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Firefly Luciferase Enzyme Fragment Complementation for Imaging in Cells and Living Animals

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Abstract

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

Luciferases are enzymes that emit light in reaction with a specific substrate in the presence of cofactors. A diverse group of organisms use luciferase-mediated bioluminescence to startle predators or to attract prey or mates. The emitted light is used as a detection system for luciferase expression, which acts as a “reporter” for the activity of any regulatory elements that control its expression. Luciferase is particularly useful as a reporter enzyme in living cells and organisms. Firefly luciferase is one among many of the sensitive luciferases and is widely used by researchers to identify different biological events of cells in culture

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and in living small animals, and it is also used by public health researchers for the detection of food contamination.[1–3]

The gene encoding firefly luciferase, cloned in 1985 from the North American firefly, *Photinus pyralis*, is now emerging as the gene of choice for in vivo and in vitro reporting of transcriptional activity in eukaryotic cells.[4] Reporter genes encoding for proteins with optical signatures, either fluorescent or bioluminescent, are a low-cost alternative for real-time analysis of gene expression in small-animal models. In fluorescent approaches an external source of light is required for excitation of the protein. In contrast, bioluminescent reporter proteins can only produce light by using the appropriate substrates. Recently, several technical advances in utilizing highly sensitive detection devices have led to the biological use of cooled charge-coupled device (CCD) cameras capable of imaging very low levels of visible light emitted from internal organs of rodents.[5,6] "Luciferase" is a family of photoproteins that can be isolated from a large variety of insects, marine organisms, and prokaryotes.[7] The emission spectrum ranges between 400 and 620 nm. In recent years, considerable work with noninvasive imaging of firefly luciferase has been performed.[6,8–12]

Functional proteins are made up of one or more polypeptides. Monomeric functional proteins can be split into two portions with resulting functionally inactive fragments. These split reporters have been used for measuring real time protein–protein interactions efficiently, both in cells and also in living animals.[11,13–15] The inactive protein fragment assisted complementation of Ubiquitin, dihydrofolate reductase, green fluorescent protein, and *β*-lactamase have been used for studying protein–protein interactions in bacteria and mammalian cells.[16–22] In our previous studies, we split bioluminescent monomeric proteins *firefly luciferase* and synthetic *renilla luciferase*, and the resulting fragments were used for studying protein–protein interactions. We demonstrated this by studying two known positive interacting proteins Id and myoD, and also by small-molecule Rapamycin mediated interaction of human proteins FRB and FKBP12 in both cell culture and with noninvasive repetitive bioluminescence optical imaging in living mice.[11,15,23] Recently, Luker et al. identified different fragments of firefly luciferase enzyme by a combinatorial library strategy and used this for studying protein–protein interactions in cells and living mice.[13] For many proteins, the backbones of the polypeptide chain have been split by chemical, proteolytic, or genetic means and have created interchain-packed active functional proteins. This include subtilisn-modified ribonuclease, phosphoribosylanthranilate isomerase, and aminoacyl tRNA synthetase.[24–26] This combination of two protein fragments to restore activity has been termed as protein fragment complementation.[19] The generation of functional proteins from a monomeric form to a heterodimeric form has been hypothesized as the phenomenon of the reversion of evolutionary process in which functional structures or domains are recruited and then fused at the genetic level.[27] *Even though many studies have reported the use of fragment-assisted complementation of optical reporter proteins for studying protein–protein interactions, we are not aware of any reports on the self-complementation of any of these reporters.*

The α-complementation of *β*-galactosidase is one of the first published enzyme assays based on protein fragment complementation and has been used for various applications.[28] Recently, Cre-recombinase with fragment-assisted complementation using a 20 amino acid

overlap has been shown to give 30% of the recovered activity of the intact enzyme, and it has been efficiently used for temporally controlled Cre-recombinase enzyme mediated recombination in cells.[29] The fragments generated by chymotrypsin enzyme digestion of topoisomerase I have also been shown to remain associated physically and retain enzyme activity.[30] Reporter proteins fragment assisted complementation may have applications in many areas of basic science research including the following: (1) the identification of cellular localization of proteins where the study protein can be linked with one fragment of the reporter protein and the second fragment can be linked with different known cellular localization signals; (2) study of macromolecular cellular delivery vehicles where one fragment will be stably expressed in the cells and the second fragment will be delivered by the delivery vehicle (this will avoid the problems associated with signals that do not allow precise knowledge of the location of the delivery vehicle); (3) development of phosphorylation sensors where conformational changes induced by a phosphorylation event can lead to complementation; (4) studying of cell–cell fusion where the cells under study can be labeled with the fragments of reporter and that gives complemented luciferase activity only if the cells fuses.

In this study we identified suitable sites from the crystal structure of the firefly luciferase enzyme to generate rational fragments of monomeric reporter protein firefly luciferase that can produce signal through complementation. We studied several different sites designed in different locations including: loops, α-helices, and *β*-pleated sheets. We also proved that the co-localization in the same cellular compartment of both fragments of the enzyme is essential for complementation. This also confirmed the necessity of either complete helices or pleated sheets of the protein should be present, at least in one of the two fragments, to generate efficient complementation assisted recovery of enzyme activity. We tested the efficiency of this system in different cell lines and the ability to image with sufficient sensitivity from cells implanted in living mice.

EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Reagents

Restriction and modification enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). PCR amplification was performed with TripleMaster Taq DNA polymerase purchased from Brinkmann Eppendorf (Hamburg, Germany). PCR amplification was used for generating the rational fragments of N and C portions of firefly luciferase gene for each split points using the primers mentioned in Table 1 and the template plasmid pG5-Luc purchased from Promega (Madison, WI). Superfect transfection reagents, plasmid extraction kits, and DNA gel extraction kits were purchased from Qiagen (Valencia, CA). Coelenterazine was purchased from Biotium (Hayward, CA) and luciferin from Xenogen (Alameda, CA). Bacterial culture media were purchased from BD Diagnostic Systems (Sparks, MD). All animal cell culture media, fetal bovine serum, antibiotics (streptomycin and penicillin), and plastic wares for growing cell cultures were purchased from Invitrogen (Carlsbad, CA). Rapamycin was purchased from Sigma (St. Louis, MO). The NCBI protein structure using the cn3D online software (Version 4.1, NCBI) was used to locate the split sites of firefly luciferase enzyme.

Construction of Plasmids

The N and C portions of firefly luciferase fragments for the selected 10 sites (generates 17 fragments: 7 Nfluc fragments and 10 Cfluc fragments), as shown in Figure 1a, were amplified using the primers listed in Table 1 and the template pG5-Luc plasmid of Promega's two-hybrid kit reporter. The forward primers of all N and C fragments were designed with the Nhe I restriction enzyme site to allow for convenient cloning, and also with the Kozak sequence and start codon, for ribosome binding and initiation of translation, respectively. The reverse primers were designed with stop codon and Xho I restriction enzyme site. The amplified fragments of each site were cloned in the corresponding restriction enzyme digested pcDNA3.1 (+) vector backbone (Invitrogen, CA-92008). The sequence-confirmed clones from each site were used for further studies. The Nfluc (1–475) and Cfluc (265–550) fragments were also cloned with nuclear localization signal peptide (NLS of SV40 T antigen) at the N-terminal using the primer NLS–NF–Nfluc-475 and NLS–NF–Cfluc-265 (Table 1). The vector expressing Nfluc (1–475) was transferred to the pcDNA vector expressing the Puromycin antibiotic selection marker for making 293T cells stably expressing the Nfluc (1–475) fragment. Similarly, we constructed an arabinose inducible prokaryotic expression vector pBAD-HisA (Invitrogen) expressing the Cfluc (265–550) fragment by linking with the N-terminal TAT peptide with two glycine amino acids as linker and C-terminal 6-histidine molecules for protein purification (Figure 1b).

Cell Culture

Human embryonic kidney cancer cells, 293T (ATCC, Manassas, VA) were grown in MEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. The N_2a cells (Mouse neuroblastoma cells) were obtained from V. P. Mauro (Scripps Research Institute, La Jolla, CA), and CHO cells were grown in DMEM-Ham-F12-glutamax supplemented with 10% FBS and 1% penicillin/streptomycin. 3T3-L1 adipose cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/ streptomycin/glutamine. HeLa cells were grown in MEM–high glucose supplemented with 10% FBS and 1% penicillin/ streptomycin. SK-N-SH, SH-SY-5Y cells (ATCC, Manassas, VA) were grown in MEM supplemented with 10% FBS/1 mM sodium pyruvate and 1% penicillin/streptomycin solution.

Cell Transfection and Luciferase Assay

Transfections were performed in 80% confluent 24 h old cultures of 293T, N_2a, HeLa, CHO, SK-N-SH, SH-SY-5Y, and 3T3-L1 cells. In 12 well plates, 250 ng/well of each plasmid was used for transfection. Volumes of Superfect used were as recommended by the manufacturer. The samples were assayed by mixing 20 μL of supernatant and 100 μL of substrate LARII from Promega by counting for 10 s in a Turner 20/20 single-tube luminometer. The readings were normalized by measuring the concentration of proteins from the cell lysates and represented as relative light units per microgram protein per minute. The transfection was normalized by cotransfecting 10 ng/well of pCMV-hRL DNA and measuring the renilla luciferase activity by using the substrate coelenterazine.

Purification of TAT-Cfluc (265–550) Protein Expressed in *Escherichia coli*

The *E. coli* strain LMG cell was transformed with the pBAD-HisA-TAT-Cfluc (265–550) for the purification of the protein. The protein was purified with use of a Ni–NTA agarose (Qiagen, Valencia, CA) column by using the manufacturer protocol. The purified protein was desalted by using PD10 column, and the estimated protein was used for transduction studies in stable 293T cells expressing Nfluc (1–475) fragment.

Imaging in Living Mice

To study bioluminescence imaging in living mice, the Nfluc fragment (1–475) was found to give the maximum signal with the different Cfluc fragments [(245–550), (265–550), (300–550), (437–550), (445–550), and (500–550)] used. Five million 293T cells transiently cotransfected with both N and C portions of the plasmid when different combinations were implanted subcutaneously in six different positions and imaged. The animals were repeatedly imaged at 0, 24, 48, and 72 h after implantation by using optical CCD camera (Xenogen IVIS) to acquire the photons 10 min after intraperitoneal injection of 3 mg of D-luciferin dissolved in 100 μL of PBS. The bioluminescence signals were estimated by using the software Living Image (version 2.12) and are in units of photons per cubic centimeter per second per steradian ($P/cm^2/s/sr$).

