Combinatorial Library Screening for Developing an Improved Split-Firefly Luciferase Fragment-Assisted Complementation System for Studying Protein–Protein Interactions

Ramasamy Paulmurugan* and Sanjiv S. Gambhir*

Departments of Radiology and Bioengineering, Bio-X Program, Molecular Imaging Program at Stanford (MIPS), Stanford University School of Medicine, James H. Clark Center, 318 Campus Drive, Stanford, California 94305-5427

Split reporter-based bioluminescence imaging is a useful strategy for studying protein–protein as well as other intracellular interactions. We have used a combinatorial strategy to identify a novel split site for firefly luciferase with improved characteristics over previously published split sites. A combination of fragments with greater absolute signal with near-zero background signals was achieved by screening 115 different combinations. The identified fragments were further characterized by using five different interacting protein partners and an intramolecular folding strategy. Cell culture studies and imaging in living mice was performed to validate the new split sites. In addition, the signal generated by the newly identified combination of fragments (NF excise 398/CF excise 394) was compared with different split luciferase fragments currently in use for studying protein–protein interactions and was shown to be markedly superior with a lower self-complementation signal and equal or higher postinteraction absolute signal. This study also identified many different combinations of nonoverlapping and overlapping firefly luciferase fragments that can be used for studying different cellular events such as subcellular localization of proteins, cell–cell fusion, and evaluating cell delivery vehicles, in addition to protein–protein interactions, both in cells and small living animals. A protein fragment-assisted complementation (PFAC) strategy for studying protein–protein interaction involves the use of a combination of split reporter gene fragments that encode for split proteins that have relatively low affinity for each other and thus produce low signal. When these same fragments are fused to interacting proteins of interest, the interaction of the two proteins drives complementation of the split reporters, leading to a detectable signal. The PFAC strategy has been developed using a variety of reporter proteins including dihydrofolate reductase,\textsuperscript{3,4} β-lactamase,\textsuperscript{5} green fluorescent protein,\textsuperscript{6} firefly luciferase,\textsuperscript{7} and Renilla luciferase.\textsuperscript{8} Several of the currently available techniques for studying protein–protein interactions are restricted to using either cell lysates or intact cells. To extend these applications to small living animals, we and others have adopted a yeast two-hybrid system\textsuperscript{9,10} using bioluminescence. To develop a more robust and generalizable strategy for imaging protein interactions in living small animals, we previously reported a split firefly luciferase fragment-assisted complementation and intein-mediated reconstitution system.\textsuperscript{11} The fragment-assisted complementation approach was subsequently also studied by others in small animals.\textsuperscript{12}

The PFAC strategies based on split bioluminescent reporters (firefly luciferase and Renilla luciferase) are particularly useful for simpler approaches to study these protein–protein interactions, particularly on a larger scale and especially in intact cells, is great. Furthermore, if these interactions could be studied in intact living subjects, this would allow additional insights into normal and diseased states. In order to understand such ubiquitous protein interactions, several techniques have been developed and are reviewed elsewhere.\textsuperscript{1,2}

* To whom correspondence should be addressed. E-mail: sgambhir@stanford.edu; paulmur8@stanford.edu.

because of their applicability in imaging protein–protein interactions in intact cells and by direct extension to small living animals. In addition, these bioluminescent reporters hold potential advantages for use in small animals over other reporters particularly due to their low background signal.13 This is in contrast to work with fluorescent reporters (e.g., green fluorescent protein and red fluorescent protein) and reporters that use fluorescent substrates as a readout (e.g., β-lactamase and β-galactosidase) due to autofluorescence and confounding increases in background signal. We8,11 and others12,13,14 have identified several combinations of split fragments for bioluminescent reporters such as firefly and Renilla luciferase that are suitable for studying protein–protein interactions in living animals.8,11,12,14 In addition, we also recently identified several combinations of firefly luciferase enzyme fragments that self-complement without assistance via protein–protein interactions.15 These types of fragments are useful for studying cellular localization of tagged proteins, evaluation of cell macromolecular delivery vehicles and also for studying cell–cell fusion. We have also recently applied split reporter strategies to study intramolecular folding of the estrogen receptor in intact cells and small living animals.16

