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Recent Developments of Biological Reporter Technology for Detecting Gene Expression

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Abstract

Reporter gene assay is an invaluable tool for both biomedical and pharmaceutical researches to monitor cellular events associated with gene expression, regulation and signal transduction. On the basis of the alternations in reporter gene activities mediated by attaching response elements to these reporter genes, one sensitive, reliable and convenient assay can be provided to efficiently report the activation of particular messenger cascades and their effects on gene expression and regulations inside cells or living subjects. In this review, we introduce the current status of several commonly used reporter genes such as chloramphenicol acetyltransferase (CAT), alkaline phos-

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Abbreviations: CAT, chloramphenicol acetyltransferase; AP, alkaline phosphatase; β-gal, β-galactosidase; GFP, green fluorescent protein; PNPP, *p*-nitrophenyl phosphate; FADP, flavin adenine dinucleotide phosphate; CSPD, chemiluminescent phenyl phosphate-substituted dioxetane; SEAP, secreted alkaline phosphatase; HSV-TK, herpes simplex virus thymidine kinase; ONPG, *o*-nitrophenyl β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl galactoside; FDG, fluorescein-di-β-D-galactopyranoside;DDAOG,9H-(1,3-dichorlo-9,9-dimethylacridin-2-one7-yl),β-galactopyranoside;DDAO,7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one); SRL, sequential reporter-enzyme luminescence; EgadMe, (1-(2-(β-galactopyranosyloxy)propyl)-4,7,10- tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane) gadolinium (III); RFP, red fluorescent protein; FRET, fluorescence resonance energy transfer; BFP, Blue GFP; $FMNH_2$, flavin mononucleotide; Rluc, *Renilla* luciferase; BRET, bioluminescence resonance energy transfer; LCI, luciferase complementation imaging; ERE-luc, estrogen-responsive element - luciferase; Id and MyoD are members of the helix-loop-helix (HLH) family of nuclear proteins; 'amp', β-lactamase (Bla) ampicillin resistance gene.

phatase (AP), β-galactosidase (β-gal), luciferases, green fluorescent protein (GFP), and β-lactamase. Their applications in monitoring gene expression and regulations *in vitro* and *in vivo* will be summarized. With the development of advanced technology in gene expression and optical imaging modalities, reporter genes will become increasingly important in real-time detection of the gene expression at the single-cell level. This synergy will make it possible to understand the molecular basis of diseases, track the effectiveness of pharmaceuticals, monitor the response to therapies and evaluate the development process of new drugs.

Introduction

Many physiological phenomena, including cell communication, cellular development, growth regulation, proliferation and oncogenesis, can be attributed to differential gene expression that is tightly and precisely regulated in response to intrinsic developmental programs and extrinsic signals. To gain insightful information about the relationship between the activation/inhibition of different pathways and their effects on gene expression, specific response elements are fused to genes encoding reporter proteins. These reporter genes can then be used as indicators in monitoring the transcriptional activity in cells (Rosenthal *et al*., 1987). Normally, the reporter gene is linked to a promoter sequence through an expression vector that is further transferred into cells. After transfer, the cells are assayed for the presence of the reporter by directly testing the amount of either the reporter mRNA, the reporter proteins, or the enzymatic activities of the reporter proteins (Gambhir *et al*. 1999). In general, reporter genes should not only have the advantages of reduced background activity (not endogenously expressed protein of interest) in cells but also amplify the signal from the cell surface to produce a response that is rapid, highly sensitive, reproducible and easily detectable. Currently, reporter genes are used extensively in both in *vitro* and *in vivo* applications. In particular, reporter systems are employed to study the promoter and enhancer sequences or trans-acting mediators for the transcription, mRNA processing and translation. They can also be utilized to monitor the transfection efficiencies, protein-protein interactions, protein subcellular localization, and recombination events as well as to screen genome-wide libraries for novel genetic regulatory elements (Ignowski *et al*., 2004; Alam *et al*. 1990; Naylor, 1999; Golzio *et al*., 2004). These applications underline their enormous significance in biology and biotechnology.

The choice of reporter depends on the cell line used (e.g. absent endogenous activity), the nature of the experiment (e.g. dynamics of gene expression versus transfection efficiency), and the adaptability of the assay for appropriate detection (e.g. cellular imaging). Some specific assays and detection methods based on the tracer level of radiolabeled probes in radionuclide imaging techniques such as positron emission tomography (PET), single photon emitted computed tomography (SPECT), planar gamma camera imaging etc have been discussed in several authoritative reviews and therefore will not be covered here (Dobrucki *et al*., 2007; Tyer *et al*., 2005). In this article, we will present an overview of the latest developments of several reporter genes including, chloramphenicol acetyltransferase (CAT), alkaline phosphatase (AP), β-galactosidase (β-gal), green fluorescent protein (GFP), luciferases and β-lactamase based on the literatures reported over the last decade. All these available genetic reporters and their potential applications in optical imaging *in vitro* and *in vivo* are also summarized.

Chloramphenicol acetyltransferase (CAT)

CAT is the first gene reporter used for the analysis of transcriptional regulation in mammalian cells. As a bacterial enzyme, CAT has a trimeric structure with three identical subunits of 25 kDa each, and is relatively stable in the context of mammalian cells. There are no endogenous proteins exhibiting similar properties with its enzymatic counterpart in mammalian cells, thus making CAT easily expressed and assayed. CAT can catalyze the transfer of the acetyl group from acetyl-coenzyme A (acetyl-CoA) to chloramphenicol. As the first reported gene reporter, CAT has been extensively utilized to monitor the delivery, location and pattern of transgene expression in some disease models such as hepatitis B, heart disease, and drug resistance in bacteria (Rajamanickam *et al*., 2005; Selbert *et al*., 2002; Arnone *et al*., 2004; Schwarz *et al*., 2004). Unfortunately, this assay relies on the radioisotopes which limit its further application in living cells.

Alkaline phosphatase (AP)

AP is a generic term for a family of orthophosphoric monoester phosphohydrolases exhibiting optimum activity at alkaline pH. It is a relatively stable protein ubiquitously expressed in bacteria and mammalians. Due to its dephosphorylation of a broad range of natural and synthetic substrates, a number of assays with varying degrees of sensitivity are available for measuring its activity. One standard spectrophotometric assay is based on the hydrolysis of the AP substrates to generate the absorbance change. The commonly used substrates for AP enzymes are *p*-nitrophenyl phosphate (PNPP) (Schenborn *et al*., 1999), flavin adenine dinucleotide phosphate (FADP) (Harbron *et al*., 1992) and chemiluminescent phenyl phosphate-substituted dioxetane (CSPD). These substrates are illustrated in *Figure 1*. AP has been widely used for analysis of a culture medium from stably transformed cells, with the wide variety of easy and sensitive assays allowing for a convenient and versatile reporter system (Gorr, 1996), for monitoring the inflammatory events (Hiramatsu *et al*., 2006), and for its high-throughput screening applications, such as G-protein-coupled receptors (Durocher *et al*., 2000). It has also been shown to monitor promoter activity (Shiraiwa *et al*., 2007), detect growth factors (Tesseur *et al*., 2006) and identify inducers/repressors of gene expression (Su *et al*., 2002). Unfortunately, AP is expressed practically in all cell types. Although, one improved reporter system was developed on the basis of secreted alkaline phosphatase (SEAP) to decrease all the mammalian AP isoenzymes activities by treatment with heat or homoarginine, the presence of the similar endogenous proteins in the mammalians decreases the effective sensitivity of the AP reporter gene system and therefore limits their extensive applications.

β-galactosidase (β-gal)

β-galactosidase (β-gal) is a well-characterized bacterial enzyme, and structurally, it contains one tetramer with a large subunit size of 1023 amino acids (the monomer is 116kDa). The individual monomer peptide chains in each β-gal tetramer structure can fold into five sequential domains, with an extended segment of ~50 amino acids

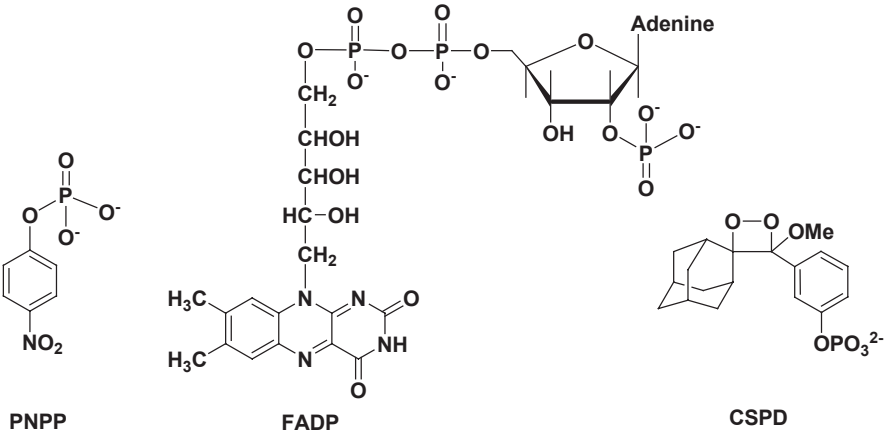

Figure 1. Structures of the substrates for alkaline phosphatase (AP).

residues at the amino terminus. About 26 amino acids can be removed and then substituted with several hundred or more residues from a variety of other proteins without influencing catalytic activity. Meanwhile, the redundancy of its two carboxy-terminal amino acids enables substitution by other coding regions producing active chimeric β-galactosidase (Fowler *et al*., 1983). On the other hand, β-gal plasmids possessing the SV40 promoter/enhancer, the cytomegalovirus (CMV) promoter/enhancer, the herpes simplex virus thymidine kinase (HSV-TK) promoter/enhancer or the major adenovirus late promoter/tripartite leader can induce the expression of the encoded *lacZ* gene in cells. Functionally, the expressed β-gal can efficiently accelerate the hydrolysis of various β-galactosides, which has been used extensively as an internal control for normalizing variability in reporter protein activity due to the different transfection efficiency or cell extract preparation.

