Current State of Imaging Protein-Protein Interactions In Vivo with Genetically Encoded Reporters

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Key Words
signal transduction, protein fragment complementation, firefly luciferase, Renilla luciferase, inteins, bioluminescence, molecular imaging

Abstract
Signaling pathways regulating proliferation, differentiation, and inflammation are commonly mediated through protein-protein interactions as well as reversible modification (e.g., phosphorylation) of proteins. To facilitate the study of regulated protein-protein interactions in cells and living animals, new imaging tools, many based on optical signals and capable of quantifying protein interactions in vivo, have advanced the study of induced protein interactions and their modification, as well as accelerated the rate of acquisition of these data. In particular, use of protein fragment complementation as a reporter strategy can accurately and rapidly dissect protein interactions with a variety of readouts, including absorbance, fluorescence, and bioluminescence. This review focuses on the development and validation of bioluminescent protein fragment complementation reporters that use either Renilla luciferase or firefly luciferase in vivo. Enhanced luciferase complementation provides a platform for near real-time detection and characterization of regulated and small-molecule-induced protein-protein interactions in intact cells and living animals and enables a wide range of novel applications in drug discovery, chemical genetics, and proteomics research.
INTRODUCTION

The complexity of the proteome and its regulation drives the need for more accurate assessment of protein activity and interactions in their native state. Tools for the measurement of these interactions have evolved greatly over the years with the development of novel probes that facilitate in-depth investigation of proteomic processes (1, 2). Older “tried and true” technologies, such as coimmunoprecipitation, and Western blots, require cell lysis to assess protein interactions. Coimmunoprecipitation generally requires a specific sequence or conformation of amino acids (an antigen) that is recognized and bound by a target antibody. A second antibody, often bound at one end to a solid matrix, such as a bead, binds to the first antibody, precipitating the complex out of solution. If the antigen is associated in a complex of proteins, other members of the complex may be coprecipitated and hence separated from the rest of the cellular milieu. This process is then followed by a Western blot, wherein proteins are separated by size using gel electrophoresis, transferred to a membrane, and probed with a primary antibody to the antigen of interest. A secondary antibody, typically fused to a reporter that emits a biochemically detectable signal, is then allowed to bind a species-specific region of the primary antibody, thus enabling measurement of levels of the original antigen (3, 4). More recently, the introduction of two-hybrid systems established novel and valuable tools for use in high-throughput screening and discovery of new protein interactions by means of transcriptional activation of a reporter. For the two-hybrid strategy, a transcription factor is split into two domains, a DNA-binding domain (required for transcription factor binding to DNA) and an activator domain (required for basal activation of
transcription). Both domains are required to induce proper transcription of a reporter gene. The domains of the transcription factor are modular and their activity can be recapitulated by close physical approximation. Each protein of a potentially interacting pair is fused to one of the partial transcription factor fragments, such that their interaction will induce reassembly of the transcriptional machinery and production of a detectable event via the reporter. However, these strategies are conventionally limited temporally (on the order of hours) and require nuclear translocation of hybrid fusion proteins, thereby limiting their analytical value (5). To overcome these limitations, several different types of reporters have been developed that utilize fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), or protein fragment complementation (6–10). These techniques use a variety of readouts, such as absorbance, fluorescence, and bioluminescence, allowing for in-depth analysis of protein interactions, screening and testing of drugs that modify those interactions, as well as in vivo experimentation. Several recent articles have presented the principles and applications of biochemical and resonance energy transfer techniques for investigating protein-protein interactions in cells and lysates (1, 8, 10–12). The goal of this review is to focus on the relevant principles, opportunities, and caveats for use of genetically encoded reporters to interrogate protein–protein interactions in whole cells and living animals in vivo. The ease with which such data are acquired using these new reporters may speed interrogation of protein networks, expedite the process of drug design and discovery, as well as aid in the analysis of bioavailability and efficacy of ligands and novel compounds in vivo.

GENERAL OVERVIEW OF STRATEGIES FOR DETECTING PROTEIN-PROTEIN INTERACTIONS

Traditional assays for measuring protein-protein interactions exhibit weaknesses, such as the requirement for cell lysis and creation of experimental conditions that are inconsistent with the natural intracellular milieu. A standard technique in the field for more than 30 years, coimmunoprecipitation is dependent on high-affinity protein interactions and may not detect proteins that function as large multiprotein complexes (13). Another caveat of this technique is the potential failure to detect transient interactions that may have potent effects on cell signaling and intracellular processes.
Figure 1

Transcriptional strategies for imaging protein-protein interactions. (a) Two-hybrid systems are applicable to protein interactions that can translocate to the nucleus. When a protein interaction is induced, the fusion proteins bring together transactivator and DNA binding domains of a transcription factor (TF), which then induces reporter transcription. (b) The split ubiquitin (Ub) system bypasses the nuclear translocation requirement by taking advantage of ubiquitin-specific proteases (UBP) that recognize only intact Ub. One fragment of the split Ub is fused to a TF that under basal conditions remains tethered outside the nucleus. Upon protein interaction and cleavage by UBP, the TF localizes to the nucleus and induces reporter gene transcription.

Finally, the key reagent that determines the success of this technique is a high-quality antibody, for which the binding affinity and specificity to the target antigen greatly influences the degree of false positives and false negatives obtained, thus impacting the reliability of the data.

Many of these caveats have been overcome by the introduction of two-hybrid systems that incorporate different reporter constructs to signal the strength and timing of protein interactions (Figure 1) (5, 14, 15). In addition to genetic complementation as a readout, new technologies include the use of fluorescent and bioluminescent reporter proteins as well as absorbance and radioactivity-based reporters (16–21). However, conventional two-hybrid systems require target protein interactions that are localized within the nucleus or at least compatible with translocation of the complexes into the nucleus because proximity to DNA and the transcriptional machinery is needed for reporter expression. Thus, membrane proteins cannot be studied in their...
Luciferase: a generic term for bioluminescent proteins that generate light by means of a chemical reaction with oxygen and a substrate.
Figure 2
(a) A two-hybrid system has been modified to include a Tet-inducible bidirectional promoter to ensure equal coexpression of the target fusion proteins. The interacting proteins consist of p53 and T-antigen (TAg), which were fused to the Gal4-binding domain and VP16 activator domain, respectively. The negative control utilized coat protein (CP) fused to VP16, a known noninteractor with p53. (b) Upon treatment with doxycycline, the bidirectional promoter is activated and the two interacting proteins subsequently activate the reporter via binding to the Gal-4 promoter region. The reporter is a fusion of a modified HSV-TK and GFP. (c) Cells activated with Dox show GFP expression by fluorescent microscopy. (d) Photograph of the anterior thorax of a nu/nu mouse with axillary xenograft tumors of CP cells (arrowhead) and TAg cells (arrow). (e) Coronal microPET image of the same mouse 48 h after starting treatment with i.p. injections of doxycycline (dox; 60 μg/g body weight for six doses) showing accumulation of 18F-FHBG only in the TAg tumor (arrow) expressing the interacting proteins Gal4-BD-p53 and VP16-TAg (representative image of three animals). Asterisk denotes excretion of radiotracer into the gallbladder. Intestinal activity from normal hepatobiliary clearance of the radiotracer is observed in the lower portion of the image (16).

