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REVIEW

Split-luciferase complementary assay: applications, recent developments, and future perspectives

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Abstract Bioluminescent systems are considered as potent reporter systems for bioanalysis since they have specific characteristics, such as relatively high quantum yields and photon emission over a wide range of colors from green to red. Biochemical events are mostly accomplished through large protein machines. These molecular complexes are built from a few to many proteins organized through their interactions. These protein–protein interactions are vital to facilitate the biological activity of cells. The split-luciferase complementation assay makes the study of two or more interacting proteins possible. In this technique, each of the two domains of luciferase is attached to each partner of two interacting proteins. On interaction of those proteins, luciferase fragments are placed close to each other and form a complemented luciferase, which produces a luminescent signal. Split luciferase is an effective tool for assaying biochemical metabolites, where a domain or an intact protein is inserted into an internally fragmented luciferase, resulting in ligand binding, which causes a change in the emitted signals. We review the various applications of this novel luminescent biosensor in studying protein–protein interactions and assaying metabolites involved in analytical biochemistry, cell communication and cell signaling, molecular biology, and the fate of the whole cell, and show that luciferase-based biosensors are powerful tools that can be applied for diagnostic and therapeutic purposes.

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Introduction

Bioluminescence has been observed in many organisms, such as bacteria, fungi, algae, fish, squid, shrimp, and insects; it is a fascinating phenomenon that produces light by a chemical reaction. In this enzyme-dependent reaction, the enzyme luciferase generates light through the release of chemical energy from its substrates, for example, in catalysis of the luciferin and ATP reaction by firefly luciferase [1–5]. Bioluminescence-based assays have been used as a powerful technique for several applications in various types of biotechnological investigations. This technique is so sensitive that just a few photons can be detected by the use of available light-measuring systems. They have been used as sensitive tools in different fields from gene expression monitoring, protein localization, and protein–protein interactions to the detection of infections, monitoring of cell death and programmed cell death (apoptosis), tumor development and metastasis in whole animals [6, 7], reporter gene assays [8], protein trafficking [7], drug screening [9], and detection of environmental contamination [10, 11]. Among the major advantages of bioluminescence-based techniques are the very low background in biological systems, flexibility, noninvasiveness, reproducibility, high efficiency, and ease of assay performance with high sensitivity and low cost [7, 8, 10].

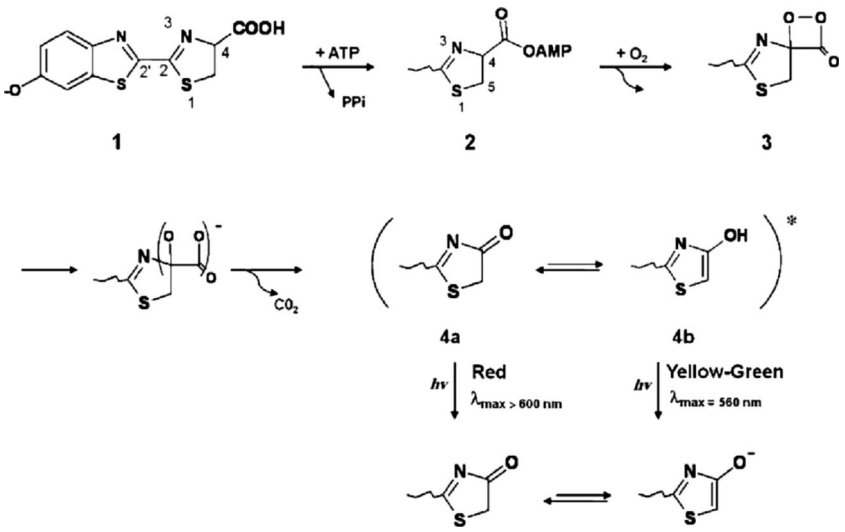
Bioluminescence in vivo imaging makes the real-time analysis of disease development at the molecular level and monitoring the course of disease and disease progression possible in living organisms. Consecutive quantification of biological processes is possible in entire animals by bioluminescence in vivo imaging [12]. As mammalian tissue lacks intrinsic bioluminescence, bioluminescence imaging could be

considered as a suitable and subtle tool for in vivo imaging [6]. Photons are both scattered and absorbed when they pass through mammalian tissue. Shorter wavelengths of light (blue and green) are mainly absorbed by the tissue. In contrast, the longer wavelengths (red light) are less affected [12, 13] and therefore can penetrate through several centimeters of tissue. This causes red light to be more easily detected than the absorbed blue or green light [6].

Bioluminescence is usually found in three main groups of insects: fireflies (Lampyridae), click beetles (Elateridae), and railroad worms (Phengodidae). Luciferases from these groups are very similar in their structures, substrates, and cofactors: luciferin, O_2, ATP, and Mg^{2+}. Firefly luciferase, characterized as an enzyme that has a high quantum yield, emits light in two successive reactions. In the first step, luciferin (a benzothiazole compound biosynthesized from cysteine) and ATP form luciferyl adenylate. In the next step, oxygen binds to the luciferyl adenylate intermediate to produce carbon dioxide and excited singlet oxyluciferin, which returns to the ground state and emits light ranging from yellow to red depending on the conditions, such as the pH and polarity of the solvent (Scheme 1) [14–16]. Regarding the crystal structure, firefly luciferase has two distinct domains, the large N-terminal domain (residues 1–436) and the small C-terminal domain (residues 440–550), connected by a flexible link between the two domains. The way the substrates are attached induces conformational changes and results in the domains being close to each other [17]. It is imagined that these two domains have no dramatic effect on each other's stability and also that they fold independently. On the basis of this hypothesis, separation of luciferase domains and their attachment to other proteins is probable and should have no disruptive effect on the domain structures. Thermosensitivity is one of the most important features of luciferase and also limits its application. The decay rate of luciferase activity is 3–5 min in vitro, whereas it is up to several hours in vivo. With this in mind, luciferase is very thermosensitive and hence unstable during in vitro assays [18]. Many strategies such as solvent engineering [19–21], site-directed mutagenesis [22–26], and disulfide bridge introduction [27–29] have been applied to achieve more favorable luciferases in order to decrease the disadvantages of this enzyme. One of the most thermostable luciferases is a mutant variant from *Photuris pennsylvanica* with improved properties, which can increase remaining activity for up to 5 h at 65 °C [30].

Renilla luciferase, which oxidizes coelenterazine in the presence of oxygen to produce oxyluciferin, CO_2, and blue light (λ_{max}=480 nm), is another known luciferase. The bioluminescence spectrum of *Renilla* is completely different from the spectrum of firefly luciferase. Moreover, *Renilla* luciferase light emission is ATP-independent, and unlike with ATP-dependent firefly luciferase, no ATP interference with other cell processes will occur during addition of ATP in different assays [2, 31]. Some advantages of luciferase emitting light of different colors are related to its application in designing new dual reporters and biosensors which provide a good means of evaluating more than one bioanalyte in a real-time manner. The gene sequence of *Renilla* luciferase cannot be expressed in mammalian cells owing to its very different codon usage. To end this, a new sequence has been designed, by substituting the nucleotides in the codons in such a way that the protein sequence remains the same, and has been exploited in mammalian cells for promoting the expression of *Renilla* luciferase [31].

Up to now, many novel genetically encoded biosensors using luciferase have been designed. These biosensors are categorized into three major groups: circularly permuted luciferase, cyclic luciferase, and split luciferase. In biosensors based on circularly permuted luciferase, a suitable peptide linker is designed to connect the original protein terminals



Scheme 1 Two-step oxidation of luciferin during the firefly luciferase reaction to produce light, oxyluciferin, CO_2, and AMP. Oxyluciferin emits light through a keto or enol tautomer

and permuted proteins. This linker may have sites for specific ligand attachment, which would induce conformational changes and alter the bioluminescent signals. Even though circularly permuted proteins are an invaluable blessing for investigating protein evolution and folding, their application is limited owing to the optimization of enzyme activity in most studies [18]. In methods based on cyclic luciferase, the terminals of luciferase are attached to two fragments of DnaE intein with a sequence for a specific protease. After protein expression in the cell, the terminals of luciferase will be ligated to each other by protein splicing and produce closed circular inactive luciferase. This inactive construct is unable to produce any bioluminescent signal until it is converted into an open active form by means of a specific protease [32]. This strategy is limited to screening just a few proteases and is not suitable for investigating many other biochemical analytes. The last kind of engineered luciferase is split luciferase; this is used in the luciferase complementation assay. Split luciferase is a powerful tool for careful examination of protein–protein interactions, protein–nucleic acid interactions, and also many bioanalytes both in vivo and in vitro. In this strategy, the two halves of luciferase are attached to two probable interacting proteins. When the two candidate proteins interact with each other, the halves of luciferase undergo complementation and luciferase activity is recovered. Additionally, by attaching the halves of luciferase to the terminals of a single polypeptide with a specific ligand site, one can design biosensors for many different ligands [33].

To achieve an appropriate split-luciferase complementation assay, the system designer must pay careful attention to some issues. The most important issue is finding appropriate sites at which to fragment luciferase and produce suitable N-terminal and C-terminal fragments, which recover most of their activity after induction of conformational changes, therefore generating a high quantum yield of photons. Paulmurugan and Gambhir used combinatorial library screening to develop an improved split firefly luciferase fragment assisted complementation system for studying protein–protein interactions. They found the best site for splitting firefly luciferase and *Renilla* luciferase, where the recovery of split luciferase peaks after interaction [15, 31]. The linker composition is another important factor that influences split-luciferase complementation in both its amino acid modules and its length. A flexible linker which contains two repeated sequences including four glycines and one serine, $(GGGGS)_2$, is the best linker to join the N-terminal and C-terminal luciferase halves to the two interacting proteins. This linker is flexible, and it has been empirically shown that the length of the linker should be sufficient to allow the assembly of reporter protein fragments [34].