The sensitivity of the split reporters in studying protein–protein interactions will in general depend on several variables including the affinity of the two interacting proteins of interest. The split firefly luciferase fragments used in our previous study showed a good sensitivity with the interacting protein partners Id/myoD,11 but they failed to produce significant levels of signal with the rapamycin-mediated interacting proteins FRB/FKBP12. In the rapamycin-mediated interaction strategy, the small molecule rapamycin binds to the proteins FRB and FKBP12 and leads to the induction of both homo- and heterodimerization between these proteins. This result contrasts with the combination of split firefly luciferase fragments utilized by others that are reported to have a greater level of signal with the rapamycin-mediated FRB/FKBP12 interaction system but at a cost of increased background signal.12 The Renilla luciferase fragments (Nrluc 229, CrLuc 229) utilized in our previous study show significant levels of protein–protein interaction assisted luciferase signal for many different interacting partners with a near-zero background.8,17 However, the wavelength of light emitted during the enzymatic reaction of Renilla luciferase with its substrate coelenterazine is in the range 480–510 nM, and this wavelength range sometimes incurs limitations of light absorption by different proteins in biological tissues (e.g., hemoglobin) when extending this system to imaging studies in small living animals.

To improve the absolute signal by increasing the protein–protein interaction assisted luciferase signal, we also reported a fusion protein strategy where a single vector encodes both interacting partners and the split reporters separated by linker sequences as a fusion protein.18 Whereas these published studies have already proven the utility of split bioluminescence reporters in studying protein–protein interactions, the focus of the current study was to use a combinatorial screening approach to identify new combinations of firefly luciferase fragments for studying protein–protein interactions with greater absolute signal and relatively low background that would be applicable to many different types of studies in cell culture and imaging small living animals.

We have collected over the last several years a library of N- and C-terminal fragments of firefly and Renilla luciferase split reporter protein fragments in the course of developing and validating various split reporter strategies. In the current study, by applying a combinatorial screening approach to this library, we screened several combinations of N- and C-terminal firefly luciferase fragments with the interacting proteins FRB/FKBP12 and identified a new set of Nfluc and Cfluc fragments that have ideal properties of low background signal (low self-complementation) and a high signal after protein–protein interaction assisted complementation. In addition, this strategy also identified a number of combinations with and without overlapping regions and with and without self-complementing properties, in a single step. The selected combinations that produced the highest level of rapamycin-induced luciferase signal were further evaluated with five other interacting protein combinations. An intramolecular folding strategy with the estrogen receptor and various ligands was also studied in cells and in mice with the identified optimal split reporters. The novel split reporters developed in this study will potentially increase the sensitivity and the generalizability of fragment-assisted complementation systems for studying protein–protein and other interactions in cells and small living animals.

**MATERIALS AND METHODS**

**Chemicals, Enzymes, and Reagents.** Restriction and modification enzymes and ligase were obtained from New England Biolabs (Beverly, MA). TripleMaster Taq DNA polymerase was obtained from Brinkmann Eppendorf (Hamburg, Germany). All the constructs were made with a pcDNA3.1 (+) backbone. PCR was used for constructing all the vectors with different fragments and different interacting proteins. Rapamycin and all the different ER ligands used in this study were obtained from Sigma (St. Louis, MO). Lipofectamine transfection reagent was from Invitrogen (Carlsbad, CA). The plasmid extraction kit and DNA gel extraction kit were from Qiagen (Valencia, CA). Coelenterazine was from Nanolight (Pinetop, AZ). Luciferin was from Xenogen (Alameda, CA). Bacterial culture media were from BD Diagnostic Systems (Sparks, MD). All cell culture media, fetal bovine serum, and the antibiotics streptomycin and penicillin were from Invitrogen. The charcoal-treated fetal bovine serum (FBS) was from HyClone (Logan, UT). Oligonucleotide synthesis and DNA sequencing were performed by the Stanford Protein and Nucleic Acid facility.

