Chapter

Protein-Protein Interaction Assays Using Split-NanoLuc

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Abstract

Protein-protein interaction assays are fundamental to basic biology, drug discovery, diagnostics, screening, and immunoassays. Protein-fragment complementation (PCA) is one of such useful protein-protein interaction assays. PCA when performed using luciferase is a reversible approach, whereas when performed using green fluorescent protein analogs is an irreversible approach. The NanoLuc technology developed in 2012 utilizes a small and structurally robust luciferase that is capable of producing very bright luminescence. NanoLuc PCA has been used to detect many protein-protein interactions and for screening purposes. Methods developed from NanoLuc PCA include the HiBiT technology and NanoLuc ternary technology. These novel technologies are promising in various fields and further developments are anticipated.

Keywords: NanoLuc, PCA, NanoBiT, protein-protein interaction, HiBiT, NanoLuc ternary technology

1. Introduction

It is predicted that there are 150,000–650,000 protein-protein interactions in the human interactome [1–3]. Protein-protein interaction assays have been developed and used for studies on basic biology, drug discoveries, diagnostics, screenings, and immunoassays.

In 1994, the first protein-fragment complementation assay (PCA) was developed using split ubiquitin [4]. PCA typically uses two-split reporter proteins that are fused to the target proteins. The interaction leads to the association of the fragments and the subsequent reconstitution of the full-length structure from the two fragments (**Figure 1**) [5–8]. More recently, fluorescent proteins and luciferase enzymes have been widely utilized for innovative PCAs. Reversible PCAs generally utilize enzymes, and the exceptions are two fluorescent proteins IFP1.4 and UnaG [9, 10]. Most other PCA systems that use fluorescent proteins, including green fluorescent protein (GFP) analogs, show irreversible behavior. In such irreversible assays, once the full-length structure is reconstituted, it is difficult to separate them into the two fragments when dissociation occurs after the interaction. On the contrary, in the reversible PCA systems, both interaction and dissociation can be detected. Therefore, PCA systems using enzymes, such as luciferase, are more suitable to detect the spatiotemporal dynamics of protein-protein interactions. However, until recently, the luminescent signal is significantly weaker than the fluorescent signal.

Recently, a novel luciferase enzyme, NanoLuc, and its furimazine substrate were developed [11, 12]. NanoLuc is small (19 kDa) and structurally stable, and produces

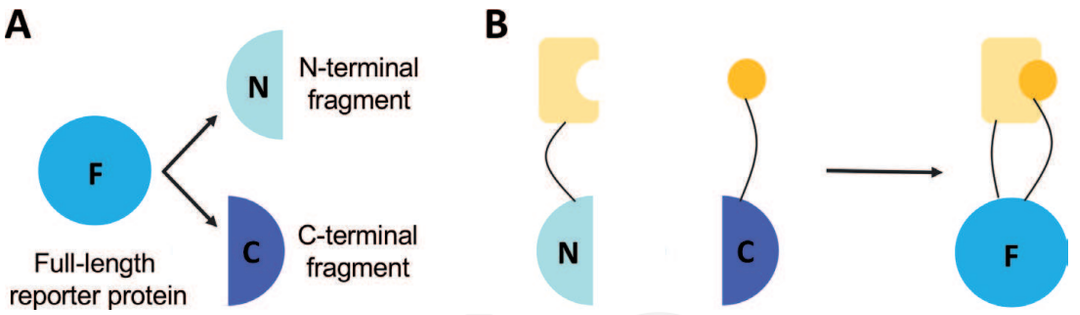

Figure 1.
Basic principle of PCA. (A) The reporter protein (F) is separated into two fragments (N and C). (B) N and C are fused to target protein (left). When the interaction occurs, N and C move to the neighboring position, and the full-length reporter protein is reconstituted.

very bright luminescence. Based on this attractive enzyme, PCA systems were developed [13, 14]. This innovation on the NanoLuc PCA improves the luminescent signal, which is markedly better than the conventional PCA signal obtained using other luciferases. Herein, we will focus on the new PCA technology and its application, and further discuss potential improvements in the system.