In vitro methods such as enzyme-linked immunosorbent assays (ELISA), surface plasmon resonance, and fluorescence polarization are extensively used to investigate protein–protein interactions and protein–DNA interactions and their antagonists. These techniques are often dependent on either antibodies or highly purified proteins, and sometimes they require chemical modifications [31, 33, 35]. On the other hand, the robust in vivo procedures, which include yeast two-hybrid assays, are more convenient as there is no need for protein purification, which increases the duration of the experiments. These methods, however, may cause false positives and false negatives because of the multifactorial nature of signal production [36, 37]. Protein-fragment-based methods, where particular intramolecular or intermolecular interactions promote the reassembly of a previously split reporter protein, are located between the two ends of the spectrum [38]. As mentioned above, red-emitting mutants of firefly luciferase act more efficiently in imaging of protein–protein interactions within small living animals. Furthermore, these bioluminescent reporters surpass other reporters in small animals because of their low background signal, whereas fluorescent reporters (e.g., green fluorescent protein and red fluorescent protein) and those that use fluorescent substrates as a readout (e.g., β-lactamase and β-galactosidase) increase the background signal owing to autofluorescence [39].

In this review, we demonstrate the traditional techniques used in different aspects of molecular biology and biochemistry and summarize the worldwide research according to the different applications of the split-luciferase complementation assay. We wish to show the importance, ability, and efficiency of this state-of-the-art split-luciferase-based method and encourage science enthusiasts to expand and apply this potential system in every branch of biological science and clinical practice. We present a variety of examples and try to compare different techniques used in the three major fields of biology—biochemical analysis, cell communication and cell signaling, and protein–protein interactions—to help readers gain better understanding of reporter proteins. At the end of this article, we present critical perspectives and try to open a new window to further studies.

Biochemical analysis

Biochemical analysis is a rapidly developing field and is a key component in new discoveries of and research into biochemical and physiological events occurring in cells. The split-luciferase complementation assay is a technique that can be used for evaluating the levels of specific small molecules, enzymes, and protein activity modification produced by chemical reactions in the cell, and for investigating whole-cell conditions [9]. Recombinant proteins can be designed using split luciferase as a reporter protein to indicate intermolecular and intramolecular interactions [40, 41].

The commonest strategy is to insert an epitope, a domain or an intact protein, into an internally divided luciferase, where

the inserted fragment acts as a response unit and its conformational changes make the split luciferase be complemented or discomplemented, thereby increasing or decreasing, respectively, the intensity of the luminescent signals [42–44]. A glucose-sensing molecule, for example, was constructed by inserting galactose/glucose-binding protein (GGBP) into luciferase and was used to design a luminescence-based glucose sensing molecule. To change the specificity to glucose, a single mutation was introduced in the glucose-sensing luciferase (Fig. 1) [45]. This was the first report of applying a periplasmic binding protein to create split-luciferase-based biosensors. Potential periplasmic binding proteins with application in biosensors have been identified for a wide range of ligands, such as monosaccharides, amino acids, and metal ions, with high affinity [46]. Previously, a fluorescence-based glucose-sensing system conjugated with GGBP had been developed for blood glucose monitoring, based on how the fluorescence intensity of fluorophores changed following the GGBP conformational changes. The most serious disadvantage of this system was the quenching effect of environmental compounds caused by the reduction of the intensity of the fluorescent signals; this problem does exist in the split-luciferase-based method [47]. Furthermore, the activity ratio of split luciferase in the presence and absence of glucose was 18, in contrast to just severalfold in the fluorescent system.

Biochemical analysis is significantly important in molecular biology studies, particularly in studies of regulation of gene expression, protein synthesis, and protein modification. It is crucial to know when the specific gene is expressed and what kind of small molecules can suppress or induce its expression. To achieve this, each domain of luciferase is fused to two different transcription factors. In the presence of a specific substance or cell condition, assembly of these transcription factors will be detected by the emitted luminescent signal, which in turn is evidence of triggering gene expression. On the basis of this strategy, a cell-based high-throughput screening assay has recently been established via a luciferase fragment complementation assay that detects nuclear-factor-E2-related factor 2 (Nrf2) activators. Nrf2 is a cytosolic protein which is involved in a major pathway for protecting the cell against oxidative damage and is inhibited by Kelch-like ECH-associated protein 1 (Keap 1). This inhibition is eliminated under stress conditions or on induction of exogenous Nrf2 activators and causes Nrf2 to be translocated into the nucleus, where it interacts with MafK to form a heterodimer, and subsequently an antioxidant response will be induced [48]. In a recent study, an N-terminal fragment of luciferase was attached to Nfr2 and the C terminal was fused to MafK and used as a biosensor for screening compounds which could act as an inhibitor in the interaction of Nfr2 and MafK. This study found that the overexpression of the protein under study may result in a high background. On the other hand, a minimum expression of protein is required for sufficient interaction between two protein partners. With this in mind, in future studies it will be necessary to calibrate the system and find the proper expression condition for the optimum assay performance [49]. In parallel to exogenous molecules, the effect of different transcription factor interactions on expression or suppression of a gene can be studied using the split-luciferase complementation assay.

Detection of various protein biomarkers in clinical research is another area where split luciferases should be considered, as there are some limitations in the conventional techniques, particularly ELISA [50, 51]. In ELISA, the fixation of an antigen or antibody on a solid base requires washing and linked-secondary-antibody attachment, which is not feasible for complex heterogeneous fluids such as blood and lysates. For that reason, a precise general method is needed for direct detection of native protein in heterogeneous solutions. In this case, fragmented luciferase halves are linked to receptor fragments or a single-chain antibody, which indicates the presence of protein targets. To evaluate this hypothesis, CD4 receptor of T lymphocytes and the Fab portion of a certain antibody were fused to the N-terminal and C-terminal halves, respectively, of firefly luciferase to see if it can recognize the presence of HIV-1 gp120 coat protein. As CD4 and the Fab portion of the antibody interact with different epitopes, luciferase complementation and light emission detect the definite presence of gp120 as a protein target. The same strategy has

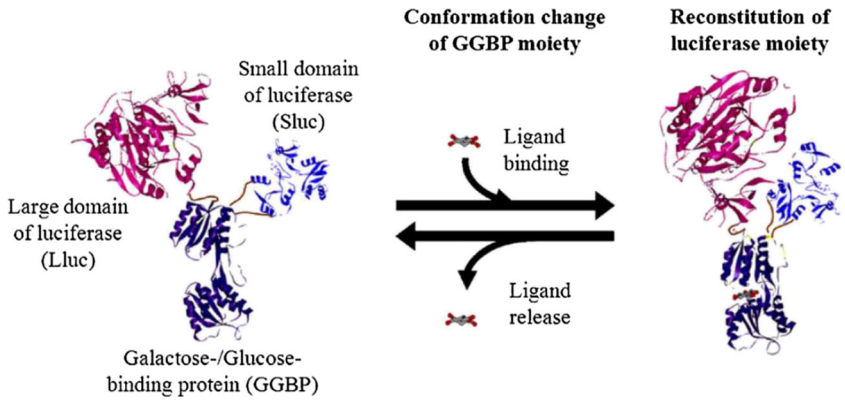


Fig. 1 The principles of ligand detection by periplasmic-binding-protein-fused luciferase (glucose-sensing luciferase). (Reprinted from [45] with permission from Elsevier)

been used for detection of human epidermal growth factor receptor (EGFR)2 (HER2; a marker for breast cancer) and vascular endothelial growth factor (a factor for angiogenesis and tumor growth) [52].

In addition to gene expression analysis and identification of the expressed protein, there is intense interest in exploring protein modification by taking advantage of the luciferase complementation assay. Initial studies were based on introducing a specific recognition sequence in luciferase and detecting the activity of a modifier enzyme such as a proteinase or a protein kinase by the change in the intensity of the luminescent signals [53]. There are some limitations in this kind of procedure; for example, the introduced sequence could have a disruptive effect on the activity of the luciferase, so just a narrow range of modifier enzymes, whose recognition sequence does not interfere with the normal activity of luciferase, can be evaluated. Moreover, that modified luciferase is less stable than native luciferase. In this case, split luciferase might be an appropriate substitute for engineered luciferase. Among the advantages of this approach is the real-time analysis of protein modification and modifier enzymes in the cell, and the most important feature is that unlike the modified luciferase, split luciferase is able to demonstrate the degree of modification.

Many identical proteins are expressed in various cells, but why are discrete behaviors and functions divulged by the cells? To answer this question, we need to look at dynamic regulation of the expressed proteins caused by posttranslational modification like what is seen in neuronal and myocardial excitability [54], in which voltage-gated Na^+ channels (Nav) are involved and regulated mainly by kinases. Protein phosphorylation is one of the commonest modifications. There are different hot spots for phosphorylation of these channels, whose regulator protein is intracellular fibroblast growth factor 14 (FGF14) [55, 56]. The traditional methods used for studying protein binding to ion channels are patch-clamp electrophysiology and ion flux assay, but these are not high-throughput methods or optimized for protein–ion channel complexes. These techniques are also costly and time-consuming as antibodies and large amounts of purified proteins are required. Split luciferase has eliminated these limitations, and in addition has some advantages, such as high signal-to-noise ratio, reversibility of luminescence, and appropriate dynamic range. According to studies of split-luciferase-attached Nav and FGF14, as selective kinase inhibitors are activated, a decrease in Nav and FGF14 phosphorylation followed as the interaction of these two phosphorylated proteins strengthened and made the fragmented luciferase be complemented more efficiently, resulting in higher light intensity [57]. Therefore, on the basis of the number of phosphorylated sites that affect the interaction strength and subsequently the complementation of luciferase fragments, the light intensity will increase or decrease.