An interesting variation of the recruitment approach applicable to mammalian cells is the cytokine-receptor-based interaction trap. Here, a signaling-deficient receptor provides a scaffold for recruitment of interacting fusion proteins that phosphorylate endogenous STAT3. Activated STAT complexes then drive a nuclear reporter (30). This system permits detection of both modification-independent and phosphorylation-dependent interactions in intact mammalian cells, but the transcriptional readout again limits kinetic analysis.

RESONANCE ENERGY TRANSFER

FRET is a quantum mechanical process in which radiationless energy is transferred over a distance, usually <80–100 Å from one fluorophore to another (8, 31–34). Upon excitation of the donor fluorophore, a radiationless energy emission, called an
exciton, is transferred from one fluorophore to the other. The exciton excites the acceptor fluorophore, which subsequently emits light that is further red-shifted than would be expected from the donor fluorophore (Figure 3). For this to work correctly and efficiently, the emission spectrum of the donor and the excitation spectrum of the acceptor should overlap sufficiently to enable energy transfer, yet be separated well enough to allow each spectrum to be resolved. The efficiency of a resonance

![Diagram of FRET, BRET, Split intein, and Protein fragment complementation]

**Figure 3**
Post-translational strategies for monitoring protein-protein interactions. (a) Fluorescence resonance energy transfer (FRET) monitors the degree of protein interaction as a change in color caused by nonradiative light transfer from the donor fluorophore (CFP) to the acceptor fluorophore (YFP) when the proteins are within proximity to each other (10–100 Å). (b) Bioluminescence resonance energy transfer (BRET) displays the same characteristics as FRET except the donor (Luc) is a bioluminescent protein, which requires no incident light, only molecular substrates. (c) Self splicing split inteins (DnaE) will splice the reporter protein together when brought within close proximity of each other. The splicing reaction is covalent and produces an intact, active reporter protein. (d) Protein fragment complementation monitors protein interactions as an increase in reporter activity caused by reversible approximation of two fragments, which when separate are inactive, yet when brought together reconstitute activity. Brown symbols are proteins of interest in an inducible interaction system. The yellow star represents an inducer of protein interaction.
energy transfer reaction depends on five factors: (a) spectral overlap between the emission of donor and the absorbance of acceptor, (b) relative orientation between the emission and absorption dipoles of the respective donor and acceptor pair, (c) distance \( r \) between the donor and acceptor, (d) quantum yield of the donor, and (e) extinction coefficient of the acceptor. Energy is transferred from donor to acceptor with an efficiency \( E \) defined by the Förster equation, \( E = \frac{R_0^6}{R_0^6 + r^6} \), where a factor, \( R_0 \) (Förster distance), is the distance at which 50% efficiency is obtained and \( r \) is the distance between the two fluorophores. \( R_0 \) typically ranges from 20 to 60 Å and small variations in \( r \) are detectable with sensitive equipment. The typical measurements of RET comprise calculation of the ratio of light emitted by the donor to that emitted by the acceptor, and a change in this ratio signifies changes in the distance between the two proteins of interest.

Of the several different RET modalities that exist, the simplest is an intermolecular interaction design consisting of separate fusion proteins with donor and acceptor conjugated to the respective interacting proteins. Problems with this technique occur when the donor and acceptor constructs are not present in an exact 1:1 ratio. The fluorescent readings may not be accurate and tend to be skewed depending on the degree of contamination produced by spectral overlap of the donor into the acceptor interrogation window. The final effective protein ratio within a cell can be impacted by genome-dependent expression levels sensitive to where each construct was incorporated, locus-dependent repressor activities, and whether expression is equivalent. Even if the ratio of protein expression is 1:1, actual ratios may be skewed owing to differences in intracellular localization. There are current methods for measuring intermolecular FRET interactions based on sophisticated mathematical algorithms. Additionally, false negative readings become a problem because interacting proteins may not produce a positive RET signal owing to excessive distance between the fluorophore pair.

Another technique, based on an intramolecular interaction design, uses a format wherein the donor and acceptor are on one polypeptide chain (35). This guarantees a 1:1 ratio and intracellular colocalization. Any variation in emission ratios will be due to changes within interacting domains and not the diffusion or localization of separate constructs. Various different interactions can be measured using this technique, such as cleavable events measuring protease activities, induced conformational changes of multidomain proteins, and inducible protein-protein interactions. This minimizes problems encountered by intermolecular RET and maximizes the measurable data output from the probe when inherent internal controls of measurable color changes are determined independent of total output of the donor. Therefore, construction of single chain biosensors consisting of multidomain proteins containing 1:1 ratios of donor to acceptor potentially eliminate many shortcomings of RET methods. However, single chain biosensors may have the negative effects of increased false positive rates and skewed measurable drug kinetics for different protein interactions owing to the increased effective concentration of the detector pair as well as the interacting proteins.