2. PCA using NanoLuc

Verhoef et al. constructed a PCA system using NanoLuc [14]. They made several pairs of NanoLuc fragments by cutting at several loop regions, and selected a pair comprised of the N-terminal 52-amino acid (aa) fragment and the C-terminal 119-aa fragment (**Figure 2A**). These fragments were used to successfully detect the interaction between the transactivation domain fragment of p53 and Mdm2.

At almost the same time, Dixon et al. developed another NanoLuc-based PCA system designated *Nano*Luc *Bi*nary *T*echnology (NanoBiT) [13]. This was devised by first identifying a dissection site from 90 candidate sites. An 18-kDa N-terminal fragment and 13-aa C-terminal fragment were selected. The K_D value between these fragments was 6 μM. This low affinity was suitable for PCA, but their use was hampered by the very low stability of the N-terminal fragment. The sequence of the N-terminal fragment was optimized from an N-terminal library containing 15,000 variants. The optimization increased the luminescent signal by 300-fold when the two fragments were interacting, which was 37% that of the wild-type NanoLuc. However, the affinity between the N- and C-terminal fragments became too strong for PCA (K_D = 900 nM). As a next step, the sequence of the C-terminal peptide was optimized from 350 variants. Finally, two fragments were obtained. They were designated LgBiT (18 kDa) and SmBiT (11 aa). These exhibited significantly low

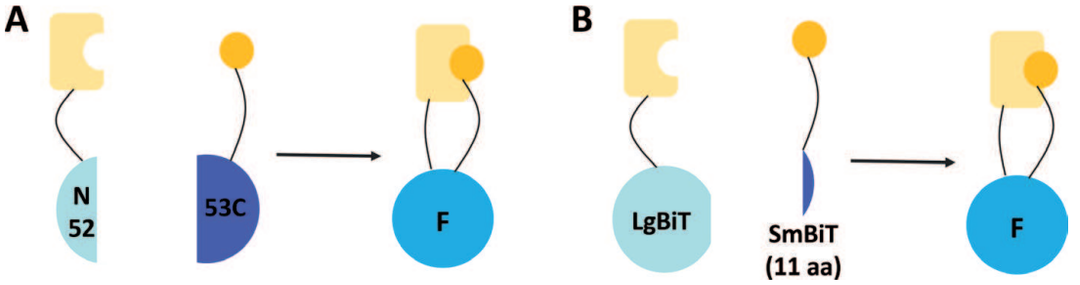

Figure 2.
Two systems of NanoLuc PCA. (A) Verhoef et al. separated NanoLuc into N-terminal 52-aa fragment and C-terminal 119-aa fragment. (B) Dixon et al. separated NanoLuc into the large fragment, LgBiT (18 kDa), and the small fragment, SmBiT (11 aa).

affinity (K_D > 10 μM) and high luminescent intensity. The very bright signal and remarkably high signal/background ratio obtained enabled the quantitative detection of several interactions. Furthermore, the luminescent signals were capable of rapid change and were reversible depending on changing interactions (**Figure 2B**).

3. Application of NanoBiT for analysis of protein-protein interaction

In spite of recent appearance, NanoBiT has been already used to analyze several protein-protein interactions. Elevation of plasma triglycerides causes various metabolic diseases. These triglycerides are digested by lipoprotein lipase [15–19]. Chi et al. used NanoBiT to demonstrate the association between lipoprotein lipase and angiopoietin-like 3 (ANGPTL3) induced by ANGPTL8 [20]. They further described that the association inhibits the digestion activity of lipoprotein lipase.