RESULTS

The nonoverlapping fragments studied at seven different split sites showed no significant fragment complementation but showed significant protein–protein interaction mediated complementation from two of the seven sites

To study the assisted (driven by two interacting proteins) and unassisted (self) complementation from different nonoverlapping fragments of the firefly luciferase enzyme, seven different sites (amino acid positions 415, 420, 437, 445, 455, 475, and 500) were selected. Nfluc and Cfluc fragments (seven each), without any interacting proteins, and with rapamycin-mediated interacting proteins, FRB and FKBP12, were constructed and studied using the cotransfected 293T cells. The cells cotransfected with all seven combinations with FRB–Nfluc and Cfluc–FKBP12 show significant signal upon exposure to rapamycin only from the combinations of vectors constructed at split sites at amino acid positions 420, 437, 445, and 455 ((10 ±2)-, (40 ±5)-, (350 ±5)-, and (30 ±4)-fold, respectively, over the cells not exposed to rapamycin). All seven combinations studied without interacting proteins and with interacting proteins but without rapamycin show signals that were not significantly different from mock-transfected cells (Figure 2).

Fragment complementation of firefly luciferase enzyme fragments was achieved from different overlapping fragments, and the highest signal was achieved from cells cotransfected with Nfluc (1–475) and Cfluc (265–550)

To identify the firefly luciferase protein fragments that give complementation-assisted recovery of luciferase enzyme activity, 13 different sites, including 7 for generating Nfluc and Cfluc fragments and 6 for generating only Cfluc fragments, were identified by analyzing the crystal structure of the protein (Figure 1a). Cotransfection of each of the Nfluc fragments

separately with all Cfluc fragments was studied using transiently transfected 293T cells. The result shows different levels of fragment complementation based luciferase activity from different combinations of Nfluc and Cfluc fragments. The maximum level of activity was achieved from the cells cotransfected with the overlapping fragments Nfluc (1–475) and Cfluc (265–550). The levels of activity achieved from different combinations of fragments cotransfected cells ranged from 0.123 RLU/(μg of protein)/min [Nfluc (1–445) and Cfluc (445–550)] to as high as 3500 RLU/(μg of protein)/min [Nfluc (1–475) and Cfluc (265–550)]. The cells cotransfected with the combination of Nfluc and Cfluc without overlapping sequences show signals that were not significantly different ($p < 0.05$) from mock-transfected cells. At the same time, 65% of overlapping fragments showed enzyme activity that was significantly ($p < 0.05$) above mock transfected cells upon cotransfection (Figure 3). The results were normalized for transfection efficiency by cotransfecting pCMV-hRL and assaying for renilla luciferase activity.

The fragment complementation of Nfluc (1–475) with different Cfluc fragments at different time points showed different levels of enzyme activity by different combinations

To assess the efficiency of complementation as a function of time, 293T cells were cotransfected with Nfluc (1–475) with different combination of Cfluc fragments (245–550, 265–550, 300–550, 437–550, 445–550, and 500–550) and assayed 24, 48, and 72 h after cotransfection. The results show the greatest activity from cells cotransfected with Nfluc (1–475) and Cfluc (265–550) at each of the different time points studied. The signal observed by this combination was significantly higher ($p < 0.05$) than all other combinations at all three time points studied. There was a slight increase in the activity with increasing time (Figure 4).

Fragment complementation based recovery of luciferase enzyme activity was efficiently achieved in all the different cell lines studied

To test the complementation-assisted recovery of luciferase enzyme activity in different cell lines, the optimal combination of fragments Nfluc (1–475) and Cfluc (265–550) were studied in seven different cell lines; 293T, N_2a, HeLa, CHO, SK-N-SH, SH-SY-5Y, and 3T3-L1 cells. The cells were assayed for luciferase enzyme activity 24 h after transfection. The results show efficient complementation-assisted luciferase enzyme activity in all the different cell lines studied. The result shows different levels of activity across different cell lines before normalizing for transfection efficiency and is found to be similar when normalized. The greatest level of activity was achieved with 293T cells. The complemented signal level, in different cell lines, was compared with the cells transfected with intact Fluc and ranged from 3.2 to 5% after normalizing for transfection (Figure 5).

The firefly luciferase fragment complementation system showed sufficient signal for optical cooled coupled device camera imaging in living mice implanted subcutaneously with cells

We achieved only 3–4% luciferase enzyme signal through complementation of optimal Fluc fragments as compare to the signal of intact firefly luciferase in transiently transfected cells in culture. Hence, to test the complementation-assisted luciferase signal intensity in living

animals and also to determine the detectable limit of different combinations, we implanted 5 million 293T cells cotransfected with combination Fluc fragments: Nfluc (1–475) and different Cfluc fragments (245–550, 265–550, 300–550, 437–550, 445–550, and 500–550) subcutaneously in six different sites immediately after transfection. The animals ($N = 6$) were imaged immediately (0 h), 24, 48, and 72 h after implanting the cells by using optical CCD camera by injecting 3 mg of D-Luciferin i.p. The signals were quantified and compared with cell culture data. No signals were detected from any of the sites imaged immediately after implanting the cells. After 24 h, the sites implanted with cells cotransfected with Nfluc (1–475) and Cfluc [(245–550), (265–550), and (300–550)] showed significant ($p < 0.01$) signals (0.3 ±0.15 P/cm^2/s/sr) compared to the animals imaged immediately after implanting cells. At the 48 h time point, the site implanted with the cells cotransfected with Nfluc (1–475) and Cfluc (437–550) also showed signal that was significantly ($p < 0.05$) above the background but not as high as the other three sites. All the sites other than the site where cells implanted with the cells cotransfected with nonoverlapping Nfluc (1–475) and Cfluc (500–550) fragments showed significant detectable signal after 72 h, and the average of photon maximum estimated across six animals by the fragment combinations Nfluc (1–475)/Cfluc (245–550), Nfluc (1–475)/Cfluc (265–550), Nfluc (1–475)/Cfluc (300–550), Nfluc (1–475)/Cfluc (437–550), Nfluc (1–475)/ Cfluc (445–550), and Nfluc (1–475)/Cfluc (500–550) respectively are 1.1 ±0.4, 1.8 ±0.5, 1.9 ±0.3, 2.1 ±0.6, 0.7 ±0.42, and 0.3 ±0.2 P/cm^2/s/sr (Figure 6).