One simple colorimetric assay based on the enzymatic hydrolysis of the substrate *o*-nitrophenyl *β*-D-galactopyranoside (ONPG) has been conducted in cell-free extracts. To observe the transfection efficiency, ONPG is added into the lysated cells in the tissue culture dishes. The color changes at 420nm are monitored upon enzyme treatment (Leahy *et al*., 2001; Holmes *et al*., 2000). Another frequently used colorimetric assay is based on the cleavage of the substrate 5-bromo-4-chloro-3-indolyl galactoside (X-Gal), as shown in *Figure 2*. This substrate is a galactose sugar with a glycosidic linkage to an indolyl molecule. It remains colorless as long as the chromophore is linked to the galactose. The glycosidic link, however, is hydrolyzed in the presence of the enzyme of β-gal. The free indolyl can be then released and further oxidized into an indoxyl which self-couples to form an indigo blue insoluble derivative (Alam *et al*., 1990). Both the ONPG and X-Gal have been used in these normalization processes with equal success. A direct comparison can be made between the number of cells transfected and the strength of the promoter through the use of colorimetric enzymatic assays to identify the transfection efficiency, cell specificity, or preference of infection, as well as evaluation of the promoter expression level. This method is predominantly useful in recognizing specific cells expressing the promoter-*lactZ* construct within a primarily heterogeneous cell population (Shimohama *et al*., 1989).

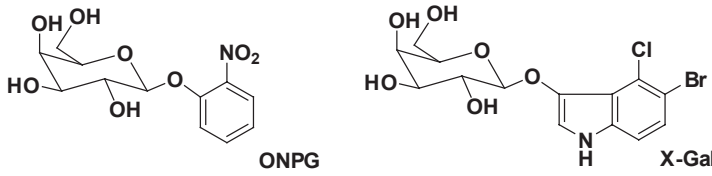

Figure 2. Structures of o-nitrophenyl β-D-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl Galactoside (X-Gal).

The X-Gal and ONPG substrates have both been consistently used for β-gal expression staining *in vitro* and *ex vivo* due to their chromogenic feature. Nonetheless, the simple colorimetric assays exhibit narrow dynamic range and poor sensitivity. Use of a single cell is likewise insufficient. As a result, more sensitive substrates with adaptable biological or chemical properties are needed to replace these chromogenic substrates. Among the sensitive substrates, fluorescein-di-β-D-galactopyranoside (FDG) is better able to detect β-gal in the mammalian cells.

As illustrated in *Figure 3*, native FDG does not have fluorescence; reaction with β-gal, however, generates two galactose equivalents. The reaction also produces one fluorescein, which exhibits intense fluorescence. This β-galactosidase-FDG system has widely been employed in reporter technology, particularly in single cell applications, due to its sensitive fluorescent signal following catalysis despite the less membrane permeability of the substrate. For example, Blau *et al* (Rossi *et al*., 1997) developed a new assay monitoring protein-protein interactions based on intracistronic β-gal complementation. Two mutants of β-gal are utilized; both contain inactivating mutations in different crucial domains and are able to reproduce an active enzyme by sharing their intact domains. To monitor the interaction between two proteins, each of the proteins was fused to one half of a pair of β-gal mutants, and the two fusion proteins were expressed at low levels in mammalian cells. The reconstitution of β-gal activity was dependent on the physical interaction of the non-β-gal of the chimeric proteins, thus allowing it to identify the enzymatic kinetics of the interactions. In line with this, an improved substrate (2-Me-4-OMe-Tokyo-Green O-β-galactoside or TG-β-gal) has recently been found superior to FDG in terms of cell imaging with single-phase kinetics (Urano *et al*., 2005). This new probe has only one β-galactoside in a molecule. In just one step of hydrolysis, it would already afford a higher rate of fluorescence increase and higher sensitivity, as well as ensure a strict linearity in the relationship between the fluorescence intensity and enzyme activity. Furthermore, this probe was relatively membrane-permeable due to its lower hydrophilicity, enabling the

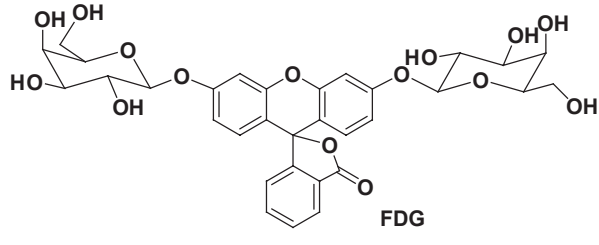

Figure 3. Structures of fluorescein di-β-D-galactopyranoside (FDG).

researchers to obtain an image of the β-gal activity in living cells without hypotonic shock for permeablizing the cell membrane (*Figure 4*).

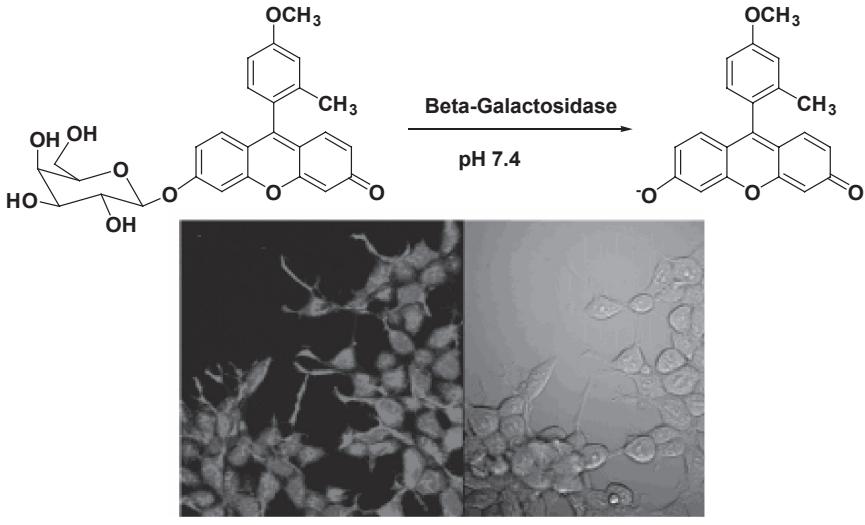

Figure 4. Reaction scheme of TG-βGal and fluorescent imaging of to β-galactosidase activity with TG- βGal in living lacZ-positive cells. (Reprinted by copyright permission of the American Chemical Society)

To expand the function of β-gal from *in vitro* and living cell to *in vivo* imaging, Tung and coworkers (Tung *et al*., 2004) demonstrated that a previously available substrate, 9H-(1,3-dichorlo-9,9-dimethylacridin-2-one 7-yl) β-galactopyranoside (DDAOG), which is a conjugate of β-galactoside and 7-hydroxy-9H-(1,3-dichloro-9,9- dimethyl-acridin-2-one) (DDAO), a far-red fluorescent compound, could be used to image β-gal expression in a living mouse (*Figure 5*). In their experiment, an HZCX-HSV amplicon vector containing the *lacZ* gene under the immediate-early IE4/5 HSV promoter is used to infect human glioma Gli36 tumor cells. The cleavage of DDAOG by β-gal released the fluorophore DDAO, which is emitted maximally at 659nm with a 50nm red shift from the substrate DDAOG. The current system proves to be active in the realm of animal imaging, albeit one downside of this probe is the limited sensitivity due to the significantly narrow separation of the DDAO emission spectrum from its excitation spectrum.

More recently, Blau and coworkers (Wehrman *et al*., 2006) have developed a sequential reporter-enzyme luminescence (SRL) technology for the *in vivo* detection of β-gal activity. Their design consists of a caged D-luciferin-galactoside conjugate (Lugal), which is a substrate that must first be cleaved by β-gal before it could undergo an enzymatic reaction by firefly luciferase (Fluc) to generate light (*Figure 6*). Therefore, Fluc-generated luminescence is dependent on the activity of β-gal. This caged galactoside-luciferin conjugate has proven significant both in the recognition of low levels of bacterial contamination in food poisoning, as well as in β-gal based high throughput screening assays. This technique has been employed as a bioluminescence approach in imaging *lacZ* reporter gene expression in transgenic mice, it can also be used for the visualization of cells using cell surface specific antibodies conjugated to recombinant β-gal (Masuda-Nishimura *et al*., 2000; Yang *et al*., 2005).

