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Bioluminescent Proteins: High Sensitive Optical Reporters for Imaging Protein-Protein Interactions and Protein Foldings in Living Animals

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1. Introduction

1.1 Bioluminescence

Bioluminescence is the production and emission of light by a living organism. Bioluminescence imaging was developed over the last decade as a tool for studying biological processes in living small laboratory animals by molecular imaging. The bioluminescence-based optical imaging is highly sensitive, low-cost, and non-invasive, enabling the real-time analysis of disease processes within the cell at a molecular level in living animals. Recent advances in protein complementation strategies have further expanded its applications by quantitatively monitoring several sub-cellular processes such as protein-protein interactions, protein dimerizations, and protein foldings. In this chapter, we provide a brief introduction to bioluminescence imaging technology and discuss its applications in studying protein-protein interactions, protein dimerizations, and protein foldings, which are some of the most important cellular processes that occur in the heart signal transduction network within the cells, by non-invasively imaging living animals.

Molecular imaging offers many unique opportunities to study biological processes in intact organisms. Bioluminescence imaging (BLI) is one of several molecular imaging strategies currently in use for studying different biological processes. It is based on the sensitive detection of visible light produced during luciferase enzyme mediated oxidation of substrate luciferin in the presence of several co-factors. The luciferase enzyme can be expressed in cells as an indicator of cellular process, and can be used to image living animals by developing tumor xenografts, or developing transgenic animals either to selectively express in a particular type of tissue using a tissue specific promoter, or in the entire animal by a constitutive promoter, to study different cellular diseases. The expressed luciferase enzyme can be imaged with an optical cooled charge coupled device (CCD) camera by injecting the substrate luciferin. Several bioluminescence reporters with a wide range of emission wavelengths are currently identified from insects and crustacean copepods (**Table 1**). Some of the proteins were even modified by changing from a few to several amino acids by *in vitro* manipulations, and achieved considerably altered proteins with

change in their emission wavelengths, which improved their detection sensitivity especially for *in vivo* imaging applications.

Bioluminescence light from firefly luciferase which emits at the ~575 nm wavelength (with several red shifted mutants) can be imaged at a depth of several centimeters within the tissues, which allows at least organ-level resolution. This technology has been applied in several studies to monitor transgene expression, progression of infection, tumor growth and metastasis, tissue acceptance/rejection in transplantation, toxicology, viral infections, and gene therapy. BLI is simple to execute, and enables monitoring throughout the course of disease, allowing localization and serial quantification of biological processes without sacrificing the experimental animal. This powerful technique can reduce the number of animals required for experimentation because multiple measurements can be made in the same animal over time, which has the added benefit of minimizing the effects of biological variation in handling different groups as control. The strengths of bioluminescence reporters are not just limited to their applications in monitoring disease progress at the cellular level. The recent development of split-reporter technology has further extended their application to monitoring sub-cellular events such as protein-protein interactions and protein-foldings that are the main focus of this chapter.

Bioluminescent Reporters	Physical Property	Source	Emission Wavelength	Substrate
Firefly Luciferase	Non-secretary	<i>Photinus pyralis</i>	575/610nm: ATP/dATP	D-Luciferin
Beetle Luciferase	Non-secretary	<i>Pyrearinus termitilluminans</i>	Red:610nm/Green: 540nm	D-Luciferin
Renilla Luciferase	Secretary	<i>Renilla reniformis</i>	482nm	Coelenterazine
Gaussia Luciferase	Non-secretary	<i>Gaussia princeps</i>	480nm	Coelenterazine
Metridia Luciferase	Secretary	<i>Metridia longa</i>	480nm	Coelenterazine
Vargula Luciferase	Secretary	<i>Vargula hilgendorffii</i>	478nm	Vargula-Luciferin
Bacterial Luciferase	Non-secretary	<i>Vibrio fischeri</i>	482nm	Fatty acids

Table 1. Bioluminescent reporters currently in use for different biological applications, and their sources and properties

1.2 Protein-protein interactions

Cells are the fundamental working units of every living system. Cells determine how a living organism functions. The complex cellular functions rely on several fundamental principles. Each cell has a nucleus that contains chemical DNA (deoxyribonucleic acid) as its genetic material, which carries all the instructions needed to direct their activities in the form of functional units called proteins. Therefore, cellular functioning ultimately depends on the performances of different proteins. Some proteins act as building blocks, such as muscle proteins, while others such as enzymes control the chemical reactions within the

cells. Protein-protein interactions are important determining factors in the regulation of many cellular processes. Signaling pathways regulating cellular proliferation, differentiation, and apoptosis are commonly mediated by protein-protein interactions as well as reversible chemical modifications of proteins (e.g., phosphorylation, acetylation, methylation, and sumoylation), which normally control sub-cellular trafficking and function of proteins. To understand these modifications in proteins, and protein modification-assisted or independent protein-protein interactions, several techniques have been developed and studied in intact cells and in cell extracts. The yeast two-hybrid system is one of the earliest techniques, which used enzyme beta-galactosidase as a reporter protein at the beginning, and later was improved by adopting bioluminescent reporters for rapid measurement. The latter is used extensively in screening for protein-protein interactions and also for identifying small molecule drugs that alter (inhibit or enhance) protein-protein interactions, which can be used as therapeutic agents for treating several cellular diseases including cancer. The major limitation of this system is that it can only study the protein-protein interactions occurring in the nucleus; otherwise it requires the study proteins to be trafficked into the nucleus. The readout of yeast two-hybrid system is based on the amount of reporter proteins produced during protein-protein interaction associated transcriptional activation of reporter proteins (see more details in section 3.2). To circumvent this limitation, other techniques have been developed, including the split ubiquitin system, Sos recruitment system, dihydrofolate reductase complementation, β -galactosidase complementation, β -lactamase complementation, the G protein fusion system, and, most recently, split-luciferase (firefly luciferase, click-beetle luciferase, renilla luciferase, and Gaussia luciferase) and split-fluorescent (GFP and RFP) complementation systems. Of these, the split-luciferase complementation system provides significant advantage over other systems, particularly in measuring protein-protein interactions in cell lysates, intact cells, and cell implants in living animals by molecular imaging. The firefly luciferase complementation imaging is robust and a broadly applicable bioluminescence approach with applications in both modification-independent (phosphorylation, acetylation, methylation, and sumoylation) and dependent protein-protein interactions.

1.3 Post genomic proteomic era

We are in a post-genomic proteomic era. The completion of the human genome project has given us knowledge of the complete nucleotide sequences of human genome, their arrangements in different chromosomes, and the number of functional genes that are present in a human cell. The information collected from the human genome project along with other bio-informatic tools have led to several major new directions in science, including the characterization of RNAs (via transcriptional profiling), microRNAs, and proteins (proteomes). The human genome project estimated the number of functional genes in a human cell to range from 30,000 to 40,000. The concept of one protein, one function can accommodate only a limited number of functions, and does not explain the vastly more proteins needed by cells than those produced from the limited number of functional genes. The management of additional cellular functions, including various house-keeping functions and other specialized functions, mainly depends on the functional organ or tissue types to which these cells are part of. It is logical and even necessary to postulate that multifunctional proteins within the cell, and/or various collaborative interactions between proteins, are needed as molecular machines to carry out the work within a cell. To illustrate, the proteomes are much more dynamic and complex than the genome; it changes during

development in response to external stimuli, and form large interaction networks through which they support and regulate each other. The genetic blueprint and the genome of human cells are well known. However, the functions that genome encodes and program through which the proteins are produced by the genetic blueprint are not well understood. New research is only beginning to uncover the incredibly rich diversity of protein structure, which is much more complex than that of DNA. One new direction has sought to isolate and structurally characterize all the proteins that exist in the cell (Skolnick et al., 2000; Tucker et al., 2001). Unlike DNA, proteins have a vast repertoire of structures to carry out the diversity of functions. Once the proteins are identified and characterized, a second major challenge to find out how they assemble into the molecular machines that perform the cellular functions. Identifying all of the protein-protein interactions is fundamental for understanding the cellular processes involved in virtually all biological interactions. The collection of protein-protein interactions can be visualized as a map, in which proteins are the nodes and the circuits are the interactions. A protein-protein interaction network or map would then represent a search grid on which biological circuits are constructed (Tucker et al., 2001; Wills 2001).

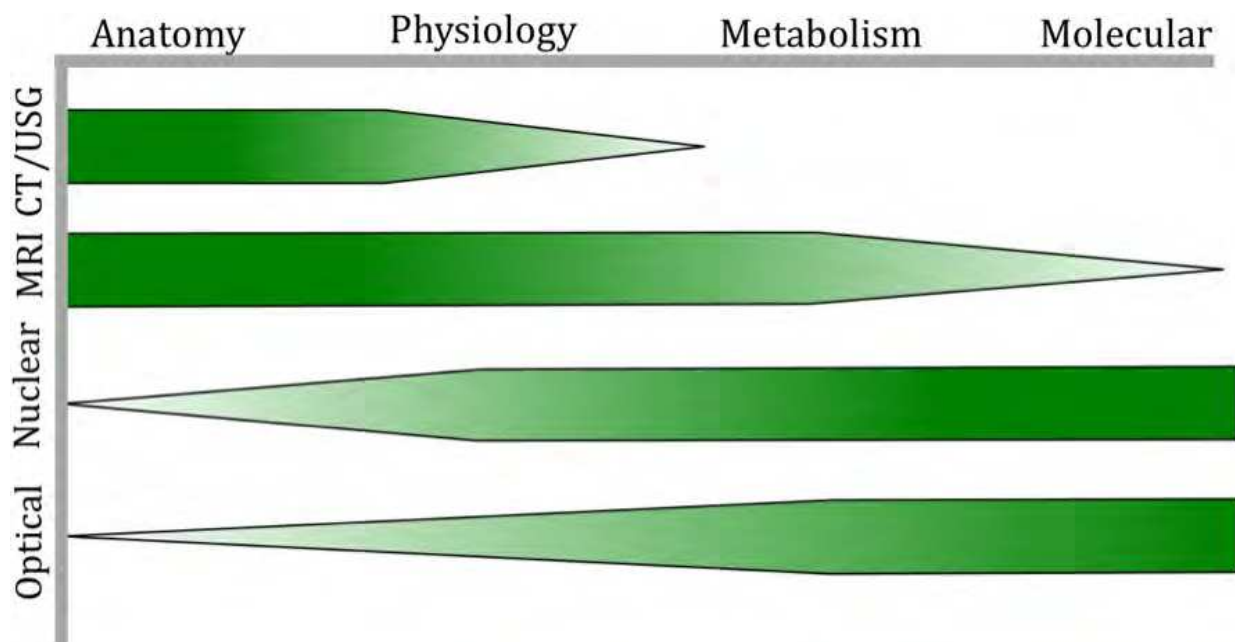


Fig. 1. Schematic illustration of current molecular imaging strategies, and their potential for providing biological informations such as anatomical details, physiological data, and metabolic status at the molecular level, for clinical applications in human. None of the current strategies is uniquely superior in independently providing different informations needed for making clinical decisions in diagnosis, staging and treatments especially in oncology, diagnosis and treatment in several other diseases; each has its strengths and weaknesses.