Cleavage of preproteins and changing them into active proteins is another form of protein modification done by proteases which influences different biological pathways, such as cell cycle regulation, programmed cell death, and maturation of polyprotein precursor [58, 59]. Therefore, techniques for measuring protease activity have been well investigated, but conventional methods are still limited to in vitro analysis in which large peptides are generally attached to and quench a fluorescent probe. These large peptides might not be able to permeate cells and so might not be ideal for studying intracellular environments. As a solution, sensors based on green fluorescent protein, whose detection needs highly sensitive instruments, were genetically designed [60]. Split luciferase is widely used for evaluating the activity of proteases, which are especially important in the retroviral life cycle, such as that of hepatitis C virus, influenza virus, and HIV-1 and HIV-2, all of which affect millions of people worldwide [61–63]. Replication of these viruses is highly dependent on the maturation of the polyprotein precursor, which is mediated by specific proteases through cleavage of this large precursor into several small active proteins. These proteases are therefore appropriate targets for therapeutic strategies which involve developing inhibitors that hamper protease activity, thus causing termination of the viral progress [64, 65]. For this reason, strategies for monitoring the retroviral protease activity in vivo should be applied in designing new drugs. Split luciferase is a state-of-the-art imaging tool for quantifying protein interactions and detecting the activity of these proteases in both cells and living animals. In 2010, the activity of hepatitis C virus protease was first reported by using a split-luciferase strategy in which two interacting polypeptides were fused to a fragmented luciferase and a protease cleavage site was inserted between the two halves [66]. By the function of the protease, the two interacting polypeptides caused the luciferase fragments to come close to each other and this resulted in optical signals being detected for caspase 3/7, where a caspase cleavage site (DEVD) was inserted between the two luciferase fragments (Fig. 2) [67]. As a result, the dose of active protease was determined, but because of the undesirable luciferase complementation, the luminescent signal was just two to three times more intense than the background. A possible reason for the high background is the presence of a short cleavage site, which cannot effectively sterically restrict the two halves of the luciferase. To prevent this, the inserted sequence must keep the luciferase fragments away from each other, in addition, to containing a cleavage site.

Autoinhibited coiled coil strategies are the next generation of turn-on protease biosensors, controlling the activity of fragmented luciferase more efficiently and increasing the intensity of the bioluminescent signals 1,000-fold compared with the background mentioned for the previous technique [68, 69]. The biosensors generated, which are used for assaying protease activity, must contain three modular

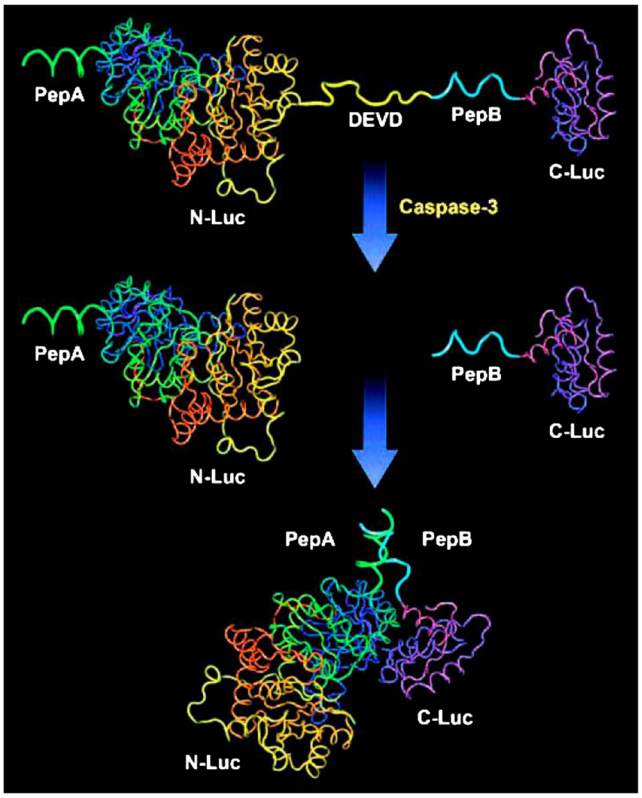


Fig. 2 Strategy for imaging caspase 3. N-terminal and C-terminal fragments of luciferase (*N-Luc* and *C-Luc*, respectively) are linked to a pair of interacting peptides, *PepA* and *PepB*, with the DEVD caspase 3 cleavage motif as a linker. When apoptosis is induced, caspase 3 proteolytically cleaves the DEVD motif, and hence by interaction between PepA–N-Luc and PepB–C-Luc it is possible to reconstitute luciferase. (Reprinted from [67] with permission from the American Association for Cancer Research)

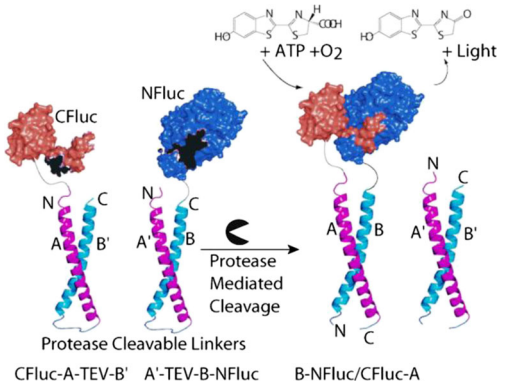


Fig. 3 Doubly inhibited coiled coil protease biosensor. Both helix A and helix A and their equivalent helices B and B are identical in sequence and have been labeled differently for clarity. With *Tobacco etch virus* protease treatment, the linker cleaves luciferase, and the two halves of luciferase reassemble and catalyze the monooxygenation of luciferin to emit light. Unlike what is shown here, if the two linkers are different, the system is a so-called dual protease sensor. *CFLuc* C-terminal of firefly luciferase, *NFLuc* N-terminal of firefly luciferase, *TEV Tobacco etch virus* protease cleavage linker. (Reprinted from [68] with permission from the American Chemical Society)

domains: a split luciferase, an antiparallel heterodimeric coiled coil, and a linker cleavage site for protease function. In this strategy, there are two sequence-identical components (B, B), one of which is attached to a fragment of luciferase (B) and the other (B) is connected to the coiled coil partner, which is fused to the other fragment of luciferase (A) through a protease-cleavable linker. Because of favorable entropy, the luciferase complementation is autoinhibited owing to the preference of A and B for intramolecular interaction. This autoinhibition is eliminated through linker cleavage of a specific protease which causes the split luciferase to be complemented owing to intermolecular coiled coil interaction (A, B). In a more efficient strategy, a doubly inhibited coiled coil system consisting of two autoinhibited coiled coils attached to split-luciferase halves (Fig. 3) [68]. This novel method takes advantage of the high signal-to-noise ratio, and the system will act as a dual protease biosensor if the linkers contain two different protease cleavage sites.

In addition to proteins, in molecular genetics, detection of different RNAs and detection of chemical changes involved in multiple DNA-damage-associated pathways have been investigated using split luciferase. DNA and RNA are two important biological targets related to a vast variety of human diseases. Fluorescently labeled oligonucleotides, as in fluorescent in situ hybridization, are commonly used for detecting nucleic acids [70]. However, there are some limitations, such as the necessity for washing steps and a reduction of sensitivity [71]. Consequently, there is great interest in using split luciferase for detecting nucleic acids. The first attempts were able to detect some specific RNAs by inserting RNA-binding arginine-rich motifs, Rev and/or Tat, into firefly luciferase and constructing several peptide-inserted firefly luciferases. Evaluation of split-luciferase activation and inactivation in the presence of different target RNAs suggested that the RNA sequence, RNA concentration, and insertion site have critical roles in split-luciferase complementation or discomplementation [72]. Similarly, two fragments of luciferase have been attached to two RNA sequence-specific binding proteins, such as Pumilio (Fig. 4a). In the presence of sequence-specific single-stranded RNA, luciferase complementation occurs. These cannot be considered general approaches for targeting a variety of RNAs, since the attachment of peptides is dependent on specific RNA sequences. To design a general approach for targeting single-stranded RNA, synthetic RNAs complementary to the target RNA and with a short sequence overhang recognized by proteins fused to luciferase fragments, as in the case of argonaute, were used recently (Fig. 4b). Although this technique was expected to be successful, in an experimental setting it was inefficient because of the minimal reassembly of the split luciferase. To avoid this limitation, a nucleic acid chain is synthesized in such a way that the overhang moiety forms a hairpin loop, which is the site for binding high-affinity specific-sequence

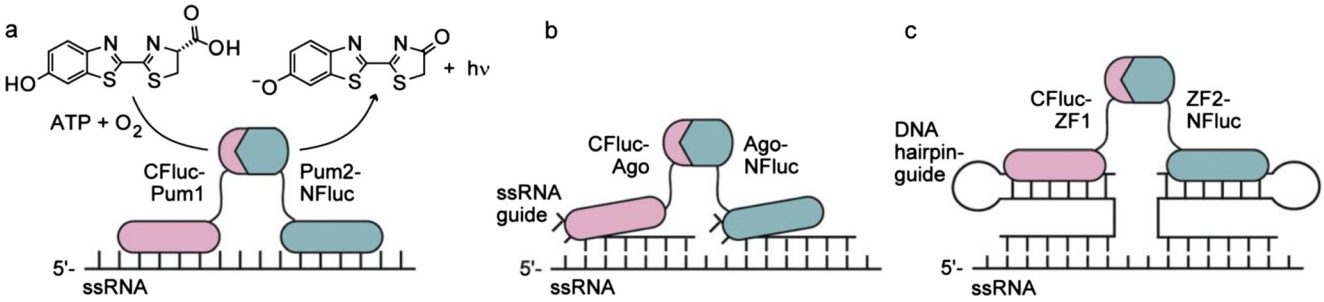


Fig. 4 Pumilio, argonaute, and zinc finger domains fused to fragmented luciferase. The presence of a specific single-stranded RNA (*ssRNA*) target causes the assembly of split luciferase. **a** Pumilio RNA binding proteins recognize a specific RNA sequence. **b** In argonaute-mediated detection approaches, a 3 two-nucleotide overhang resulting from binding of a short guide sequence to the complementary target is recognized. **c** Double-stranded DNA hairpins connected to RNA target bind to zinc fingers. Double-stranded DNA hairpins are connected to ssRNA through the hybridization of the attached guide sequence. *Ago* argonaute, *CFLuc* C-terminal fragment of luciferase, *NFLuc* N-terminal fragment of luciferase, *Pum* Pumilio, *ZF* zinc finger. (Reprinted from [73] with permission from the American Chemical Society)

binding motifs such as a zinc finger. On the other hand, these motifs are linked to luciferase fragments (Fig. 4c) [73].