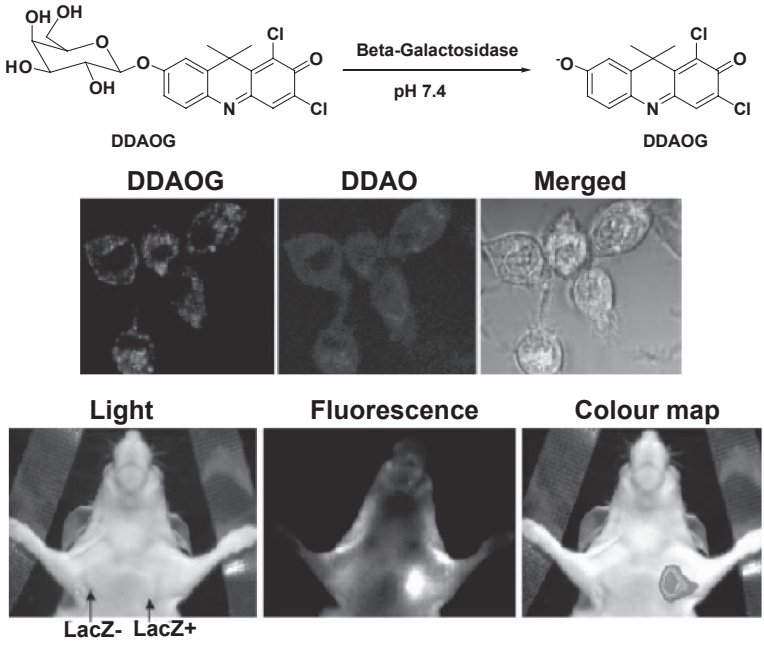

Figure 5. A structure and reaction of TG-β-Gal with β-galactosidase, fluorescent imaging of DDAOG to β-galactosidase activity in living 9L-lacZ-positive cells and in vivo animal imaging of β-Gal expression. (Reprinted by copyright permission of The American Association for Cancer Research)

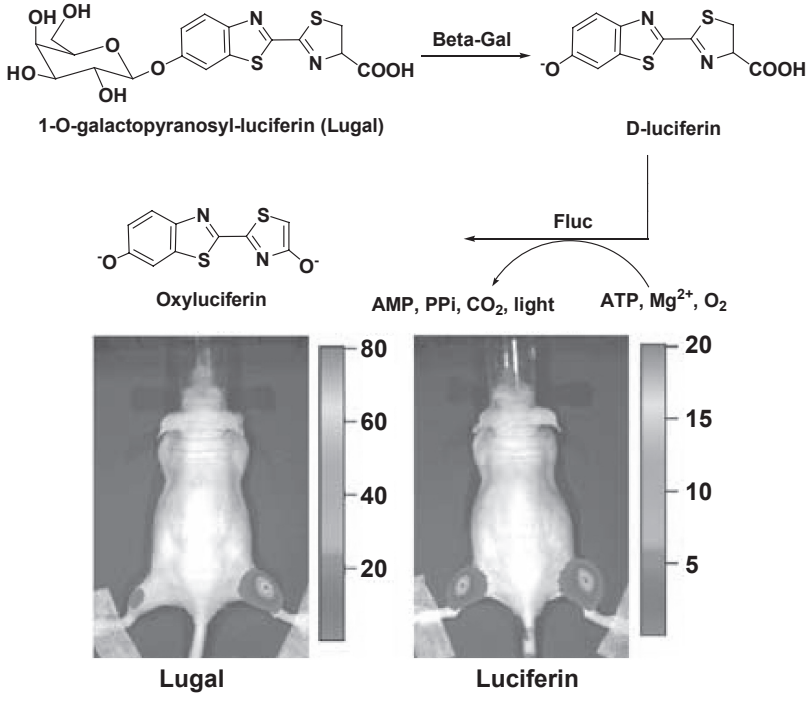

Figure 6. The structure of the galactoside-luciferin conjugate Lugal. β-Gal expressing cells can be imaged in living subjects by using Lugal. (Reprinted by copyright permission of the Nature Publishing Group)

Recently, Fraser and coworkers have reported a β-gal based magnetic resonance imaging (MRI) contrast agent (1-(2-(β-galactopyranosyloxy) propyl)-4,7, 10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane) gadolinium (III) (abbreviated as EgadMe) for high resolution *in vivo* imaging of gene expression in opaque animals (Louie *et al*., 2000). EgadMe consists of a chelate with bounded gadolinium that is linked to the 2-postion of β-galactoside (*Figure 7*), and displays low T1 relaxitivity due to the blocking of the remaining coordination site on the gadolinium ion from water by the galactose residue. The β-galactosidase hydrolysis cleaves the galactose and renders the gadolinium chelate more water accessible, thus leading to an increase in the T1 relaxitivity. These results document the ability of EgadMe for 3D imaging of the mRNA expression in *Xenopus larvae* and offer the promise of *in vivo* mapping of reporter gene expression in living subjects with MRI (*Figure 8*) (Louie *et al*., 2000).

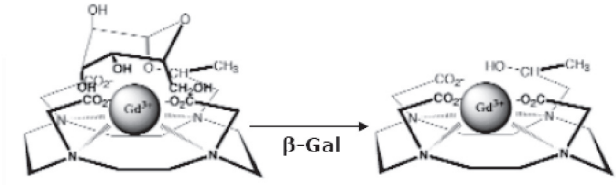

Figure 7. Schematic of the transition of Egadme from a weak to a strong relaxitivity**.** (Reprinted by copyright permission of the Nature Publishing Group)

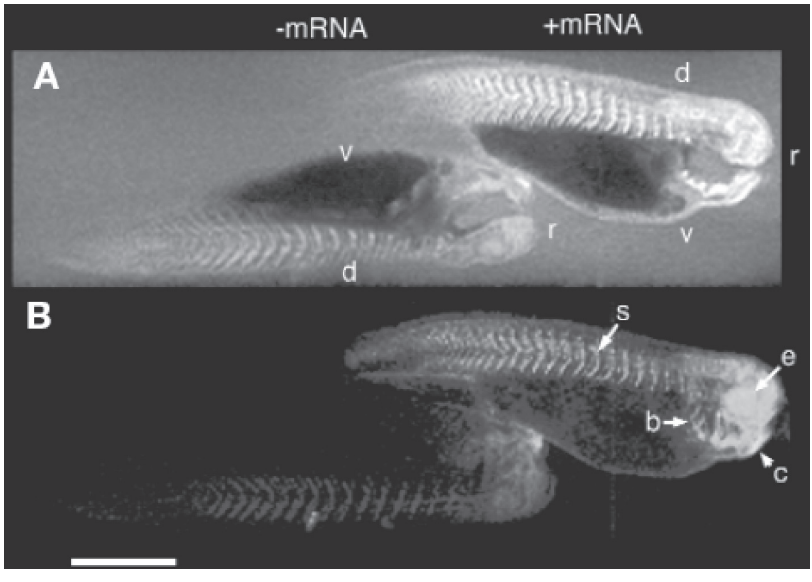

Figure 8. MRI detection of β-galactosidase mRNA expression in living X. laevis embryos. MR images of two embryos injected with EgadMe at the two-cell stage**.** A). Unenhanced MR image. B). Pseudocolor rendering of same image in A) with water made transparent. (Reprinted by copyright permission of the Nature Publishing Group)

Green fluorescent protein (GFP)

Green fluorescent protein (GFP) is one of the most frequently used reporter genes in biological systems. Green fluorescent proteins exist in a variety of coelenterates, both hydrozoa such as *Aequorea, Obelia* and *Phialidium* and *Anthozoa* such as *Renilla* (Tsien, 1998; Prasher *et al*., 1992). Among these GFPs, only the GFP gene from the jellyfish *Aequorea victoria* has been cloned. This cloned GFP gene has been the most studied green fluorescent protein, and is a 27kD monomer with 238 amino acids. The wild type *Aequorea* protein shows a complex spectrum with a major excitation peak at 395nm, about three times higher in amplitude than its minor peak at 475nm. The excitation at 395nm gives emission peaking at 508nm in normal solution. GFP has high extinction coefficient, is resistant to pH-induced conformational changes and denaturation, and has tendency to dimerize under a higher protein concentration (Tsien, 1998). As illustrated in *Figure 9*, the wild type GFP is an 11-strand β-barrel threaded by an α-helix running up the axis of the cylinder. The chromophore is attached to the α-helix, and is burred almost perfectly in the center of cylinder and forms one β-can structure. Almost all the primary sequence is used to build the β-barrel and axial helix. There are no apparent places where one could design large deletions and reduce the size of the protein by a significant fraction (Yang *et al*., 1996). The chromophore of the GFP is a *p*-hydroxybenzylideneimidazolinone formed from residues Ser65, Tyr66, and Gly67. *Figure 10* shows the proposed mechanism for chromophore formation. First, GFP folds into a nearly native conformation, the imidazolinone is then formed by nucleophilic attack of the amide of Gly67 on the carbonyl of residue Ser65, followed by dehydration. Next, molecular oxygen dehydrogenates the α-β bond of residue Tyr66 to put the aromatic group into conjugation with the imidazolinone and thus the chromophore acquires visible absorbance and fluorescence (Cubitt *et al*., 1995).