1.4 Complexity of protein interaction networks

There are thousands of different proteins active in a cell at any time. Many of these proteins are working as enzymes that catalyze the chemical reactions of metabolism, while others work as components of cellular machineries, such as ribosomes that read genetic information and synthesize proteins. Still proteins are involved in the regulation of gene

expression. Many proteins play their functional roles only in specific cellular compartments, whereas others move from one compartment to another, acting as "signals". By directly interacting with one another, proteins continually influence other functions (Wills 2001). In addition, proteins are constantly produced and degraded in cells. The rates at which these processes occur depend on how much of each protein is already present, how they interact with each other, and with other macromolecules such as DNA and RNA, and regulate the cellular mechanisms. One protein can speed up or slow down the rate of production of another by interacting with DNA or RNA, which is needed for making that particular protein. The interactions between different proteins that control different cellular functions are therefore interdependent. When a mutation causes the loss of one of these essential protein functions, then this can significantly affect the function of many other proteins, even leading to cell death (Tucker et al., 2001). Clearly the interactions between different proteins in a cell are much more complex than previously thought, and it is vital to understand their fundamental interlinked networks. Protein-protein interactions are important determining factors in the control of many cellular processes such as transcription, translation, cell division, signal transduction, and oncogenic transformation. To modulate many of these cellular events, it is essential to delineate which proteins are involved and how they interact with one another, their precise roles in executing cellular functions, and techniques and mechanisms needed to manipulate these interactions for novel drug development or treatment strategies relevant to particular diseases. Biochemical pathways and networks require many different systems of dynamic assembly and disassembly of proteins with other proteins and nucleic acids (Michnick 2001). Much of modern biological research is concerned with how, when, and where proteins interact with other proteins involved in biological processes in the intact cellular context. The completion of the human genome project has added a major impetus in research that can provide simple approaches to study protein-protein interactions on a large scale in diseases, including cancer.

1.5 Cellular signaling pathways

The cellular regulatory mechanisms are interlinked. To understand the complex biological processes, and disease states at a molecular level, a systematic approach is necessary to illustrate signaling pathways. Efforts to elucidate the cellular mechanisms for different pathological conditions have significantly increased after the Human Genome Project. Each signaling pathway reacts to specific external stimuli that can be regulated by changes in proteins and chemicals. Recent advances in large-scale and high-throughput techniques, including functional genomics, proteomics, RNAi technology, and genomic-scale yeast two-hybrid and protein complementation assays, have provided a tremendous amount of information on signaling pathways. To extract the biological significance from the vast data, it is necessary to develop an integrated environment for a formal and structured organization of the available information, in a format suitable for analysis with bioinformatics tools. To present a signaling pathway, a database must include information on 1) the molecules involved in signaling in response to each external stimulus, 2) which direction the signal is being conveyed, and 3) how the activities and sub-cellular localizations of molecules are changed by protein modifications and/or protein-protein interactions. Analyses of the first database containing such information should made it possible to further expand the database to understand the signaling results in processes such as proliferation, differentiation, and apoptosis, and to explicate how a network can be

composed of various signaling pathways in response to multiple external inputs. Signaling entities ranging from small molecules and proteins-to-protein states and protein complexes should be studied. It must be noted that these entities are not independent of one another. For instance, protein complexes are composed of proteins, and a protein binding to a small molecule can define a protein state. It is not surprising to find many gaps in the current knowledge about any particular signaling pathway. In order to organize such diverse yet incomplete information into a structured and coherent database, the use of a formal model is indispensable. Differing levels of abstraction are inter-related so that essentially the same signaling event can be described in detail at multiple levels. As model systems that implement all the parameters become available, the sharing of models with integrated biological data will be essential to fill in the gaps in our current knowledge base.

1.6 Complexity in studying protein interaction networks

There are no methods currently available to test protein-protein interaction networks, which occur within a cell without introducing a constructed system that mimics the function of its endogenous protein. It is to be expected that when a new protein of endogenous origin is introduced in a cell in addition to the level of its counterpart expressed inside a cell, it will have some direct physical effect on a number of other proteins. These new interactions may cause some changes in the functional aspects of several other proteins. Such effects can be felt right across the protein interaction network, most often becoming less significant as the distance of the new protein from the other protein increases. It is also possible for genetically modified cells to produce a new protein that will display completely new patterns of protein interactions. This may not be evident until the cells find themselves in some unusual circumstances. They may then respond in a very different way from wild-type cells. Although the genetically engineered cells may appear to behave just like wild-type cells, this cannot be guaranteed under all circumstances (Becker et al., 1990; Beeckmans 1999; Bode and Willmitzer 1975). However the techniques currently available for inserting new DNA into the chromosomes of cells do not have any specific control mechanisms, capable of directing the point of insertion in the organism's existing genome without producing significant impact on the expression level of any of the endogenous proteins. Of the gene delivery systems currently available, the adeno-associated virus is the only viral mediated vector which can normally introduce and integrate a single copy of the transgene specifically into human chromosome loci at 19 (19q13.3-qter). Otherwise, it is customary to produce millions of cells with the new DNA inserted at essentially random positions in the hope of producing at least some "hits." Screening is then conducted to find those cells, which must survive the engineering process and also express the newly inserted gene. These survivors are then subjected to further screenings to find those that seem to behave most like the wild-type, and yet possessing the new, desired, engineered properties. It is generally assumed that any harm to an organism as a result of inserting a new gene will be observed as a change in gross characteristics of the organism (Stopeck et al., 1998).

1.7 Biological importance in studying protein folding

As discussed in the previous sections, proteins are cellular macromolecules with complex structural and functional properties. Dysfunctional protein folding represents the

molecular foundation of a growing list of diseases in humans and animals. Proteins undergo several levels of structural alterations executed by active chaperon complexes (e.g., Hsp90, Hsp70) and indirectly by the inherent amino acid sequences, before they become a biologically active functional entity of a cell. There is significant supporting evidence that associates the misfolding of proteins with several cellular diseases, including cancers (**Table 2**). Biologically representative *in vitro* and *in vivo* studies of these abnormal events are best suited to the discovery of molecular mechanisms to prevent or ameliorate such diseases. There is an active search for small molecules which assist refolding of misfolded proteins into their biological functional forms, as equal or at near equal levels of native forms, for the treatment of several biochemical disorders. However, thus far no current technique can be optimally extended to imaging assays in intact living subjects. The development of novel imaging techniques to quantitatively measure the level of protein misfolding in cells and in living animals, and also of small molecule mediated refolding, will be very useful for screening and pre-clinical evaluation of drugs which rectify or cure these diseases. Normally, the conformational changes in protein folding result in the close approximation of amino and carboxy termini in a great majority of native proteins, at their functionally active forms. The 'protein folding problem' has remained one of the more perplexing quandaries in fundamental biological research ever since the classic work of Anfinsen some four decades ago on the hydrophobic-collapse mechanism. How to predict the three-dimensional, biologically active, native structure of a protein from its primary sequence, and how a protein reaches this native structure from its denatured state are still unresolved questions. The intellectual conundrum of the folding pathway of proteins, underscored by the Levinthal paradox, has been addressed to some extent over the last twenty years by various proposed mechanisms for protein folding, including the framework model (diffusion-collision and nucleation mechanisms) (Anfinsen 1973; Levinthal 1969).

There is accumulating evidence that the conditions used for refolding proteins *in vitro* are only distantly related to those found *in vivo*, where the physiological environment in living cells exerts a profound influence on protein folding owing to the involvement of the intracellular macromolecular background, which also contains folding catalysts and molecular chaperones. Aside from the relevance of the protein-folding problem to deciphering fundamental processes in cell biology, it is becoming clear that dysfunctional protein folding represents the molecular foundation of a growing list of diseases in humans and animals. There is mounting interest in such diseases arising from protein misfolding and aggregation, including Alzheimer's disease, amyloidosis, Creutzfeldt-Jakob disease, cystic fibrosis and cancer, to name a few. Molecular chaperones are involved in the protection of cells against protein damage through their ability to hold, disaggregate, and refold damaged proteins or their ability to facilitate degradation of damaged proteins. Many of the proteins implicated in the pathogenesis of misfolding diseases escape the diverse chaperoning pathways that are in place to assist and assure the fidelity of correct protein folding. More biologically representative *in vivo* structural and functional studies of these abnormal events, carried out in the context of living cell environments, are likely best suited to the discovery of molecular mechanisms to prevent or ameliorate such diseases (**Table 2**)(Goetz et al., 2003).

Disease	Mutant Protein/Protein involved	Molecular Phenotype
<u>Inability to fold</u>		
Cystic fibrosis	CFTR	Misfolding/ altered Hsp70 and calnexin interactions
Marfan syndrome	Fibrillin	Misfolding
Amyotrophic sclerosis	Superoxide dismutase	Misfolding
Scurvy	Collagen	Misfolding
Maple syrup urine disease	α -Ketoacid dehydrogenase complex	Misassembled/Misfolding
Cancer	p53	Misfolding/ altered Hsp70 interaction
Osteogenesis imperfecta	Type I procollagen pro α	Misfolding/ altered BiP expression
<u>Toxic folds</u>		
Scraple/Creutzfeldt-jakob/ familial isomonia	Prion protein	Aggregation
Alzheimer's disease	β -Amyloid	Aggregation
Familial amyloidosis	Transthyretin/ lysozyme	Aggregation
Cataracts	Crystallins	Aggregation
<u>Mislocalization owing to misfolding</u>		
Familial hypercholesterolemia	LDL receptor	Improper trafficking
α 1-Antitrypsin Deficiency	α 1-Antitrypsin	Improper trafficking
Tay-Sachs disease	β -Hexosaminidase	Improper trafficking
Retinitis pigmentosa	Rhodopsin	Improper trafficking
Leprechinism	Insulin receptor	Improper trafficking

Table 2. Examples of some putative protein misfolding associated diseases and proteins involved in these diseases

2. Molecular imaging

2.1 Role of molecular imaging in cancer research

Molecular imaging, a new field of pharmacology that exploits the multidimensional approaches of light energy, has paved the way for easy understanding, interpretation, and manipulation of biological events at the molecular level. Molecular imaging is instrumental in the diagnostic aspects of various pathological conditions, and also in the evaluation of drugs which target specific molecular and biochemical processes in living cells and in intact living animals. This new field of research has flourished with the introduction of many novel molecular imaging probes, and genes which emit light either by reacting with a

substrate or with induction of light waves, as well as the advancement of sensitive imaging instrumentations. Imaging techniques such as positron-emission tomography (PET), single-photon emission tomography (SPECT) with the use of radioactive tracers, and magnetic resonance imaging (MRI), are now widely used in the clinical observation of cancer pathology. Recently, the use of combinatorial techniques like PET-CT and PET-MRI are rapidly replacing conventional imaging methods. Positron Emission Tomography (PET) is an imaging technique that produces three-dimensional images of the functional processes of living subjects by capturing a pair of gamma rays emitted indirectly upon the injection of a positron-emitting radionuclide into the living body with a biomolecule.