The firefly luciferase fragment complementation can be efficiently achieved by localizing both fragments in the same cellular compartment

To use the firefly luciferase fragment complementation efficiently for the identification of interacting protein partners with unknown cellular localization and also for the identification of nuclear, mitochondria, and membrane transport of particular interacting protein pairs, we simply, as proof of principle, studied the interaction of the fragments by expressing nuclear localizations signals. Nfluc (1–475) and Cfluc (265–550) fragments were expressed with NLS signal peptide of SV40 T antigen (PKKKRKVD) at amino terminal, and firefly luciferase activity was determined by cotransfecting 293T cells in different combinations as described above. The cells cotransfected with the combinations of plasmid vectors containing Nfluc (1–475) + Cfluc (265–550) and NLS–Nfluc (1–475) + NLS–Cfluc (265–550) showed fragment complementation signals that were significantly ($p < 0.01$) greater than the mock-transfected cells and the cells transfected with all four fragments individually. At the same time, the cells cotransfected with the combination fragments containing one fragment with NLS and the other without NLS showed signal that was significantly less ($p < 0.01$) than cells transfected with Nfluc + Cfluc or NLS–Nfluc + NLS–Cfluc (Figure 7a).

The complementing firefly luciferase fragments can be used for studying cellular macromolecular delivery vehicles by delivering a TAT-linked Cfluc fragment (265–550) to cells stably expressing Nfluc (1–475)

To demonstrate the use of complementing Fluc fragments for studying a macromolecular delivery system, we made 293T cells stably expressing Nfluc (1–475) and purified N-terminal TAT-linked Cfluc (265–550) fragment expressed in *E. coli*. The cells were exposed to different concentrations of TAT-Cfluc (265–550) for 2 h in PBS and for 24 h in MEM

with 10% FCS and 1% penicillin and streptomycin. The cells were then assayed for complementing luciferase activity. The stable cells exposed to TAT-Cfluc (265–550) show Fluc activity that is (7 ±3)-fold higher ($p < 0.01$) than unexposed cells. The complemented luciferase signal seen with the stable cells exposed to TAT-Cfluc (265–550) is (10 ±2)-fold less than the cells transfected with Cfluc (1–475) (Figure 7b).

DISCUSSION

Even though we studied 63 different combinations of N and C fragments of firefly luciferase, a significant ($p < 0.05$) amount of luciferase activity through fragment complementation was achieved only from 18 combinations of overlapping fragments that include different Nfluc fragments (1–415, 1–420, 1–437, 1–445, 1–475, and 1–500) and Cfluc fragments (245–550, 265–550, and 300–550). The Nfluc and Cfluc fragments utilized without any overlap for all split points showed activity that was not significantly different from mock transfected cells. A few other Nfluc fragments selected next to amino acid position 400 or before showed no significant complementation-assisted luciferase signal (data not shown). The fragments with greater overlap showed a higher signal, but this result did not hold true for combinations of fragments examined. For example, in both cell culture and animal study, the luciferase signal from the combination of fragments selected with the greatest degree of overlap (Nfluc 1–475)/(Cfluc 245–550) was 2-fold less than that of the combination with less overlap [(Nfluc 1–475)/(Cfluc 265–550)]. Therefore, there does not appear to be any correlation between the degree of overlap and signal generated from complementation-assisted luciferase signal.

Although the use of complementing fragments of reporter proteins are not very suitable for applications of studying protein–protein interactions,[11,15] they can potentially be useful in many different areas of basic cell biology research. The complementing fragments of the Cre recombinase enzyme have been efficiently used for temporally controlled recombination processes in cells.[31] Even though the basic principles of splitting monomeric proteins for fragment complementation and protein–protein interaction assisted complementation are the same, the utility of these protein fragments is entirely different. In the current study, for the first time, we generated a dimeric active firefly luciferase enzyme by generating many NH_2 and COOH terminal fragments. The firefly luciferase enzyme is a reporter molecule with many sensitive detection systems available for the quantification of protein activity both in cells and in living animals; the developed fragment complementation system will be useful in many different applications. A number of studies have been conducted for investigating the structural folding and the noncovalent interaction of complementing fragments of a protein using enzymes RNase A, cytochrome *c*, staphylococcal nuclease, and *β*-galactosidase for different applications, e.g., to see the evolutionary origin, to temporally control enzyme activity, and to study protein–protein interactions.[32–35]

Advances in bioluminescence imaging using cooled CCD cameras are providing a highly sensitive means for imaging and quantifying very low levels of visible light from intact small living subjects.[9] We previously reported an inducible yeast two-hybrid system with firefly luciferase[12] and a split firefly luciferase complementation system[11] to study protein–protein interactions in cell lines and to noninvasively image interactions in living mice.