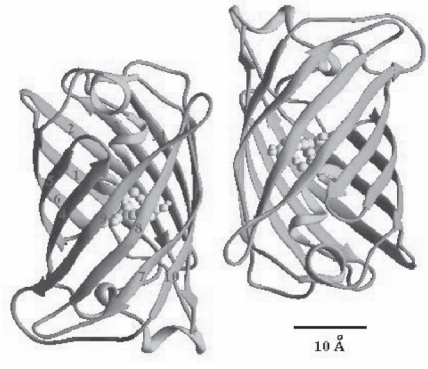

Figure 9. Ribbon structure of the green fluorescent protein (GFP). (Reprinted by copyright permission of the Nature Publishing Group)

GFP is also noted to possess unique structural properties, in which accurate protein folding is necessary to reveal its entire fluorescent activity. Temperature at or below 25˚C is fairly efficient for wild-type jellyfish GFPs to fold, but this folding efficiency will sharply decrease at higher temperatures. A triple mutant (Phe99Ser, Met153Thr, and Val163Ala) can improve 37˚C-folding, reduce protein aggregation at high

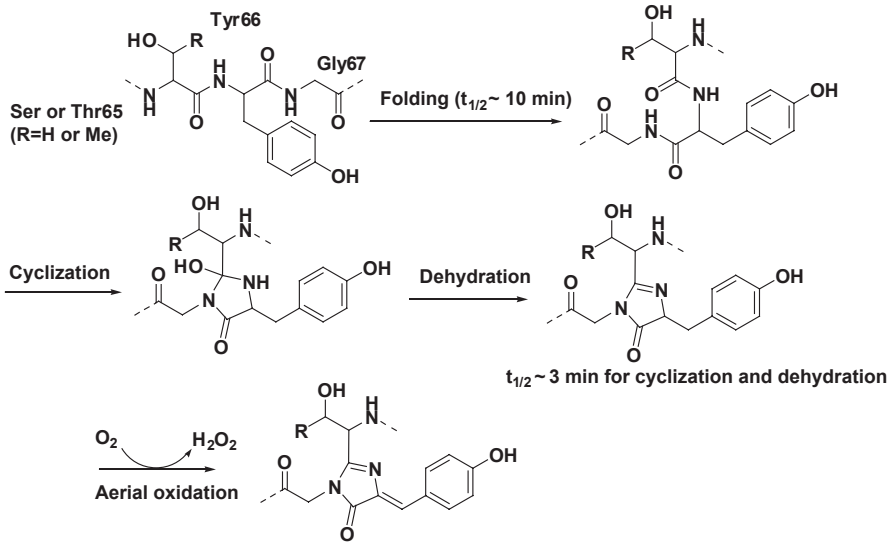

Figure 10. Mechanism for the intramolecular biosynthesis of the GFP chromophore.

protein concentrations, and increase the diffusibility of proteins inside cells. Such folding mutations do not increase the intrinsic brightness of properly matured GFP molecules; nevertheless, they can improve the solubility and folding ability, and also provide evidence of being mainly advantageous in *E. coli* and mammalian cell culture utilization (Yang *et al*., 1996). The GFP expression level and detectability in mammalian cells are influenced by many factors, such as total amount of GFP, efficiency of posttranslational fluorophore formation, molecular properties of mature GFP, and the noise and background signals. In addition, the presence of molecular oxygen can also affect the maturation of GFP expression in living cells. Unless O_2 dehydrogenates the α, β-bond of residue Tyr66, no fluorescence will exhibit in obligate anaerobes. Once GFP is matured, O_2 has no further effect (Heim *et al*., 1994).

The scientific potential of a fluorescent protein has been rapidly recognized after GFP cloning. In principle, fluorescent proteins are primarily used as a tracer for the detection of labeled cells *in vivo*. FPs encoded in plasmid are available for cloning proteins at either the N- or C-terminal. These plasmids permit the controlled expression in a variety of cells and organisms, including bacteria, yeast, and mammalian cells. However, some of the properties of wild-type GFP are not suitable with respect to fluorescent intensity, fluorophore formation kinetics and biphasic excitation spectrum. Therefore, in order to improve the fluorescent properties of GFP and stabilize the translation and expression in mammalian cells, extensive researches have been exploited to design the variant GFPs as enhanced green fluorescent protein (EGFP). Improved and stable FPs of different color fluorescence such as blue, cyan, and yellow fluorescent proteins (BFP, CFP, YFP) have been produced through mutation and selection (Rizzo *et al*., 2004) (*Figure 11*). New efforts have also extended the range of fluorescence further into the red region. In 1999, the red fluorescent protein (DsRed) was cloned possessing 3D structure very similar to that of GFP (Yarbough *et al*., 2001), and exhibited maximum emission at 583nm with excitation at 558nm. However, DsRed suffers from a significant disadvantage: high tendency for aggrega-

tion as a tetramer in order to be fluorescent (Baird *et al*., 2000)**.** Extensive targeted mutagenesis by Tsien *et al* has successfully eliminated the oligomerization of DsRed, leading to a monomeric variant of the red fluorescent protein, mRFP1 with an excitation peak at 584nm and an emission peak at 607nm, but the fluorescent properties of mRFP1 with respect to quantum yield and molar extinction coefficient have not been satisfactory for standard application (Campbell *et al*., 2002).

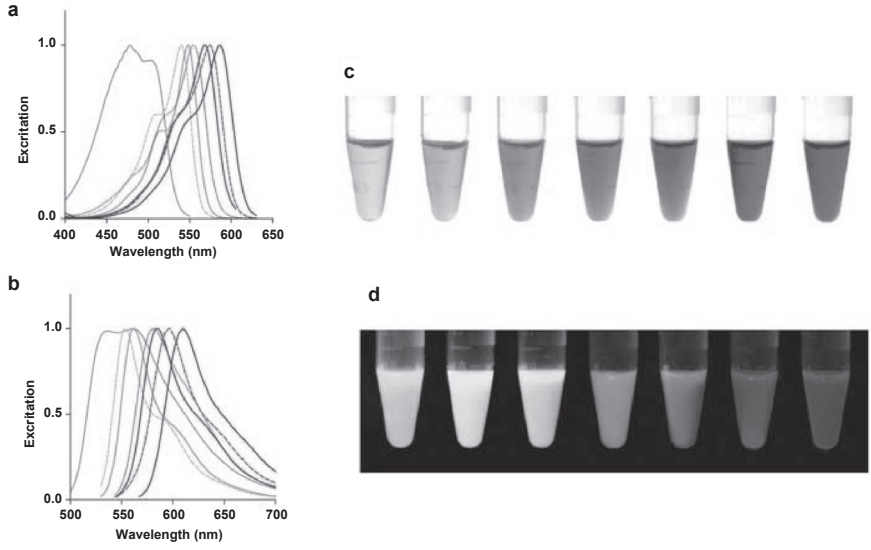

Figure 11. Excitation and emission spectra for new RFP variants. Excitation (a) and emission (b) curves are shown as solid or dashed lines for monomeric variants and as a dotted line for dTomato and tdTomato, with colors corresponding to the color of each variant. (c,d) Purified proteins (from left to right, mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, and mCherry) are shown in visible light (c) and fluorescence (d). The fluorescence image is a composite of several images with excitation ranging from 480 nm to 560 nm. (Reprinted by copyright permission of the Nature Publishing Group)

The GFP mutants with longer wavelength (e.g. near infrared) emission will facilitate newer applications for *in vivo* fluorescent imaging (Wang *et al*., 2004). Recently Shkrob *et al*. developed some far-red fluorescent proteins with maximal emissions reaching the 650nm barrier (Shkrob *et al*., 2005). Chudakov *et al* created another far-red fluorescent protein with high brightness and photostability, named Katushka by using directed and random mutagenesis. Direct comparison with existing red and far-red fluorescent proteins demonstrated that Katushka was strongly preferred for imaging the living animals (*Figure 12*) (Shcherbo *et al*., 2007). Fluorescent proteins have been used to monitor neoplastic processes such as primary tumor growth, cancer cell motility and invasion, metastatic seeding and colonization, angiogenesis, and tumor-microenvironment interaction. A transgenic mouse transplanted with tumor cells expressing red fluorescent protein (RFP) enabled the real-time visualization of interaction between the tumor cells and host cells (Hoffman, 2005). Likewise, non-invasive real-time fluorescent imaging techniques could be utilized to assess the efficacy of the therapeutics, discover new medications and determine the latest genes mitigating cancer growth and progression (as showed in *Figure 13*) (Zhao *et al*., 2005). These techniques enable scientists to elucidate on what stage tumors become