Guanine nucleotide-binding (G) protein-coupled receptors (GPCRs) bind G proteins or β-arrestins, and initiate several cellular signaling events. Regulator of G protein signaling (RGS) proteins regulate G proteins. The regulation has been implicated in several disease states, including various cancers, Parkinson's disease, and cardiomyopathy [21–25]. Several reports have described the use of NanoBiT to analyze the mechanisms of GPCRs. These included the interaction of several sets of RGS proteins and G proteins [26] and the interaction between the galanin receptor 2 GPCR and β-arrestin2 [27]. Furthermore, the LgBiT-fused galanin receptor 2 was modified with a fluorescent dye, and the conformational changes of galanin receptor induced by the binding of ligands, including galanin, spexin, and Fmoc-dA4-dQ14, were analyzed by bioluminescence resonance energy transfer (BRET). Stome et al. applied NanoBiT to analyze the interaction between the GPCR adenosine receptor 3 and β-arrestin2, and observed that the 1-deoxy-1-[6-[[(3-iodophenyl)methyl] amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (2-CI-IB-MECA) agonist recruited β-arrestin2 [28]. In addition, the authors described the importance of the phosphorylation site of adenosine receptor 3 for the association. The site was implicated as a potential clinical target. Melanocortin receptors are also categorized as GPCRs. Melanocortin 4 receptor (MC4R) binds one of melanocortins α-melanocyte-stimulating hormone (α-MSH), which is considered is important in obesity. Habara et al. isolated melanocortin receptor 4 and its regulator Melanocortin 2 receptor accessory protein 2 (MRAP2) from cats and analyzed the heterodimerization of these proteins [29]. Leory et al. characterized several mutants of Janus Kinase 2 in signaling by a transmembrane cytokine receptor, erythropoietin receptor [30].

Interactions with other membrane proteins implicated as important drug targets were also analyzed by NanoBiT. Folding and steric hindrance are problematic for many membrane proteins. The small size of SmBiT could eliminate these problems. O'Neil et al. revealed the amino acids of NADPH that were important for the interaction with p22 using NanoBiT [31]. Chaudhri et al. applied NanoBiT to analyze the association between programmed death ligand 1 (PD-L1) and B7–1 [32]. Peptide hormone, a member of the relaxin family of peptides, participates in reproduction, food intake, stress response, and glucose homeostasis [33–36]. Hu et al. demonstrated the association between the relaxin family peptide receptors peptide 3 and peptide 4 using NanoBiT [37]. The same group further reported that the interaction is electrostatic by analyzing the association between several mutant ligands and their receptors [38]. Equilibrative nucleoside transporters regulate the levels of adenosine and hypoxanthine level, and are crucial in purinergic signaling in the central nervous system, cardiovascular and renal systems, and in pathophysiological conditions including myocardial ischemia, inflammation, and diabetic nephropathy [39–41]. Grañe-Boladeras et al. analyzed the homo- and

hetero-oligomerization of ENT1 and ENT2, and revealed that the phosphorylation by protein kinase C promotes oligomerization [42].

4. Application of NanoBiT for screening

Several groups have successfully used NanoBiT in highly accurate drug screening, including illegal drugs [43, 44]. In the latter studies, β-arrestin2 was fused to SmBiT, and the CB1 and CB2 GPCRs of cannabinoid (the neurologically active component of cannabis) were fused to LgBiT. As cannabinoid induces the interaction between β-arrestin2 and these receptors, the luminescent intensity was increased by adding synthetic cannabinoids and their metabolites. The synthetic cannabinoids and metabolites were detected in subnanomolar concentrations in authentic urine samples with an accuracy rate of 73%.

Next, the authors tried to detect synthetic opioids, which act similarly to heroin or morphine. The μ-opioid receptor and β-arrestin2, which interact in the presence of opioid, were fused to LgBiT and SmBiT, respectively [45]. The system was nearly 100% successful in detecting subnanomolar levels of the synthetic opioids in blood samples.

Aggregation of TDP (transactivating response region DNA binding protein)-43 occurs in approximately 95% of amyotrophic lateral sclerosis patients [46, 47]. Oberstadt et al. constructed a screening system for inhibitors of aggregation by the fusion between the LgBiT and SmBiT probes and TDP-43 [48]. Aurorafin, chelerythrine, and riluzole were identified as inhibitors from the Library of Pharmacologically Active Compounds (LOPAC1280).

Stomes et al. selected agonists of the interaction between adenosine receptor 3 and β-arrestin2 and revealed the structural features of the selected ligands [28].

The NanoBiT screening system is not only effective for drug screening but can be valuable to screen enzyme substrates. Peptide ligases, which can connect two polypeptides, are powerful tools for protein engineering [49–52]. Li et al. performed the screening of substrates of the peptide ligase Sortase A by fusing this enzyme to SmBiT and the candidate peptides to LgBiT [53]. In addition to known substrate sequences, they rapidly identified some previously unknown substrates with varying activities. In addition, the measurement was very stable, and the signal was maintained for more than 16 h.