**Construction of Plasmids.** All PCR-amplified reporter fragments with corresponding restriction enzyme sites were inserted in the plasmids by replacing the Nrluc and CrLuc portions of the vectors pcDNA-Nrluc-FRB and pcDNA-FKBP12-CrLuc used in our previous study.17 Similarly the vectors with the interacting protein partners Id/myoD, HIF1α/pVHL, ER/ER, and TK/TK were constructed by replacing the FRB and FKBP12 portions of the vectors. The ER intramolecular folding vector was constructed by first generating the vector pcDNA-Nfluc-CrLuc, followed by

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insertion of PCR-amplified ER (amino acids 281–595) with BamHI restriction enzyme sites on either side. The digested dephosphorylated vector was used for inserting the BamHI-digested ER fragment and constructed pcDNA-Nfluc-hER-Cfluc. The orientations of the insert were initially confirmed by activity assay and further by sequencing.

**Cell Culture.** Human 293T embryonic kidney cancer cells (ATCC, Manassas, VA) and MCF7 human breast cancer cells were grown in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were used in the experiments with estrogen receptors, we used the cells grown supplemented with 10% FBS and 1% penicillin/streptomycin. For uterine carcinoma cells were grown in DMEM-high glucose supplemented with 10% FBS and 1% penicillin/streptomycin. MDA-MB-231 breast cancer cells and RL95 human uterine carcinoma cells were grown in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. MDA-MB-231 breast cancer cells and RL95 human uterine carcinoma cells were used.

**Cell Transfection and Luciferase Assay.** Transfections were performed in 24 h old cultures of 293T, MCF7 and RL95 (~80% confluent) cells. For transfections and cotransfections, 200 ng or 200 ng of each/well of DNA was used in 12 well culture plates. Lipofectamine transfection reagent was used as recommended by the manufacturer. For cell culture heterodimerization experiments, 40 nM rapamycin was added immediately after transfection. Similarly for ER ligand-induced folding and homodimerization studies, 1 µM of each ligand dissolved in DMSO was added immediately after transfection. DMSO was used as solvent control. Cells were assayed for luciferase activity by using LARII (Promega) assay reagent and for Renilla luciferase activity as by the method previously published. Light measurements were performed in Turner Designs, 20/20 luminometer (Sunnyvale, CA) for 10 s. Bio-Rad protein assay reagent was used for measuring the protein concentrations in the cell lysates. The luciferase activities are represented as relative light units (RLU) per microgram of protein per minute.

**Combinatorial Screening by Optical CCD Camera Imaging.** To screen the combinations of fragments, 50 000 cells (293T) plated in 96 well plates were transfected with different combinations of plasmids. For transfection, 12.5 ng of each plasmid/well was used. The cells were exposed to 40 nM rapamycin immediately after transfection and imaged after 48 h by adding 1 µg/well β-luciferin in 25 µL of PBS with 1 min acquisition. For each sample, one well served as control and another well was with rapamycin. The plates were analyzed for rapamycin-mediated complementation of luciferase enzyme fragments.

**Optical CCD Camera Imaging of Ligand-Induced Intramolecular Folding of the Estrogen Receptor in Living Mice.** All animal handling was performed in accordance with Stanford University Animal Research Committee guidelines. For imaging in living nude mice (nu/nu), 293T and RL95 cells stably expressing sensors 2 and 3 (Supporting Information Figure S3) were used. Mice were anesthetized by ip injection of ~40 µL of a ketamine and xylazine (4:1) solution, and 5 million cells of each stable 293T and RL95 cells expressing the sensors were implanted on either side of the animals' hind limbs. The animals were repetitively imaged (with and without ip injection of 0.5 mg of the ER ligand antagonist raloxifene) by injecting 3 mg of the substrate β-luciferin. All mice (n = 5 each) were imaged using a cooled charge-coupled device (CCD) camera (Xenogen IVIS; Xenogen Corp. Alameda, CA), and photons emitted from the mice were collected and integrated for a period of 1 min. Images were analyzed using Living Image software (Xenogen) and Igor image analysis software (Wavemetric). To quantify the number of emitted photons, regions of interest were drawn over the area of the implanted cells and the maximum photons s⁻¹ cm⁻² steradian⁻¹ (sr) were obtained as previously described.

