely approximated by the dimerization of proteins FRB and FKBP12 only in the presence of the small molecule rapamycin, and this, in turn, leads to recover the activity of the hRluc protein. Optical CCD camera imaging of living mice carrying IV injected 293T cells transiently cotransfected with Nhrluc-FRB and FKBP12-Chrluc (Fig. 9.2b). The animals not receiving rapamycin showed only a mean background signal of $4 \pm 1 \times 10^3$ p/s/cm^2/sr at all of the four time points studied. The animals receiving repeated injections of rapamycin emitted signals in the region of abdomen that were three fold (mean, 1.6×10^4 p/s/cm^2/sr) and five-fold (mean, 3.0×10^4 p/s/cm^2/sr) higher than background ($p < 0.05$) at 24 and 48 h after the injection of rapamycin, respectively. (left-sided animals did not receive rapamycin; right-sided animals received rapamycin). Reproduced with permission from Paulmurugan et al. [54]

essential steps in the preclinical evaluation of candidate pharmaceutical agents targeting PPIs, including signaling pathways in cancer cells.

In another study adopting a different strategy, we evaluated the rapamycin-mediated interaction of the human proteins FK506-binding protein (FKBP12) rapamycin-binding domain (FRB) and FKBP12 by constructing a fusion of these proteins with a split-Rluc or a split enhanced green fluorescent protein (split-EGFP) such that complementation of the reporter fragments occurs in the presence of rapamycin [58]. Different linker peptides in the fusion protein were evaluated for the efficient maintenance of complemented reporter activity. This system was studied in both cell culture and xenografts in living animals. We found that peptide linkers with two or four EAAAR amino acid repeats showed higher PPI-mediated signal with lower background signal compared with having no linker or linkers with amino acid sequences GGGGSGGGGS, ACGSLSCGSF, and ACGSLSCGSFACG SLSCGSF. A 9 ± twofold increase in signal intensity both in cell culture and in living mice was seen compared with a system that expresses both reporter fragments and the interacting proteins separately. In this fusion system,

rapamycin-induced heterodimerization of the FRB and FKBP12 moieties occurred rapidly even at very lower concentrations (0.00001 nmol/L) of rapamycin. For a similar fusion system employing split-EGFP, flow cytometry analysis showed significant level of rapamycin-induced complementation.

We also evaluated small molecule-mediated inhibition of PPIs [59]. Heat shock protein 90 alpha (Hsp90 alpha)/p23 and Hsp90 beta/p23 interactions are crucial for proper folding of proteins involved in cancer and neurodegenerative diseases. Small molecule Hsp90 inhibitors block Hsp90 alpha/p23 and Hsp90 beta/p23 interactions in part by preventing ATP binding to Hsp90. The importance of isoform-selective Hsp90 alpha/p23 and Hsp90 beta/p23 interactions in determining the sensitivity to Hsp90 was examined using 293T human kidney cancer cells stably expressing split Rluc reporters. Interactions between Hsp90 alpha/p23 and Hsp90 beta/p23 in the split Rluc reporters led to complementation of Rluc activity, which was determined by bioluminescence imaging of intact cells in cell culture and living mice using a cooled charge-coupled device camera. The three geldanamycin-based and seven purine-scaffold Hsp90 inhibitors led to different levels of inhibition of complemented Rluc activities (10–70 %). However, there was no isoform selectivity to either class of Hsp90 inhibitors in cell culture conditions. The most potent Hsp90 inhibitor, PU-H71, however, led to a 60 and 30 % decrease in Rluc activity (14 h) in 293T xenografts expressing Hsp90 alpha/p23 and Hsp90 beta/p23 split reporters, respectively, relative to carrier control-treated mice. Molecular imaging of isoform-specific Hsp90 alpha/p23 and Hsp90 beta/p23 interactions and efficacy of different classes of Hsp90 inhibitors in living subjects have been achieved with a novel genetically encoded reporter gene strategy that should help in accelerating development of potent and isoform-selective Hsp90 inhibitors.

Homodimeric protein interactions are potent regulators of cellular functions but are particularly challenging to study in vivo. We also used a split hRluc complementation-based bioluminescence assay to study homodimerization of herpes simplex virus type 1 thymidine kinase (TK) in mammalian cells and in living mice [28]. We quantified and imaged homodimerization of TK chimeras containing N-terminal (n-hRluc) or C-terminal (c-hRluc) fragments of hRluc in the upstream and downstream positions, respectively, (tail-to-head homodimer). This was monitored using luminometry (68-fold increase, and was significantly [$P < 0.01$] above background light emission) and by CCD camera imaging of living mice implanted with ex vivo transfected 293T cells (2.7-fold increase and was significantly [$P < 0.01$] above background light emission). We also made a mutant-TK to generate n-hRluc mutant TK and mutant TK-c-hRluc by changing a single amino acid at position 318 from arginine to cysteine, a key site that has previously been reported to be essential for TK homo-dimerization, to support the specificity of the hRluc complementation signal from TK homodimerization. Ex vivo substrate (8-3H Penciclovir) accumulation assays in 293T cells expressing the TK protein chimeras showed active TK enzyme. We also devised an experimental strategy by constructing variant TK chimeras (possessing extra n-hRluc or c-hRluc 'spacers') to monitor incremental lack of association of the tail-to-head TK

homodimer. Application of this potentially generalizable assay to screen for molecules that promote or disrupt ubiquitous homodimeric PPIs could serve not only as an invaluable tool to understand biological networks but could also be applied to drug discovery and validation in living subjects.