More recently, we developed an inducible hRLUC protein-fragment-assisted complementation-based bioluminescence assay to quantitatively measure real time protein–protein interactions in mammalian cells in the presence of the substrate coelenterazine,[15] and also we extended this system for studying small-molecule drug mediated protein–protein interaction in cells and in living animals.[23] The design of a protein fragment assisted complementation assay entails the interaction of two protein chimeras: each composed of one protein partner fused in frame with a split segment of a reporter protein. The interaction is driven by, and brings together, the two protein partners, leading to the recovery of reporter activity through protein complementation of the two trailing split reporter segments. The developed system in the current study does not require protein–protein interaction in order to complement the reporter fragments, but they should be in the same cellular compartment to achieve complementation. The complementing fragments developed in this study produce signal intensities that were enough to efficiently assay and image in cells and mice quantitatively. We also confirmed that the developed complementing firefly luciferase system could be efficiently used for studying macromolecular delivery vehicles, which is an important area of research in molecular pharmacology for the specific delivery of therapeutic molecules such as nucleic acids, proteins, and drugs to different tissues in living animals.[36,37] Further extensions of the strategy used should allow assays to verify if a given cargo fused to the split reporter protein is able to cross the cell membrane and be delivered into cytosol or a given cellular compartment where it would complement with its partner, which could be constitutively expressed by the cell of interest.

Even though split mutant GFP and quantum dot tagged proteins or peptides may also be useful in studying the co-localization of cellular proteins and other intracellular events, these approaches may be more difficult to use in living small subjects and may be more difficult to quantitate due to autofluorescence. Future studies will need to perform head-to-head comparisons between the available systems to determine which strategy has greater sensitivity in cell culture and in small living subjects. It is also important to determine the binding affinity of the fragments identified in the current study. This will help to determine how much of each split protein is necessary for complementation and the potential for false negatives.

Intercellular, intracellular and nucleocytoplasmic trafficking of functional proteins plays a major role in regulating many cellular functions.[38,39] Even though the functions of different proteins are determined by posttranslational modification, the only way by which proteins trafficking within and between cells can be currently studied is by immunocytochemistry or by tagging with fluorescent proteins.[39,40] However, the subcellular localization of proteins by these microscopic techniques is not suitable for quantitative analysis and for the high-throughput screening of a larger number of samples. Hence, the strategy developed in this study compensates for both of these drawbacks and also provides an additional advantage for the use of this system in living animals via noninvasive imaging. This strategy may also have additional applications including the identification of protein cellular localization, studying macromolecular delivery systems, and developing intracellular phosphorylation sensors.

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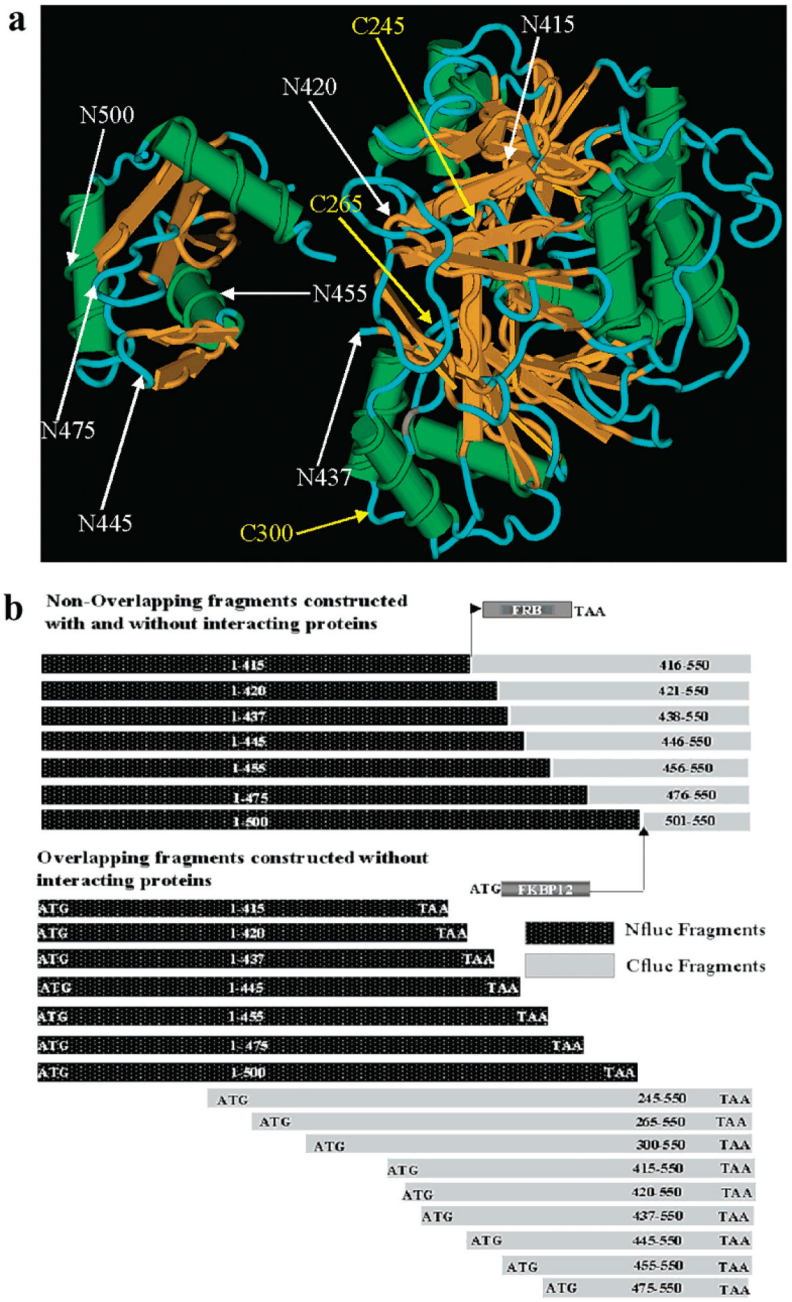

Figure 1.