PET was introduced by David E. Kuhl and Roy Edwards of the University of Pennsylvania in late 1950s, and has been continually updated and modified to correspond to the clinical and research needs to work as an independent or a combinatorial device (Ter-Pogossian et al., 1975). It has made invaluable contributions in cancer diagnosis, treatment, and research by revealing tumor progression both in clinical and preclinical applications, especially in the diagnosis and detection of tumor metastasis. Advances in PET scanner devices and the introduction of novel radiotracers have fueled the progress of PET imaging. Fluorodeoxyglucose (^{18}F -FDG), an analogue of glucose, is the most common radiotracer used for PET imaging, because it can reveal specific tissue metabolic activity. However, ^{18}F -FDG is phosphorylated by hexokinase and the phosphate cannot be cleared in most tissues, which can result in intense radiolabeling of tissues with high glucose uptake (Burt et al., 2001). FDG-PET is widely used in clinical oncology for diagnosis, staging and follow-up after treatment of tumors. Tissue and also molecule-specific radiotracers have been introduced to monitor the expression level of structural and functional proteins (Torigian et al., 2007). Steroid receptors have been associated with the growth of breast tumors, and thus understanding the receptor status is essential for the treatment of breast cancer. Radiolabeled ligands and their analogues are in preclinical application for receptor imaging. ^{18}F -fluro-17 β -estradiol (FES) has been used in PET imaging to examine the estrogen receptor status in different tissues of living subjects (Mintun et al., 1988). Bombesin, a peptide isolated from the frog *Bombinas bombina*, binds with gastrin-releasing peptide (GRP) receptor and has been implicated in breast cancer. This property has led the development of radiolabeled bombesin for peptide receptor imaging in breast cancer diagnosis (Scopinaro et al., 2002).

Single Photon Emission Computed Tomography (SPECT) is another imaging technique that is similar to PET imaging. Unlike PET, however, the tracer used in SPECT emits gamma rays that can be measured directly. In SPECT, the 2-D view of 3-dimensional images is acquired by a gamma camera and eventually 3-D data set is generated with the use of computer based tomographic reconstruction algorithm. Magnetic Resonance Imaging (MRI) is another widely used imaging technique, but unlike PET and SPECT, MRI can be used to view the anatomical nature of living subjects by generating data about the functional status of tissues (MacDonald et al., 2010). Magnetic Resonance Imaging (MRI) is a well-established diagnostic method to detect cancer. It has been widely used to detect breast cancer as it produces the highest sensitivity in spatial resolution of all imaging modalities. Guinea et al. (2010) investigated and analyzed the possible relationship between the magnetic resonance imaging (MRI) features of breast cancer and its clinicopathological and biological factors such as estrogen and progesterone receptor status, and expression of p53, HER2, ki67,

VEGFR-1, and VEGFR-2 (Fernandez-Guinea et al., 2010). Their results did not show a significant association between the MRI parameters and any of the biological factors included in the study. By contrast, other reports have shown that high spatial dynamic MRI of morphological or kinetic analysis are associated with prognostic factors such as expression of estrogen receptor (ER), expression of progesterone receptor (PR), and expression of p53, c-erbB-2, and Ki-67 (Fernandez-Guinea et al., 2010, Szabo et al., 2003) found that rim enhancement pattern, early maximal enhancement, and washout phenomena are associated with the poor prognostic factors of histological differentiation, high Ki-67 index, and negative PR expression status (Szabo et al., 2003). It was suggested that these MR images could be useful in the prognosis of breast cancer. The MR signals may also be used to noninvasively identify highly aggressive breast carcinomas and help differentiate between benign and malignant lesions. The difference in contrast enhancement has been associated mainly with a higher vascular permeability in tumors, and the overexpression of c-erbB2 in tumor cells is closely linked to increased expression of vascular endothelial growth factor (VEGF) and Ki-67 in proliferation (Szabo et al., 2003).

Similar to nuclear and magnetic imaging modalities, optical imaging has also made a sizable contribution in medical imaging (Weissleder and Pittet 2008). Fluorescence and bioluminescence methods are used as a source of contrast in optical imaging, but a major setback in optical imaging is the lack of penetration depth, which prevents its wide clinical applications in humans. Near infrared (NIR) imaging has been identified as a useful optical imaging technique because of the lower absorption coefficient of tissue to light in near infrared region. Radiolabeled antibodies have been evaluated for breast cancer diagnosis since 1978 with tumor associated antigens such as carcinoembryonic antigen (CEA) and the polymorphic epithelial mucin antigen (MUC1) (Goldenberg et al., 1974). They were widely used in studies of immunolocalization and radiosciintigraphy. Clinically, affinity purified ¹³¹I-labeled goat anti-CEA IgG was subjected in selective breast tumor targeting (Goldenberg and Sharkey 2007). CEA-Scan with Arcitumomab, an US FDA approved anti-CEA antibody, could detect breast cancer that has been missed by mammography. In addition, antibodies against the HER2/neu receptor have also been investigated in detail either as therapeutic and/or diagnostic agent. Biotinylated anti-HER2/neu antibodies have been used to increase the contrast of MR images when they bind with avidin-gadolinium complexes (Artemov et al., 2003). The ¹¹¹In labeled Trastuzumab (Herceptin) Fab has been identified as a selective imaging agent to localize HER2/neu receptor in small BT-474 tumors (Tang et al., 2005).

2.2 Reporter gene imaging in living animals

Molecular imaging is a rapidly expanding field that attempts to visualize fundamental molecular/cellular processes in living subjects (Gambhir 2002; Massoud and Gambhir 2003; Weissleder 2002). Imaging molecular events in cells in their native environment within the living subjects probably result in the least amount of perturbation of normal signaling processes. However, this advantage of non-invasive imaging has a trade-off. For example, most current techniques do not have single cell resolution at any significant depth within the animal. Instead, bulk signals from large numbers of cells (hundreds to millions) are needed. Newer methods that allow the observation of single cells within living subjects are under active investigation, but are more invasive in nature (Jung and Schnitzer 2003; Mehta et al., 2004). To produce a signal detectable outside the animal subject, the cells located

inside the subject must produce a signal of sufficient intensity. The signal may come from a fluorescent protein excited at the correct wavelength, the interaction of a bioluminescent protein with its substrate (**Figure 2**), or from radiolabeled substrates that emit a signal in the form of gamma rays. For optical signals, red light and near infrared light have the best tissue penetration, and are therefore preferred. For radiation-based signals, the use of single photon emitters and positron emitters generating gamma rays are favored. It is not possible to use beta emitters (e.g., ^3H), due to their minimal tissue penetration. The focus of this chapter is primarily on optical technologies for imaging protein-protein interactions. Although other approaches such as microPET can be used for imaging protein-protein interactions (Luker et al., 2002a; Massoud et al., 2010), the much lower cost, higher throughput, and greater sensitivity of optical imaging in small animals favor its use for imaging protein-protein interactions. Additional discussions of other small animal imaging technologies including microPET may be found elsewhere (Cherry and Gambhir 2001; Massoud and Gambhir 2003).

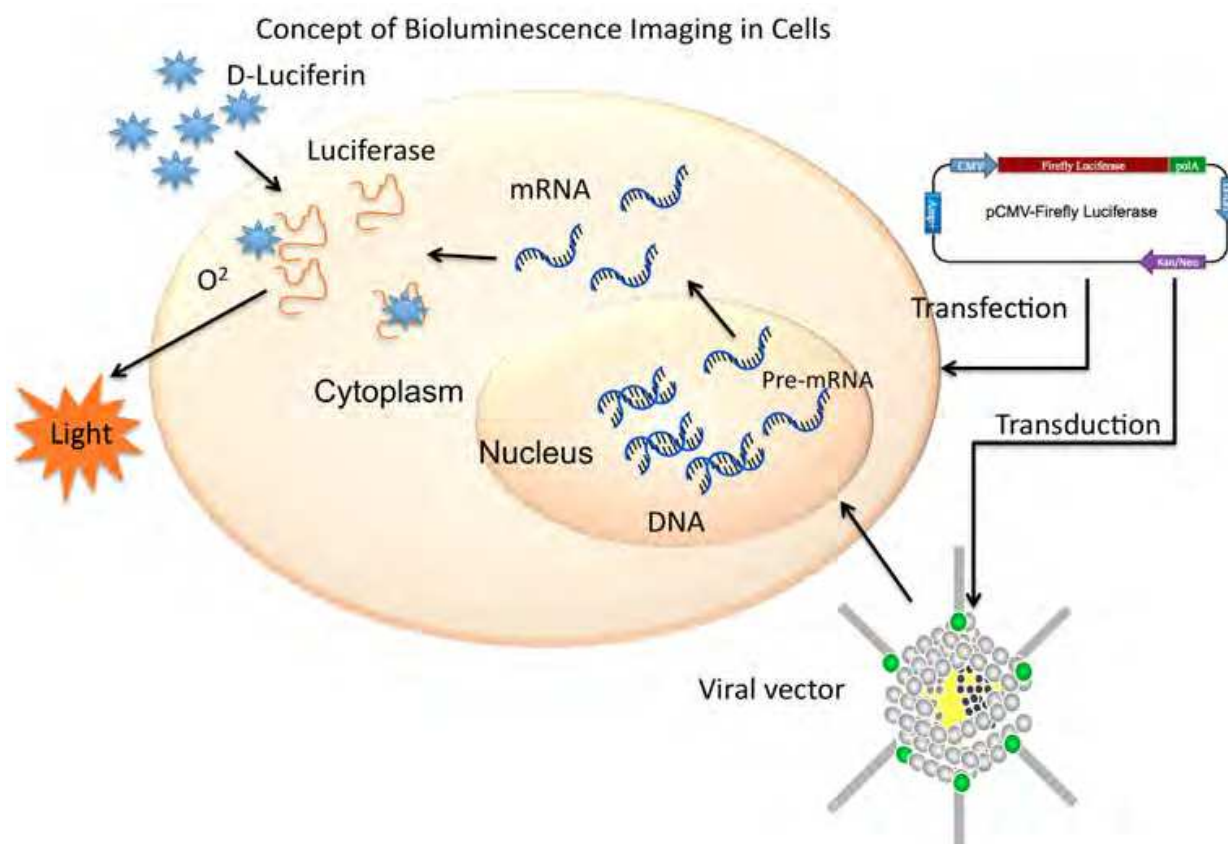


Fig. 2. Schematic illustration of the principle of optical bioluminescence imaging in cells. In this strategy mammalian cells are labeled to express bioluminescent protein under a constitutive (CMV, LTR, Ubiquitin, or CAG) or tissue-specific or an inducible promoter, either by transfecting a plasmid with chemical agent (Liposome) or transduced with a viral vector (Lentivirus, Adenovirus, Retrovirus, or Adeno-associated virus). The cells can be allowed to express luciferase protein for a particular period of time and imaged by exposure to substrate luciferin in intact cells, or can be measured by luminometer in cell lysates by adding luciferin and other co-factors.