As an offshoot to our designing of strategies for PPI imaging, we used the split Renilla system to help devise newer approaches to high-throughput analysis of interactions between various hormones and drugs with the estrogen receptor (ER) [60]. These are crucial for accelerating the understanding of ER biology and pharmacology. Through the careful analyses of the crystal structures of the human ER (hER) ligand-binding domain (hER-LBD) in complex with different ligands, we hypothesized that the hER-LBD intramolecular folding pattern could be used to distinguish ER agonists from selective ER modulators and pure antiestrogens. We therefore constructed and validated intramolecular folding sensors encoding various hER-LBD fusion proteins that could lead to split Rluc/Fluc reporter complementation in the presence of the appropriate ligands. A mutant hER-LBD with low affinity for circulating estradiol was also identified for imaging in living subjects. Cells stably expressing the intramolecular folding sensors expressing wild-type and mutant hER-LBD were used for imaging ligand-induced intramolecular folding in living mice. This is the first hER-LBD intramolecular folding sensor suited for high-throughput quantitative analysis of interactions between hER with hormones and drugs using cell lysates, intact cells, and molecular imaging of small living subjects. The strategies developed can also be extended to study and image other important protein intramolecular folding systems. A subsequent further development of this system allowed for in vivo molecular imaging of ERα and ERβ homo- and heterodimerization using split Fluc or Rluc [61].

In an interesting combined application of both split Fluc and split Rluc systems, we demonstrated the feasibility of imaging ER-ligand-modulated multiprotein interactions [human estrogen receptors (ER-alpha/beta), p53 tumor suppressor protein and the human equivalent of mouse double minute 2 (HDM2)] [R Paulmurugan and SS Gambhir: Imaging ER-ligand-modulated multiprotein interactions [(human estrogen receptors (ER-alpha/beta), p53 tumor suppressor protein and the human equivalent of mouse double minute 2 (HDM2)] with a split-luciferase system. Presented at the Joint Molecular Imaging Conference, Providence, Rhode Island, September 2007]. The background impetus to this arises from knowledge that cancers develop when accumulated genetic defects cause cells to proliferate unchecked and escape from programmed cell-death. p53, HDM2, and ERs are a few among several prime targets that generate genetic abnormalities that lead to cancer. These proteins function independently and interact together by forming a ternary complex that regulates the expression level of different proteins required for normal cell death/growth. The advantages of these targets over other proteins are that they can be controlled by small-molecule ligands that bind to any one of these three (p53/HDM2/ER) to modulate the functional role of all three proteins. The authors showed for the first time simultaneous interaction of these multiproteins and their modulation in response to different ER-ligands. Plasmids expressing fusion-proteins nFluc-ER-alpha/beta-chRluc, p53-cFluc and nhRluc-HDM2 were transiently

cotransfected in 293T cells and complemented Fluc (ER/p53-interaction) and Rluc (p53/HDM2-interaction) activities were determined before and after exposure to 13-different ligands that bind to ER. The results showed significant levels of modulation by ER-ligands for the Fluc signals (ER/p53-interaction). There was no significant change in the Rluc signal (p53/HDM2-interaction). Studies with both ER-alpha and beta showed similar levels of interaction with p53. The system with the ER/p53 interaction showed significant levels of reversibility upon removal of the ligand. These results demonstrated that the interaction between ER and p53 was ligand-dependent. Studying this multiprotein interaction system with ligands specific to p53 or/to HDM2, in the presence and the absence of ER-ligands, may further identify the complexity behind the interactions between these three proteins. This was the first demonstration of multiprotein-interactions studied with multiple-split-reporters and this strategy should be useful for also studying other similar types of complex interactions.

Of note, the split protein strategies described previously are based on absolute stereospecific and regiospecific requirements for complex formation among interacting sequences. Although no head-to-head comparison is available, this strategy would therefore appear more specific than the modified yeast two-hybrid system, which suffers from many false-positive outcomes, at least in its standard (nonimaging) laboratory use. Moreover, the split protein strategies can be used to image interacting proteins anywhere in the cell.