**RESULTS**

Comparison of Previously Reported Split Renilla (Nrluc 2229/Crluc 2229) and Split Firefly Luciferases (NfLuc 437/Cfluc 437 and NfLuc 416/Cfluc 398). Use of a fragment-assisted complementation strategy shows that the split Renilla luciferase strategy has a higher fold induction with near-zero background signal. The split Renilla luciferase fragments identified from our previous study (Nrluc 2229/Crluc 2229), the split firefly luciferase fragments used by us in our previous studies (NfLuc 437/Cfluc 437), and those used by others (NfLuc 416/Cfluc 398) were compared using vectors constructed to express the rapamycin-mediated protein–protein (FRB/FKBP12) interaction system. All three split reporter systems were studied in transiently cotransfected 293T cells. The cells were assayed for luciferase activity before and 24 h after exposure to 40 nM rapamycin. The results show relatively low levels of firefly luciferase activity before exposure to rapamycin and a (60 ± 10)-fold ((6 ± 2) × 10⁴ RLU µg of protein⁻¹ min⁻¹) signal increase upon exposure to rapamycin from the combination of split firefly luciferase enzyme fragments (NfLuc 437/Cfluc 437) used previously by our laboratories. The split firefly luciferase enzyme fragments identified by others (NfLuc 416/Cfluc 398) show luciferase signal both in the presence and the absence of rapamycin, with a (6 ± 2)-fold greater signal after exposure to rapamycin (3.2 ± 0.4) × 10⁵ RLU mg of protein⁻¹ min⁻¹) (Figure 1).

The split Renilla luciferase system developed by us (Nrluc 2229/Crluc 2229) shows a very low signal without rapamycin;
Combinatorial Screening with Optical CCD Camera Imaging. The screening establishes that ~25% of the combinations studied show a significant level of firefly luciferase signal after exposure to rapamycin. To identify N- and C-terminal firefly luciferase enzyme fragments that further improve the ability to study protein–protein and other interactions, 115 different combinations were screened by constructing vectors with rapamycin-mediated interacting proteins FRB/FKBP12. 293T cells cotransfected with different combinations in a 96 well format were imaged after 48 h of exposure to rapamycin by optical CCD camera imaging by adding the substrate d-luciferin to identify combinations of fragments that lead to luciferase signal through complementation. The results show bioluminescence signal from a significant number of combinations (29/115 or 25%) only after exposure to rapamycin. Similarly, 3 out of 115 combinations (2.6%) produce bioluminescence signal both in the presence and in the absence of rapamycin (self-complementation) (Supporting Information Table 1 and Figure S1). From among the 29 combinations that generated rapamycin-mediated bioluminescence, 20 combinations with the highest signals were selected for further evaluation by luminometry.

Selected Combinations Identified by Optical CCD Camera Imaging and Evaluated Further by Luminometry. These combinations show three split firefly luciferase combinations that are sensitive in producing a relatively high level of rapamycin-mediated protein–protein interaction associated bioluminescence signal with an extremely low background signal. Twenty leading combinations of split N- and C-terminal firefly luciferase fragment candidates were selected from an initial screen for further evaluation. These fragments were studied by luminometry in cotransfected 293T cells with and without exposure to rapamycin. Three combinations of N- and C-terminal luciferase enzyme fragments produce a significant amount of rapamycin-mediated luciferase signal (NFluc 398/CFluc 394, (800 ± 150)-fold with an absolute signal of (1.7 ± 0.5) × 10⁸ RLU µg of protein⁻¹ min⁻¹; NFluc 398/CFluc 398, (2550 ± 500)-fold with an absolute signal of (0.6 ± 0.2) × 10⁶ RLU µg of protein⁻¹ min⁻¹; NFluc 416/CFluc 415, (250 ± 60)-fold with an absolute signal of (1.6 ± 0.4) × 10⁸ RLU µg of protein⁻¹ min⁻¹) (Figure 2). Of these three, we selected NFluc 398/CFluc 394 for further experimentation because of its greater overall fold induction and absolute signal. Cotransfection experiments using RL95, MCF7, and MDA-MB-231 cells with all three fragment combinations show a similar pattern of results seen with the 293T cells (data not shown).