There are two primary types of optical imaging systems for living subjects: a) fluorescence imaging, which use emitters such as green fluorescent protein (GFP), wavelength-shifted GFP mutants, red fluorescent protein (RFP), “smart” near-infrared fluorescent (NIRF) probes, and b) bioluminescence imaging, which utilizes a specific enzyme-substrate reaction such as Firefly luciferase/D-Luciferin, Renilla luciferase/coelenterazine (Bhaumik and Gambhir 2002a; Contag and Ross 2002; Tung et al., 1999) and several other bioluminescent proteins with the respective substrates (Substrates and properties are shown in **Table 1**). Emission of light from fluorescent markers requires external light excitation, while bioluminescence systems generate light *de novo* after an injectable substrate is introduced. In both cases, emitted light can be detected with a thermoelectrically cooled charge-couple device camera (CCD), which can detect light in the visible light range (400 nm to 750 nm) to near-infrared range (~800 nm) (**Figure 3**). Cooled to -120 to -150°C, these cameras are exquisitely sensitive to even weak luminescent sources within a light-tight “black-box”

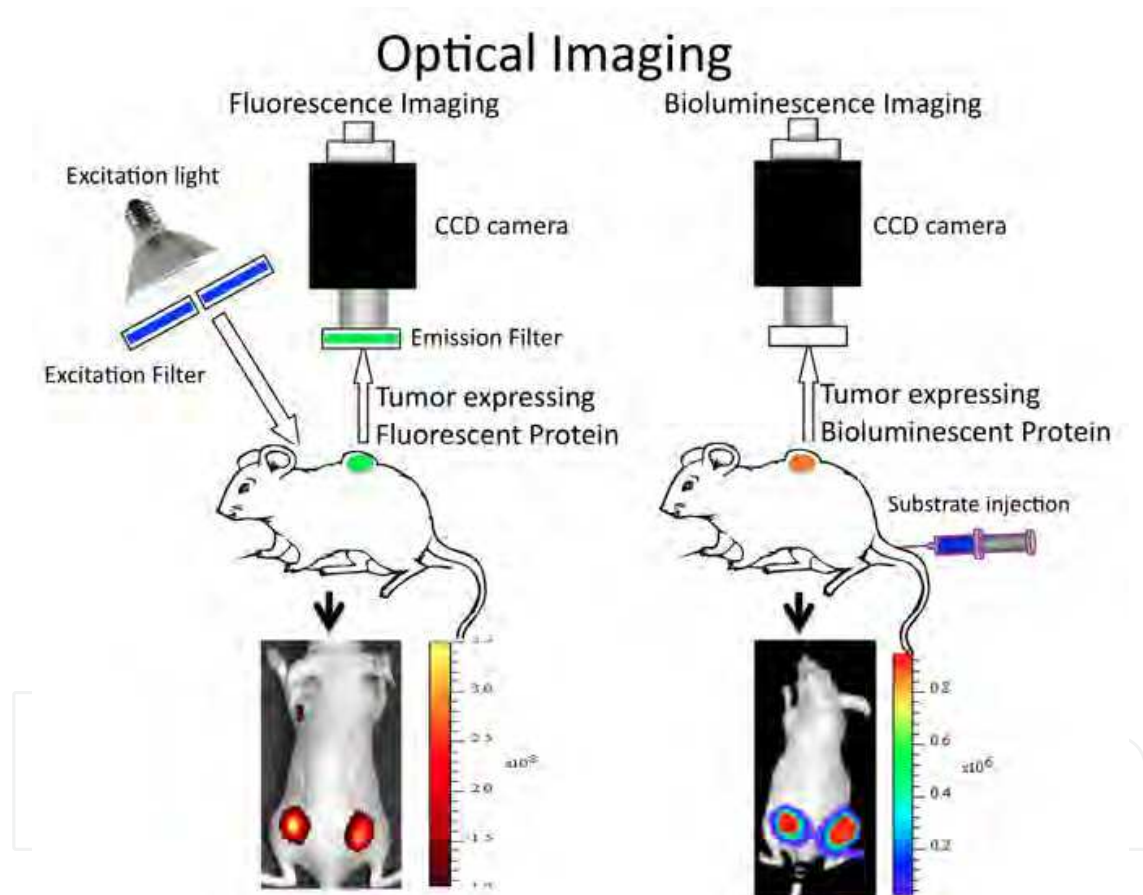


Fig. 3. Scheme of optical imaging (bioluminescence and fluorescence) in living animals. In this strategy, mammalian cells stably expressing bioluminescent or fluorescent proteins are implanted in animals (orthotopic or xenograft) and allowed to grow the tumors. The animals were imaged by exciting with respective excitation wavelength of the protein used for labeling in fluorescence imaging. The emitted light was captured by an optical cooled charge coupled device camera, and quantitated by compatible software provided with the system. Similarly, in bioluminescence imaging, the animals were injected with the respective substrate of the bioluminescent reporter used for labeling the cells, and light emitted from the tumor cells is collected without passing through filters.

chamber, allowing for quantitative analysis of the data. The method of imaging bioluminescence sources in living subjects with a CCD camera is relatively straightforward: the animal is anesthetized, injected intravenously or intraperitoneally with the substrate, and placed in the light-tight chamber for a few seconds to minutes. A standard light photographic image of the animal is obtained, prior to a bioluminescence image captured by the cooled CCD camera positioned above the subject within the confines of the dark chamber. A computer subsequently superimposes the two images on one another, and relative location of luciferase activity is inferred from the composite image. An adjacent pseudocolor scale indicates relative or absolute number of photons detected. This scale does not reflect the color (wavelength) of the emitted photons, but only the number of such photons, measured in relative light units per minute (RLU/min). At present, the primary disadvantage of fluorescence as compared to bioluminescence is the background level of auto-fluorescence of tissues in the former approach. However, methods are being developed to correct for auto-fluorescence and may allow greater use of fluorescence in the study of protein-protein interactions in living subjects. To date, the initial validation of *in vivo* imaging has been based primarily on bioluminescence. If auto-fluorescence issues can be minimized, then it may be possible to image multiple interactions using different fluorescent reporters. Comparison of optical-based imaging systems with the other imaging modalities, such as the radionuclide-based or MRI-based systems, reveals important differences. One major advantages of optical-based reporter systems is that they are at least an order of magnitude more sensitive than the radionuclide-based techniques at limited depths (Ray et al., 2003). Furthermore, the direct and indirect costs of optical systems are generally less than radionuclide-based techniques or MRI. However, significantly less spatial information is obtained from optical imaging, and the signal obtained from light-emitting reporter systems is limited by the tissue depth from which it arises. Furthermore, while significant progress has been made to localize fluorescent signals tomographically to obtain distribution of fluorochromes in deep tissues (Ntziachristos et al., 2002), there are currently only prototype instruments to obtain three-dimensional localization of the targeted optical probes.

2.3 Optical reporter genes to image cellular process in small animal models

Luciferases are enzymes that emit light when they react with a specific substrate. A diverse group of organisms make use of luciferase-mediated bioluminescence. Luciferases that catalyze the light emitting reactions of fireflies, coelenterates, or bacteria show no nucleotide homology to each other. The substrates (i.e., luciferins) of these reactions are also chemically unrelated (Wilson and Hastings 1998). All these bioluminescent reactions combine molecular oxygen with luciferin, to form a luciferase-bound peroxy-luciferin intermediate. This, in turn, releases photons of visible light (Wilson and Hastings 1998) over an emission spectrum range between 400 and 620 nm. Experimentally, the emitted light is used as a "reporter" for the activity of any regulatory elements that control expression of luciferase. Firefly luciferase (FLUC), cloned in 1985 from the firefly *Photinus pyralis*, is now emerging as the gene of choice for *in vivo* and *in vitro* reporting of transcriptional activity in eukaryotic cells (de Wet et al., 1985). FLUC emits light from green to yellow in the presence of D-Luciferin, ATP, magnesium, and oxygen. The short half-life and fast rate of turnover of FLUC ($T_{1/2} \sim 3$ h) in the presence of D-Luciferin allows for real-time measurements, because the enzyme does not accumulate intracellularly to the extent of other reporters; thus, the

relationship between the enzyme concentration and the intensity of emitted light *in vitro* is linear up to 7-8 orders of magnitude. These properties potentially allow for sensitive non-invasive imaging of FLUC reporter gene expression in living subjects (Massoud et al., 2004; Wu et al., 2002). In recent years, considerable work with non-invasive imaging of firefly luciferase has been carried out (Bhaumik and Gambhir 2002b; Contag et al., 2000; Contag et al., 1997; Contag et al., 1998; Jawhara and Mordon 2004; Paulmurugan et al., 2002b; Ray et al., 2002a; Wu et al., 2002; Wu et al., 2001).

The use of a second bioluminescent reporter [(Renilla Luciferase (RLUC)] with different substrate utility than firefly luciferase, has allowed for monitoring of more than one process at a time in mammalian cells. Renilla luciferase (RLUC), originally cloned and sequenced from the sea pansy, *Renilla reniformis*, by Lorenz et al. (Lorenz et al., 1991), has been used as a marker of gene expression in bacteria, yeast, plant and mammalian cells (Lorenz et al., 1996). RLUC is widely distributed among coelenterates, fishes, squids and shrimps (Hastings 1996). The enzyme RLUC catalyzes oxidation of its substrate, coelenterazine, leading to bioluminescence. Coelenterazine has an imidazolopyrazine structure [2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-8-benzylimidazo [1, 2-a] pyrazin-3- (7H)-on], and upon oxidation, releases blue light across a broad range, peaking at 480 nm (Wilson and Hastings 1998). However, the native RLUC protein has some inherent limitations when used in mammalian cells. Ten percent of its codons are associated with poor translation in mammalian cells, limiting expression efficiency. Also, the presence of a large number of potential transcription factor binding sites within RLUC sequences can cause anomalous transcriptional behavior in mammalian cells. For many *in vivo* imaging applications, researchers have utilized a synthetic *Renilla* luciferase reporter gene (hRLUC) that has been codon optimized for efficient translation in mammalian cells. In addition, deletion of poly (A) signals (AATAAA) and incorporation of a Kozak sequence at the beginning of the gene contributed to a better expression. The resulting reporter gene has a higher transcriptional efficiency, which enhances the detection of the reporter enzyme in cell culture and living animals (Bhaumik et al., 2004).