5. Application of NanoBiT using self-assembling NanoLuc fragments

Self-assembling NanoLuc fragments have been used to detect protein aggregation, to detect the edited protein by CRISPR/Cas9, to monitor viral entry, release, and propagation, and to analyze clathrin-dependent internalization (**Figure 3**).

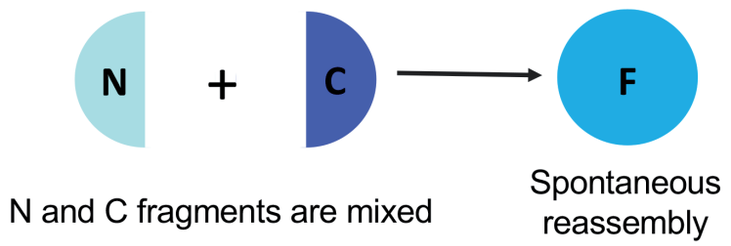

Figure 3.
Scheme for protein fragments self-assembly. Since the affinity between N and C is high, the full-length reporter protein is reconstituted by just mixing N and C.

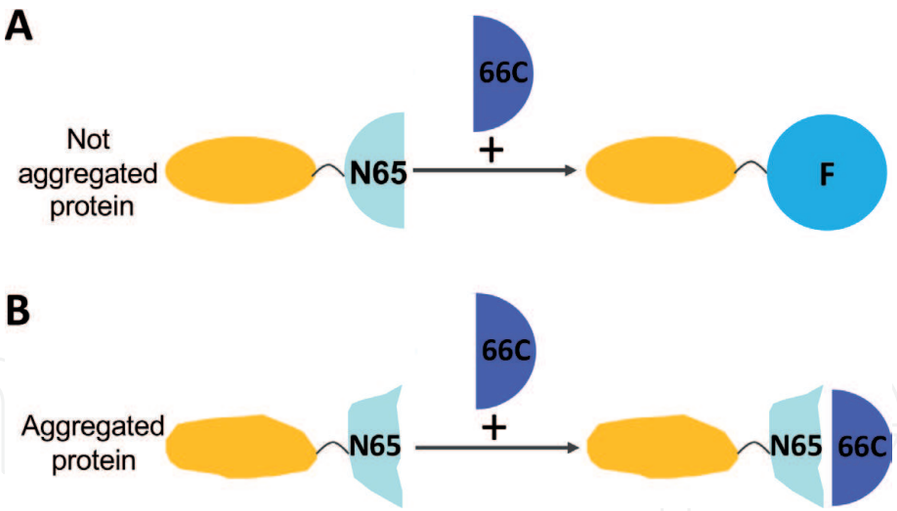

Figure 4.
Self-assembling Nluc fragment as a probe for protein aggregation. (A) N65 is fused to the target protein. When the fusion protein is not aggregated, NanoLuc is reconstituted by the addition of 66C. (B) When the target protein is aggregated, N65 is also aggregated, and, then, the reassembly does not occur.

The first description of the use of self-assembling NanoLuc fragments was provided by Zhao et al [54]. NanoLuc was separated into two fragments, N65 (1–65 aa) and 66C (66–171 aa). NanoLuc was rapidly reconstituted when N65 and 66C were mixed. Next, N65 was fused to the target proteins. When the target protein was soluble, N65, which had the correct structure, could reassemble with 66C, resulting in recovery of the luminescence. On the other hand, the insoluble target protein did not induce the recovery of the luminescence, because the aggregated N65 could not assemble with 66C (**Figure 4**). The aggregations of amyloid-β mutants were assessed using the system. Similar monitoring systems of protein aggregation using split-GFP and conventional split-luciferase systems had been previously reported [55–60]. However, a time lag occurred for the chromophore formation in the split-GFP system, and other luciferases were relatively unstable compared with NanoLuc. Zhao et al. succeeded the robust measurement of amyloid-β in this study.