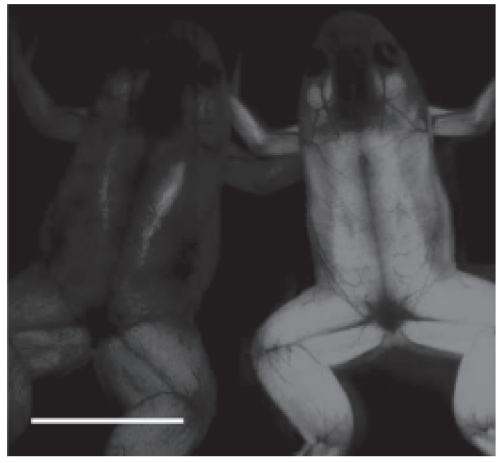

Figure 12. Fluorescence image of 2.5-month-old frogs expressing DsRed –Express (Left) or Katushka (Right) from the dorsal side, scale bars, 10 mm. (Reprinted by copyright permission of the Nature Publishing Group)

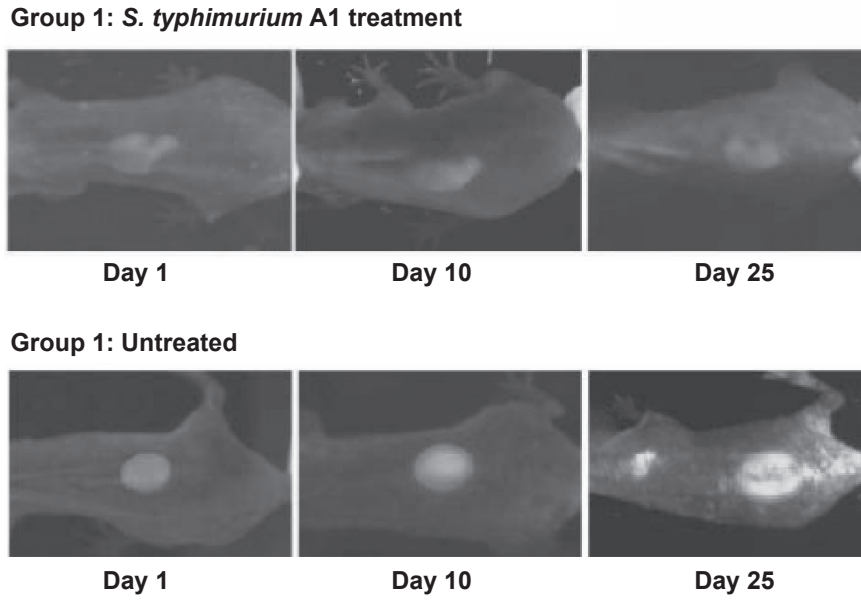

Figure 13. Whole body imaging of the antitumor efficacy of S. typhimurium A1 on the growth of a PC-3 human prostate tumor after i.v. injection**.** NCR nude mice (aged 6-8 weeks) were implanted s.c. on the mid-right side with 2×10^6 RFP-labeled PC-3 human prostate tumor cells (2×10^6) with a 22-gauge needle. *S. typhimurium* were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-logarithmic phase and washed with PBS, then diluted in PBS and injected directly into the tail vein (10^7 cfu per 100 μl of PBS). Tumors were visualized by fluorescence imaging at indicated time points after injection. (Copyright (2008) National Academy of Sciences, U.S.A.)

resistant to specific treatments, which in turn, may help discover means to evade drug resistance (Katz *et al*., 2003; Schmitt *et al*., 2002). Fluorescent proteins based imaging techniques can be used not only in the visualization of cancer *in vitro* and in living subjects, but also for noninvasive monitoring the status of other diseases such as HIV and heart failure (Hübner *et al*., 2007; Shirani *et al*., 2007).

The availability of spectrally distinct fluorescent proteins can also open up new avenues for imaging protein-protein interactions *in vitro* and *in vivo* based on the quantum physical phenomenon, namely, fluorescence resonance energy transfer (FRET) between FPs of different color as showed in *Figure 14*. FRET is a quantum-mechanical phenomenon that occurs when two fluorophores are in molecular proximity (<100 Å) and the emission spectrum of one fluorophore, the donor, overlaps the excitation spectrum of the second fluorophore, the acceptor. This principle further elaborates that excitation of the donor can produce emission from the acceptor at the expense of the emission from the donor, which would normally occur in the absence of the acceptor. FRET has been extensively used, both in the study of protein interactions as well as in the development of FRET-based genetically encoded fluorescent sensors (Miyawaki, 2003; Rizzo *et al*., 2004; Nguyen *et al*., 2005).

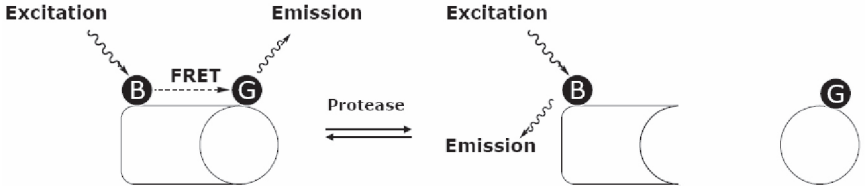

Figure 14. The scheme of energy transfer from a blue-emitting mutant (Y66H) to an improved green mutant (S65T) of GFP depends on the distance between the fluorophores and thereby might facilitate monitoring the proximity of the host protein subunits or domains.

The first-mentioned example to achieve and modulate FRET between FPs was to fuse a Blue GFP mutant (e.g. BFP) to a phenolate containing green GFP through an intervening protease-sensitive spacer (Heim *et al*. 1994). The broad emission spectrum of the donor BFP, peaking at 447nm, overlaps fairly well with the excitation spectrum of the GFP with a peak at 489nm. The tandem fusion consequently exhibits FRET, which is disrupted when a protease is added to cleave the spacer and allow the FPs to diffuse apart. In the investigation, P4-3 and Ser65Cys or Ser65Thr GFP mutants and a trypspin- or enterokinase- sensitive 25 residue linker have been used. The protease action has achieved a 4.6-fold increase in the ratio of blue to green emissions (Heim *et al*., 1996). Mitra *et al* used BFP5 (Phe64Met, Tyr66His) and RSGFP4 with a factor X_a-sensitive linker and have obtained a 1.9-fold increase in the analogous ratio (Mitra *et al*., 1996). In another study based on FRET, Periasamy *et al* demonstrated a static homodimerization of the transcription factor Pit-1 by coexpression of BFP-pit-1 and GFP-pit-1 fusion in HeLa cells (Periasamy *et al*., 1997). Following that study, Mahajan and coworkers (Mahajan *et al*., 1999) developed two novel fluorescent substrates that were specifically cleaved by caspase-1 or caspase-3. In their system, four-amino-acid recognition sequences, YVAD for caspase-1 and DEVD for caspase-3, have been introduced between BFP and GFP. Both of these fusion proteins have been expressed in bacteria. In addition, YVAD and DEVD have also

been introduced between cyan fluorescent protein and yellow fluorescent protein, and expression has been monitored in mammalian cells. These substrates have allowed the spatial activation of the specific caspase family members deciphered during the initiation and execution phase of programmed cell death. This technology is also likely to be useful for high-throughput screening of reagents that modulate caspase activity (*Figure 15a*). Moreover, the FRET-based reporter probe is designed to evaluate the changes in kinase and phosphatase activities within cells (Nagai *et al*., 2000; Ting *et al*., 2001; Zhang *et al*., 2001; Johnson *et al*., 2007). For example, a peptide with phosphorylation consensus sequence is inserted between the acceptor and the donor fluorescent proteins. The conformation change induced by phosphorylation leads to the FRET shift (*Figure 15b*). Another similar approach is based on the phosphorylation of peptide, which promotes the interaction of the two domains resulting in a shift in the fluorescence properties of the FRET construct (*Figure 15c*).