After a long lag time, recently several other luciferases with different properties have been isolated. Gaussia luciferase (GLUC) with its secretory property has been recently used for secretory biomarker identification and validation in human cancers. As GLUC uses the same substrate (coelenterazine) as RLUC and emits light in a similar wavelength (480nm), but differs in its secretory property, its use has been limited in several applications. Similarly, Metridia luciferase, with similar substrate utilization, emission wavelength and secretory property as GLUC, has been identified, but it has not added much to the field of bioluminescence imaging in living animals. Recent isolation of another luciferase (Vargula luciferase) with similar physical properties (secretory, emits light at 480nm) as GLUC, but uses a different substrate (Vargula luciferin), has the advantage of adding another bioluminescent protein for multiplexing different bioluminescent reporters to enable simultaneous monitoring of several biological processes in cells and in living animals.

3. Bioluminescent assays to study protein-protein interactions

As we discussed briefly in section 1.6 the different assays currently available for studying protein-protein interactions, in this section we further explain how these assay systems

work, and discuss the advantages most of these assays have over other complementary systems, and their contributions to the field of molecular imaging in disease monitoring and drug development processes.

3.1 Yeast two-hybrid system to study protein-protein interactions

Protein-protein interactions play vital part in almost all biological processes. Large networks of interacting proteins control many important regulatory pathways. A full understanding of any pathway or cellular processes will require a map of the binary interactions among the proteins involved. One of the most widely used methods to detect biologically important protein-protein interactions is the yeast two-hybrid system (Fields and Song 1989). In a two-hybrid assay, the two proteins are expressed in yeast one fused to a DNA-binding domain (BD) and the other fused to a transcription activation domain (AD). If the two proteins interact, they activate transcription of one or more reporter genes that contain binding sites of the BD. Investigated first in yeast, this classical two-hybrid system was later adopted for mammalian cells with certain modifications (Luo et al., 1997). When expressed simultaneously in the same cell, the interactions between the two mammalian proteins bring the activation domain (VP16) and binding domain (Gal4) together that in turn bind to the Gal4 domain binding sequences followed by a reporter gene. High levels of reporter gene expression will indirectly indicate the physical interactions between the proteins of interest. Most of the approaches used for high-throughput two-hybrid studies have been limited to *in vitro* assays and cultured cells that do not represent the actual scenario in intact animal, as well as being unable to predict the kinetics of the interaction. Thus, the development of methods to non-invasively and repetitively image protein-protein interactions using the mammalian two-hybrid system in living animal would significantly increase our knowledge on the intricacy and complexity of different regulatory pathways.

To overcome these problems, we and others have developed a modified mammalian two-hybrid system to detect protein-protein interactions in living mice using bioluminescence and positron emission tomography (PET) imaging techniques (Luker et al., 2002b; Luker et al., 2003; Ray et al., 2002b). To demonstrate the use of this system for imaging in living animals, the two known interacting proteins, ID and MyoD, were used. These two proteins strongly interact *in vivo* during muscle generation (Ray et al., 2002b). To modulate the expression of these two fusion proteins (ID-GAL4 and MyoD-VP16), we used the NF- κ B promoter to drive expression of the Id-gal4 and/or myoD-vp16 fusion genes, and utilize TNF- α to induce the NF- κ B promoter that controls the expression level of fusion proteins in response to TNF- α dose. The reporter construct comprised of five GAL4 binding sequences followed by the firefly luciferase reporter gene. In cell culture, co-transfection with the effector and reporter plasmids with variable levels of expression regulated by TNF- α show induction in FLUC activity. The FLUC activity directly correlate with the interaction between the proteins used, which are Id and myoD in this case. By replacing these two proteins, it is possible to look for other proteins that are currently not known for their interactions (**Figure 4**).

A similar system was developed for a tetracycline inducible bi-directional vector carrying two other proteins (the tumor suppressor p53 gene and SV40-T antigen) by other groups (Luker et al., 2002b; Luker et al., 2003). Interactions of p53 and SV40-Tag proteins after

doxycycline induced expression resulted in formation of the VP16-Gal4 trans-activator complex that binds to the Gal4 binding sequences driving expression of a HSV1-sr39TK-GFP reporter fusion protein. Expression of GFP (fluorescence imaging) is detected at the level of single cell, and expression of HSV1-sr39TK is imaged in living mice with microPET. Clones of HeLa cells stably expressing both the reporter plasmid and the bi-directional effector plasmid are isolated, and levels of interacting proteins are measured with increasing doses of doxycycline and further confirmed by western blots, thymidine kinase radiotracer assays, and fluorescence microscopy. Promising cell culture studies have led to further investigations into the protein-protein interactions in living mice using different modalities in cells and *in vivo* in living animals.

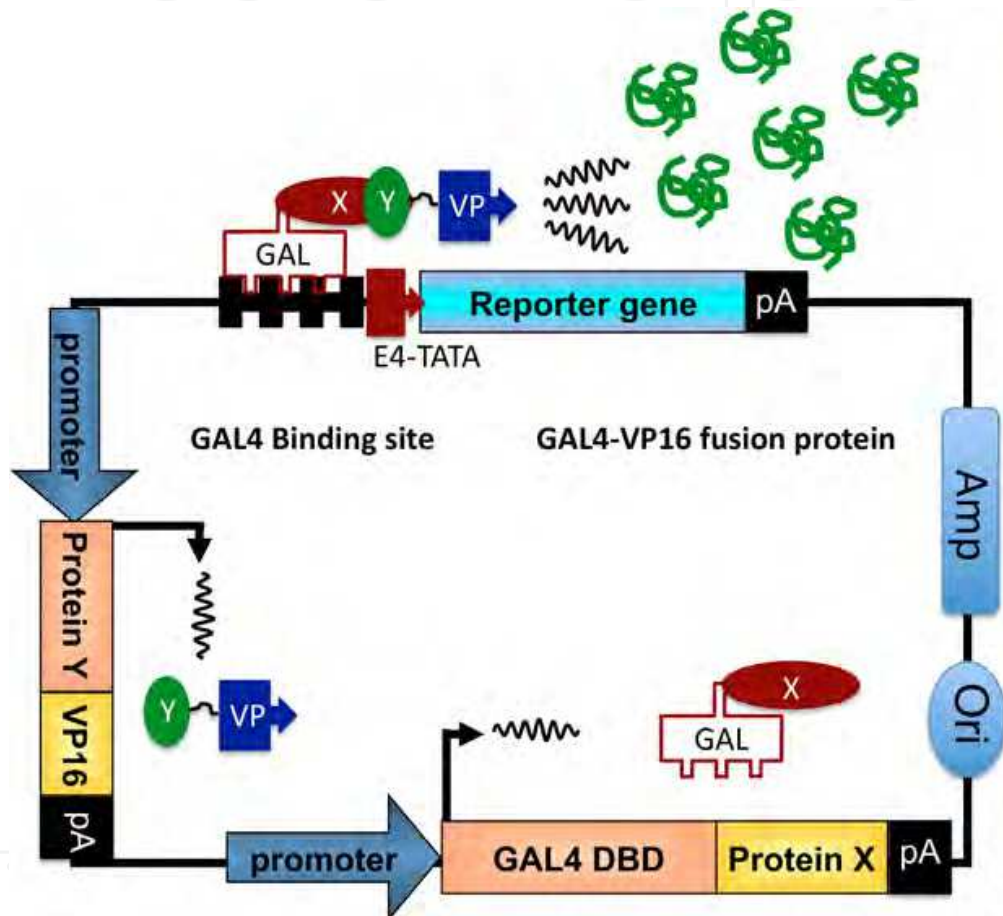


Fig. 4. Scheme of a single vector mammalian two hybrid system to study protein-protein interactions. The vector is designed to have several components needed for measuring protein-protein interaction. In this strategy, the vector expresses two fusion proteins under two separate promoters. They can be either constitutive or inducible. One fusion protein contain Gal4-DNA binding domain (Gal4-DBD) expresses with one of the study protein (Gal4-DBD-Protein-X), and the other one expresses a small transactivating peptide derived from herpes simplex virus thymidine kinase (HSV1) with another protein (Protein-Y-VP16). The same vector has a specific DNA sequence repeated five times on which the Gal4-DBD can bind, and a minimal promoter (TATA box), followed by a reporter gene (Luciferase). This minimal promoter will not express any reporter protein until the Gal4-DBD binds to it and brings the VP16 domain fused to another fusion protein by protein-protein interactions. The amount of luciferase expression directly relate to the interaction which occurs between proteins X and Y.

However, both the two-hybrid (TNF- α and tetracycline inducible) approaches have several limitations. Both use strong and constitutively (spontaneous) interacting proteins and are unable to fully address weakly associated proteins or proteins with differential binding affinity, and also to decipher the time-kinetics of protein-protein interactions. Moreover the two-hybrid system could only detect the interacting proteins in the nucleus, not in the cytoplasm where the largest pool of protein-protein interactions responsible for many regulatory pathways occur. It is hoped that by combining non-invasive imaging approaches involving detection of cytoplasmic protein-protein interaction (through protein complementation study, split reporter strategy, FRET/BRET study etc.) with the two-hybrid system, it will be possible to measure the complete spectrum of the pharmacokinetics and pharmacodynamics of protein interactions in different regulatory pathways, as well as to perform screening and pre-clinical evaluation of small molecule drugs for therapeutic applications.

3.2 Bioluminescent reporter protein complementation assays to study protein-protein interaction

Protein complementation assays with split luciferases (split Firefly, split Renilla, and split-Gussia luciferases) are highly useful techniques for studying protein-protein interactions. Functional proteins can be assembled from one or more non-covalently attached polypeptides, with the efficiency of assembly a measure of real time protein-protein interactions, both in cells and in living animals. As discussed before, β -galactosidase from *Escherichia coli* is one of the first enzymes used extensively as an experimental reporter, long before the discovery of several other reporter proteins such as Chloramphenicol Acetyl Transferase (CAT), luciferases, and fluorescent proteins. Active β -galactosidase is a tetramer that hydrolyzes terminally non-reduced β -galactose residues in sugars, glycoproteins, and glycolipids (Hucho and Wallenfels 1972; Johnsson and Varshavsky 1994a; Johnsson and Varshavsky 1994b; Loontjens et al., 1970; Nichtl et al., 1998; Stagljar et al., 1998). The identification of the α -complementation process of β -galactosidase opened up the idea of using protein fragments coupled with an enzymatic assay to gauge protein interactions (Hodges et al., 1992; Ullmann et al., 1968). Each monomer of the tetramer can be cleaved into a small N-terminal α -fragment (50-90 residues) and a large (135 kDa fragment) ω -fragment. Addition of purified α -fragment to dimers of enzymatically inactive purified ω -fragments forms an active tetrameric enzyme, in a process called α -complementation (Hodges et al., 1992; Kippen and Fersht 1995; Smith and Matthews 2001; Ullmann et al., 1968). This process suggests that synthetically separated fragments of a single polypeptide might complement each other, and give rise to an enzymatically active protein, particularly if the interaction is aided by fusion of the halves to strongly interacting moieties. In the "split protein" strategy, a single reporter protein/enzyme is cleaved into N-terminal and C-terminal halves; each half is fused to one of two interacting proteins, X- and Y. Physical interactions between the two proteins reconstitute the functional reporter protein, leading to enzymatic activities that can be measured by *in vitro* or *in vivo* assays. This split protein strategy can work either through protein-fragment complementation assays, or intein-mediated reconstitution assays (Ozawa et al., 2001). To date, a number of different reporter proteins (β -lactamase, β -

galactosidase, ubiquitin, dihydrofolate reductase, firefly luciferase, renilla luciferase, Gaussia luciferase, etc.) have been adapted for split-protein strategies. Advantages of this approach include its ability to monitor the interaction of proteins occurring in their specific cellular compartments, utility for drug evaluation, low background, ability to measure real-time interactions, applications in protein array analysis, and potential adaptability to study both cells and in living animals. However, not every reporter protein can be used for this strategy (Ozawa et al., 2001; Paulmurugan et al., 2004; Pelletier et al., 1999; Remy and Michnick 1999; Rossi et al., 1997; Wehrman et al., 2002) (Figure 5).