Other self-assembling NanoLuc fragments were described [13]. The SmBiT sequence was optimized using peptides with different affinities to LgBiT. Of the candidates, the HiBiT peptide displayed high affinity (K_D = 700 pM) although the affinity of SmBiT was very low (K_D > 100 μM). HiBiT (11 aa) and LgBiT assembled spontaneously, allowing the construction of NanoLuc. HiBiT is a useful tag due to the small size as further described below.

In one of the split-GFP systems, GFP was split into two fragments [57]. The C-terminal fragment of GFP contains 16 aa (GFP11). Waldo et al. found that this and the other fragment (GFP1–10) expressed in the cell assembled spontaneously, and the GFP fluorescence was recovered. Leonetti et al. described the synthesis of the donor DNA templates encoding GFP11 and the tagging of endogenous proteins using CRISPR/Cas9 (**Figure 5A**) [61]. The formation of full-length GFP was induced by coexpression with GFP1–10, and the tagging endogenous protein by GFP11 could be detected. Instead of GFP11, Schwinn et al. used HiBiT as the tag for endogenous proteins and were successful in achieving the highly efficient integration and monitoring of the expression dynamics of the tagging proteins without the time lag, which occurs in the split-GFP system due to the chromophore formation (**Figure 5B**) [62].

Ryes-Alaraz et al. analyzed the internalization of galanin receptor 2, which is dependent on the binding of the endogenous ligand, using an HiBiT-fused galanin receptor 2 [27]. LgBiT could bind to the HiBiT-fused receptor on the cell surface.

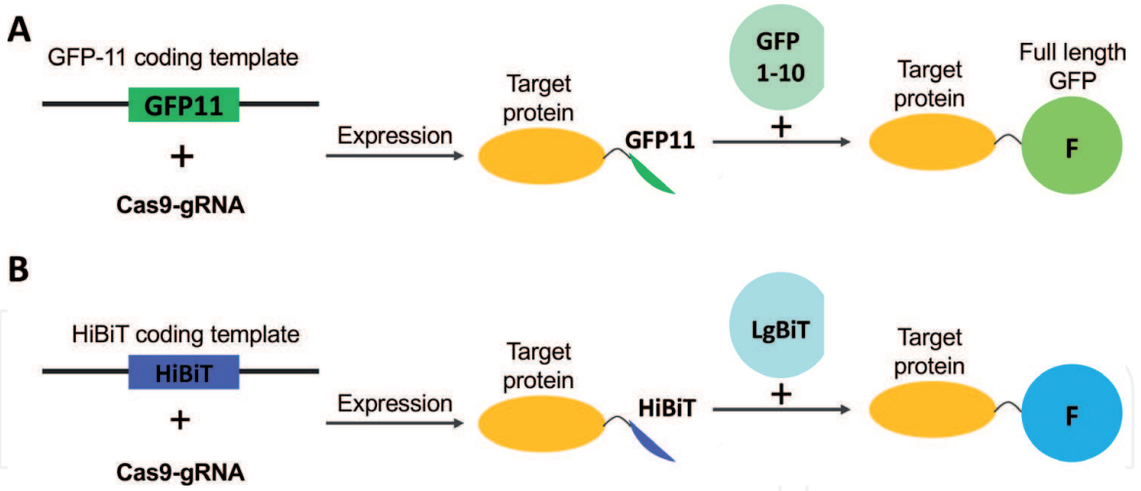

Figure 5.
Application of self-assembling fragment for Crispr-Cas9 system. (A) GFP11-encoding template is inserted at the end of the genome encoding target protein by Crispr-Cas9 system. GFP11-fused target protein is expressed. By the addition of GFP1–10, full-length GFP is reconstituted and the target protein was detected. (B) HiBiT is used instead of GFP11, and higher sensitivity is attained.

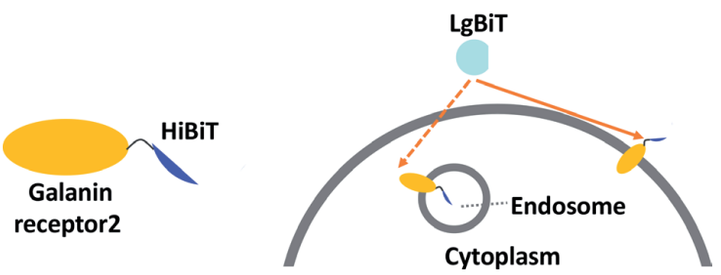

Figure 6.
Application of self-assembling fragment for the analysis of internalization. LgBiT can bind to HiBiT-galanin receptor 2 on the cell membrane, while it cannot access HiBiT-galanin receptor 2 in the endosome.