For use in vivo, the FRET technique has many disadvantages. First, fluorescent probes require the use of incident light to activate the donor protein. Given that even
the most red-shifted FRET probes (GFP-RFP) require an excitation wavelength in the green, little light will travel through tissues, as most tissues suffer from high light attenuation below a wavelength of 600 nm (36–38). Absorption of light at 489 nm (maximal excitation of EGFP) is approximately 500-fold greater than absorption at 630 nm. Furthermore, relative molar ratios of FRET pairs that are not 1:1 can cause calibration and detection problems that are amplified in vivo. If the donor fluorophore is overexpressed relative to the acceptor fluorophore, then spectral overlap will complicate deconvolution of desired signal in vivo. Also, the intense monochromatic incident light in vivo often produces many other side reactions, first of which is photobleaching. Many fluorescent proteins, when overexposed to light photons, lose their ability to fluoresce due to photochemical decomposition. Secondly, in the green-blue range of the spectrum (<500 nm), there tends to be significant amounts of background fluorescence in tissues and cells causing autofluorescence. This significantly reduces signal-to-noise ratios, which degrades the sensitivity of the assay in vivo. Finally, as the intensity of light increases, shorter, more energetic wavelengths can cause tissue damage as well as lower fluorescence efficiency. Despite these caveats, FRET remains a powerful tool for real-time imaging of cellular events owing to its sensitivity and mode of action.

BRET works much like FRET except that the donor molecule is not a fluorescent protein or dye, but a bioluminescent protein (Fig. 3) (10, 39, 40). This offers many distinct advantages over FRET owing to its extremely high quantum yield and the high sensitivity for measurement of interactions. Currently used BRET pairs consist of Renilla luciferase coupled with either modified EYFP or GFP. The commercial construct BRET2 uses Renilla luciferase and a modified coelenterazine substrate (DeepBlueC) that emits with a $\lambda_{\text{max}}$ of 395 nm (41). This emission activates a modified GFP2 characterized by a significantly red-shifted Stokes shift that emits transferred energy at 508 nm. Although other known pairs use Renilla (475 nm) and EYFP (530 nm), the emission of these BRET pairs is still not red-shifted sufficiently for optimal utility in vivo (42). Therefore, for molecular imaging of protein-protein interactions in vivo, an optimized technique using resonance transfer methods would utilize a single chain biosensor (to fix molar ratios of donor and acceptor) with a bioluminescent donor in conjunction with a red-shifted fluorophore. Such a system has yet to be engineered and validated.

**PROTEIN FRAGMENT COMPLEMENTATION**

Protein fragment complementation assays depend on division of a monomeric reporter protein into two separate inactive components that can reconstitute function upon association. When these reporter fragments are fused to interacting proteins, the reporter is reactivated upon association of the interacting proteins. Protein fragment complementation (sometimes called split reporter) strategies based on several proteins and enzymes, including dihydrofolate reductase (DHFR), β-galactosidase, β-lactamase, green fluorescence protein (GFP), and firefly luciferase have been used to monitor protein-protein interactions in mammalian cells (Table 1) (7, 43–49). Several of these are reviewed below.
Table 1 Strategies for detecting protein-protein interactions and their applications to various biological systems

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Lysates</th>
<th>Bacterial cells</th>
<th>Mammalian cells</th>
<th>Living animals</th>
<th>Readout</th>
<th>Assay type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-immunoprecipitation</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Immunoblotting, fluorescence</td>
<td>Biochemical</td>
<td>(3, 4)</td>
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<tr>
<td>Two hybrid</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Fluorescence, bioluminescence, PET, SPECT</td>
<td>Transcriptional</td>
<td>(5, 14–19)</td>
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<tr>
<td>Split ubiquitin</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>Fluorescence, bioluminescence, PET, SPECT</td>
<td>Transcriptional</td>
<td>(24–27)</td>
</tr>
<tr>
<td>FRET</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>Fluorescence</td>
<td>Post-translational</td>
<td>(8, 31–34)</td>
</tr>
<tr>
<td>BRET</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>Bioluminescence</td>
<td>Post-translational</td>
<td>(10, 39–42)</td>
</tr>
<tr>
<td>Split DHFR</td>
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<td>−</td>
<td>+</td>
<td>−</td>
<td>Survival assay, fluorescence, fluorescence</td>
<td>Post-translational</td>
<td>(45, 53)</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>Fluorescence, absorbance</td>
<td>Post-translational</td>
<td>(44, 47)</td>
</tr>
<tr>
<td>Split beta-galactosidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>Fluorescence, absorbance</td>
<td>Post-translational</td>
<td>(56–58)</td>
</tr>
<tr>
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<td>+</td>
<td>−</td>
<td>Fluorescence</td>
<td>Post-translational</td>
<td>(49, 59–62, 84)</td>
</tr>
<tr>
<td>Split luciferases</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Bioluminescence</td>
<td>Post-translational</td>
<td>(7, 74–78, 81)</td>
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<tr>
<td>Split luciferase inteins</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Bioluminescence</td>
<td>Post-translational</td>
<td>(12, 70–73)</td>
</tr>
</tbody>
</table>

*Details on each method can be found in the selected references.

**SPLIT DIHYDROFOLATE REDUCTASE**

DHFR is the primary source of folate in mammalian systems. It converts dihydrofolate acid into tetrahydrofolate acid, which in turn is transformed into many cellular cofactors, including thymine. Many drugs inhibit DHFR, causing disruption in nucleoside synthesis and hence exhibiting anticancer (methotrexate, pemetrexed) and antibiotic (trimethoprim) properties (50–52). Remy et al. have described a protein fragment complementation assay in which DHFR is split and fused to different interacting proteins (Figure 3) (45, 53). The approximation of the interacting proteins brings the partially folded split DHFR fragments together so they can properly fold and recapitulate DHFR activity. Two models exist for the mechanism behind protein fragment complementation (54). The protein fragments can either be partially folded and become fully folded upon approximation or they may be mostly folded and only reconstitute protein activity upon approximation with its protein pair. The split DHFR protein fragments are reported to be unfolded prior to association (55). However, such a case may be disadvantageous as folding of the reporter protein upon
protein association may skew the actual kinetics and thermodynamics of target protein interaction by the $\Delta G$ of DHFR folding.

The DHFR system allows two types of strategies for measuring protein interactions. A survival assay can be devised by using null cells lacking DHFR. Following transfection with the fusion reporters, the null cells are grown on nucleoside-free medium. Those cells without induced DHFR do not survive, whereas those with interacting proteins provide DHFR activity and survive. Another method to measure protein interactions is to use a fluorogenic substrate, methotrexate, which binds intact DHFR. Unbound methotrexate is washed out of cells, whereas bound methotrexate can be fluorescently measured. This system, however, is limited by the timescale of the readout. When used in a survival assay, one can only measure a binary on/off signal after many hours to days. Even in the fluorescence assay mode, addition of pharmacologically relevant quantities of methotrexate severely affects the function of the cells being tested. This limits the kinetics of the assay and the capacity for repetitive measurements in the same cells. Nonetheless, it should be noted that this marks an early use of protein complementation as a reporter for protein interactions.