Orientation-Specific Interaction of Proteins and Their Associated Reporter Fragment Complementation. Study of the interaction shows a preferred orientation for optimal signal production. We next wanted to determine the optimal orientation of NFluc- and CFluc-reporter fragments and the interacting partners required for efficient protein–protein interaction mediated complementation. Eight different vectors were constructed to express fusion proteins with the reporter fragment and the interacting proteins in different orientations by using the most optimal reporter fragments NFluc-398/CFluc-394 and the previously published reporter fragments NFluc-416/CFluc-398 (NFluc 416-FRB, NFluc 398-FRB, FRB-NFluc 416, FRB-NFluc 398, CFluc 394-FKBP12, CFluc 394-FKBP12, FKBP12-CFluc 398, and FKBP12-CFluc 394). All these vectors in different combinations were studied in cotransfected 293T cells with and without rapamycin. The specific orientation of the fused reporter fragment/protein determines the level of signal produced. The fusion proteins NFluc398-FRB/ FKBP12-CFluc 394 generated (800 ± 200)-fold induction with an absolute level of luciferase signal of (1.7 ± 0.5) × 10⁸ RLU µg of protein⁻¹ min⁻¹. Other combinations of the same expressed reporter fragment and interacting proteins, but in different orientations, (NFluc 398-FRB/CFluc 394-FKBP12, (20 ± 4)-fold/(1.5 ± 0.3) × 10⁶ RLU µg of protein⁻¹ min⁻¹; FRB-NFluc 398/ FKBP12-CFluc 394, (320 ± 40)-fold/(0.8 ± 0.2) × 10⁷ RLU µg of protein⁻¹ min⁻¹; FRB-NFluc 398/CFluc 394-FKBP12, (2 ± 1)-fold/(0.3 ± 0.2) × 10⁶ RLU µg of protein⁻¹ min⁻¹) show lower levels of induction with different absolute levels of signal (Figure 3).

Study of the Optimal New Combination of Luciferase Enzyme Fragments with Different Interacting Protein Part-
ners or Intramolecular Folding. The study demonstrates the robustness of the new split sites. To evaluate the efficiency of the newly identified combination of N- and C-terminal luciferase enzyme fragments (NFluc 398/CFluc 394) with different interacting proteins, we constructed vectors expressing N- and C-terminal fragments with four different interacting partners other than the one used for initial screening, with different types of interaction patterns including the (i) Id/myoD interaction system,11 (ii) TK-TK homodimerization system, 20 (iii) ER ligand-induced homodimerization system (unpublished data), and (iv) hypoxia-induced interaction system (HIF1-α/pVHL) system (unpublished data) and the ER ligand induced intramolecular folding system. 16 All these systems were studied in transiently transfected and cotransfected 293T cells under appropriate conditions. The results show significant levels of protein–protein interaction and intramolecular ER folding associated the luciferase signal with expected specificity for all the systems using the newly identified NFluc 398/CFluc 394 fragments (Figure 4).

In the Id/myoD interaction system and the TK/TK homodimerization system, the expressed interaction proteins with the reporter protein fragments undergo protein–protein interaction mediated complementation and generate signal. So we modulated the level of protein by varying the concentration of DNA used for the study. These results show a protein concentration-dependent increase in the complemented luciferase signal (Figure 4 a, b). Whereas in the ER–ER homodimerization system and ER ligand-induced intramolecular folding systems, the binding of ER ligands induces the level of luciferase fragments complementation. The result show specific ER ligand-induced ER homodimerization and the ER intramolecular folding and subsequent complemented luciferase signal (Figure 4c, e). Similarly, in the hypoxia-induced interaction system, the level of protein hydroxylation controls the level of protein–protein interaction. This system shows efficient reduction in the complemented luciferase signal when the cells are exposed to hypoxic conditions (CoCl2 and DFO) that lead to reduction in the level of HIF1-α hydroxylation (Figure 4d).

Optical CCD Camera Imaging of HIF1-α/pVHL Interaction. The interactions shows a hypoxia-induced reduction in complemented luciferase signal from the cells. To show that the signal level generated by the new combination of luciferase enzyme fragments was significant enough for imaging cells in culture and living animals, we selected a hypoxia-inducible interaction system. The protein HIF1-α is phosphorylated by the enzyme prolyl hydroxylase in cells under normoxic conditions. The phosphorylated form of this protein interacts with the von Hippel-Lindau (pVHL) tumor suppressor protein. Hypoxic conditions lead to change in the phosphorylation level of HIF1-α, which in turn reduces its interaction with pVHL. Exposures of cells to either CoCl2 or DFO have been reported to generate hypoxic conditions. Hence, 293T cells cotransfected with vectors expressing fusion proteins NFluc 398-HIF1-α and pVHL-CFluc 394 were imaged after treatment with either CoCl2 or DFO for 24 h. These results show a significant level of protein–protein interaction assisted luciferase signal as compared to the cells not exposed to either CoCl2 or DFO (normoxia). Cells exposed to either CoCl2 or DFO exhibit induction of hypoxia that in turn leads to a drop