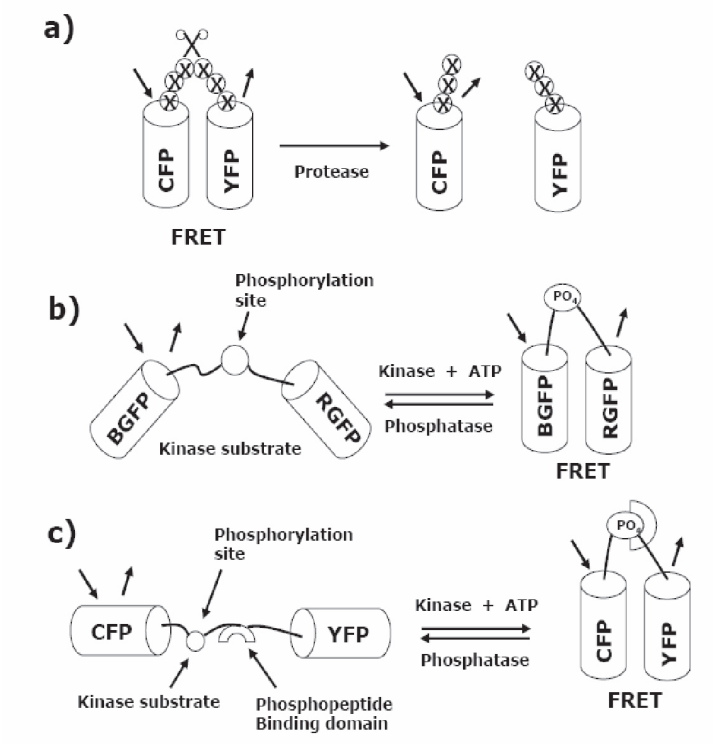

Figure 15. Several examples for FRET based on mutant variants of GFP. a). A FRET based reporter for the detection of protease activity. b). A FRET based reporter for kinase activity. c). FRET inducing conformational change of a CFP-YFP chimera as a result of the binding of the phosphorylation site of the kinase-substrate to a recombinant phosphopeptide domain following phosphorylation.

Since then, extensive efforts have been made to develop genetically encoded GFPs-based fluorescent sensors for efficient monitoring of various intracellular analytes such as Ca^{2+}, pH, Cl^{-}, membrane potential or specific proteins as well as to measure the activity of specific enzymes. (Miyawaki *et al*., 1997; Miyawaki *et al*., 1999). In these systems, GFP is fused to a target protein, which can undergo structural rearrange-

ment in the presence of these analytes, and hence induce the change in the fluorescent properties of GFP. Such sensors can be constructed by combing a FP with a detector such as Flash, which is one membrane potential sensor designed by inserting GFP into a voltage dependent Shaker K^+ channel (Siegel *et al*., 1997; Guerrero *et al*., 2002) or by inserting targeted domains into a FP (such as the Ca^{2+} sensor termed Camgaroo (Griesbeck *et al*., 2001) with calmodulin insertion). Other methods can be utilized by fusing targeted domains to the so-called "circularly permuted" FP (such as Pericam, GCaMP, MAOK activity sensor (Nakai *et al*., 2001; Kawai *et al*., 2004). The permutation of a FP places on fusion-sensitive domains closer to the chromophore can facilitate the transmission of any conformational changes to the chromophore environment. These permuted FRET-based GFP sensors have also been developed for measuring glucose (Fehr *et al*., 2003), maltose (Fehr *et al*., 2002), cAMP (Dipilato *et al*., 2004), cGMP (Honda *et al*., 2001), protease (Nagai *et al*., 2004) as well as EnvZ/OmpR (King *et al*., 2007).

Luciferase

Luciferase is a generic term for bioluminescent proteins that generate light by means of a chemical reaction with oxygen and a substrate. This enzyme is now emerging as one of the most commonly used reporters. The most commonly exploited luciferases are the terrestrial and marine bacterial luciferases, the eukaryotic firefly luciferases and *renilla* (sea pansy) luciferases (Hastings, 1996). The bacterial luciferases are the heterodimeric enzymes consisting of one 40KD α-subunit and one 37KD β-subunit. These two subunits are coded by the *luxA* and *luxB* genes, respectively (Baldwin *et al*., 1992; Stewart *et al*., 1992). These bacterial luciferases catalyze the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde in the presence of oxygen to yield blue light (Bronstein *et al*., 1994) (as showed in *Figure 16a*). This type of luciferase, however, is unable to generate continuous emission light as $FMNH_2$ can be rapidly oxidized in air. Furthermore, these genes are difficult to express in mammalian cells, bacterial luciferases have therefore found limited application as reporters for the gene expression.

Firefly luciferase is the most well known members of the luciferase family. With the molecular weight of 62KD, this enzyme catalyzes oxidation of a specific substrate, D-luciferin, in the presence of oxygen and ATP as the energy source. During the reaction, the chemical energy is released as visible light (blue to yellow-green in color) with broad-band emission spectra (530nm-640nm), peaking at wavelengths of 560 nm (Rice *et al*., 2001) (*Figure 16b*). *Renilla* luciferase (Rluc), another member of luciferase family originally comes from sea pansy (*Renilla reformis*), one bioluminescent soft coral that emits blue-green bioluminescence upon mechanical stimulation. Rluc is also widely distributed among coelenterates, fishes, squids and shrimps (Hastings, 1996). Compared with firefly luciferase, *renilla* luciferase does not require ATP and uses a different substrate, coelenterazine, as showed in *Figure 16c*. *Renilla* luciferase catalyzes the oxidation of coelenterazine and emits blue light with a spectral peak at 480 nm.

Both the firefly and *renilla* luciferase are excellent markers for gene expression, as they lack post-translational modifications, have absent endogenous proteins or enzymes and exhibit the fast enzymatic interactions. All these attributes render the

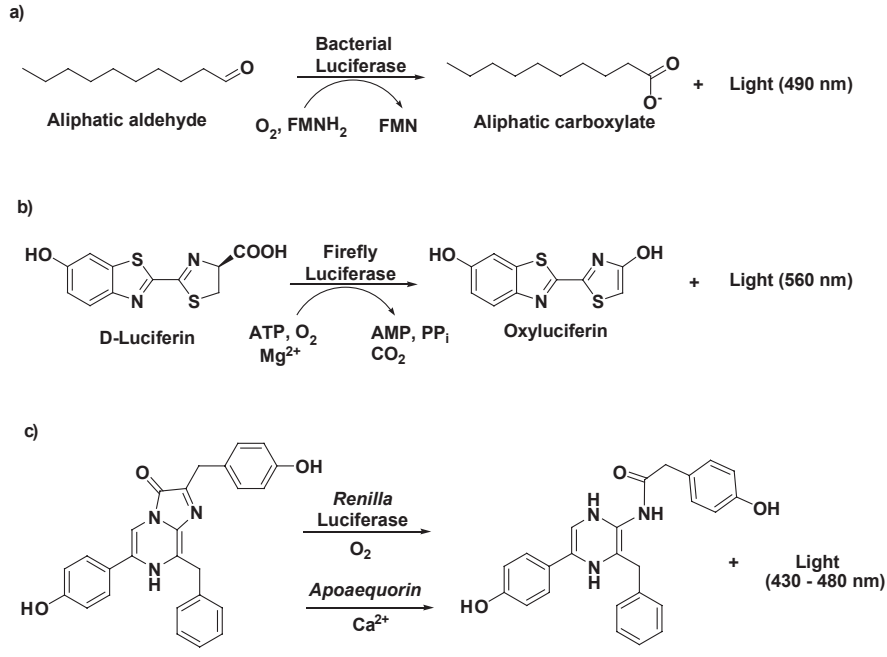

Figure 16. Bioluminescence substrates and enzymatic reactions of several common luciferases. a). the aliphatic aldehyde substrate of bacterial luciferase; b). structure and reaction of luciferin, the substrate of firefly luciferase; c). colenterazine, the substrate for Renilla luciferase and also part of apoaequorin.

luciferase reporter system a good choice for detecting the low-level gene expression such as analyzing of promoters with no or transient activity, and inspecting promoter function in cells that do not readily take up or express exogenous DNA (Selden *et al*., 1986). The broad-band emission spectra of luciferase-catalyzed reactions can also produce the longer wavelength of light (especially with a spectral content above 600 nm) that can penetrate through several centimeters of tissue (de Boer *et al*., 2006). Therefore, it is possible to detect light emitted at different timeframes for the internal organs of animals expressing luciferase as a reporter gene.

Luciferase activity is now widely used to track cells *in vivo* (Choy *et al*., 2003, Doyle *et al*., 2004), to monitor gene transcription (Jeffrey *et al*., 1985) in living animals and to detect protein–protein interactions (De *et al*., 2005). For example, hematopoietic stem cells (Edinger *et al*., 2003) and mature immune cells (Hildebrandt *et al*., 2004) could be monitored when they traverse lymphoid organs and other tissues in animal models in the presence of inflammatory or immune-related disorders. Another interesting application is real-time monitoring of tumor progression in animal models of cancer (Choy *et al*., 2003). Luciferase-labeled tumor cells could be tracked to follow all tumor behavior, including growth, regression and metastasis, which could be further used to monitor efficacy of therapeutic interventions. Several animal models of cancer, such as prostate (Kalikin *et al*., 2003; Scatena *et al*., 2004), breast (Caceres *et al*., 2003) and colon cancer (Zeamari *et al*., 2004), have been studied through this method. Recently, Yu *et al.* developed non-invasive and cell-label-free 'tumor-finding' luminescent viruses, which could potentially be adapted for clinical application (Yu *et al*., 2004).