As previously mentioned, split luciferase is also used for investigation of pathways involved in DNA repair systems. In damaged sites of DNA, some proteins are recruited and subsequently subjected to poly(ADP-ribosylation), which in turn precedes the DNA relaxation. Now it is possible to generate fused proteins, containing luciferase fragments and the interacting protein, with ribosylated proteins [74]. Therefore, developing and using these biosensors has the potential to help better our understanding of the chemical biology of DNA damage response.

Cell communication and cell signaling

For a multicellular organism to develop, as in humans, cells must necessarily communicate. There are complex intracellular mechanisms that control the type and duration of emitted signals. These mechanisms are involved in interpretation of extracellular chemical signals and guidance of the cell behavior, and cause specialization and differentiation of cells to occur. Regardless of the essence of the signal, cells respond to signals by means of their receptors. Cell receptors are in turn categorized into cell surface receptor proteins that trigger the signaling pathway through hydrophilic signal molecules and intracellular receptor proteins which respond to small hydrophobic signal molecules. The function of surface receptors is executed by their coupling with an enzyme, G-protein-gated ion channel, in contrast to intracellular receptors, which are commonly ligand-modulated gene regulatory proteins and are a member of the large nuclear receptor superfamily.

Different cells of all organisms are continuously sending and receiving signals. But what will happen if a cell lacks the ability to send a signal at the proper time? And what will happen if a target cell is unable to receive or respond to a signal or if a cell responds even though it has not received a signal? These are just a few ways in which cell communication can go wrong, possibly resulting in disease. In fact, most diseases involve at least one defect in cell communication. Therefore, studying cell signaling pathways and the proteins involved as therapeutics targets is one of the most important issues in the field of medical sciences.

Förster resonance energy transfer (FRET) is a common technique used for visualizing signal transduction in living cells by describing the energy transfer between two chromophores, a donor chromophore in its electronic excited state transferring energy to an acceptor chromophore. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between the donor and the acceptor. However, there are some limitations in this technique, such as low sensitivity compared with the split-luciferase complementation assay (the gain of FRET probes rarely exceeds 50 %) [75], interference with internal signaling pathways, difficulties in choosing probes for contemporary detection of two FRET probes in a single cell, stable expression of probes, and the need for an external excitation source [76]. This is where split luciferase emerges to overcome these problems. Early examples of the sensors developed were based on the estrogen receptor (ER) ligand binding domain, a common nuclear receptor and gene regulatory protein, which was inserted into *Renilla* luciferase or firefly luciferase. Highly conformational changes following ligand attachment cause changes in the separation and orientation of luciferase fragments and hence the luminescent signals. The effect of mutations in human ERα, the affinity of receptors for different agonists and antagonists, and interaction with other proteins, such as Src, were evaluated through split luciferase [43, 44, 77]. Moreover, there are some advantages to the split-luciferase complementation assay that may play an important role in the development of multicolor biosensors. As reported, multiple activities of receptors, depending on their localization, can initiate genomic or nongenomic signaling pathways and are determined by the specific type of ligands. So, it is rational to

determine multifacial activity of ligand action simultaneously. For this, a polypeptide was designed by fusing the ligand binding domain of a receptor and the two probable different interacting motifs of proteins involved in genomic and nongenomic pathways into a single-chain polypeptide. At the same time, two N-terminal fragments of luciferase emitting light of different colors (red and green) and one C-terminal fragment are alternatively located among the single chain polypeptide. Depending on the type of ligand (agonist or antagonist) and initiation of either the genomic or the nongenomic pathway, red or green bioluminescent signals will be detected as a result of the C-terminal fragment of luciferase complementing each of the two N-terminal fragments. This technique has been applied to investigate the interaction between the ER ligand binding domain and either the Src homology 2 domain of the Src protein or the LXXLL motifs, as illustrated in Fig. 5. The specific points in this study were the split sites and cell lines, which should be regarded in

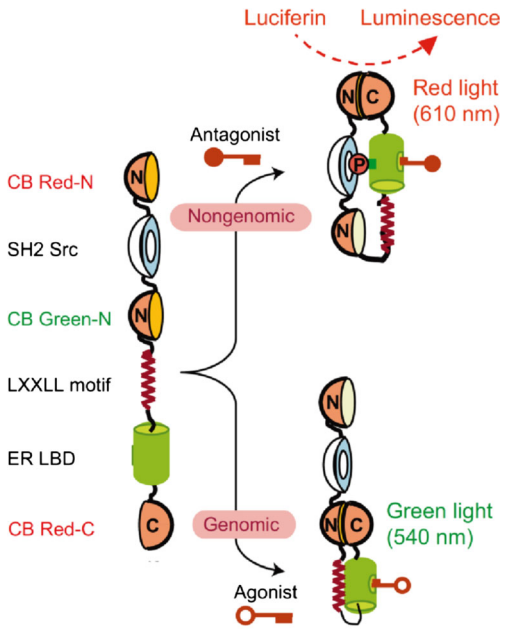


Fig. 5 Integrated-molecule-format multicolor probe for detecting multiple activity of an active small molecule. Here, a multicolor probe is designed by fusing the ligand binding domain of an estrogen receptor (ER) and the two probable different interacting motifs of the SH2 domain and LXXLL. Two N-terminal fragments of luciferase emitting different colors (red and green) and one C-terminal fragment are alternatively embedded among the fragments mentioned. Depending on the type of agonist or antagonist and initiation of either genomic or nongenomic pathway, red or green bioluminescent signals will be detected as a result of complementation of the C-terminal fragment and each of the two N-terminal fragments. *SH2 Src* SH2 domain of Src protein, *ER LBD* ER ligand binding domain, *CB Red-N*N-terminal domain of red click beetle luciferase, *CB Red-C* C-terminal domain of red click beetle luciferase, *CB Green-N*N-terminal domain of green click beetle luciferase, *LXXLL motif* amino acid sequence interacting with The ER ligand binding domain for genomic activities of ligands. (Reprinted from [79] with permission from the American Chemical Society)

future studies. In this study, several probes with different luciferase fragment attachment sites were designed, and small variations in luciferase split points produced astonishing results. On separation of the N-terminal and C-terminal fragments of luciferase at the 439 position, the maximum luminescent signal was small, whereas when splitting was done at the 412 position, an incredibly intense signal was produced. Moreover, in this study the efficiency of the biosensor was evaluated in the presence of different ligands in different cell lines, and the results highlighted the role of cell lines in assessing the efficiency of the biosensor [78].

The importance of this technique is that the activation of the genomic and nongenomic pathways and their ratio is detectable. This integrated-molecule-format probe can provide a high-throughput imaging scheme for determining multifacial activity of ligand action in living cells [79]. This procedure is used for determining the activity of ligands of ER. In estrogen signaling, attachment of estrogen to the ligand binding domain precedes the dimerization process. As there are two types of ER (ERα and ERβ), the distinction between homodimerization and hetereodimerization of these ERs is possible by using split luciferase. As recently reported, three constructs were designed using firefly luciferase and *Renilla* luciferase, which emit light of different colors. ERβ and ERα were fused to the C-terminal fragment of firefly luciferase and the N-terminal fragment of *Renilla* luciferase, respectively, and the C-terminal fragment of *Renilla* luciferase and the N-terminal fragment of firefly luciferase were flanked with one ERα molecule. On the basis of homodimerization or hereodimerization, *Renilla* luciferase or firefly luciferase complementation will occur and light of different colors will subsequently be emitted (Fig. 6) [33].

In some intracellular signaling pathways, activation of a transcription factor may regulate the expression of many genes. Demonstrating the degree of real-time activation of these transcription factors is important and can provide a significantly better understanding of the cellular process during development as well as of various cancers arising from dysfunctionality in the regulation of specific gene expression. Spilt luciferase is helpful to study the activation of these transcription factors through the attachment of a luciferase fragment to the given transcription factor and the interacting protein. This approach has recently been exploited to investigate the Myc protein, the regulation factor for up to 15 % of all vertebrate genes [80, 81], and the Notch receptor, which acts as a transcription factor following proteolysis [82, 83]. Split luciferase has not been applied as it deserves to be, and it might be a good candidate for investigating the key transcription factors in future studies.