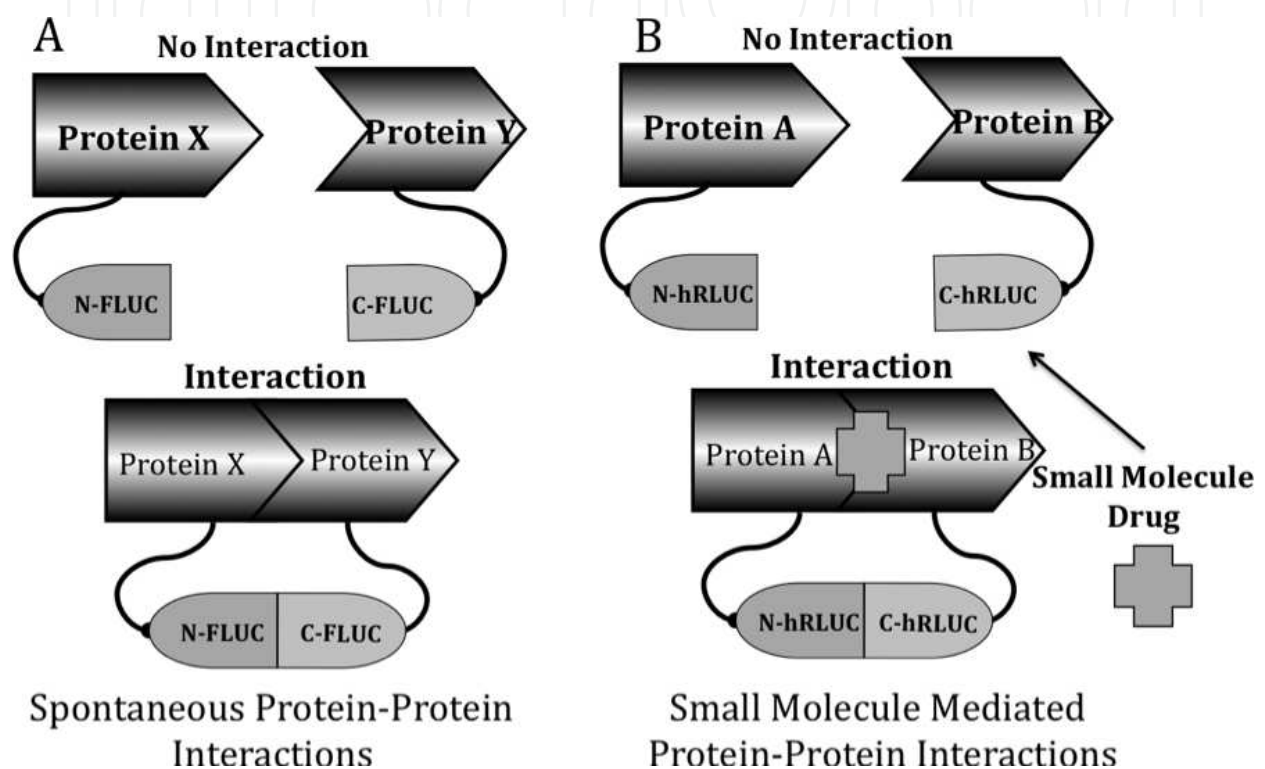


Fig. 5. Scheme of a split-reporter protein complementation system for studying protein-protein interactions. (A). *Complementation system to study spontaneous protein-protein interactions.* In this strategy, N- and C- terminal firefly luciferase reporter protein fragments are attached to two study proteins and are expressed as individual fusion proteins (N-FLUC-Protein-X and Protein-Y-C-FLUC). When cells are transfected to co-express these two fusion proteins, the interaction between protein-X and protein-Y brings the N-FLUC and C-FLUC fragments to a close proximity and induces the complementation to produce reporter signal. (B). *Complementation system to study small molecule mediated protein-protein interactions.* In this strategy N- and C- terminal Renilla luciferase reporter protein fragments are attached to two study proteins and are expressed as individual fusion proteins (N-hRLUC-Protein-A and Protein-B-C-hRLUC). When cells are transfected to co-express these two fusion proteins, the interaction between protein-A and protein-B is induced by a small molecule drug, which brings the N-hRLUC and C-hRLUC fragments to a close proximity and causes the complementation to produce reporter signal. In both strategies, the amount of luciferase signals produced through complementation directly relates to the interaction which occur between proteins X and Y or A and B (Paulmurugan and Gambhir 2003; Paulmurugan et al., 2009).

We made several combinations of N- and C- terminal fragments of the FLUC, RLUC, and GLUC enzymes by a semi-rational dividing approach and used these fragments for protein-protein interactions and protein folding studies. These fragments have been efficiently used in complementation assays for the detection of insulin-mediated phosphorylation, as gauged by the subsequent interaction of insulin receptor substrate peptide and its interacting partner SH2 domain of PI-3kinase (Ozawa et al., 2001). They have also been used to detect the interactions of the myogenic differentiation proteins Id and MyoD, in both cell culture and non-invasive repetitive optical imaging in living mice (Paulmurugan et al., 2002b). Separately, Luker et al., have also described a systematic truncation library yielding alternative complementary N- and C- fragments of FLUC. These fragments were used to monitor rapamycin-mediated interactions of rapamycin binding proteins (Luker et al., 2004). We also have used our FLUC fragments to study Rapamycin-mediated interactions, and found the complementation to be too weak for imaging in living animals by optical CCD camera.

RLUC and GLUC are the smallest optical bioluminescent reporter proteins, and we have identified several split sites for these proteins and found selective combinations that are efficient for studying protein-protein interactions through a protein fragment complementation strategy. This reporter protein, when rationally split (RLUC: between residues 229 and 230 or 235 and 236; GLUC: between 105 and 106) (Paulmurugan and Gambhir 2003), functions efficiently in both cell culture and in living animals, as we have demonstrated with several different protein partners. Fragments generated by splitting between residues 229 and 230 for RLUC and 105 and 106 for GLUC, were used to study rapamycin-induced interactions of human proteins FRB (FKBP12 Rapamycin Binding domain) and FKBP12 (FK506 Binding protein) (Paulmurugan et al., 2004), and also the inherent homodimerization property of mutant HSV1-sr39TK (Massoud et al., 2004). One limitation associated with the use of both RLUC and GLUC is their relatively rapid reaction kinetics, requiring early time-point measurements (Bhaumik and Gambhir 2002a). Nevertheless, this split reporter system appears highly suitable for studying protein-protein interactions in cell culture and in living animals owing to its strong optical bioluminescence, generating a signal that is amplifiable through an enzymatic process.

3.3 Bioluminescent reporter protein complementation assay to study protein folding

As we discussed in section 1.7, techniques for studying protein foldings are crucial in measuring the functionality of important biologically active proteins. Several experimental techniques are currently available for *in vivo* evaluation of protein folding within intact cells, mainly relying on intramolecular fluorescence resonance energy transfer (FRET) (Morris et al., 1982; Russwurm et al., 2007; Tsien 2009) of labeled residues or attached variants of green fluorescent protein (GFP), but none of these techniques can be optimally extended to imaging assays in intact living subjects. Protein complementation assays based on bioluminescent reporters are highly sensitive in measuring not only protein-protein interactions, they can also be efficiently used for studying protein foldings in intact cells and in living animals by imaging. The application of protein complementation assays based on bioluminescent reporters are more generalizable for measuring protein folding, but can be used for imaging of protein folding in intact living subjects. The ability to detect, locate, and quantify protein folding in the setting of a whole living animal model has important

implications: (1) to characterize the functional aspects of the fundamental process of protein folding, including the study of biologically relevant and important factors, within realistic and relatively undisturbed confines of cells that are also present in the midst of fully functional and intact whole-body physiological environments; and (2) to accelerate the evaluation in living animal models of emerging novel classes of drugs that promote folding and conformational stability of proteins (e.g., those directed at the molecular chaperone heat shock protein 90 (Hsp90)). Emerging strategies for molecular imaging of biological processes in living small animal models of disease offer many distinct advantages over conventional *in vitro* and cell culture experimentation.

In conclusion, we previously identified suitable split sites in the molecule of hRLUC that generated an N-terminal 229-residue fragment (N-hRLUC) with minimal independent activity and an inactive C-terminal 82-residue fragment (C-hRLUC) of the reporter protein. Together, they were able to produce significant recovered activity through assisted complementation. Later we used these reporter fragments along with reporter fragments of firefly luciferase (FLUC) to test protein-protein interactions mediated in different experimental settings: 1) proteins that spontaneously interact; 2) proteins that interact when a small molecule is present; 3) protein-protein interactions that are blocked by small molecules; 4) proteins-protein interactions that are mediated by phosphorylation; and 5) protein hydroxylation that is mediated protein-protein interactions (**Table 3**). After a thorough investigation of the system for studying protein-protein interactions, we demonstrated that the normal conformational changes during protein folding, which result in the close approximation of amino and carboxy termini of the proteins, can be measured by using intramolecular complementation of correctly oriented chimeric split imaging reporters in a strategy to detect, locate, time, quantify, and image protein folding in living subjects. As we have extensively studied ligand induced estrogen receptor (ER) folding in cells and in living animals, the following section will explain in detail the intramolecular folding of ER in response to different steroidal and non-steroidal ligands in cells and in living animals.