**SPLIT β-GALACTOSIDASE**

β-galactosidase can be used as a measure of protein interactions by utilizing a pair of weakly complementing deletion mutants, $\Delta \alpha$ and $\Delta \omega$ (56–58). When expressed as a fusion with two interacting proteins, the interacting proteins drive β-gal complementation, enabling the development of an optical signature of β-gal activity upon exposure to substrate (galactoside). This method exhibits several advantages, including measurement of compartmentalization, utility in mammalian cells, signal amplification through β-gal enzyme activity on its cognate substrates, and the ability to quantify interactions via histochemical, biochemical, fluorescent, and chemiluminescent readouts. The weakness of this assay lies in the requirement for substrate treatment ex vivo during readout and thus is not routinely applicable in vivo nor can it provide a reliable real-time output. In addition, the signal amplification, which is a function of substrate concentration and time of exposure, can lead to false positives because the product accumulates over time. If exposed to substrate long enough, a single active β-gal reporter enzyme could in principle produce enough fluorescent product to accumulate ex vivo and be measurable, but would not be an accurate reflection of the true underlying protein interaction profile.

**SPLIT GFP**

Many different split fluorescent proteins have been designed as protein fragment complementation reporters (49, 59–62, 84). Using previously reported variants of GFP, Hu et al. were able to create split fluorescent proteins with colors in the blue, cyan, green, and yellow ranges (Figure 4). The fluorescent proteins were split along amino acids 155 and 173 and activity was recapitulated by fusing each fragment to basic region leucine zipper family (bZIP) proteins, bFos and bJun. The color of these different combinations of split fluorescent protein was dependent primarily on the N-terminal fragment, yet was still modifiable to a smaller degree by the C-terminus of
the fluorescent protein. These split proteins allow simultaneous imaging of multiple protein interactions within cells as well as differential compartmentalization. Because maturation involves covalent rearrangement of bonds within the fluorophore inside the β-barrel of the fluorescent protein, once formed and matured (23), the split fluorescent proteins do not stop fluorescing until the intact fusion proteins are degraded. This increases the total fluorescence output greatly, but limits the utility of this technique as a real-time assay because off-reactions are kinetically delayed and difficult to detect. Of note, the advent of several fluorescence colors allows for the imaging of complex interactions that may involve several partners. Several advantages of this technique over bioluminescent probes include the lack of a required substrate, sufficient signal for high-throughput analysis via FACS, and subcellular localization measurements via microscopy.

**SPLIT LUCIFERASE STRATEGIES**

The two luciferase reporters most commonly used in optical imaging are *Renilla* luciferase (coelenterazine substrate) and firefly luciferase (D-luciferin substrate) (63). *Renilla* luciferases generally emit blue (\(\lambda_{\text{max}} = 475\) nm), a property less favorable for in vivo imaging, whereas firefly luciferases generally emit yellow to red (\(\lambda_{\text{max}} = 575–600\) nm), enhancing their utility in vivo. Coelenterates are sea animals and require the presence of oxygen and the bioluminescent substrate, coelenterazine, to generate bioluminescence. Coelenterazines are imidazolopyrazines containing a variety of substitutions that generate bioluminescence with a variety of characteristics in different organisms. The coelenterazine used in *Renilla* and apoaquorin, the most commonly used optical reporters, is unstable in air and somewhat hydrophobic. By modifying the chemical substrate, the spectral output of the luciferase can be altered substantially. Note that several coelenterazines have been found to be substrates for the efflux transporter **MDR1** P-glycoprotein, including coelenterazine f, h, and hcp (64). This raises general concern for the indiscriminant use of coelenterazine and *Renilla* luciferase 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, i.e., protein-protein interactions.

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**Figure 4**

Visualization of complementation between fragments of different fluorescent proteins fused to \(b\)Fos and \(b\)Jun. (a) Diagram of amino acid substitutions among enhanced green fluorescent protein variants and the positions where they were fragmented (155 and 173). (b–k) Fluorescent images of COS-1 cells transfected with plasmids expressing the protein fragments indicated in each panel fused to the bZIP domains of Fos and Jun. The C-terminal fragments were fused to \(b\)Fos and the N-terminal fragments were fused to \(b\)Jun. Structural models of bimolecular fluorescent complexes shown to the right of each image are based on the X-ray crystal structure of full-length GFP. The positions of fragmentation are indicated by arrows in the structures. The position and structure of the duplicated β-strand shown in (b–j) is unknown. The excitation/emission maxima of each complex are shown below the images. The bar represents 10 μm in all images. Reprinted by permission from Macmillan Publishers Ltd., Hu et al. 2003. *Nat. Biotechnol.* 21:53945.
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. The other main group of luciferase reporters is firefly and click beetle luciferases. This group of enzymes uses a benzothiazolyl-thiazole reaction in the presence of Mg, ATP, and O$_2$ to generate light (65–67). The substrate is first adenylated and then reacts with O$_2$ to create a dioxetanone ring. This ring is broken and CO$_2$ released to form the singlet state product, which consequently releases light upon returning to the ground state. The emission of these luciferase proteins (green to red) is modifiable by changing amino acid side chains within the active site and other areas of the protein. According to crystal structure data (68), firefly luciferase is composed of two distinct domains, a large N-terminal and small C-terminal domain. These domains encompass a large cleft within the active site and are hypothesized to work as a clamshell mechanism, opposing each other to exclude water upon the binding of the substrate D-luciferin and cofactors, thereby enabling the bioluminescent reaction.

**SPLIT LUCIFERASE INTEIN-MEDIATED PROTEIN COMPLEMENTATION**

Intein-mediated protein complementation relies on post-translational protein splicing reactions that facilitate precise excision of an intein (internal protein segment) followed by ligation of flanking exteins (external proteins) (69). The intein peptide itself is split into N- and C-terminal halves and fused in frame to each half of a reporter gene, which are in turn fused in frame to protein partners of interest (48). When the two interacting proteins come together, the intein is reconstituted and spliced out, leading to reconstruction of an intact reporter gene (Figure 3).