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Figure 3. Luminometer assay results from the lysates of 293T cells cotransfected with different combinations of N- and C-terminal firefly luciferase fragments with interacting proteins FRB and FKBP12 constructed with different orientations. Orientation of an interacting protein with the reporter fragments is important for achieving efficient complementation. The combination containing NFluc-FRB/FKBP12-CFluc produced a greater level of rapamycin-mediated protein–protein interaction signal over other orientations.

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in protein–protein interaction and consequently a decrease in luciferase signal. The signal level decreases by \((3 \pm 2)\)-fold with DFO, and \((6 \pm 2)\)-fold with CoCl₂ (Supporting Information Figure S2).

**Imaging of Ligand-Induced ER Intramolecular Folding with the New Combination of N- and C-Terminal Firefly Luciferase Enzyme Fragments.** The imaging shows a greater level of complemented luciferase signal upon induction by either the injected or endogenous ligand in living animals. To check the efficiency of the newly identified firefly luciferase fragments in living animals, the ER ligand-induced intramolecular folding system was selected. We constructed three different sensor systems containing the estrogen receptor ligand binding domain (amino acids 281–595) and different combinations of firefly luciferase enzyme fragments as shown in Supporting Information Figure S3a (NFluc 416, NFluc 398, CFFluc 398, and CFFluc 394). These three different sensors were transiently transfected using 293T cells and studied by exposure of cells to different ER ligands (Supporting Information Figure S4). The results show efficient ligand-induced intramolecular folding for all three sensors with different levels of absolute signal. The sensor constructed with N- and C-terminal luciferase fragments having a higher level of overlap (sensor 3, NFluc 416/CFFluc 394) shows significant level of background before the addition of ligands and a higher level of signal after introduction of ligand. The sensor constructed with different interacting protein partners. 293T cells cotransfected with vectors constructed to express the reporter fragments with interacting proteins (a) Id/myoD, (b) TK-TK homodimerization, (c) ER ligand-induced homodimerization, (d) hypoxia-induced interacting proteins (HIF1α/pVHL), and (e) ER ligand-induced intramolecular folding were studied under appropriate conditions. A significant level of protein–protein interaction associated luciferase signal is seen with expected specificity from all the different systems used.

**Molecular Imaging in Living Animals Using the ER Ligand-Induced Intramolecular Folding Strategy.** This strategy, with different sensors constructed using the new split sites, demonstrates a high sensitivity for in vivo applications. To demonstrate the use of newly identified fragments with improved sensitivity for applications in living animals, we selected the ER ligand-induced intramolecular folding strategy. The 293T cells stably expressing sensors 2 and 3, and the RL95 cells stably expressing sensor 3, were used for the study (Supporting Information Figure S3b). In a first set of experiments, male nude mice \((n = 5)\) subcutaneously implanted with 5 million each of 293T cells expressing sensors 2 and 3 were imaged for several days with and without the ligand antagonist raloxifene (20 μg/g in 50 μL of sesame oil). The results show no significant signal from the implanted cells expressing the sensors. This is due to the unavailability of ER ligands to induce complementation. When these animals received the ER ligand raloxifene (Figure 5a, day 3), a significant level of complementation-mediated luciferase signal is detected through the intramolecular folding induced by the ligand (Figure 5a, day 4).

Similarly in a second set of experiments, female nude mice implanted with 5 million each of 293T and RL95 cells expressing sensors 2 and 3, respectively, were imaged for several days without administering any ligands to observe the complementation induced by the endogenous estradiol that usually changes during different phases of the estrous cycle. The result shows estrous-phase-dependent complementation with a peak signal approximately once every 4–5 days (Figure 5b, c). The absolute level of signals and the day at which these animals attained peak luciferase
signals are different for each animal used for imaging. This reflects variations in the level of ligand produced by each animal.