3.4 Protein complementation assays to study ligand- induced estrogen receptor folding

Estrogens are responsible for the growth, development, and maintenance of the reproductive, skeletal, neuronal, and immune systems as well as several other systems of the body. The physiological effects of these hormones are mediated by the estrogen receptor (ER), which is a ligand-inducible nuclear transcription factor (Tsai and O'Malley 1994). In the classical pathway of steroid hormone action, 17β -estradiol (E2), hormones, and a variety of other estrogens bind to the ligand-binding domain (LBD) of ER, leading to its dimerization and subsequent binding to a specific regulatory sequence in the promoters of ER target genes known as the estrogen response elements (Gronemeyer 1991; Schuur et al., 2001) that then trigger activation or repression of many downstream target genes (Brzozowski et al., 1997). The deficiency or excess of estrogens can lead to various pathological conditions, including osteoporosis and breast carcinomas (Beck et al., 2005), making ER a major cellular therapeutic target. ER activity in regulating target genes is modulated by the binding of both steroidal and synthetic non-steroidal ligands. The ligand

binding with ERs induces various conformations that control their interactions with transcriptional co-regulators. Estrogen receptors (ER α and ER β) regulate the expression of a number of gene products required for the growth of cells in response to the endogenous estrogen 17 β -estradiol (E₂). The crystal structures of ER ligand-binding domains (ER-LBDs) complexed with different ER ligands provide useful insight into the design and synthesis of new ligands. Although computer modeling and structure-based design can help predict molecular interactions and structure-activity relationships, the pharmacological actions of these ligands are unpredictable and require further biological evaluation. Thus, it remains important to fully characterize nuclear hormone receptor ligands in cells and in animal models before considering their use in humans. Several assay systems are currently available to characterize ER ligands for their biological activity through ER *in vitro* and in cell-based assays, but only a few can be directly extended for use in animals. We approached this issue by using bioluminescence imaging to study estrogen biology in living animals. Our system involves monitoring luminescence that derives from intramolecular complementation of a split luciferase gene that is activated by ligand-induced folding of the ER-LBD, and the responsiveness of various versions of this ligand sensor system to selected ER ligands was validated in cellular systems.

Split-Reporter Proteins	Imaging Modality	Protein-Protein Interactions	Nature of interaction
Firefly Luciferase (FLUC)	Bioluminescence	Id/myoD	Spontaneous interaction
Renilla Luciferase (RLUC)	Bioluminescence	FRB/FKBP12	Rapamycin mediated interaction
Gaussia Luciferase (GLUC)	Bioluminescence	pVHL/HIF1- α	Hydroxylation mediated interaction
Green Fluorescent Protein (GFP)	Fluorescence	Ras/Raf	Phosphorylation mediated interaction
Red Fluorescent Protein (RFP)	Fluorescence	Hsp90/p23	Spontaneous interaction
Thymidine Kinase (HSV1-TK)	PET	p53/SV40-Tag	Spontaneous interaction
		ER/ER	Estrogen mediated homodimerization
		TK/TK	Spontaneous dimerization
		ER-Folding	Estrogen mediated intramolecular folding
		IRS1/SH2	Phosphorylation mediated interaction
		c-myc/GSK3 β	Phosphorylation mediated interaction

Table 3. List of optical and PET based split reporter complementation systems developed and protein-protein interactions and protein foldings studied

The crystallographic studies with ER-LBD have shown that conformation of helix 12 (H12) is critical in responses observed with various ER ligands (Brzozowski et al., 1997; Pike et al., 2000; Shiau et al., 1998). The conformation of H12 behaves as a “molecular switch” that

either prevents or enhances the binding of ER to an array of co-activator proteins, which then activates transcription of many downstream estrogen-regulated genes responsible for cell growth. Given the critical role of H12 in ER signaling, we reasoned that it might be feasible to develop an intramolecular ER folding sensor with specific split reporter complementation patterns to study ligand pharmacology based directly on the conformational changes of H12 in response to different ligands (Figure 6). We used a split synthetic Renilla luciferase (RLUC) and Firefly luciferase (FLUC) complementation system, which we previously developed and validated (Paulmurugan and Gambhir 2007; Paulmurugan et al., 2002a), to test this hypothesis by assaying ligand-induced RLUC/FLUC complementation in cell lysates, intact cells, and cell implants in living mice by noninvasive bioluminescence optical imaging. The validated ER intramolecular folding sensors can also be used to distinguish ligand pharmacology in cell culture studies and cell implants in living animals treated with different ER ligands, agonists, selective ER modulators (SERMs), and pure antiestrogens. In adapting this bioluminescence ER ligand sensor system for *in vivo* use, we developed a version that contained a carefully developed single amino acid

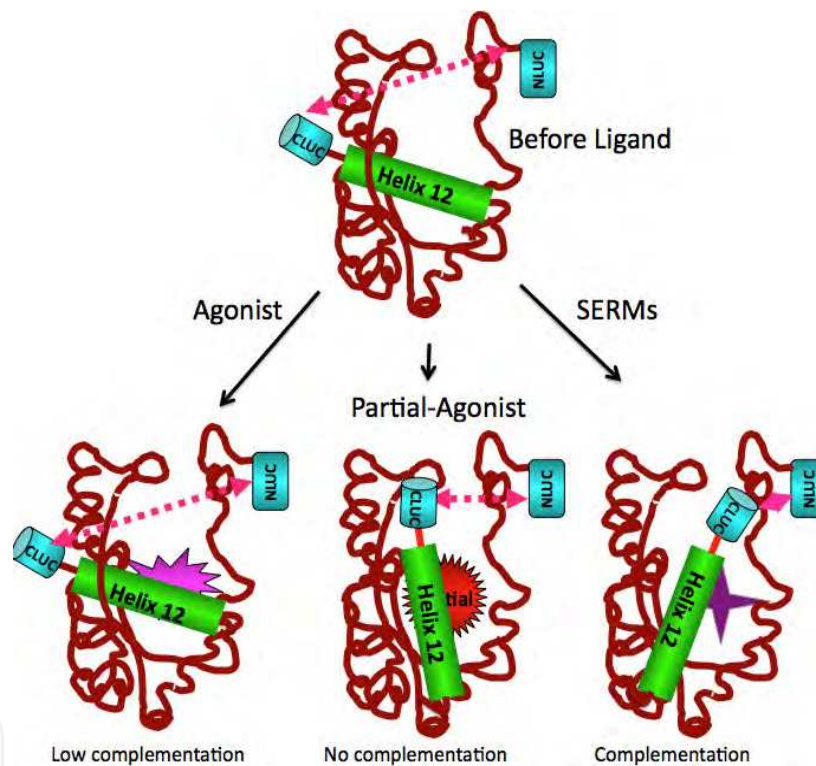


Fig. 6. Schematic representation of the hypothetical model of ligand-induced intramolecular folding of ER that leads to split firefly luciferase (FLUC) complementation. The N- and C-terminal fragments of split-FLUC were fused to the N- and C-terminus, respectively, of the hER α -LBD of various lengths (amino acids 281–549 or 281–595). Binding of ligands to the LBD of ER in the intramolecular folding sensor (N-RLUC-hER-C-RLUC) induces different potential folding patterns in the LBD based on ligands properties of potency and biocharacter. This folding leads to split-FLUC complementation for ER antagonist/SERMS (C), low complementation for ER agonist (A), and no complementation for partial ER agonist/antagonist (B) with the selective folding sensor. Even though the distance between the N- and C-FLUC fragments after binding with partial agonist (B) is smaller than that of agonists (A), this model depicts the importance of the orientations of the split-FLUC fragments in achieving complementation.

mutation in the ER α -LBD, G521T. This ER mutant was selected to be essentially unresponsive to E₂, so that it could be used in mice without interference from the endogenous ligand, while being responsive to certain non-steroidal estrogens such as diethylstilbestrol (DES) and SERMs, making it possible to study their activity *in vivo* by bioluminescence (Paulmurugan and Gambhir 2006; Paulmurugan et al., 2008).

We have developed and validated two hER intramolecular folding sensors that can be used to distinguish ER ligand pharmacology. These receptor sensors can be directly translated from cell culture studies to molecular imaging in small living subjects. In this study we used an ER-based split reporter complementation strategy to follow the position of H12 within the ER-LBD to detect changes in the receptor structural folding in response to ligand binding. The longer construct with the F domain (281–595) appears ligand-pharmacology-independent, whereas the shorter construct without the F domain (281–549) leads to the highest levels of split luciferase complementation in response to SERMs, moderate levels for agonists, and minimal levels for pure antiestrogens (Paulmurugan and Gambhir 2006). We validated these intramolecular folding sensors with various ER ligands in both transiently and stably transfected 293T kidney cells, and MDA-MB-231 (ER-negative) and MCF-7 (ER-positive) breast cancer cells. To extend the folding sensor for applications in living animals, we incorporated a previously undescribed mutant of hER (G521T) into the folding sensor that was insensitive to circulating endogenous estrogen but retained its ability to distinguish SERMs from synthetic agonists. Alternatively, ovariectomized mice can likely be used with the wild-type hER with minimal competition from endogenous estrogens while retaining the ability to study estrogen-like drugs.

To date, several *in vitro* assays have been developed for screening ER ligands by using either purified ER α protein or ER isolated from cell lysates (Inoue et al., 1983; Krey et al., 1997; Nasir and Jolley 1999; Nichols et al., 1998). Limited fluorescence-based assays (Zhou et al., 1998) have been developed to measure receptor conformational changes (23) and recruitment of coactivator peptides (Bai and Giguere 2003; Weatherman et al., 2002; Zhou et al., 1998) in the full-length hER α within cell culture (Michalides et al., 2004). Other assays have been designed to study the effects of synthetic ligands on ER transcription through the activation of downstream target genes (Awais et al., 2004). However, most of these reported assays are not suitable for quantitative, high-throughput screening of ER ligands in intact cells and especially in living subjects through noninvasive molecular imaging. A nontranscriptional assay containing fusion chimeras of either Flp recombinase (Logie et al., 1998) or Cre recombinase (Kemp et al., 2004) with a truncated mouse ER α (amino acids 281–599) has been reported and used for regulating the recombination of reporter genes in cells and living animals. This system demonstrates high background activity even before the addition of ER ligands, mainly through enzymatic amplification, thus limiting its dynamic range in response to different ER ligands. We developed an analogous fusion chimera by fusing a truncated version of hER (amino acids 281–595) with FLUC, which leads to luciferase activity that is 10⁴-fold greater than background (mock-transfected cells) even before the addition of ligands. To our knowledge, only one study has reported the construction of mutant versions of hER (G521R and G521V) for selective ER ligand binding using a fusion chimera containing hER_{251–595} with Flp recombinase enzyme (28). Incorporation of the same mutation into our intramolecular folding sensor (N-RLUC-hER_{281–595}-C-RLUC) led to nearly complete abolishment of signal for all ER ligands (hER_{G521R}) and a

significant reduction in signal (77–89%) for all agonist activities (hER_{G521V}) relative to wild type hER. We constructed intramolecular folding sensors using the hER_{G521} mutants with 19 different possible amino acids. We found that the replacement of hER_{G521} with threonine leads to nearly complete abolishment of the E2-induced RLUC complementation but only a 10–20% reduction for all other ER ligands studied. Subsequently, 293T cells stably expressing this intramolecular folding sensor (N-RLUC-hER_{281–549/G521T}-C-RLUC) were generated for imaging hER α /ligand complexes in living animals.