Luciferase reporter gene can likewise be used as a proper system for monitoring gene expression *in vivo*. Ciana P *et al*. generated transgenic mice, which possessed the luciferase reporter gene under the control of an estrogen-responsive element (ERE-luc) (Ciana *et al*., 2003). Luciferase activity in individual mice paralleled estrogen cycles, which indicated that the peak transcriptional activity of the estrogen receptor occurred during proesterus in reproductive tissue (*Figure 17*). These ERE-luc mice facilitated kinetic monitoring and quantitative analysis of estrogen receptor activity in specific tissues. Zhang *et al*. also monitored inhibition of ubiquitin ligase Cdk2 with the use of the luciferase reporter system (*Figure 18*) (Zhang *et al*., 2004).

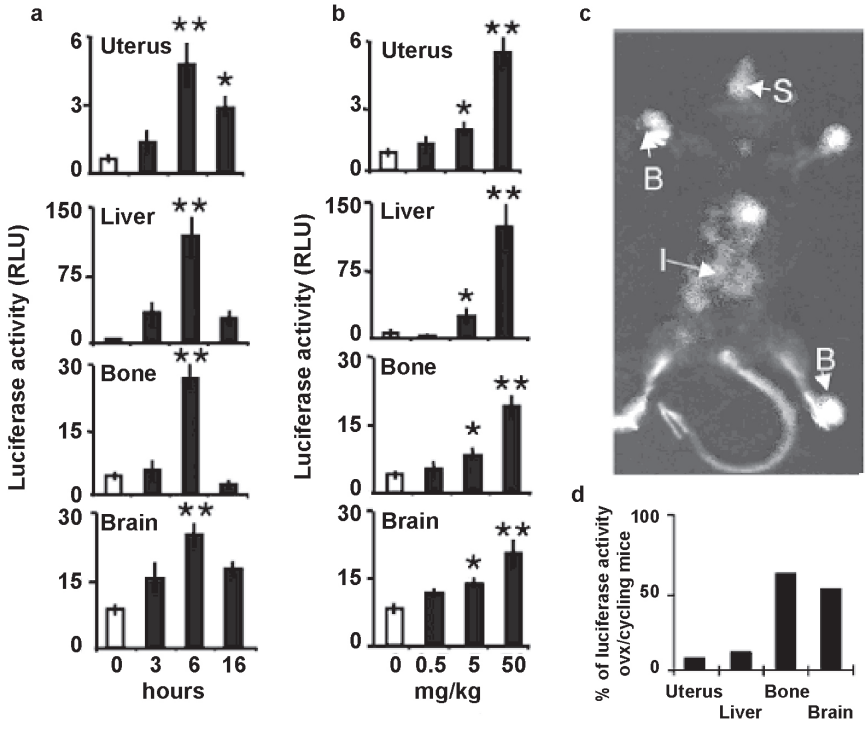

Figure 17. Effect of administration of exogenous 17 β-estradiol on ER activitiy. Mice were ovariectomized 3 weeks before the experiment**.** a). Time course of luciferase enzymatic activity after administration of 50 μg/kg estradiol. b). Dose dependency at 6 hr of treatment. Each bar represents the mean value ± s.e.m. of 5 animals. *p≤0.05; **p≤ versus controls (□). c). The bioluminescence of a paradigmatic untreated mouse 3 weeks after ovariectomy. d). Luciferase activity in ovariectomized (ovx) mice expressed as the percentage of the maximal activity detected in cycling female mice (proestrus for uterus and liver and diestrus for bone and brain). *n*=5. (Reprinted by copyright permission of the Nature Publishing Group)

Generally, regulation of various physiological and pathological processes in many biological signal pathways is based on protein-protein interactions (Ryan *et al*., 2005). Recently, several clever adaptations of luminescence imaging, such as bioluminescence resonance energy transfer (BRET) and luciferase complementation imaging (LCI) have been designed to facilitate the study of regulated protein-protein interactions in cells and living animals. BRET was first observed as a natural phenomenon in the jellyfish *Aequorea*, in which green fluorescent protein accepted the blue light emitted by the calcium-sensitive luminescent protein aequorin, resulting in emission of green

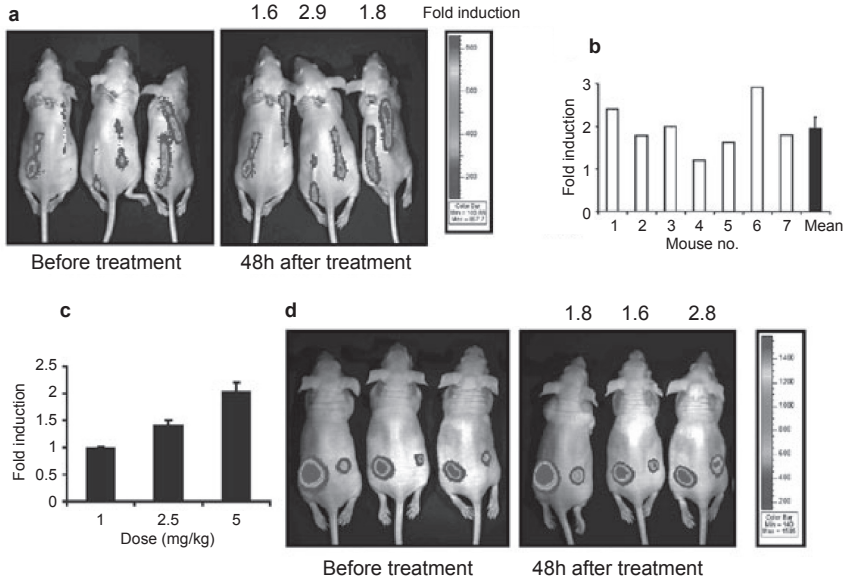

Figure 18. Monitoring Cdk2 inhibition in vivo using bioluminenscent imaging. (a) Hollow fibers filled with polyclonal U2OS cells producing luciferase (Luc; left flank) or p27Luc (right flank) were implanted subcutaneously into nude mice. Left, baseline bioluminescent images taken 7d later. Right, repeat images obtained after two doses of flavopiridol (5 mg/kg, once a day by intraperitoneal injection). (b) Normalized fold induction (p27Luc/Lucpost-treatment ÷ p27Luc/Lucpretreatment) of p27Luc in seven mice after treatment with flavopiridol. (c) Normalized fold induction of p27Luc in mice treated with the indicated doses of flavopiridol (eight mice per treatment group) and imaged as in a. (d) Polyclonal H1299 cells producing luciferase (left flank) or p27Luc (right flank) were injected subcutaneously into nude mice. Left, bioluminescent images were obtained 6 weeks later, when tumors of comparable size (5 mm) had formed bilaterally. Right, repeat images were obtained after two doses of flavopiridol (5 mg/kg, once a day by intraperitoneal injection). Fold induction was calculated as p27Luc/Lucpost-treatment ÷ p27Luc/Lucpretreatment. Error bars indicate standard error (s.e.m.). (Reprinted by copyright permission of the Nature Publishing Group)

light rather than blue light specific for aequorin (Gorokhovatsky *et al*., 2004). Coupled with protein fusion technology, BRET has been applied to detect protein-protein interactions as showed in *Figure 19*. Two proteins are fused to *renilla* luciferase and fluorescent protein (e. g. Yellow FP), respectively. If there is no interaction, only one emission from the luciferase can be detected after addition of its substrate coelenterazine. If they interact, then resonance energy transfer occurs between the luciferase and the YFP, and an additional signal, emitted by YFP, can be detected (Boute *et al*., 2002). Recently, BRET has been used for real-time monitoring the association of V2-vasopressin receptor and β-arrestin induced by a receptor agonist (Coulon *et al*., 2007), measuring interaction between full-length ER and coregulator proteins (Koterba *et al*., 2006) and detecting the dimerization of G-protein-coupled receptors *in vivo* (Angers *et al*., 2000). More recently, Gambhir *et al* employed the BRET system with *renilla* luciferase (Rluc) as the bioluminescent donor and the mutant GFP as the fluorescent acceptor to demonstrate the ability for detecting the signal from protein-protein interactions in cultured cells, as well as from the surface to deeper tissues of living animals (as showed in *Figure 20*) (De *et al*., 2005; Massoud *et al*., 2007).

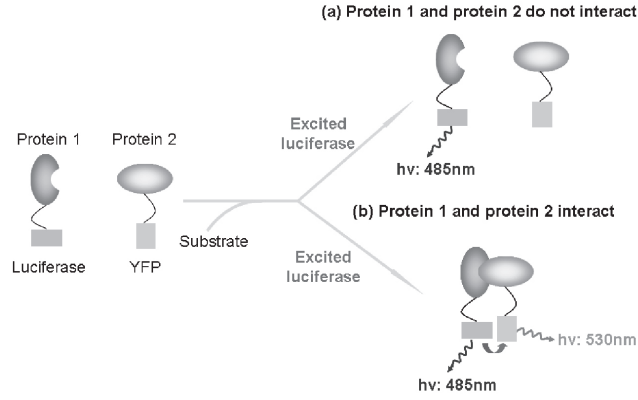

Figure 19. Principle of the bioluminescence resonance energy transfer (BRET) technology**.** To study the interaction between two proteins, protein 1 is fused to a luciferase and protein 2 is fused to a fluorescent protein [e.g. yellow fluorescent protein (YFP)]. The reaction is initiated by addition of the substrate of luciferase, coelenterazine. (a) If the distance between protein 1 and protein 2 is greater than 100 Å, light is emitted with an emission spectra characteristic of the luciferase. (b) If the distance between protein 1 and protein 2 is 10–100 Å, energy (not photons) is transferred from the luciferase to the YFP, resulting in an additional signal emitted by the YFP.