**DISCUSSION**

In this study, we used a combinatorial screening approach to identify a new combination of N- (NFluc 398) and C- (CFluc 394) terminal firefly luciferase enzyme fragments that work efficiently in studying protein–protein interactions and intramolecular folding with a near-zero background signal (without producing self-complementation-associated signal). As we have been working for more than 5 years in developing split reporter-based optical imaging techniques to study different cellular events, we have available to us several different combinations of split luciferase fragments with different complementation properties.11,15 We used all these fragments for this combinatorial screening study by constructing vectors expressing fusion proteins with different N- and C-terminal firefly luciferase fragments from the library and the rapamycin-mediated interacting proteins FRB (FKBP12 rapamycin binding domain) and FKBPI2 (FK506 binding protein). The rapamycin-mediated interacting proteins FRB and FKBPI2 were selected for this study because in a single step it is possible to distinguish between signals generated from self-complementing reporter fragments, and through protein–protein interaction-assisted reporter fragment complementation, using high-throughput optical CCD camera imaging.

Although we have previously reported split *Renilla* luciferase fragments with near-zero background signal for studying protein–protein interactions, the blue-green emission spectrum of *Renilla* luciferase (480–510 nM) penetrates tissues less efficiently, sometimes limiting its sensitivity in some small living animal applications.12,17,18 The split firefly luciferase fragments (Nfluc 437/Cfluc 437) used in our previous study have limited signal, thereby precluding their generalizability. The split firefly luciferase enzyme fragments used by others (Nfluc 416/Cfluc 398) show a relatively high level of signal before protein–protein interactions occur, leading to potential difficulty in differentiating from the low level of protein–protein interaction-mediated signal generated from weak interacting partners.12 Hence, the combination of fragments identified in this study (Nfluc 398/Cfluc 394) and evaluated using several different protein partners and an intramolecular folding strategy overcome many of the problems encountered from previously reported systems. Even though the identified fragments of this study are more optimal than any previous ones, future studies may still identify better split reporter combinations.

In addition, in this study, we also evaluated the relative orientation between the interacting protein partner and the split reporter that leads to optimal PFAC. Different studies from our laboratory have found that attaching small peptides or proteins of different lengths to the NH$_2$ terminus of the firefly luciferase
protein significantly reduces the luciferase enzyme activity. The study by Luker et al. achieved a significant level of complemented luciferase signal by attaching the protein FRB to the NH₂ terminus of the NfLuc fragment. Hence, in this study to further confirm the orientation that is essential for efficient PFAC, we constructed several different vectors expressing all the different possible orientations by using selective N- and C-terminal luciferase enzyme fragments. The preferred orientation identified from this study is NfLuc–FRB/FKBP12–CFLuc. We also found that the newly identified fragments showed consistent results across several cell lines used for the study. In addition, the newly identified fragments showed a similar level of sensitivity to the split Renilla luciferase fragments, without resulting in any steric hindrance for the estrogen receptor intramolecular folding system.

The ER ligand-induced intramolecular folding system utilizing the newly identified luciferase fragments was used to evaluate the complementation-induced by the injected ligand antagonist (raloxifene) in living mice. Similarly, by using this system, we also estimated the level of complementation induced by circulating endogenous ligand in female nude mice. This study also identified many different combinations of nonoverlapping and overlapping luciferase fragments that can be used for studying different cellular events such as subcellular localization of proteins, cell–cell fusion, and evaluating cell delivery vehicles where self-complementing split reporters are needed.

In summary, in this study we compared previously published split sites for firefly and Renilla luciferase. We sought to overcome limitations of previous split reporters and used a combinatorial approach to identify a new split site for firefly luciferase with optimal characteristics. We tested this new split site with several different interacting proteins and with an intramolecular folding strategy in cell culture. Optical imaging in small living animals further demonstrates the utility of the new split sites. This new split reporter complementation system with greater absolute signal and lower background than previous systems can be further extended to study PFAC and other intracellular interactions. The developed system can also be used for high-throughput screening of new protein–protein interaction targeted drugs in cells along with further evaluation in small living animals.

**Abbreviations:** Fluc, firefly luciferase protein; fluc, firefly luciferase gene; RLuc, Renilla luciferase protein; rLuc, Renilla luciferase gene; FKBP12, FK506 binding protein; FRB, FKBP12-rapamycin-binding domain; ER, estrogen receptor; LBD, ligand binding domain.

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**SUPPORTING INFORMATION AVAILABLE**

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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