Unlike the intracellular signaling pathways mentioned above, extracellular signal molecules mostly bind to surface receptors and initiate signal transduction, in which the production of small secondary messenger molecules and

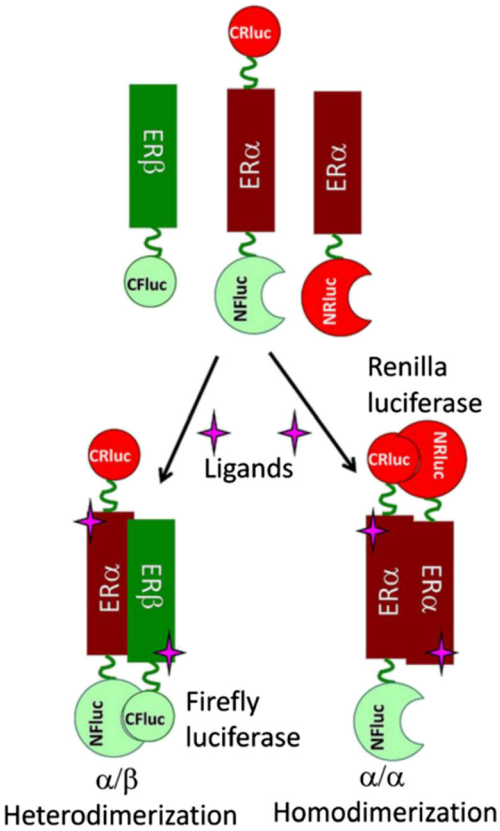


Fig. 6 Simultaneous split firefly and *Renilla* luciferase reporter protein complementation system. This system is designed to study ER-ligand-induced homodimerization and heterodimerization (ERα and ERβ) in cells. Here, the ligand binding domains of ERα and ERβ are fused to the N-terminal fragment of *Renilla* luciferase (*NRluc*) and the C-terminal fragment of firefly luciferase (*CFluc*), respectively. On the other hand, another construct is designed through attachment of CRluc and NFluc to ERα. Depending on the presence of ligands and homodimerization or heterodimerization, light of different colors is emitted. *CRluc* C-terminal fragment of *Renilla* luciferase, *NFluc* N-terminal fragment of firefly luciferase (Reprinted from [33] with permission from the Endocrine Society)

subsequent activation of effector proteins occur. Accordingly, three strategies can be imagined in surface-receptor-mediated signaling pathways based on split luciferase.

Protein–protein interactions involved in signaling pathways

For studying a specific signaling pathway, various optional interacting proteins can be taken into consideration in signaling cascades for fusing luciferase fragments. In the first approach, luciferase fragments are attached to each monomeric unit of a dimer receptor, where ligand binding induces the dimerization of the receptor. Luciferase fragments act more efficiently when they fuse to a monomeric unit of a heterodimer, since in a homodimer receptor two identical luciferase fragments (either N-terminal fragments or C-terminal fragments) may be placed in the correct position, and as a result no optical signal will be detected, decreasing the efficiency of the split-luciferase system. This is what was examined in the homodimerization and heterodimerization of an EGFR, where each luciferase fragment was attached to an EGFR molecule, or an EGFR molecule or an ErbB2 molecule and homodimerization and heterodimerization were investigated in response to a specific ligand binding. In routine receptor tyrosine kinases, such as EGFR, two phenomena should occur before the effector protein recruitment, i.e., ligand-dependent dimerization and autophosphorylation, each of which induces certain conformational changes in the receptor [84]. So in studying these kinds of receptors, we find light emission is first detectable as a result of receptor dimerization, which indicates the time needed for dimerization, and then changes in the emitted light related to autophosphorylation are observed, and originate from changes in the orientation of the luciferase fragments caused by a change in receptor conformation. In earlier studies, β-galactosidase was used as a split reporter protein, but the efficiency was fivefold to tenfold lower than that obtained in split-luciferase complementation, and this might be due to product accumulation and out-of-place β-galactosidase fragmentation. For EGFR, in addition to what was mentioned above, different mutations were introduced, and their impact on receptor function was determined using the split-luciferase complementation assay [85].

In the second approach, luciferase halves are attached to a receptor and the interacting protein in the signaling pathway, as reported for interaction of G-protein-coupled receptor with β-arrestin 1 and β-arrestin 2. In this system, the most sensitive fragments of click beetle luciferase with the highest signal-to-noise ratio were used. These fragments were selected by using semirational library screening and were separately fused to G-protein-coupled receptor and β-arrestin, and the behavior and activity of the receptor were characterized in cell-based assays [86, 87]. Surprising results were obtained following this study, where data from native click beetle and firefly luciferases were not expanded to red-emitting click beetle and firefly luciferases, respectively. Previous studies had shown that the absorbance of hemoglobin was drastically decreased beyond 600 nm, raising the possibility that red-emitting luciferases would be suitable for in vivo imaging in animals, but unexpectedly it was demonstrated that the sensitivity severely decreased and the signal-to-noise ratio greatly increased [88]. On the basis of these results, it was suggested that in spite of a variation of a few amino acids in the sequences of common and redshifted luciferases, it was not feasible to generalize the data obtained from one specific type to the other types and these data might occasionally be significantly different. This approach, i.e., the study of receptor attachment to interacting protein, was used for the ErbB2/HER2/neu pathway as well. ErbB2/HER2/neu is a member of the EGFR family, and its inhibition influences tumor growth and metastasis [89]. Such systems are powerful tools for sensitive

and rapid screening of large chemical libraries in the field of drug development.

In the third approach, the two selective proteins are neither receptors nor their interacting proteins, although these proteins are executioners of the cell surface receptor's downstream interacting proteins. The two interacting proteins must include one of two important characteristics, either to be the first point in the downstream signaling pathway or to be one of the following common intermediates. One of the procedures which was used for studying the two interacting proteins in the first point of an insulin signaling pathway was an interesting but less efficient method of a so-called protein-splicing-based split-luciferase enzyme system which was designed by Ozawa et al. [90] in 2001. In this system, the N terminal of an intein (such as DnaE) is linked to an N-terminal fragment of luciferase, and the C terminal of the same intein is fused to the rest of the luciferase. Each of these fusion proteins is attached to one of the two interacting proteins of interest. Because the interaction occurred between the two interacting proteins, intein segments were brought close together and this caused the inteins to be refolded, which in turn caused the initiation of splicing events; subsequently, the attachment of the two halves of the luciferase through a peptide bond made a complete and functional luciferase (Fig. 7). As a criticism of this system used for the insulin signaling pathway, it should be mentioned that as a result of complete luciferase formation and hence lack of steric limitation due to separation of luciferase from the interacting proteins, the signal intensity is much higher than that observed in conventional split luciferase. On the other hand, as the connection of the complete luciferase with interacting proteins is disrupted, the emitted light is not necessarily indicative of the continuation of interaction between the two proteins of interest. Designing such systems is difficult, as two pair of complementation fragments must be set up, one for inteins and one for luciferase. To solve these problems, the conventional split-luciferase complementation assay is used and makes the real-time activity assay of the insulin signaling pathway possible [91].

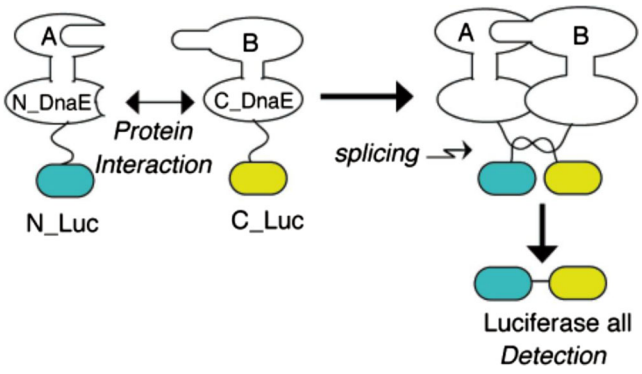


Fig. 7 Protein-splicing-based split-luciferase system. An N-terminal fragment of DnaE (*N_DnaE*) and a C-terminal fragment of DnaE (*C_DnaE*), respectively, are fused to the N and C terminals of luciferase. The opposite ends of DnaE attached to interacting proteins A and B. Interactions of proteins A and B facilitates the folding of N_DnaE and C_DnaE. As a result of protein splicing, the activity of the luciferase recovers. (Reprinted from [90] with permission from the American Chemical Society)

Rho GTPase is an example of a protein that functions as a common downstream intermediate of many signaling pathways investigated using split firefly luciferase. Rho GTPase and the GTPase-binding domain of one of its effector were, respectively, attached to the C terminal and the N terminal of firefly luciferase. Emission of light indicated the rate of Rho GTPase activation [92]. When a common intermediate is selected for investigation, it should be taken into consideration that the resulting activity cannot be positively attributed to a specific signaling pathway, as it is possible for common intermediates to be involved in several signaling pathways derived from different cell surface receptors.

It is noteworthy that the attachment of luciferase fragments to two selected interacting proteins in a signaling pathway may affect the interaction of the proteins with upstream regulatory proteins and downstream effectors. As the interaction of native proteins involved in a signaling pathway might be distinct from that of those attached to the luciferase fragments, the data resulting from split luciferase are not too reliable. This interference cannot be ignored, but the configuration of luciferase fragments and the linker should be optimized in a trial-and-error manner to obtain the most efficient constructs with the least interference [76, 92].