However, LgBiT could not bind to the HiBiT-fused receptor in cells due to the impermeability of LgBiT to cells (**Figure 6**).

This technology has often been used to quantify targets. Oh-hashi et al. used HiBiT to quantify the expression of transcription factor ATF4 that was induced by endoplasmic reticulum stress [63]. Sasaki et al. developed a quantitative detection system of viral entry and release using HiBiT fused to subviral particles and flavivirus-like particles of West Nile virus [64]. Tamura et al. constructed recombinant viruses carrying HiBiT [65]. Viral amplification and propagation were rapid and comparable with the parental viruses, due to the small size of HiBiT. The techniques proved useful to study the viral life cycle and pathogenesis.

6. NanoLuc ternary technology

In PCA, the reporter protein is generally separated into two fragments. To our knowledge, PCA using 3-split reporter protein was first reported by Cabantous et al. [66]. In the study, GFP was split into two peptides, GFP10 and GFP11, and the remaining part. The two peptides were each fused to an interacting partner. When the interaction occurred, the peptides came into close proximity with one another and then assembled to form the full length of GFP with the remaining part (**Figure 7**).

Possibly inspired by this GFP ternary technology, Dixon et al. developed the NanoLuc ternary technology, NanoLuc is consisted of 11 β-strands [67]. The

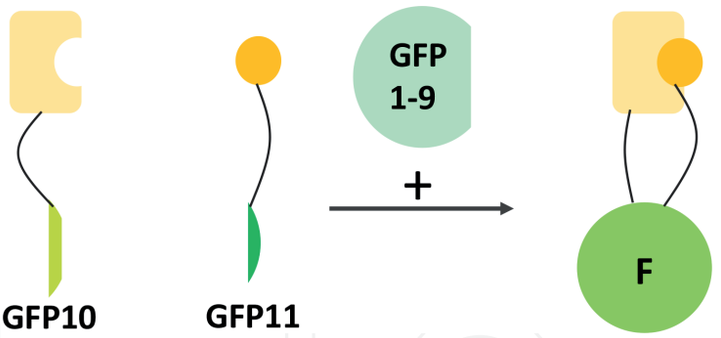

Figure 7.
GFP ternary split technology for the detection of protein-protein interaction. The two small fragments GFP10 and GFP11 are fused to the interacting proteins, respectively. When the interaction occurs, GFP is reconstituted from GFP10, GFP11, and externally added GFP1–9.

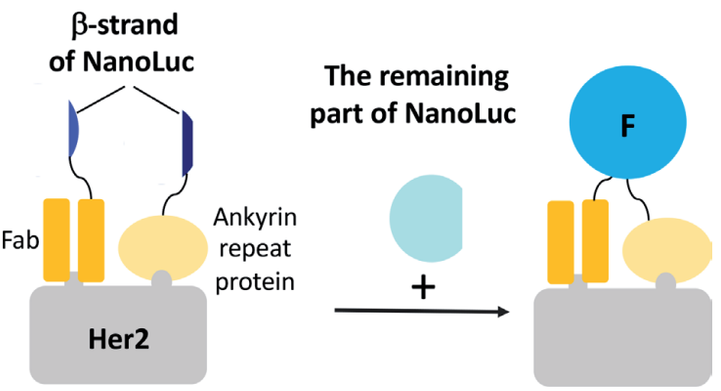

Figure 8.
Sandwich immunoassay based on NanoLuc ternary technology developed by Dixon et al. The two β-strands are fused to a Fab and an ankyrin repeat protein, respectively, which bind to two distant parts of Her2 protein. NanoLuc was reconstituted from the two strands and externally added remainder of NanoLuc.

authors dissected two β-strands and the remaining part. Each strand was fused to a Fab fragment of an antibody and an ankyrin repeat protein, which bound to distinct areas of the cancer marker, HER2. When both antibodies recognized HER2, the two strands came close together and the full length of NanoLuc was reconstituted from the three fragments of NanoLuc (**Figure 8**). The sensitivity was similar to the sensitivity detected using the commercially available AlphaLISA HER2 kit (Perkin Elmer) and NanoBiT. Furthermore, the detectable concentration range of HER2 was broader compared to the range detected by NanoBiT.