(a) Schematic diagram showing the three-dimensional structure of firefly luciferase enzyme with indicated sites used for generating different NH_2 and COOH (white) and only COOH (yellow) terminal fragments for the fragment complementation strategy. (b) Schematic diagram showing different vector constructs generated for the fragment complementation study for overlapping and nonoverlapping luciferase enzyme fragments with and without interacting proteins (FRB/FKBP12). The split sites are indicated after the amino acid positions.

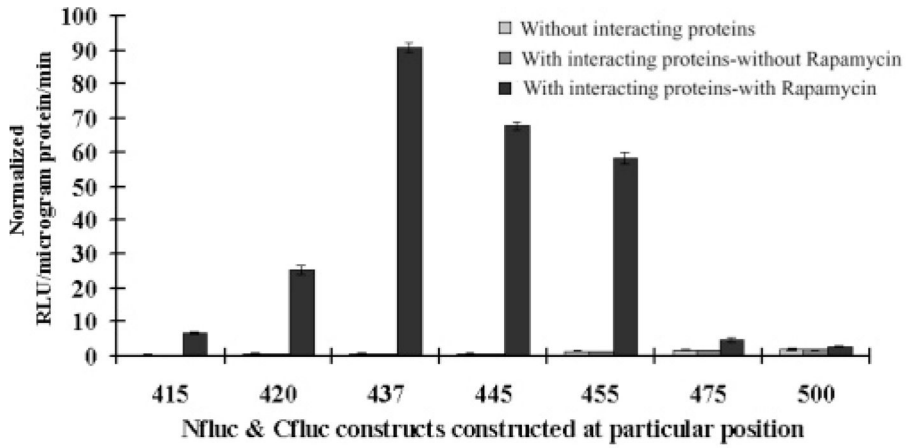

Figure 2.

Luminometer results of different nonoverlapping fragments with and without interacting proteins (FRB/FKBP12) studied in transiently transfected 293T cells and assayed 24 h posttransfection. The results are normalized by cotransfection of renilla luciferase. The result shows no significant fragment complementation from different nonoverlapping Nfluc and Cfluc fragments (fragments generated at split sites next to amino acid positions 415, 420, 437, 445, 455, 475, and 500). Four among the seven combinations showed significant signal upon exposure to rapamycin that brings Nfluc and Cfluc protein fragments together by interacting FRB and FKBP12 (split fragments generated at sites 420, 437, 445, and 455). The error bar is the standard error of the mean for three samples.

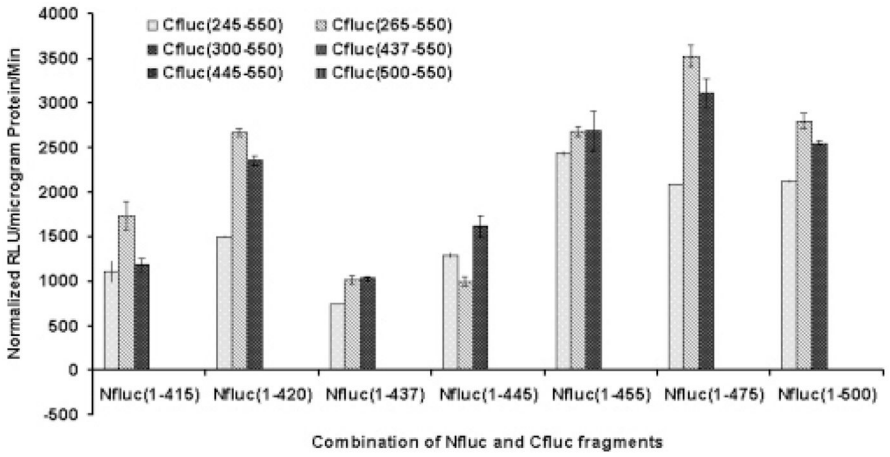

Figure 3.

Luminometer assay conducted for different fragments generated for the fragment complementation assay using 293T cells. The results are normalized for transfection efficiency using cotransfection of renilla luciferase. The result shows different levels of fragment complementation associated luciferase activity resulting from different combinations of Nfluc and Cfluc fragments. The maximum level of activity was achieved from the cells cotransfected with Nfluc (1–475) and Cfluc (265–550). The luciferase signals from cells cotransfected with the combination of Nfluc and Cfluc without overlapping sequences were not significantly different from mock-transfected cells. At the same time, many combinations of overlapping fragments showed enzyme activity that was significantly ($p < 0.05$) above mock-transfected cells upon cotransfection. The error bar is the standard error of the mean for three samples.

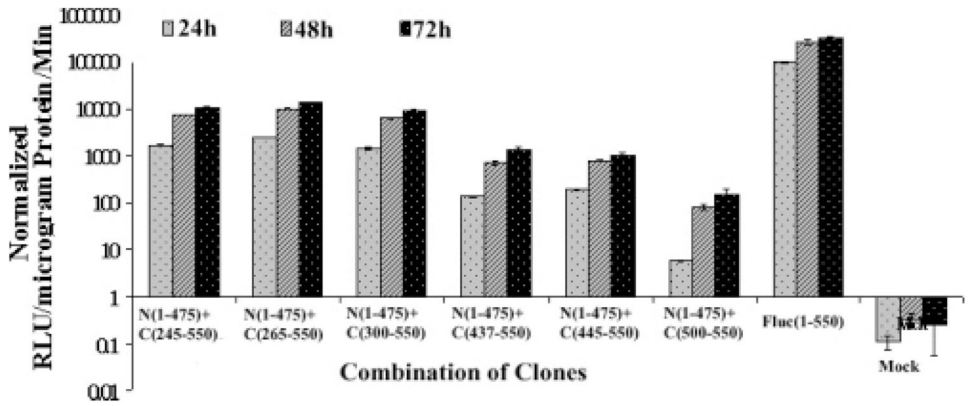

Figure 4.