Ozawa et al. designed an intein-mediated split-firefly luciferase reporter system based on DnaE, an intein derived from a strain of *Cyanobacterium synechocystis* (12, 48, 70–73). This is a naturally split intein that can be reconstituted in *trans* to ligate N- and C-terminal ends of exteins. Two interacting proteins were fused, one to a fusion protein consisting of the N-terminal end fragment of DnaE and an N-terminal fragment of firefly luciferase (N-FLuc) and the other to a fusion protein consisting of the C-terminal end fragment of DnaE and a C-terminal fragment of firefly luciferase (C-FLuc). An interaction between the two proteins of interest allows for formation of an intact DnaE, leading to protein splicing and formation of a mature firefly luciferase. Addition of D-luciferin thus allows for measurement of interactions between the proteins of interest. This technique was used to study insulin-stimulated phosphorylation of IRS-1 (insulin receptor substrate-1) and its target PI3-kinase-derived SH2N (p85 subunit of phosphatidylinositol 3-kinase) domain in living cells (48). Although five- to sixfold bioluminescence inducibility was obtained in this assay, this was only observed beginning at 3 h post insulin stimulation, whereas insulin is documented to trigger the kinase function of the insulin receptor within minutes. Thus, the split-intein system suffers from slow kinetic response rates. In addition, the background luminescence signal from unstimulated cells was remarkably high (0.15 RLU as compared to 2.1 × 10^{-4} RLU from untransfected cells), suggesting
that any partial or transient association of the DnaE fragments with each other was sufficient to initiate the splicing reaction and reconstitute luciferase, thereby exhibiting a high false-positive rate and limiting the quantification of protein interactions. Furthermore, while split-intein strategies can kinetically detect “off-to-on” reactions or compartmental shifts in protein translocation, the readout is fundamentally an irreversible reaction, and thus split-intein systems pose problems for quantitative interrogation of reversible biochemical reactions, drug-induced protein associations, or shifts in equilibrium states of interacting proteins. In principle, if given sufficient time, the split-intein system will irreversibly tether all available protein partners into a single population of spliced heteromers.

To eliminate the problem of high background signal without compromising detection sensitivity, the next generation of split-intein mediated luciferases used split Renilla luciferase (RLuc) instead of firefly luciferase (FLuc) (72). This system was used to study ligand-induced protein trafficking into the nucleus both using cell-based in vitro assays and in vivo imaging of mice. Specifically, translocation of the androgen receptor (AR) from the cytosol to the nucleus was monitored upon stimulation with 5α-dihydrotestosterone (DHT) by fusing the N terminus of RLuc to the N terminus of DnaE and a nuclear localization sequence (NLS). The AR was fused to the C terminus of RLuc, which in turn was fused in frame to the C terminus of DnaE. Upon DHT stimulation, AR would translocate to the nucleus, allowing the split inteins to associate, resulting in formation of an active RLuc protein. Addition of DHT showed two- to eightfold induction in signal in a concentration-dependent manner, which could be inhibited using antagonists. The potential utility of this bioluminescence-based technique for high-throughput screening of chemicals was also simulated using 13 different chemicals. Finally, the utility of this technique was demonstrated in vivo using either subcutaneous (s.c.) or intracranial (i.c.) implantation of cells cotransfected with each split fusion protein. The s.c. implantation showed three- to fivefold increases in signal upon DHT injection as compared to vehicle, whereas the i.c. implantation showed complete inhibition with a DHT antagonist. Using this technique in combination with animals that are genetically engineered to express split fragments in specific tissues, it may be possible to monitor translocations of proteins of interest in target tissues as well as monitor activity of agonist and antagonist drugs mediating those translocations specifically in various organs. However, as pointed out above, quantitative pharmacokinetic analysis will not be possible with this strategy.

In addition to monitoring ligand-mediated interactions, the intein-mediated split-RLuc reporter was also used to study endogenous stress hormone activity in mice (70, 71). Nuclear translocation of the glucocorticoid receptor (GR) was visualized in response to stress by fusing the GR to the C termini of RLuc and DnaE, whereas a fusion of the N termini of DnaE and RLuc was localized to the nucleus. Only modest induction in signal was observed in reporter cells cotransfected with the two constructs and implanted s.c. in mice subjected to various stress inducers, such as physical swimming or elevated blood corticosterone. The utility of this technique might be greatly enhanced by using tissue-specific inducible reporters, as several factors can contribute to elevated stress of the animals, reducing day-to-day consistency in experimentation.
Overall, split-intein-mediated split-luciferase assays for detection of protein-protein interactions were developed to overcome several limitations of techniques inherent to two-hybrid and FRET systems. Split-intein strategy traversed the limitations of detecting protein interactions occurring solely in the nucleus or the need for the partners to be in exacting close proximity to each other. Although several approaches have generated levels of success within that goal, the split-intein strategy itself has limitations. The self-catalytic nature of the DnaE gene, while forming the basis of the trans splicing, also results in very high background luminescence, purportedly owing to the splicing event occurring even when there is partial association of the DnaE fragments (48). In addition, reconstitution of spliced luciferase is permanent, providing only an “on” signal. Even a fleeting interaction of the proteins of interest will result in luminescence signal, and furthermore, disassociation of protein complexes occurring subsequent to their association cannot be monitored. Thus, although certain initial rates of kinetic reactions could be estimated, quantitative titration curves of protein- and drug-mediated equilibrium reactions cannot be accurately measured. Finally, the truly important aspects of studying protein-protein interactions 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 this 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, and the split-intein strategy precludes the study of these important protein-protein interactions in cells and live animals.