At almost the same time, we developed the NanoLuc ternary technology for use as an open-sandwich immunoassay (OS-IA), because OS-IA could not be performed using NanoBiT [68]. For OS-IA, two antigen-binding regions, the heavy-chain variable region (V_H) and the light-chain variable region (V_L), were isolated from the full-length antibody. OS-IA is based on the antigen-dependent interaction affinity between V_H and V_L, which is dependent on the antigen (**Figure 9**) [69]. The advantage of OS-IA is that small antigens can be noncompetitively detected with high sensitivity. V_H and V_L were fused to LgBiT and SmBiT. However, the signal was not increased by the addition of the small peptide antigen (7 aa) named BGP-C7. We suspected that fusion with LgBiT sterically hindered the interaction, or prevented the folding of these antibody fragments due to the relatively large size of LgBiT.

The next step was to split LgBiT in two. The C-terminal strand (11 aa) was named LcBiT, and the remaining part was named LnBiT. LcBiT and SmBiT were fused to V_H and V_L, respectively. When LnBiT, V_H-LcBiT, and VL-SmBiT were mixed, the signal was increased depending on the concentration of BGP-C7. The

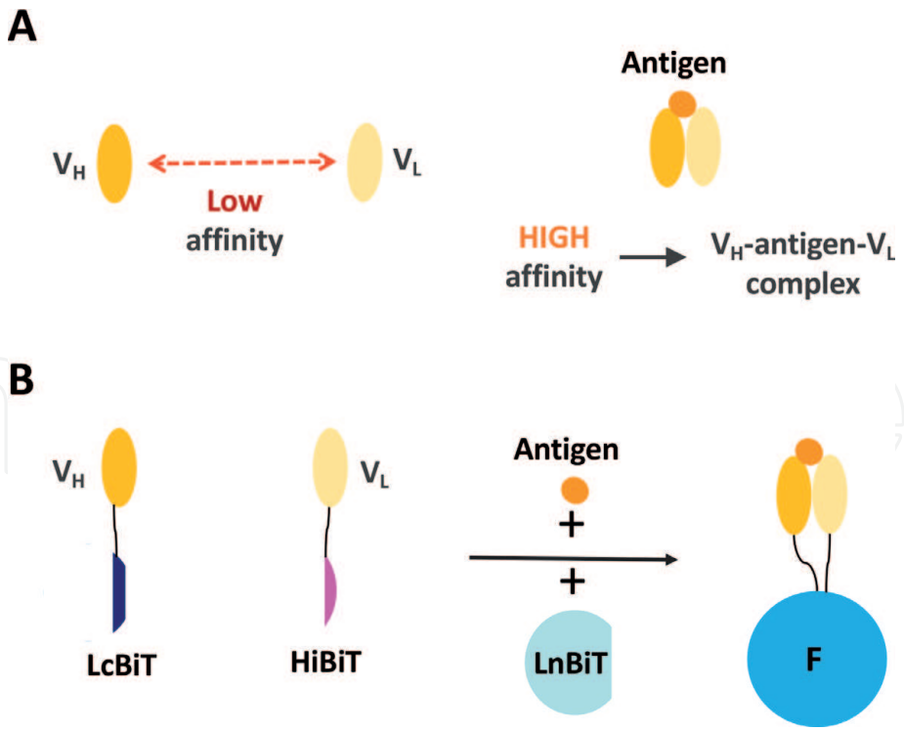

Figure 9.
Open sandwich immunoassay (OS-IA) and NanoLuc ternary technology. (A) Principle of OS-IA. Small antigens (MW < 1000) can be noncompetitively detected. (B) Noncompetitive detection of small antigen by NanoLuc ternary technology. Visual detection was possible when sufficient amount of antigen was present.

background signal without BGP-C7 was lower than the background signal of V_H-LgBiT and V_L-SmBiT.