Production of secondary messengers

Cell surface receptors, particularly enzyme-coupled receptors and G-protein-coupled receptors, activate intracellular effector proteins through the production of numerous small intracellular species named second messengers or small intracellular mediators. These species include cyclic AMP (cAMP), Ca^{2+}, and inositol 1,4,5-trisphosphate (IP_3). Each of these second messengers may attach to certain domains of effector proteins and switch them on and off. As demonstrated in Fig. 8 for rapid monitoring of IP_3, these domains change the activity of proteins by intensive conformational induction. For assaying the content of small molecules by split luciferase, it is not necessary to use the entire effector protein. Indeed, that specific domain is sufficient for designing intramolecular biosensors. So the sensitivity to the second messenger as a ligand and the ability to undergo extensive conformational changes are two important properties for domains that are going to be selected. Considering this, intramolecular biosensors were designed for IP_3, cAMP, and Ca^{2+} [42, 93–96] to demonstrate the second messengers' content. In previous studies, the radiolabeling method was chosen for investigation of many secondary messengers [97]. Not only is this technique unsafe and difficult to use, but labeling in living cells is also challenging owing to the absence of an appropriate transport

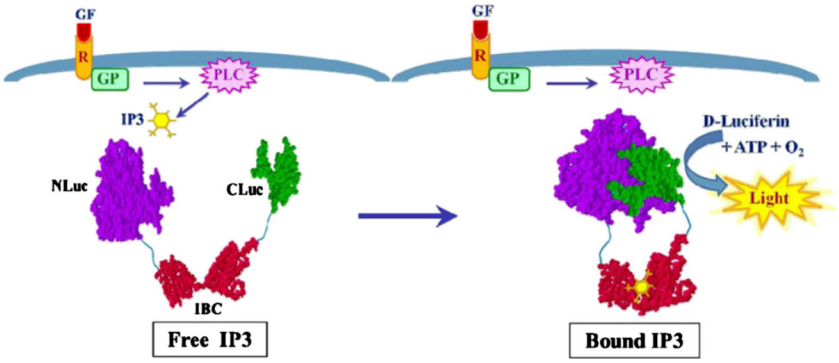


Fig. 8 Principle of inositol 1,4,5-trisphosphate (*IP3*) interaction using the split-luciferase complementation assay. N-terminal and C-terminal halves of luciferase (*NLuc* and *CLuc*, respectively) are attached to the N and C terminals, respectively, of the IP3-binding core (*IBC*). In IP3-free conditions, the biosensor is in an open state. Since IP3 molecules are generated following the activation of G-protein (*GP*)-coupled receptors (*R*) and phospholipase C (*PLC*) in response to biological stimuli such as growth factor (*GF*), it binds to IBC and induces conformational changes, resulting in the luciferase fragments being close to each other and restoring the activity. (Reprinted from [93] with permission from Elsevier)

system and inhibitory culture conditions. Moreover, exposing tissues in live animals to sufficiently high concentrations of radioactive precursor is very difficult [98]. Making use of split luciferase resolves these limitations, by substituting for the conventional radiolabeling method. Moreover, this new system has high throughput, which would save time, and requires only several minutes in comparison with several hours for traditional radiolabeling methods. For example, in an IP_3 intramolecular biosensor, just 30 s after the stimulation of a ligand is enough for the bioluminescent signal to peak. However, one of the commonest disadvantages of these intramolecular biosensors, such as in IP_3 intramolecular biosensors, is the high background luminescent signal in the absence of a ligand. Although the luminescent signal increased by about 9.5 times to a maximum of 6.7×10^5 relative luminescence units per second, the signal detected in the absence of a ligand was considerable (7×10^4 relative luminescence units per second). To solve this problem, a library of different linkers and of luciferase splitting sites should be constructed in order to minimize background noise [93].

In vivo studies, the emitted light is not necessarily indicative of the second messengers' content, as the permeability of the cell membrane to luciferin and ATP (luciferase substrates) is different in various conditions and can cause the photon counts to be affected. Instead, dual-bioluminescence ratiometric indicators have been used. Such indicators have a ligand binding domain, where a C-terminal fragment of luciferase and two differently colored N-terminal fragments of luciferase are fused through a flexible linker, making dynamic motion possible. For assaying the content of cAMP, this type of biosensor was designed using click beetle luciferase. In the absence of cAMP, the C-terminal fragment is complemented with an N-terminal fragment of red-emitting luciferase. Conversely, in the presence of cAMP, the conformational changes in the ligand binding domain cause the C-terminal fragment to be complemented with the N-terminal fragment of green-emitting luciferase (Fig. 9). Here, the emitted light is not representative of the second messenger molecule, and the green light to red light ratio indicates the cAMP content [96].

Ca^{2+} is a messenger with critical roles in muscle contraction, cell growth and differentiation, and cell signaling. To clarify the Ca^{2+} dynamics in living cells, a number of synthetic fluorescent markers have been designed which are observable under a fluorescence microscope, but it is difficult to target the desired subcellular locations. For this, to take advantage of fluorescence readout and subcellular targeting, a genetically encoded fluorescent marker based on FRET was developed [99, 100]. In spite of the fact that it was able to demonstrate Ca^{2+} dynamics in translucent live cells, it underwent much autofluorescence in in vivo assays. Now the designed intramolecular biosensor based on split luciferase is able to unravel the puzzle, as it lacks autofluorescence and can be used in either living cells or in in vivo experiments. Moreover, this novel system has great sensitivity, showing a 14-fold increase in luminescent signal after ligand induction [94].

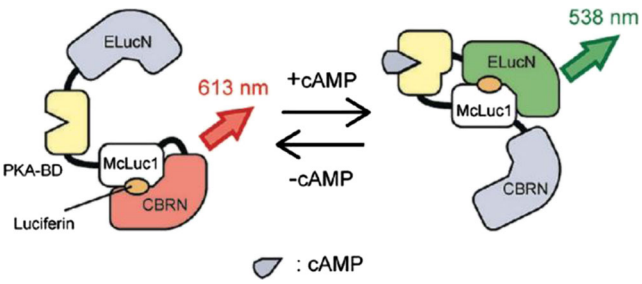


Fig. 9 A complementary AMP (*cAMP*) indicator. In the absence of cAMP, multiple-complement luciferase fragment (*McLuc1*) is complemented with the N-terminal fragment of click beetle red luciferase (*CBRN*) and emits red light. On increase of the cAMP concentration and its binding to cAMP binding domain of protein kinase A (*PKA-BD*), the resulting conformational changes trigger the complementation of the N-terminal fragment of Emerald Luc (green luciferase) (*ELucN*) and McLuc1 and green light is produced. (Reprinted from [96] with permission from the American Chemical Society)

Activation of effector proteins

The activity of protein kinases is severely regulated in signal transduction. All the protein kinases show approximately identical conformational changes in their activated state, which is consistent with ATP and substrate binding and enzyme catalysis [101–103]. One of the commonest methods developed for detecting kinase activation in cells is based on phosphate-specific antibodies, in which activation loop phosphorylation or downstream substrate phosphorylation is detected [104]. There are two important limitations in this technique, one is related to antibody interference with undesirable epitope binding and the other is that it does not make real-time assay possible. Moreover, the activation of kinase cannot be quantified. For assaying the activation of protein kinases using fragmented luciferase, three approaches might be considered. In the more general approach, which can be used for nearly all protein kinases, the regulatory domain of protein kinase is flanked with luciferase fragments. In the presence of small or protein modulators, conformational changes in the regulatory domain cause the two fragments of luciferase to be either far away from or close to each other. Therefore, according to the intensity of the luminescent signal, the activation of kinases and the effects of different allosteric factors can be quantifiably detected in a real-time manner. These allosteric factors can be therapeutically valuable, as they may upregulate or downregulate the activity of kinases. As shown in Fig. 10, this approach is exploited for assaying the AbI protein kinase activity using firefly luciferase [105].

In the second approach, the activation of protein kinase is evaluated through protein–protein interactions. In this approach, we refer to the interaction between protein kinase A (PKA) and its regulatory subunit using N-terminal and C-terminal fragments from split *Renilla* luciferase. When PKA is inactive, these proteins are in contact and cause a bioluminescent signal to be emitted. Binding of cAMP to a regulatory subunit and separation from PKA causes the two fragments of luciferase to be far away from each other, and so the intensity of the emitted light decreases [106]. It is noteworthy that the decrease in light intensity is an indicator of activity enhancement. As the lack of emitted light is a signal for the presence of activity, we should be careful that this lack of light is not due to experimental errors. So, it is doubly crucial to have validated control samples. A similar procedure can be used to detect the dimerization and activation of extracellular-signal-regulated kinase 2 (ERK2), in which light is emitted by the interaction between two ERK2 molecules and luciferase complementation [107].

In the last approach, which was first used for evaluation of the Akt signaling pathway, a hybrid polypeptide bioluminescent reporter containing a specific protein kinase consensus substrate peptide, consisting of a domain bound to a phosphorylated amino acid residue, was constructed. By the function of that protein kinase, the domain undergoes conformational changes and hence changes the luciferase activity [108]. Here, we must select a substrate peptide which is phosphorylated by just one specific protein kinase in order to prevent the unspecific phosphorylation of the substrate peptide through other kinases.