**SPLIT RENILLA LUCIFERASE COMPLEMENTATION**

*Renilla* luciferase does not require ATP and uses a different substrate than that of firefly luciferase. However, the bursting behavior of *Renilla* luciferase places significant constraints on the timing and duration of image acquisition, especially when applied in vivo. Nonetheless, Paulmurugan et al. designed a novel split *Renilla* luciferase pair utilizing sites that would be applicable for use in intein-mediated systems (74). Six cut sites were explored by choosing positions close to cysteins for easy transfer of the constructs into an intein-based system. The investigators also based their choice on the presence of flanking glycines to act as flexible peptide linkers. A system analogous to previously published work with split firefly luciferase in which the N-terminal fragment was under the control of an NFκB-α promoter was developed and shown to be activated upon treatment with TNF-α (75). Two of the six fragment pairs produced significant signals; the split *Renilla* luciferase cut between nucleotides 687–688 (amino acid residue 229) resulted in the best signal. Although significant signal induction was observed, the high background of the N-terminal fragment alone was a cause for concern. The potential for in vivo utility of this system was demonstrated by transfecting HEK293T cells and injecting them into mice via different routes (i.p., s.c., and i.v.). This split *Renilla* luciferase construct showed nominal activity, but low total photon output, and only fivefold activity over background was demonstrated. Fold inducibility over untreated cells in vivo was not reported.
Using the same split Renilla luciferase constructs, the researchers went on to design a single chain biosensor using FKBP and FRB as the interacting proteins, generating one large fusion protein in frame with the N-terminal and C-terminal fragments (76). This construct introduced a new avenue of measuring protein interactions mostly applicable to lysates. In standard lysate experiments, two separate complementation fragments typically generate very low activity owing to the low concentration of the fragments and lack of proximity of the diluted proteins in the assay buffer. By creating a single chain biosensor, only the inducing ligand or drug is limiting because the apparent local concentration of the protein fragments is extremely high. The general drawback of this strategy is a high background signal, which is expected owing to the proximity of the split luciferase halves. This background signal can be minimized to some extent by creating different interprotein linker lengths and configurations. The best linker sequence exhibiting the lowest levels of background in this reporter was (EAAAR) × 4, which was predicted to code for an α-helix domain. This was thought to keep the uninduced protein partners sufficiently separated, yet allow flexibility for induced interactions to still occur.

The single chain biosensor, using FRB and FKBP as interacting proteins, was then used to demonstrate increases in overall signal output in an in vivo setting, but the background continued to be significantly high. In cellulo, a better signal-to-noise ratio was obtained using two separate constructs instead of a single chain biosensor. This is a practical consideration because any measurement of protein interactions will be highly dependent on protein compartmentalization as well as large complex formation. Thus, a single chain biosensor shows great promise as an in vitro assay for drug-mediated interactions when protein dilution is of concern, but may not be the ideal format for monitoring interactions in cellulo or in vivo. It is readily conceivable to create a recombinant bioluminescent single chain biosensor based on these strategies to detect protein interactions in high-throughput drug screens.

Kaihara et al. also created a split Renilla luciferase with greater success (77). These investigators chose to split intact Renilla luciferase based on positions proximal to serine, tyrosine, and cysteines, producing eight different split luciferases. The reporter with the highest inducibility and best overall signal was Renilla luciferase split after serine residue 91 (RL91). Other combinations showed modest activity and underperformed relative to RL91. To demonstrate protein complementation, the researchers utilized the Y941 peptide, a peptide derived from insulin receptor substrate 1 (IRS-1), and the N-terminal SH2 domain, part of the p85 subunit of phosphatidylinositol 3-kinase. Phosphorylation of the Y941 peptide occurs via the insulin receptor upon insulin stimulation. Additionally, use was made of a modified Renilla luciferase containing a C124A mutation that greatly increased the activity of the intact protein compared to wild-type Renilla luciferase. The RL91 construct exhibited 25-fold induction upon treatment, whereas the rest of the split RLuc fragment pairs only exhibited two- to fourfold induction. Insulin inducibility was completely abrogated by mutating the 941 tyrosine peptide (Y941F) to prevent phosphorylation. This assay also enabled time course studies of the interactions, showing peak protein interaction at 5 min postinduction, with slow degradation of signal, even with very low levels of insulin (10 pM to 100 nM) (Figure 5). In addition, sites of interactions within cells were
Figure 5
Split Renilla luciferase reporter. (a) Time course of luminescence upon sRL91 complementation. Cells expressing sRL91 were incubated in culture medium supplemented with 100 nM or 10 pM insulin for 1, 5, 10, 30, and 60 min at 37°C. The luminescence of these cells was immediately assessed. (b) Immunoblot analysis of tyrosine phosphorylation and dephosphorylation on Y941 in sRL91. Cells expressing sRL91 were incubated in culture medium supplemented with 100 nM insulin for 1, 5, 10, 30, and 60 min at 37°C. Whole-cell lysates were immunoprecipitated with anti-myc antibody. Immunoblot analysis was made using anti-phosphotyrosine antibody and anti-myc antibody. Figure modified from Kaihara et al. 2003. Anal. Chem. 75:4176–81.

mapped as occurring primarily on the plasma membrane using a supercooled CCD camera and Zeiss microscopic optics. This split Renilla luciferase shows significant promise for use in in cellulo assays as a measure of protein interactions. However, as discussed above, the generally unfavorable properties of Renilla luciferase for imaging applications in vivo may preclude use of this construct in animal models.

SPLIT FIREFLY LUCIFERASE COMPLEMENTATION
Paulmurugan et al. describe the construction of a split firefly luciferase, based on the cut sites originally developed by Ozawa et al. (48) for use with split-intein mediated
reporters. Ozawa et al. chose to split the luciferase enzyme at the tether region between the two domains of intact luciferase, i.e., between residues 437 and 438. Using this split site, protein complementation was achieved by creating fusion constructs to the constitutively binding protein products myoD and Id, members of the helix-loop-helix family of nuclear proteins (75). Id was fused to the C terminus of N-FLuc(1–437) and driven by an inducible NFκB promoter or a constitutive CMV promoter. MyoD was introduced into the N terminus of C-FLuc (438–554) and driven by a constitutive CMV promoter. By showing a protein interaction readout that was immediately detectable, this study confirmed the possibility of using a bioluminescent reporter as a protein fragment complementation pair for the rapid imaging of different proteins. They also were able to reconstitute this activity in animals by implanting HEK293T pseudotumors subcutaneously. These constructs, however, exhibit extremely high background activity of the N-FLuc fragment alone, generating poor signal-to-noise ratios. Also, when treated with TNFα, the inducible system showed a large induction of the N-FLuc fragment alone when driven by a constitutive CMV promoter. This massive increase in signal can be attributed to the NFκB binding sequence found in the CMV promoter. Induction of the N-FLuc fragment alone appeared comparable in magnitude to the combined N-FLuc and C-FLuc fusion proteins, suggesting that this reporter strategy exhibited a very low dynamic range. Finally, the use of a transcription-dependent expression cassette for one fragment, in this case, the NFκB promoter activated by TNFα, limited use of this system as a real-time reporter because the delay of several hours upon ligand stimulation would not reflect the true temporal resolution of the target protein interactions.