Next, the signal was enhanced by optimizing the sequence of SmBiT. The signal was increased 288-fold using the sequence, which has higher affinity to LgBiT. The enhancement was high enough to permit detection by the naked eye. The detection limit of BGP-C7 was comparable with the limit detected by OS-ELISA. Furthermore, the strong signal was maintained for more than 1 h.

The small tags of the NanoLuc ternary system have proven to be very useful when both target proteins have complex structures. Furthermore, the system exhibits a robust and bright signal.

7. Discussion and conclusion

The described NanoLuc binary and ternary technologies are superior compared with other PCAs using other luciferase enzymes. The signals obtained were almost the strongest among the signals of PCAs using luciferase enzymes. The most important advantage is the small size of the fusion tags, SmBiT and LcBiT.

As mentioned in Section 4, NanoBiT has been used as a screening tool. In several studies, SmBiT was fused to membrane proteins, which are important targets of drug discovery. The brightness of NanoLuc increased the hit ratio, and SmBiT was validated as a tag for the fusion with proteins having complex structures, such as membrane proteins. Although the measurements were very accurate, researchers should pay attention to the influence of low-molecular weight compounds on enzymatic activity [70–72]. Some compounds increase enzymatic activity, while others decrease it. The TurboLuc system reported by Audi et al. is somewhat smaller in molecular weight (16 kDa) than NanoLuc [73]. Compared with NanoLuc

and Firefly luciferase, the activity of TurboLuc was less affected by low-molecular weight compounds. Ho et al. examined the influence of 42,460 PubChem compounds on enzymatic activities of several luciferases [74]. NanoLuc, *Renilla* luciferase, firefly luciferase, and *Gaussia* luciferase were affected by 2.7, 10, 4, and 0.02% of the compounds, respectively. The relationship between the chemical similarity and the inhibition profile showed that the compounds varied depending on the luciferase used. While NanoLuc has several advantages for screening, in some cases, researchers should select other enzymes or more than two enzymes. Furthermore, we previously cautioned using a mathematical model that the comparison between the affinity of interacting proteins and the signal detected by luciferase-based PCA can cause misinterpretation of the quantitation [75]. In addition, we suggested that the geometry of the interacting proteins influences the luminescent signal. In other words, the structures of the interacting proteins can affect the reconstitution of luciferase. Quantitative measurement with PCA using luciferase is possible, but careful examinations are needed.

One of the other problems is the unstable luminescence in cells. When we use the standard furimazine ester for live cell assay, the light intensity decreases within 1 h, and it will not be suitable for large screenings in cellulo. Recently, live cell substrates with longer half-life (Vivazine and Endurazine, Promega) have become available. These substrates can maintain the luminescent signal for several hours, although the peak luminescent intensity is significantly lower compared with that detected using the conventional furimazine ester. However, the luminescent intensity of unmodified furimazine can be maintained for several hours in vitro. Oxygen in cells and the culture medium will also be an important factor for the stable luminescence because the NanoLuc-catalyzed reaction requires oxygen, especially when the light emitted is strong. The last problem is the prices of these substrates, which tend to be costly especially when larger scale screening is intended.

Detection of the interaction among more than three proteins, and the simultaneous detection of more than two interactions will become more important in future. For the detection among three proteins, the combination of NanoBiT and BRET might be useful. The brightness of NanoLuc often disturbs simultaneous detection using both NanoLuc and other luciferases. Therefore, NanoLuc inhibitors were developed [76]. After the measurement of NanoLuc luminescence, the luminescence can be diminished by the inhibitors, which enables detection of the luminescence of another luciferase. For the simultaneous detection by multicolors, several color variants of eNano-Lantern (a fusion protein of NanoLuc and fluorescent protein) can be applied to NanoBiT. In eNano-Lantern, the luminescence at longer wavelength can be observed by the efficient intramolecular BRET mechanism [77].

Dixon et al. and the authors developed a novel PCA using 3-split NanoLuc [67, 68]. The pair of the small tags is very effective to avoid misfolding and steric hindrance of target proteins. Our next challenge will be to further improve the efficiency and stability of the reconstitution for its wider use.

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