Luminometer assay conducted for the 293T cells cotransfected with different combinations of Nfluc and Cfluc fragments studied at different time points (24, 48, and 72 h). The results are normalized using cotransfection of renilla luciferase. The result shows significant ($p < 0.05$) complementation-assisted firefly luciferase enzyme activity from different Nfluc fragments with three Cfluc fragments (245–550, 265–550, and 300–550) at all three time points studied. There is a slight increase in the activity from 24 to 48 h (significant $p < 0.05$) time point and no significant increase at 72 h. The error bar is the standard error of the mean for three samples.

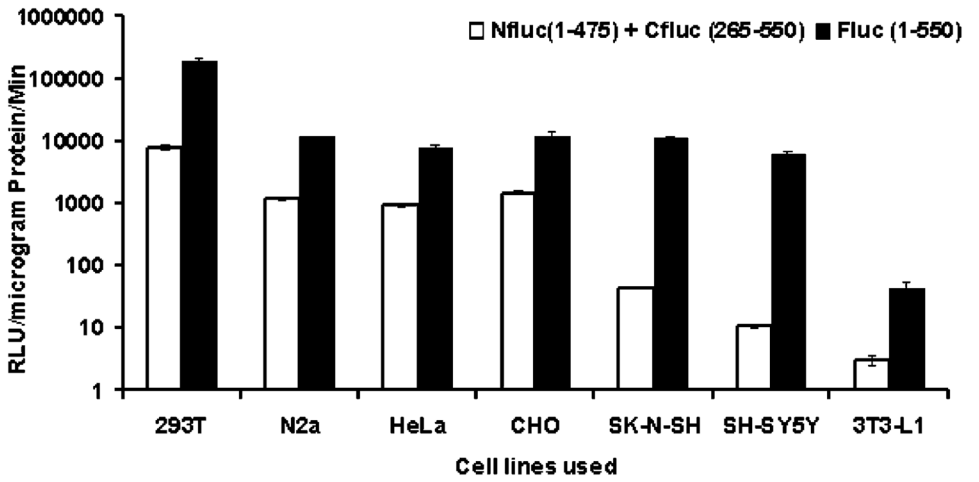

Figure 5.

Luminometer assay conducted for the combination of Nfluc (1–475) and Cfluc (265–550) as compared to fully intact fluc (1–550) in 293T, N_2a, HeLa, CHO, SK-N-SH, SH-SY-5Y, and 3T3-L1 cells. The result shows significant ($p < 0.05$) fragment complementation firefly luciferase enzyme activity in all cell lines as compared to mock-transfected cells. The error bar is the standard error of the mean for three samples.

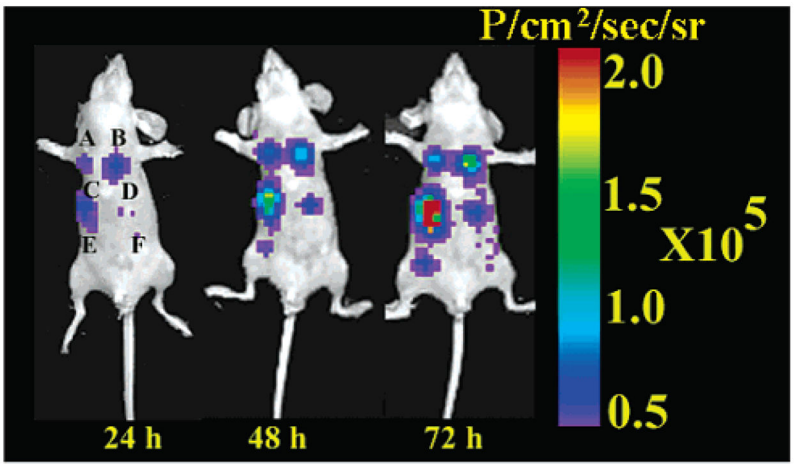

Figure 6.

In vivo imaging, using the optical CCD camera, conducted in mice with implants of 5 million cells cotransfected with Nfluc (1–475) and different Cfluc fragments in six different places: A (245–550), B (265–550), C (300–550), D (437–550), E (445–550), and F (500–550). The results show significant signal from the site implanted with the cotransfected Nfluc (1–475) with Cfluc (245–550), (265–550), and (300–550) in all time points studied. The other two combinations, Nfluc (1–475)/Cfluc (437–550) and Nfluc (1–475)/ Cfluc (445–550), showed minimum signals at the two higher time points studied. The cells cotransfected with the combinations of Nfluc (1–475) and Cfluc (500–550) showed no detectable signal at any of the time points studied.