In a more recent publication by the same investigators, changes were made to the split site of the firefly luciferase (78). They conducted a small survey of split luciferase fragments based on the crystal structure of the enzyme and created various combinations of fragments to find the best interacting pairs. The N-FLuc fragment was fused to the N terminus of FRB (FKBP12-rapamycin binding domain of mTOR) and C-FLuc was fused to the C terminus of FKBP12 (FK506 binding protein). Complementation could then be induced by the addition of rapamycin. Rapamycin binds first to FKBP12 and the Rap-FKBP complex in turn binds to FRB (79, 80). This complex brings the luciferase fragments together, recreating the active site and facilitating a bioluminescent reaction in the presence of D-Luciferin. The investigators created seven nonoverlapping split luciferases at residues 415, 420, 437, 445, 455, 475, and 500. Four of the seven (420, 437, 445, and 455) showed significant induction over untreated cells when treated with rapamycin. From this survey, the best nonoverlapping complementation fragments were the previously described residues 1–437 and 438–550. However, controls included untreated cells, which showed comparable activity to split Luc constructs that were not fused to interacting proteins. Furthermore, it is difficult to ascertain from the published data the magnitude of the background activity for these constructs relative to mock transfected cells, potentially limiting the utility of these constructs. While showing some promise, further validation of these constructs is required.

Paulmurugan et al. also designed several overlapping sequences that were able to self-associate to produce high light output (78). They were able to reconstitute
activity in 65% of the overlapping fragment combinations with a range from 0.123 RLU/μg protein/min to as high as 3500 RLU/μg protein/min. The optimal self-complementing overlap fragments are N-FLuc(1–475) and C-FLuc(265–550), a pair that is able to reconstitute 3%–4% of the activity of intact luciferase, notable for a split enzyme. To show the capacity of this system to measure compartmentalization of proteins, the researchers cotransfected cells with nuclear localization sequences on either split N-FLuc, split C-FLuc, both, or neither. The cells cotransfected with both or neither had high constitutive photon output, whereas the cells transfected with only one nuclear-localized fragment showed greatly reduced activity. The potential utility of these constructs is highlighted by an experiment using a purified permeation peptide conjugate, TAT-C-FLuc, transduced into cells stably expressing N-FLuc. The TAT-C-FLuc transduced cells showed sevenfold higher activity than untransduced cells. This interaction is tenfold less than when C-FLuc is transiently transfected in the stable cell line expressing N-FLuc. The self-associating split luciferase could potentially be used to elucidate macromolecular delivery vehicles, measure bilayer transport of tagged proteins, and explore protein compartmentalization. These self-associating constructs, however, are not intended for and have limited utility in the measurement of regulated or titratable protein-protein interactions.

**GENERATION OF ENHANCED SPLIT FIREFLY LUCIFERASE COMPLEMENTATION**

To identify an enhanced pair of firefly luciferase fragments that reconstituted an active (bioluminescent) heterodimer only upon association, we constructed and screened a comprehensive combinatorial incremental truncation library that employed a well-characterized protein interaction system: rapamycin-mediated association of the FRB domain of human mTOR (residues 2024–2113) with FKBP-12. Initial fusions of FRB and FKBP with N- and C-terminal fragments of luciferase, respectively, were designed to minimize enzymatic activity of the individual fragments and maximize induced complementation, to yield the highest fold inducibility (7, 81). Fragments of *P. pyralis* luciferase (derived from pGL3; Promega) were fused to FRB and FKBP by a linker containing a flexible Gly/Ser region. From these constructs, N- and C-terminal incremental truncation libraries were generated by unidirectional exonuclease digestion and were validated as described. The libraries were then coexpressed in *Escherichia coli* and screened for bioluminescence in the presence of rapamycin. From this screen, an optimal pair of minimally overlapping amino acid sequences for the N-FLuc and C-FLuc fragments was identified that produced very low signal in an uninduced state and abundant bioluminescence when the fused protein interactions were induced by the addition of rapamycin.

Optimal bioluminescence was seen with the FRB-N-FLuc(2–416) and C-FLuc(398–550)-FKBP pair, netting greater than 1200-fold signal over background, whereas the individual fragments exhibited undetectable signal as compared to background. The rapamycin-inducible FRB-FKBP interaction was titrated as a function of rapamycin concentration, yielding values for apparent Kd of rapamycin binding (1.5 nM ± 0.3 nM) comparable to literature values for the isolated protein in vitro.
Furthermore, inhibition of rapamycin-induced interactions with FK506, a potent inhibitor of rapamycin binding to FKBP, yielded an apparent $K_i$ value (4.2 nM) that was also comparable to literature values, demonstrating that this reporter equilibrium interaction is reversible and quantitative. FRB can be mutated at position S2035I to abrogate FRB binding to the rapamycin-FKBP complex. Introduction of the mutation into the FRB-N-FLuc fragment totally abrogated light output to levels comparable to cells not treated with rapamycin (7). Drug-induced protein interactions were readily visualized in mice in vivo by generating intraperitoneal pseudotumors of HEK293T

![Figure 6](image)

**Figure 6**
Optimization of firefly luciferase protein-fragment complementation imaging. (a) Schematic representation of the optimized N- and C-terminal fragments of luciferase (as revealed by screening of incremental truncation libraries), fused to FRB and FKBP-12, respectively. (b) Rapamycin-induced association of proteins FRB and FKBP-12 brings inactive fragments of luciferase into close proximity, thereby producing bioluminescence activity. (c) Monitoring rapamycin-induced FRB/FKBP12 association in live cells. HEK-293 cells transfected with FRB-NFLuc + CFluc-FKBP-12 (upper) or mutant S2035I FRB-NFLuc + CFluc-FKBP-12 (lower) were treated for 6 h with 50 nM rapamycin. Note that the S2035I mutation of mTOR/FRB is known to abrogate the rapamycin-induced association of FRB and FKBP-12. A pseudocolor IVIS bioluminescence image of live cells in a 96-well plate is shown. (d) Luciferase complementation imaging of two representative $nu/nu$ mice, one implanted with HEK-293 cells expressing FRB-NFLuc + CFluc-FKBP (upper) and the other with cells expressing mutant S2035I FRB-NFLuc + CFluc-FKBP-12 (lower). Images were taken 18 h before treatment with rapamycin (left) and 2.5 h after receiving a single dose of rapamycin (4.5 mg/kg, i.p., right). Reprinted from Reference 1, with permission from Elsevier.
cells transiently transfected with the fusion constructs and treating the mice systemically with rapamycin. Real-time measurement of rapamycin-induced FRB/FKBP association over time provided a noninvasive readout of target-specific rapamycin pharmacodynamics with a maximum 23-fold signal amplification upon treatment in vivo (Figure 6).