The advantages of the intramolecular folding sensor strategy that has been developed and validated include the following: (i) it is real-time (because RLUC exhibits flash kinetics) and quantitative; (ii) it can be used to distinguish binding of agonists, SERMs, and pure antiestrogens; (iii) it can be adapted for studying ligand binding to hER in living animal models by molecular imaging, and thus pharmacokinetic properties of each drug/ligand can be examined; (iv) it allows for a high-throughput strategy for screening/comparing different ER ligands and drugs in multiple cell lines; (v) it allows direct transition from cell culture studies to small living subjects because it is based on a bioluminescence split reporter strategy; and lastly, (vi) it will allow for applications using transgenic models that incorporate the intramolecular folding sensor. In addition, the availability of other split reporters with different properties and substrate specificities should allow multiplexing with other reporter assays.

The limitations with using split RLUC as the reporter gene regarding efflux of its substrate coelenterazine were resolved by showing experiments that resulted in no significant relation between the RLUC complementation and the multidrug resistance systems (Pichler et al., 2004). In addition, the intramolecular folding system was also studied with the improved split FLUC fragments by replacing RLUC fragments. Both systems showed equal sensitivity in different cell culture experiments. The FLUC fragments showed more detectable signal in mouse experiments than RLUC because of more light penetration through tissues, due to the more red-shifted wavelengths of FLUC. Also, the FLUC-based folding system showed greater efficiency in differentiating ER ligands in living mice. It is also possible that the exact locations (cytosolic vs. nuclear) of our fusion reporter proteins may affect the results obtained, and this will need to be explored in future studies. In addition, for some applications *in vivo*, the developed strategies may have difficulty in distinguishing agonists from background, and this potential problem needs to be investigated with testing of additional drugs.

3.5 Reporter protein complementations to monitor multi-protein interactions

The availability of multiple bioluminescent reporters (FLUC, RLUC, GLUC, and possibly VLUC) can be easily adopted for studying multiple proteins involved in a cellular network (e.g., Hsp90 chaperon multi-protein complex has more than 50 proteins) (Goetz et al., 2003) by multiplexing reporter combinations. The use of multiple reporters not only provides the interaction between more than two proteins, but can also provide more precise information to modulate the effect that one set of protein may exert on other sets of proteins involved in the same complex. In addition, it can also be used to extract the distances among different proteins involved in a complex based on the amount the complementation signals produced from each set of proteins (**Figure 7**). We are actively exploring multi-protein interactions by multiplexing several combinations, and we hope to soon publish results about this strategy and its feasibility.

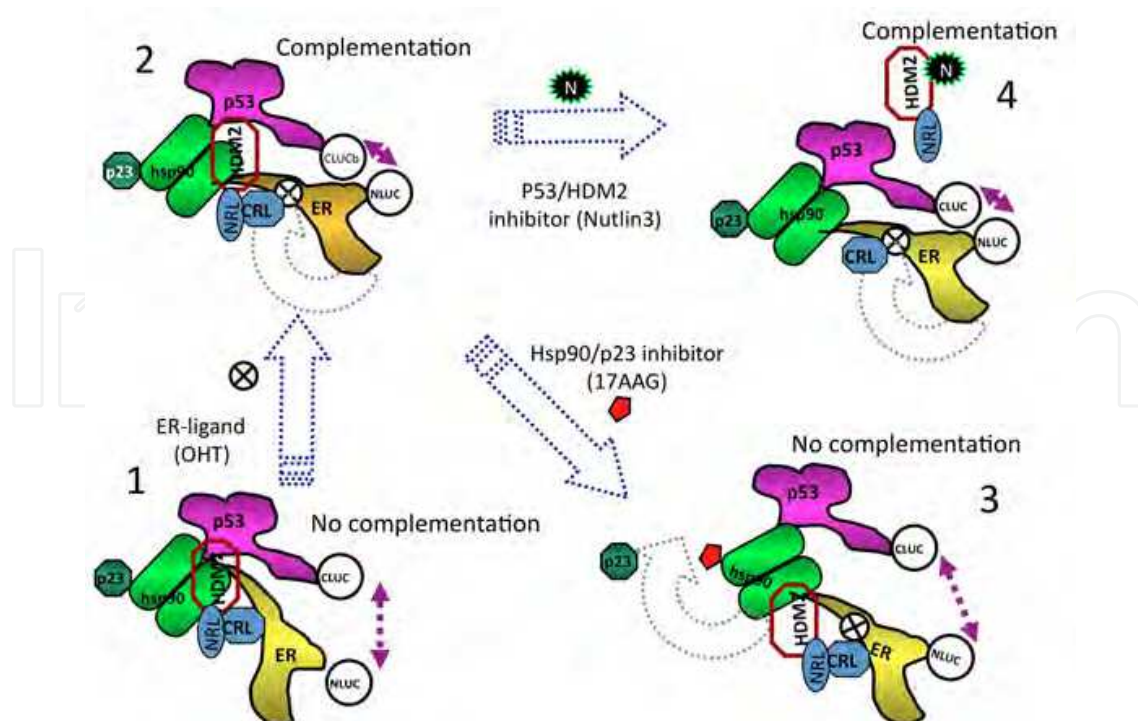


Fig. 7. Schematic representation of two split-reporter complementation systems to detect the interactions between several proteins involved in a multi-protein interaction complex. In this strategy, the results of two reporter protein complementations measured simultaneously between two reporters (Split-FLUC and Split-RLUC) induced by small molecules can predict possible arrangements of different proteins involved in this complex (Hsp90, p23, Estrogen Receptor, p53, and HDM2). Four different hypothetical models have been proposed based on possible theoretical complementations by these reporters in response to drugs which modulate Hsp90/p23 interaction (17AAG), p53/ER interaction (E2), and p53/HDM2 interaction (Nutlin 3).

4. Bioluminescence resonance energy transfer

Bioluminescence Resonance Energy Transfer (BRET) technology involves the nonradioactive transfer of energy between donor and acceptor molecules by the Förster mechanism (46). The energy transfer primarily depends on the following: (1) an overlap between the emission and excitation spectra of the donor (bioluminescence) and acceptor (Fluorophore or a fluorescent protein) molecules, respectively; (2) the proximity of $< 100 \text{ \AA}$ between the donor and the acceptor entities; and (3) the conformational orientation light emission with the acceptance end of the fluorescence entity. As BRET-based technology assumes more prominent roles in the field of studying PPIs, many commercial vendors are developing new instrumentations for measuring BRET ratios, which are generally low-intensity signals. BRET measurements are usually obtained with a microplate reader equipped with specific filter sets for detection of the donor and acceptor emission peaks. This cellular assay has been applied to real-time imaging of cells, high-throughput screening of drugs, and small animal and plant models. There are several combinations of BRET involving Renilla luciferase and green fluorescent protein and Firefly luciferase with variants of red fluorescent proteins developed for studying protein-protein interactions. The BRET2 system (Biosignal Packard Montreal, Canada) using renilla luciferase (RLUC) as a bioluminescent donor and mutant GFP2 as a fluorescent acceptor was

adapted for expression in mammalian cells and characterized by a significantly red-shifted Stokes shift that emits transferred energy at 508 nm. The resonance energy transfer from the reaction of the reconstructed RLUC protein with its substrate Deep Blue Coelenterazine (DBC) excites the GFP2 protein, as the two fused proteins Id and MyoD, or FKBP12 and FRB which interact in the presence of a small molecule mediator (rapamycin). Our lab also demonstrated the ability to detect signal from PPIs in cultured cells, as well as from the surface and deeper tissues of small living animals with implanted cells over expressing the fusion constructs (For further details, read (De et al., 2009; Dragulescu-Andrasi et al., 2011).

Our lab has recently showed that the BRET2 assay sensitivity can be significantly improved by using RLUC mutants with improved quantum efficiency and/or stability (eg, RLUC8 and RLUCM) as a donor. To extend the time of light measurement, we also developed CLZ400 (also known as bisdeoxycoelenterazine) analogs, showing that signal from our improved BRET2 vector can be monitored for up to 6 hours. This approach, currently undergoing continuing validation, should have important implications for the study of PPIs in cells maintained in their natural environment, particularly if it can be effectively applied for the evaluation of new pharmaceuticals. Most recently, further advances in this field have led us to develop a high photon efficiency, self-illuminating fusion protein combining a mutant red fluorescent protein (mOrange) and a mutant RLUC (RLUC8). This new BRET fusion protein (BRET3) exhibits a several fold improvement in light intensity in comparison to existing BRET fusion proteins. BRET3 also exhibits the most red-shifted light output (564 nm peak wavelength) of any reported bioluminescence protein that uses its natural coelenterazine substrate, a benefit that can be demonstrated at various tissue depths in small animals.

5. Future directions in bioluminescence imaging

Molecular imaging has been recognized as an important and exciting area of bio-medical research, mainly because of its ability to visually represent, characterize, and quantify biological processes in living subjects. Techniques such as Positron Emission Tomography (PET), Single-photon Emission Computed Tomography (SPECT), and Magnetic Resonance Imaging (MRI) have been extensively used in the clinic for several diagnostic and disease monitoring processes; all these systems explore intracellular proteins or other molecules as probes for the signal. Reporter genes (Bioluminescence, fluorescence, and PET), on the other hand, are capable of precisely monitoring sub-cellular processes and their native functional actions in cells, and imaging them in living animals. Challenges, however, remain in delivering these proteins in cells without perturbing the cellular microenvironments. Another obstacle is in generating sufficient sensitivity to measure these signals, especially in living animals. Problems associated with the modulations in the cellular microenvironment are also tricky, but may be minimized by expressing few copies and weighing the sensitivity. The continuing development of new high-sensitivity instruments with tomographic imaging capabilities and improved spatial resolutions will play an important role in expanding the applications of bioluminescent reporters and exploiting their unique ability to precisely image the sub-cellular processes in their native microenvironment.

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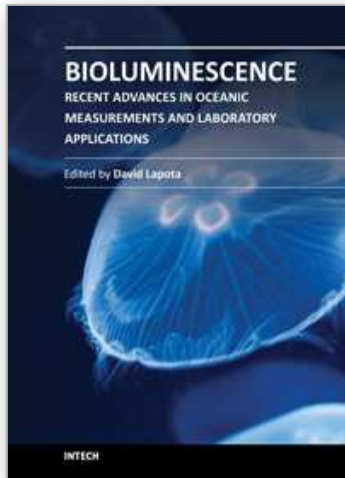
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We now find ourselves utilizing luciferase - luciferin proteins, ATP, genes and the whole complex of these interactions to observe and follow the progress or inhibition of tumors in animal models by measuring bioluminescence intensity, spatially and temporally using highly sophisticated camera systems. This book describes applications in preclinical oncology research by bioluminescence imaging (BLI) with a variety of applications. Chapters describe current methodologies for rapid detection of contaminants using the Milliflex system, and the use of bioluminescence resonance energy transfer (BRET) technology for monitoring physical interactions between proteins in living cells. Others are using bioluminescent proteins for high sensitive optical reporters imaging in living animals, developing pH-tolerant luciferase for brighter in vivo imaging, and oscillation characteristics in bacterial bioluminescence. The book also contains descriptions of the long-term seasonal characteristics of oceanic bioluminescence and the responsible planktonic species producing bioluminescence. Such studies are few and rare.

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