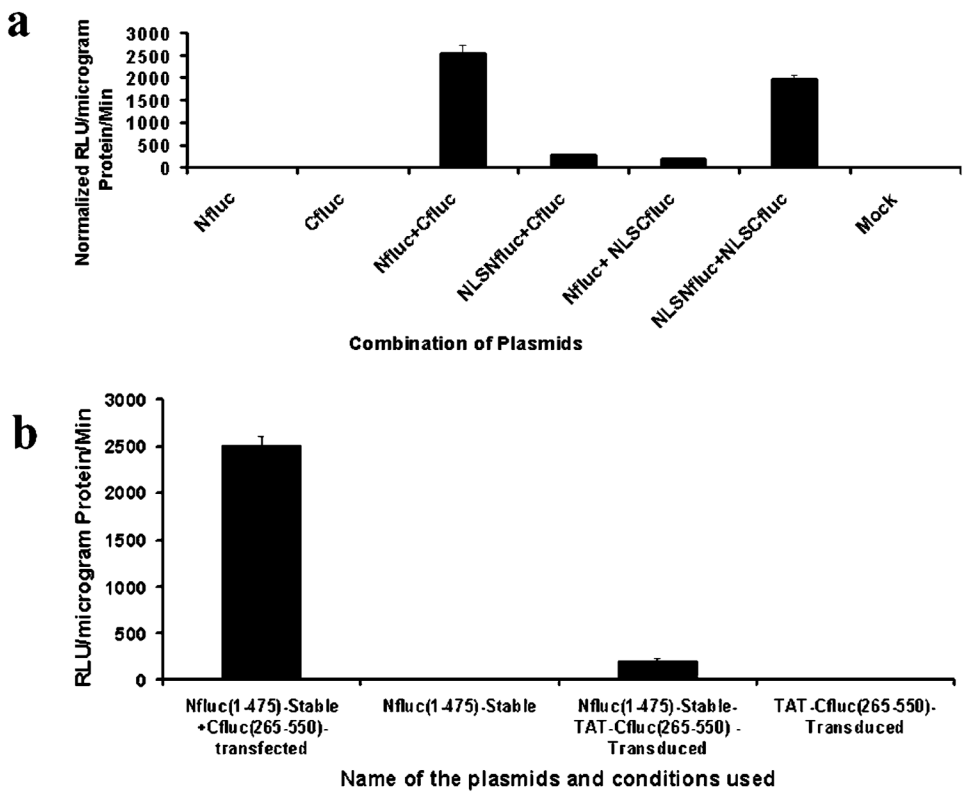

Figure 7.

(a) Luminometer assay conducted for the firefly luciferase enzyme fragment complementation by compartmentalizing the fragments in the same cellular localization. The cells cotransfected with either both the fragments without NLS or with NLS showed significant signal (N + C). The cells cotransfected with the combination fragments containing one fragment with NLS and the other without NLS showed signal that was significantly less ($p < 0.01$) than the previous one (N + NLS – C and NLS – N + C). The error bar is the standard error of the mean for three samples. (b) Fragment complementation mediated luciferase activity of 293T stable cells expressing Nfluc (1–475) with the *transfection* of Cfluc (265–550) and with the *transduction* of TAT–Cfluc (265–550). The results show significant complemented luciferase enzyme signal from the cells transfected with Cfluc (265–550) and transduced with TAT–Cfluc protein. The cells expressing only Nfluc (1–475) or Cfluc (265–550) show no significant luciferase enzyme signal. The error bar is the standard error of the mean for three samples.

Table 1

Nucleotide Sequence and the Positions of PCR Primers with Linkers Used for Constructing Different Fragments of Firefly Luciferase Enzyme Generated for the Study[a]

primer name	primer sequence (5′ →3′)	position
N-forward primer	CTAGCTAGCATGGAAGACGCCAAAAACATAAA G	1–24
	Nfluc Fragments	
415-reverse primer	CCG**CTCGAGTTA**ATCCTTGTCAATCAAGGCGT TGGT	1245–1222
420-reverse primer	CCG**CTCGAGTTA**AGAATGTAGCCATCCATCCT TGTC	1260–1237
437-reverse primer	CCG**CTCGAGTTA**GCGGTCAACGATGAAGAAG TGTTC	1311–1288
445-reverse primer	CCG**CTCGAGTTA**TTTGTACTTAATCAGAGACTT CAG	1335–1312
455-reverse primer	CCG**CTCGAGTTA**TTCCAATTCAGCGGGAGCCA CCTG	1365–1342
475-reverse primer	CCG**CTCGAGTTA**ATCGTCGGGAAGACCTGCG ACACC	1425–1402
500-reverse primer	CCG**CTCGAGTTA**ATCCACGATCTCTTTTTCCGT CAT	1500–1477
	Non-Overlapping Cfluc Fragments	
415-forward primer	CTA**GCTAGCATG**GGATGGCTGCATTCTGGAGA CATA	1246–1269
420-forward primer	CTA**GCTAGCATG**GGAGACATAGCTTACTGGGA CGAA	1261–1284
437-forward primer	CTA**GCTAGCATG**GTGAAGTCTCTGATTAAGTAC AAAG	1312–1336
445-forward primer	CTA**GCTAGCATG**GGCTATCAGGTGGCTCCCGC TGAA	1336–1359
455-forward primer	CTA**GCTAGCATG**GCCATCTTGCTCCAACACCC CAACA	1366–1390
475-forward primer	CTA**GCTAGCATG**GACGCCGGTGAACTTCCCGC CGCC	1426–1449
500-forward primer	CTA**GCTAGCATG**GACGTCGCCAGTCAAGTAAC AACCG	1501–1525
	Overlapping Cfluc Fragments	
245-forward primer	CTA**GCTAGCATG**GGTTTTGGAATGTTTACTACA CTCG	736–760
265-forward primer	CTA**GCTAGCATG**GATAGATTTGAAGAAGAGCT GTTTC	796–820
300-forward primer	CTA**GCTAGCATG**GTTGACAAATACGATTTATCT AATT	901–925
C-reverse primer	CCG**CTCGAGTTA**CACGGCGATCTTTCCGCCCT TCTT	1653–1627
NLS-Nfluc-forward primer	CCGGCTAGCATG-NLS-GAAGACGCCAAAAACA TAAAG	1–24
NLS-Cfluc-forward primer	CCG**CTCGAGTTA-**NLS-ATCCTTGTCAATCAAG GCGTT	217–236

[a]Note: Bolded bases indicate restriction enzyme site with either start codon or stop codon.