To demonstrate the versatility of this reporter system, the split-FLuc fragments were used to measure the phosphorylation-dependent interaction of Cdc25C and 14-3-3ε, members of the DNA replication and damage checkpoint pathways. Up to 50% inhibition of this interaction could be measured using a staurosporine analogue, UCN-01 (82), and the signal was totally abrogated by a S216A mutation in Cdc25C, a substitution known to block 14-3-3ε binding to Cdc25C.

This new reporter now allows for real-time assessment of cellular protein-protein interactions, regardless of subcellular localization of the binding partners. This robust reporter of protein interactions allows for detailed analyses of proteins in their normal environment and can also be performed in living animals. The fold-induction of this enhanced split luciferase system is exceptional, whereas the background activity of the individual fragments is indistinguishable from untransfected background when used at low to modest expression levels, providing a powerful tool for elucidating protein function and kinetics in living systems.

**CONCLUSIONS**

Accurate measurement of protein interactions represents the next step to deciphering the genetic and proteomic code. The intricacies of cellular events require assays that can measure different types of associations using a vast array of methods. The advent of these new complementation systems for the optical readout of real-time interactions now allows for deconvolution of previously undetectable cellular events. Firefly luciferase complementation systems are quite powerful in that they allow for the real-time assessment of protein-protein interactions within the cytosol, nucleus, and plasma membrane, not limited to analysis-specific compartments, for example, as with two-hybrid strategies. Complementation strategies may provide a reversible and responsive system to detect transient events, which no doubt constitute a major fraction of all protein-protein interactions. For use in vivo, the split firefly luciferase with small overlapping sequences excels (7) because it consistently yields high signal and excellent inducibility with a variety of protein-binding partners. The reaction kinetics and ease of delivery of the substrate, D-luciferin, also allows for facile application of optical assays. Although single chain biosensors potentially increase the total photon output of the constructs, these reporters also tend to increase the background activity to such an extent that the inducible differences in bioluminescence are often more difficult to distinguish and quantify. Single chain biosensors can, however, be used in vitro as a rapid drug-binding assay for use in high-throughput systems. As long as the proteins in question are not post-translationally modified and do not require higher order complex formation for activity, the biosensor could essentially be overproduced in bacteria and purified to generate large quantities of a versatile and sensitive reagent.
While selected Renilla luciferase systems function quite efficiently (77), the inherent weakness of any Renilla luciferase-based assay is its substrate, coelenterazine. Coelenterazine exhibits poor reaction profiles for long-term kinetic experiments, and the hydrophobicity of the molecule renders use in vivo difficult. Even so, the development of systems utilizing different substrates now allows investigators the opportunity to measure two different protein interactions at the same time by spectrally unmixing output colors. 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 (83). Simultaneous imaging of multiple interactions should allow deconvolution of complex protein interactions. Future developments may involve the creation of better, more effective split proteins with further increases in output and inducibility that would facilitate kinetically robust, long-term, and accurate measurement of protein interactions in vivo. These new systems would expand the arsenal used to assess protein interactions that current technologies are not capable of detecting and quantifying. The current systems and anticipated advances will not only lead to a better understanding of the underlying mechanisms of signal transduction, protein interaction cascades, and drug interactions, but provide investigators with novel tools for expediting drug discovery and characterization in vivo.

**SUMMARY POINTS**

1. New tools have been developed that allow for more efficient and effective quantification and analysis of signal transduction and protein-protein interactions in living animals in vivo. These assays should expedite the study of these protein interactions in their most appropriate and native milieu.

2. There exist many assays for measuring protein interactions, including FRET, BRET, two-hybrid systems, and protein fragment complementation systems (split inteins, DHFR, β-galactosidase, β-lactamase, Renilla, and firefly luciferase).

3. Firefly luciferase requires its cognate substrate (D-luciferin), ATP, Mg, and O₂ to produce light photons, whereas Renilla luciferase requires its substrate coelenterazine and O₂. The kinetics of emission and characteristics of the luciferase substrates favor the use of firefly luciferase in living animals owing to the prolonged emission and signal stability (hours). Renilla luciferase exhibits unfavorable spectral characteristics and fast bursting activity that render signal acquisition difficult to quantify and compare to other measurements in vivo unless performed on very short timescales (seconds to minutes).
4. Split inteins based on the self-splicing DnaE inteins have been incorporated into split firefly and split *Renilla* luciferase constructs. A splicing reaction triggered by an interaction between the proteins of interest reconstitutes the intact active reporter. However, the slow kinetic response rates and the irreversible nature of the splicing reaction does not allow for analysis of real-time or reversible protein interactions. Nonetheless, within cells, reduction of the excessive background signal found in typical split intein assays was achieved via differentially compartmentalized split intein protein fragments.

5. Several iterations of *Renilla* and firefly luciferase protein complementation pairs have been produced. One split *Renilla* luciferase showed promise with regard to inducibility and total photon output. A split firefly luciferase screened from an incremental truncation library exhibited the greatest levels of inducibility and signal intensity as well as applicability in vivo of all split luciferase systems described. The benefits and limitations of each of the various intact luciferases apply to their corresponding split protein fragments.

6. A novel method using a single chain biosensor illustrates the versatility of the split luciferase strategy. Although the interaction kinetics may not be biochemically accurate owing to the extremely high effective concentration of the protein interaction partners of interest, nonetheless, as a tool for screens in vitro, large amounts of recombinant protein can be produced and used as an inexpensive and effective biosensor. This could lead to new discoveries of drug interactions not previously measurable in large-scale screens.

7. Future work within this field includes the generation of second-generation complementation reporters with improved signal-to-noise ratios, inducibility, and red-shifted spectral properties for use in vivo. The ideal reporter would act not only as an “on-off” signal, but also give a graduated response with minimal background signal and excellent induced signal output.

**ACKNOWLEDGMENTS**

We thank colleagues of the Molecular Imaging Center for insightful discussions and excellent technical assistance. Work reviewed herein was supported by a grant from the National Institutes of Health (P50 CA94056).

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