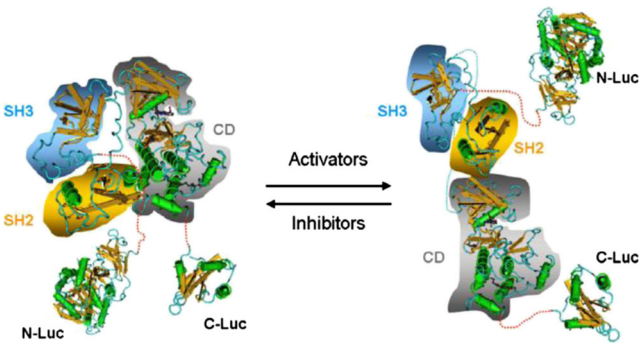


Fig. 10 Effects of activators and inhibitors on an AbI biosensor in active (*right*) and inactive (*left*) states. *SH2* Src homology domain 2, *SH3* Src homology domain 3, *CD* catalytic domain, *N-Luc* N-terminal fragment of luciferase, *C-Luc* C-terminal fragment of luciferase. (Reprinted from [105] with permission from the American Chemical Society)

Protein–protein interactions

It is conjectured that there are up to 300,000 protein–protein interactions in human cells [109, 110]. Many techniques have been developed to investigate protein–protein interactions, such as co-immunoprecipitation, far-western blot, and antibody microarrays, all of which are limited to in vitro assays. For in vivo assays, several genetic techniques, such as two-hybrid systems, have been established. Such systems are used only for proteins close to reporter genes and located in the nucleus. To overcome this limitation, the mammalian protein–protein interaction trap was developed, in which protein partners are attached to cytokine receptor chimeras deficient in signaling. When two protein partners interact, intramolecular signaling is initiated and causes the reporter gene to be expressed. But it is difficult to investigate the membrane protein and interactions within a particular organelle through the mammalian protein–protein interaction trap [111].

One of the most conventional applications of split luciferase is the investigation of protein–protein interactions. Almost all of the strategies and examples mentioned involve a kind of protein–protein interaction. In the early days of the split-luciferase complementation strategy, its applications were limited to animal cells, but as time went by it was generalized to plant cells [112–115] and more recently it was exploited in evaluating the interaction between the transcription factors GzMCM1 and FST12 in filamentous ascomycetes [116] and chemotaxis-associated response regulator CheY and its phosphatase CheZ in bacteria [117]. So this technique is

compatible with all types of organisms, from prokaryotes to eukaryotes.

Protein–protein interactions might be influenced by numerous components in the cell such as an inhibitor, a mediator, or an enhancer, all which may affect the information obtained from split luciferase. Therefore, to avoid these possible disturbances, it is suggested that the fused proteins be purified through various protein purification methods, such as immobilized metal affinity chromatography, and in vitro investigation of protein–protein interactions be done with a prefused protein [118]. Furthermore, in the comparison of the data obtained from purified and unpurified fused proteins, it is possible to discover the presence of different but equally impressive components. In some cases, the protein–protein interactions are not necessarily allocated to a pair of proteins, and there might be a different interacting partner for each protein. Here, it is preferred to use a luciferase heteroprotein fragment complementation system, which makes possible the dual-color quantification of two distinct interacting proteins [119].

Most viral and bacterial diseases are the consequence of protein–protein interactions. It is important to diagnose the stage of the disease in which certain proteins interact [120, 121]. Split luciferase can help us to determine the early stage of a disease and focus on that specific point in designing therapeutic procedures. Moreover, by knowing these key points, we can design and develop new drugs that may affect the interaction between the two proteins.

In 1999, French scientists headed by Bernard Jacq [122] coined the word "interactome," which refers to all the interactions in the cell. Sometimes "interactome" is defined as a biological network. The complexity of organisms makes the prediction of their behavior impossible by studying just a few protein–protein interactions. So, for better understanding of cell behavior and function, the interactome should primarily be determined. Utilizing the knowledge and equipment available today, we are unable to determine the complete interactome even in a single prokaryotic cell. That is why we try to determine the interactome in the pathway of interest, which itself is time-consuming and costly. For this, a high-throughput split-luciferase complementation system is invaluable for investigating protein–protein interactions. One of the first experiments performed with the interactome approach investigated 132 binary protein–protein interactions among eight auxin response factors and 12 auxin/indole-3-acetic acid proteins in *Arabidopsis* [123]. This method was also used for determination of part of the human papilloma virus interactome [124]. As numerous constructs are needed to investigate the interactome, some quick procedures are needed to provide a large number of interacting constructs. Gateway technology cloning, based on recombination, is the commonest procedure for production of such constructs [125, 126].

Split luciferase has been used in two areas of cell biology: supramolecular structure and subcellular architecture and behavior. In spite of the significant role of split luciferase in studying the complex protein assemblage, which forms a supramolecular structure, and fusion–fission of organelles, only a few experiments applying this efficient tool have been reported, and there remains a lot to be done in the case of the application of split luciferase in cellular studies. One of the most interesting examples of the formation of a supramolecular structure is the detection of an apoptosome complex

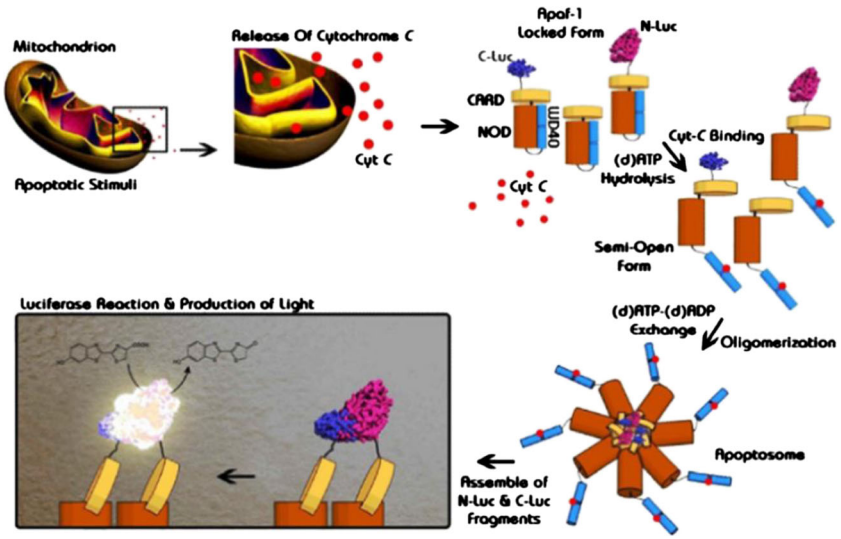


Fig. 11 Apoptosis biosensor mechanism of action. On the induction of apoptosis, cytochrome*c* (*Cyt C*) is released from the intermembrane of mitochondria and causes the apoptotic procaspase activating factor 1 (*Apaf-1*) molecule to switch from an autolock state into an open form, through which an apoptosome complex is formed. By formation of the apoptosome, fragments of luciferase are placed in the right position and emit a luminescent signal. *C-Luc* C-terminal fragment of luciferase, *N-Luc* N-terminal fragment of luciferase. (Reprinted from [128] with permission from Elsevier)

Table 1 The main applications of the split-luciferase complementation system and comparison with traditional methods

Applications of the split-luciferase complementation assay	Attachment site of N-terminal and C-terminal luciferase fragments	Assay format	Advantages (+)/disadvantages (−)/ traditional methods if available (*)	Reference
Biochemical analysis				
Galactose/glucose sensor	N-terminal and C-terminal luciferase fragments are attached to 2 ends of galactose/glucose-binding protein	In vitro	+High sensitivity (K_d is 3.9 μM for glucose and 11 μM for galactose) *Fluorescence-based glucose-sensing system conjugated with galactose/ glucose-binding protein (signals are easily quenched with environmental compounds)	[45]
Receptor-and-antibody-targeted detection of native proteins	N-terminal and C-terminal halves of firefly luciferase are, respectively, fused to CD4 receptor of T lymphocytes and the Fab portion of a certain antibody	In vitro	+Direct detection of native proteins in heterogeneous solutions +High-throughput screening of various diseases such as HIV/AIDS and breast cancer *ELISA (laborious for heterogeneous solutions and requires antibody fixation on a solid base, washing, and linked secondary antibody)	[52]
Evaluation of protein binding to ion channels	2 complementary N-terminal and C-terminal fragments of firefly luciferase are, respectively, fused to the voltage-gated Na^+ channel C-tail and intracellular fibroblast growth factor 14	In vivo	+Demonstration of high signal-to-noise ratio, reversibility of luminescence, and appropriate dynamic range *Patch-clamp electrophysiology and ion flux assay (time-consuming as large amounts of purified proteins are required and also do not have high throughput or are not optimized for channelosome investigations)	[57]
Measurement of protease activity (autoinhibited coiled coil strategy)	2 fragments of firefly luciferase are attached to 2 different coiled coil partners	In vitro	+High sensitivity and high throughput (increasing bioluminescent signals 1,000 fold compared with the background) +The system can act as a dual protease biosensor if the linkers contain 2 different protease cleavage sites *GFP-based sensors (need highly sensitive instruments) *Large peptides are attached to and quench a fluorescent probe (cannot permeate cells and so is not ideal for intracellular studies)	[68]
Cell communication and cell signaling				
Rapid monitoring of IP_3	The IP_3-binding core domain of IP_3 receptor is flanked with complementary fragments of firefly luciferase	In vitro/in vivo	+High throughput (30 s is enough for the maximum bioluminescence, after the stimulation of a ligand) −High background luminescent signal in the absence of ligands *Radiolabeling methods (require several hours for assay completion)	[93]
Intramolecular bioluminescent indicator for Ca^{2+}	C-terminal and N-terminal fragments of *Renilla* luciferase are attached to M13 (a synthetic peptide with a sequence of calmodulin binding) and calmodulin, respectively	In vivo	+High sensitivity (14-fold increase in luminescent signal intensity after ligand induction) *Synthetic fluorescent markers (difficult to target the desired subcellular locations and highly autofluorescent in vivo assays)	[94]
Protein–protein interactions				
Analysis of large-scale protein–protein interactions in *Arabidopsis*	132 binary protein–protein interactions were investigated among 8 auxin response factors conjugated with the C-terminal	In vivo	+Quantitative, high throughput, and real time *FRET (low sensitivity and the gain of probes rarely exceeds 50 %)	[123]