9.4.4 Protein-Fragment Complementation Assays Using Other Split Reporters, and Other Reporter Complementation Strategies

Although not yet at a stage of application in living subjects, Kim et al. [62] have recently evaluated the Click beetle luciferase (CBluc) and its luminescence signal as a bioanalytical index reporting the magnitude of a signal transduction of interest. CBluc is insensitive to pH, temperature, and heavy metals, and emits a stable, highly tissue-transparent red light with luciferin in physiological circumstances. They validated a single-molecule-format complementation system of split CBluc to study signal-controlled PPIs. First, they generated 10 pairs of N- and C-terminal fragments of CBluc to examine, respectively, whether a significant recovery of the activity occurs through intramolecular complementation. The ligand binding domain of androgen receptor (AR LBD) was connected to a functional peptide sequence through a flexible linker. The fusion protein was then sandwiched between the dissected N- and C-terminal fragments of CBluc. Androgen induces the association between AR LBD and a functional peptide and the subsequent complementation of N- and C-terminal fragments of split CBluc inside the single-molecule-format probe, which restores the activities of CBluc.

The examination about the split sites of CBluc revealed that the dissection positions next to the amino acids D412 and I439 can admit a stable recovery of CBluc activity through an intramolecular complementation. The ligand sensitivity and kinetics of the single molecular probe with split CBluc were studied in various cell lines and in different protein-peptide binding models. The probe may be applicable to developing biotherapeutic agents relevant to AR signaling and for screening adverse chemicals that possibly influence the signal transduction of proteins in living cells or animals, although the latter setting is yet to be verified.

Gaussia Luciferase (GLuc) is another high-sensitive optical reporter protein of 19.9 kDa (185 amino acids) that emits blue light (at a peak of 480nM) and uses the substrate coelenterazine. Optimal split sites identified between amino acids Gly93 and Glu94 by screening a library of N- and C-terminal fragments using a rapamycin-mediated FRB/FKBP12 interaction system were successfully evaluated for leucine zipper interaction and TGFβ-mediated interaction of serine-threonine kinase PKB/Akt and the transcriptional activator Smad3 **[**63**]**. Later, Kim et al. in 2009 **[**64**],** identified fragments generated by a split site between amino acids 105 and 106 can successfully reconstitute enzymatic activity through PPIs driven by calcium (calmodulin and M13 peptide interaction) in cell cultures. Even though GLuc is highly sensitive, the secretary nature of this protein limits its application for in vivo studies.

Importantly, there is a pressing need to develop better techniques for noninvasive imaging of PPIs using split reporters. We recently described the molecular engineering rationale and construction of a novel positron emission tomography (PET)-based reporter (the herpes simplex virus type 1 thymidine kinase [TK]) split into two fragments between Thr-265 and Ala-266 after demonstration of preserved enzymatic activity (85.2 %, as compared to intact TK) in a circularly permuted variant based on this cleavage site [65]. We used this split TK in a PCA to quantitatively measure PPIs in mammalian 293T cells using an in vitro [8-^{3}H]Penciclovir cell uptake assay. We showed a greater extent of TK fragment complementation when using FRB/FKBP12 than Id/MyoD as test proteins. We determined that co-expression of nTK-FRB together with FKBP12-cTK gave the optimal orientation of chimeras for evaluation in this PCA assay. We also demonstrated the use of this split TK in a PCA to quantitatively microPET image PPIs in 293T cells subcutaneously implanted in living mice. For this, we prepared 293T cells stably expressing nTK-FRB and FKBP12-cTK in a single vector. Cell uptake studies using these stably transfected cells demonstrated that the competitive inhibitor ascomycin (FK506) prevented rapamycin-induced TK activity in a dose-dependent manner. Prior to imaging we also established that adequate levels of protein expression were present by western blot. The designing of this novel split TK reporter and its application in an in vivo PET-based PCA potentially allows for the first time a more precise fully quantitative and tomographic localization of cytosolic or nuclear PPIs in pre-clinical small and large animal models of disease than has been possible to date.

9.5 Future Outlook and Conclusions

We have reviewed the main methods currently available for imaging PPIs in living subjects based on use of the split reporter systems. Unlike fluorescence microscopy-based techniques, studies of the kinetics of PPIs, including analysis of complementation reversibility, are not possible at present, although this will be an area of future active investigation. These future experiments will also require assessment in several cell lines, as well as with a greater variety of protein partners of different sizes and interaction affinities (weak transient to strong obligate), to establish the general widespread applicability of this technique.

The opportunity to measure two different protein interactions at the same time by spectrally unmixing output colors will be useful in attempts to multiplex image protein interaction networks [66]. Recent advances in processing of two color imaging now allows for the total spectral deconvolution of multicolored bioluminescent images, assuming the spectra are different enough to reliably calculate the contribution of each individual emitter within each detection window, based on their published spectra. Simultaneous imaging of multiple interactions should allow deconvolution of complex protein interactions and, eventually, protein interactomes.

The high sensitivity of these assays for detecting, locating, and quantifying PPIs, combined with the advantages of doing so in a living subject environment, should make them of potential value in many areas of biological investigation and future clinical molecular medicine applications (especially with the now available PET-based split TK PCA system). Indeed, endpoints in molecular imaging of PPIs can be quantified and therefore are particularly useful for translational research. Ultimately, we foresee innovative molecular imaging tools, such as the one presented, enhancing our appreciation of entire biological pathway systems and their pharmacological regulation, and accelerating the achievement of a ‘systems biology’ understanding of biological complexity [67].

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