Table 1 (continued)

Applications of the split-luciferase complementation assay	Attachment site of N-terminal and C-terminal luciferase fragments	Assay format	Advantages (+)/disadvantages (−)/ traditional methods if available (*)	Reference
	fragment and 12 auxin/IAA proteins conjugated with the N-terminal fragment		*Co-immunoprecipitation, far-western blot, and antibody microarrays (all are limited to in vitro assays) *2-hybrid systems (only used for proteins close to reporter genes and located in the nucleus)	
Detection of the early stage of apoptosis	The N-terminal and C-terminal fragments of firefly luciferase were linked to the N terminal of individual Apaf-1 molecules	In vivo/in vitro	+Efficient, time- and dose-dependent *MTT assay (does not necessarily indicate the cells undergoing apoptosis) *Caspase assay (limited to in vitro assays and is able to detect only the late stage of apoptosis)	[128]

Apaf-1 apoptotic procaspase activating factor 1, *FRET* Förster resonance energy transfer, *GDP* green fluorescent protein, *IAA* indole-3-acetic acid, IP_3 inositol 1,4,5-trisphosphate, *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

using split firefly luciferase. The apoptosome is a heptameric complex involved in the intrinsic pathway of apoptosis and is made of seven apoptotic procaspase activating factor 1 (Apaf-1) molecules, each of which switches from an autoinhibited state to an active form by releasing cytochrome*c*, the main apoptosis stimulator, from mitochondria [127]. In this experiment, the N-terminal and C-terminal fragments of firefly luciferase were linked to the N terminal of individual Apaf-1 molecules. By induction of apoptosis and apoptosome formation, these constructs are placed close to each other, and split-luciferase complementation occurs, which causes luminescent signals to be emitted (Fig. 11). With use of this system, a 15-fold and a 155-fold increase were observed in 4 h and 10 h, respectively, after apoptosis induction [128]. There are two common techniques for investigating apoptosis in cell lines: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and the caspase assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, which is traditionally used for measuring cellular viability and the effects of various drugs on cell death, does not necessarily indicate the cells undergoing apoptosis. The caspase assay is limited to in vitro studies and is able to detect the late stage of apoptosis [129]. The biosensor, in contrast, is able to detect apoptosis in the early stage and is also compatible with both in vitro and in vivo assays. Furthermore, this strategy can be considered as a tool for screening potential chemotherapeutic drugs, as well as a supersensitive and dynamic system in time-dependent and dose-dependent studies of apoptosis. This strategy has also been used for characterizing the oligomer formation of amyloid-β peptide, which plays an important role in Alzheimer disease, and evaluation of the impact of various factors on amyloid-β integration [130].

The other application of split luciferase in cell biology concerns the investigation of subcellular organelles. The morphology of organelles, such as mitochondria, relies on their fusion and fission. This fusion phenomenon has been studied by the using split-luciferase complementation assay. Large mitochondria produce energy more efficiently, whereas small mitochondria seem to migrate better to sites where local production of ATP is needed. For this, two constructs were designed, each containing one fragment of split *Renilla* luciferase fused to a mitochondrial-matrix-targeting sequence and a leucine zipper. Distinct cell populations were transfected with each construct. Certain chemicals were then used to induce the fusion of those different cells. In the fused cells, the relative rate of mitochondria fusion can be calculated through the reconstitution of luciferase fragments and light generation. It is noteworthy that protein synthesis must be inhibited before cell fusion in order to let the reporter proteins migrate into the mitochondria and prevent the production of a false-positive signal caused by newly synthesized proteins [131–133]. The advantages of this strategy compared with the use of fluorescent proteins, which is labor-intensive and with which quantification is difficult, are that it is a real-time method and quantification is possible.

Concluding remarks and perspectives

According to the general information reported here, it may be concluded that the split-luciferase complementary assay is one of the key strategies for monitoring protein–protein interactions, real-time analysis, and live imaging of biological phenomena. The most important applications of split-luciferase-based systems and the overall characteristics are summarized

in Table 1. Efforts have been made to overcome the limitations of previous split reporters, and combinatorial approaches have been used to identify new split sites for firefly luciferase with optimal characteristics [134]. New split sites with several different interacting proteins and with an intramolecular folding strategy in cell culture have been successfully tested. Optical in vivo imaging in small living animals further demonstrates the utility of the new split reporters. New split reporter complementation systems with a greater absolute signal intensity and a lower background than in previous systems can be further extended to study protein-fragment-assisted complementation and other intracellular interactions. The system developed can also be used for high-throughput screening of new drugs targeted at protein–protein interactions in cells along with further evaluation in small living animals. Moreover, the newly discovered protein structures and conformational properties will make possible the design of new split reporters for analysis of the interactome of specialized pathways [123].

In recent years, the number of published articles related to spilt luciferase has increased substantially. Suitable dynamic range, high signal-to-noise ratio, and reversibility of luminescence are the most important features that have affirmed split-luciferase complementation as a high-performance assay for evaluation of protein–protein interactions, intercellular and intracellular protein dynamics, protein localization, cell compartmentalization, and protein activity in real time and in a variety of living cells, plants, animals, bacteria, and fungi [15]. However, to further the application and development of more methods using the split-luciferase complementation assay some critical limitations owning to intrinsic features of wild-types luciferase, such as low thermal stability of the enzyme, low turnover number, and high K_m for the substrate ATP, must be addressed [16]. Moreover, luciferase is very sensitive to degradation by both intercellular and intracellular proteases, causing a decrease in the half-life of the enzyme and accordingly reducing its sensitivity, precision, and signal-to-noise ratio [135]. Therefore, luciferase-based methods, such as the split-luciferase complementation assay, have all the limitations mentioned above, restricting their acceptance for bioanalytical purposes. So, the more the limitations of luciferase are studied, the more solutions will be found for reducing these shortcomings, and accordingly the applications of split luciferase will gradually prevail in different fields of basic science and for diagnostic, therapeutic and clinical purposes.

To overcome the aforementioned limitations, many studies whose subjects are related to the structural biochemistry of luciferase have been performed in recent years. Previously, in our laboratory we designed and characterized a novel trypsin-resistant firefly luciferase by site-directed mutagenesis. We engineered luciferase by substituting two tryptic sites, Arg213 and Arg217, and the next residue with another amino acid [135]. Our data revealed the trypsin-resistant feature of this novel manipulated luciferase and also a slight increase in thermostability. In other studies we introduced different single disulfide bridges in wild-type luciferase by site-directed mutagenesis, in one of which the optimum temperature of enzyme activity was increased by up to 10 °C above that in the wild type [28]. By extending our structural and functional studies, we wish to eliminate the disadvantages of luciferase and may achieve a new engineered high-performance luciferase for use in related areas, such as split luciferase, circularly permuted luciferase, and cyclic luciferase for bioanalytical approaches.

There is another salient factor for bioluminescence-related methods that deserves some words here. The bioluminescence technique, which involves emission of light by a chemical reaction, is dependent on two components: a luciferase and its substrate, luciferin. Even though luciferase has been investigated in many studies, there is only little valuable information related to the biosynthesis of luciferin. Nowadays, for evaluation of the bioluminescence activity of the enzyme, the luciferin substrate should be continuously added to the sample buffer during the experiment. This laborious procedure causes some limitations, particularly in the use of the split-luciferase complementation system in transgenic animals. Scientists have worked seriously in this area to find a set of genes by which they might produce luciferin from normal cell metabolites. If and when this happens, a new window will open to a wide range of applications of luciferase-related methods, especially if they are compatible with both animal and plant cells [136]. Moreover, in recent years inhibitory effects of luciferyl adenylate and oxyluciferin, two products of luciferases, on the luciferase reaction have been shown [137]. The above-mentioned inhibitory effects cause the relatively fast initiation of light emission to decrease during the split-luciferase complementation assay. On one hand, this phenomenon can be omitted by using coenzyme A, which modifies the kinetic profile of firefly luciferase, and luciferin-regenerating enzyme, but on the other hand, the interference effects of these two components in eukaryotic cells have not been studied [136]. Accordingly, this critical problem, the decrease in light emission during the split-luciferase complementation assay, has not yet been solved.

Apart from the aforementioned explanation, without a shadow of doubt for many important reasons the split-luciferase complementation assay would be helpful in unraveling many mysteries and problems in future studies. Cell-based bioanalytical applications can be categorized into genetic assays and nontranscriptional assays. In genetic assays, long-term ligand stimulation is required in order to achieve an appropriate concentration of reporter protein. In contrast, in nontranscriptional assays, such as a split-luciferase complementation method, split luciferase conjugates with protein expressed and localized in the cell. Therefore, the assay is more reliable and easier to perform. Furthermore, in

the not so distant future, different engineered luciferases with different colors could pave the way to producing many potential integrated-molecule-format multicolor probes for monitoring multiple activities of bioactive small molecules. This split-luciferase-based strategy was introduced for the first time by Kim et al. [78] in 2008, and according to many favorable potential applications, the idea may inspire the design of many futuristic biosensors. Moreover, in recent years, systems biology approaches focusing on complex interactions within biological systems have become more interesting to many scientists, and split-luciferase complementation systems have attracted lots of attention for use as a sensitive and robust tool in studying the cell's interactome. Systems biology is dramatically dependent on such experimental methods which provide invaluable data on interactome areas. The whole set of molecular interactions in a particular metabolism pathway has been determined recently by using the spilt-luciferase complementation assay in just a few studies [124]. It is clear that this strategy has the potential to help in the investigation of other pivotal pathways related to cellular and molecular biology.

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