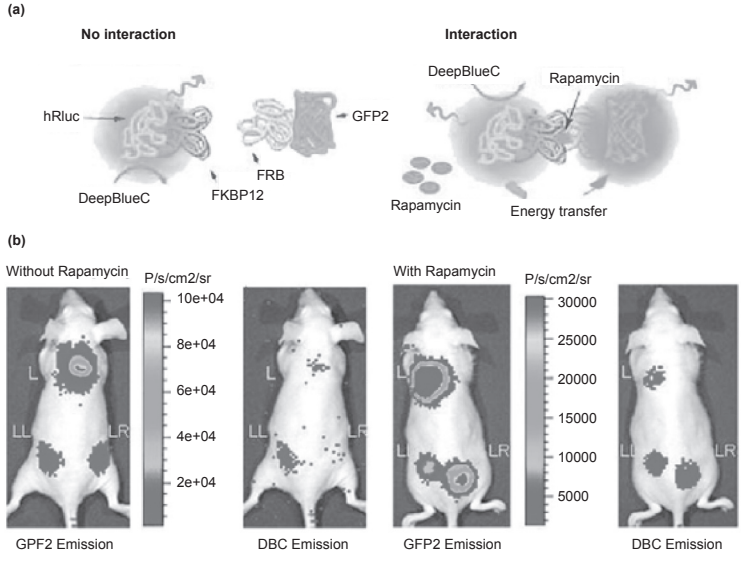

Figure 20. Imaging protein-protein interactions in living mice using BRET**.** a) Schematic showing small molecular mediated protein-protein interactions leading to bioluminescence resonance energy transfer (BRET). FKBP12 is fused to the N-terminus of Rluc donor protein, and FRB is fused to the C-terminus of GFP acceptor. When the genes encoding both of these fusion proteins are expressed inside cells and rapamycin is present to mediate the FRB-FKBP12 interaction, then resonance energy transfer occurs. This BRET signal can be detected using the deep blue coelenterazine (DBC) substrates for Rluc. b) Detection of the in vivo BRET signal from specific protein-protein interactions. Dorsal view of a nude mouse implanted subcutaneously with 5×10^6 293T cells either transiently transfected with pBRET (L) or with pFKBP12-hRluc (LL) alone or co-transfected with pFKBP12-hRluc and pGFP-FRB (LR) in the presence of rapamycin. Mice that received the small-molecule mediator drug, rapamycin (5mg/kg) were injected intraperitoneally immediately after cell implantation. The scan was performed 7h after drug administration. Mice were scanned for 5 min integration time using either GFP or DBC filters in succession by injecting with 25 μg DBC intravenously. (Reprinted by copyright permission of the Elsevier Limited)

Rao *et al*, demonstrated that in addition to fluorescent proteins, quantum dots, a type of fluorescent semiconductor nanocrystals, can also serve as BRET acceptor. By this approach, they created a novel self-illuminating conjugate that is suitable for *in vivo* imaging. Compared with traditional organic fluorophores, quantum dots (QDs) have extreme advantages in their high quantum yield and photostability, size tune-up fluorescence, extensive applications in near infrared windows as well as their broad excitation features but narrow emission properties which are suitable for optical imaging and multiplexing (Michalet *et al*., 2005). In their design, the conjugates were prepared by coupling carboxylate-presenting quantum dots to a mutant of the bioluminescent protein *renilla reniformis* luciferase (as showed in *Figures 21* and *22*). They showed that the conjugates emitted long-wavelength (from red to near-infrared) bioluminescent light in cells and in the living animals, even in deep tissues, and thus were suitable for *in vivo* imaging. Compared with existing quantum dots, self-illuminating quantum dot and luciferase conjugates exhibited greatly enhanced sensitivity in small animal imaging, with an *in vivo* signal to background ratio of > 10^3 for 5pmol of the conjugate.

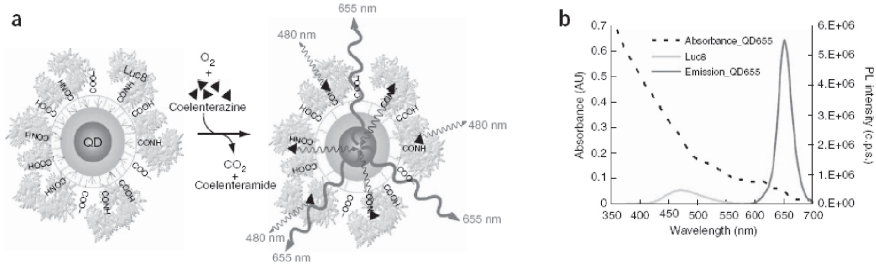

Figure 21. Design and spectroscopic characterization of bioluminescent quantum dot conjugates based on BRET. a). A schematic of a quantum dot that is covalently coupled to a BRET donor, luc8. The bioluminescence energy of Luc8-catalyzed oxidation of coelenterazine is transferred to the quantum dots, resulting in quantum dot emission. b). Absorption and emission spectra of QD655 (λ_{ex} =480nm), and spectrum of the bioluminescent light emitted in the oxidation of coelenterazine catalyzed by Luc8.

Aside from BRET, luciferase complementation imaging (LCI) is another powerful approach for detection of protein–protein interactions. In this method (shown in *Figure 23*), two proteins are tagged with complementary fragments of a luciferase, which are inactive when separate, yet when brought together, reconstitute luminescence activity (Luker *et al*., 2004). LCI has been applied to monitor protein-protein interactions, not only in cell culture, but also in living subjects. Paulmurugan *et al.* firstly imaged the interactions of two nuclear proteins Id and MyoD in nude mice implanted with transiently transfected cells. The signal of the MyoD–Id interaction has been detected through reconstitution of split firefly luciferase attached to them (Paulmurugan *et al*., 2004). In addition, a similar *renilla* luciferase reporter-based complementation strategy has been used to monitor rapamycin-induced protein-protein interactions in living mice (Paulmurugan *et al*., 2002). More recently, they developed a novel fusion protein approach to investigate the rapamycin-mediated interaction of fused FRB and FKBP12 with either split hRluc or split EGFP, to archive a system with greater sensitivity for detecting lower levels of drug-mediated protein-protein interactions *in vivo* (Paulmurugan *et al*., 2005). Separately, Lucker *et al*. described a systematic

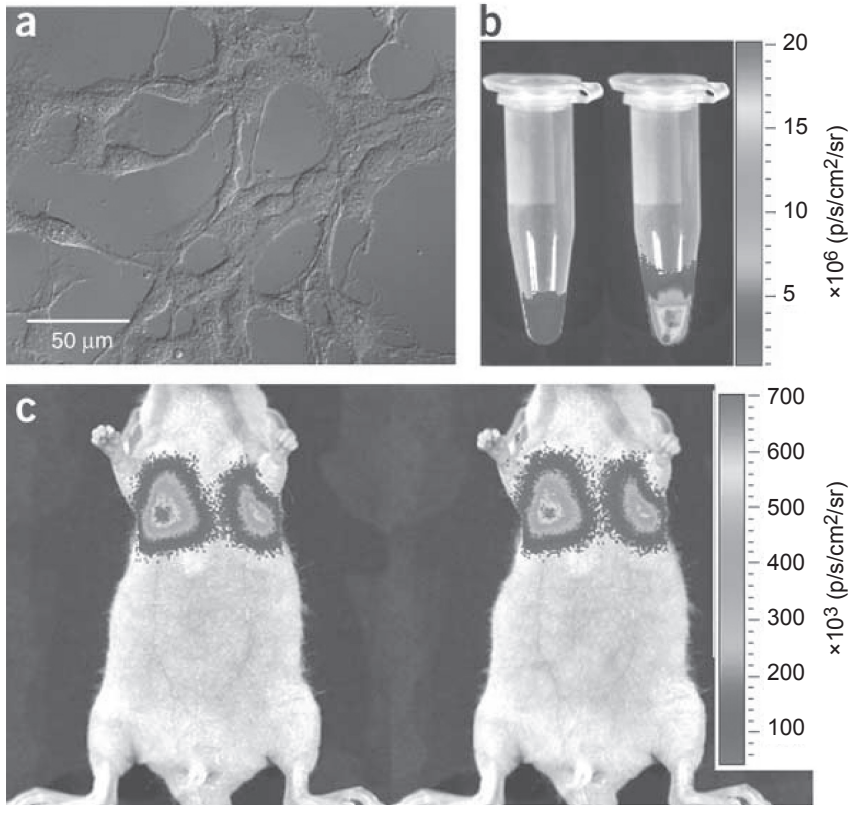

Figure 22. Imaging C6 glioma cells labeled with QD655-Luc8-R9 in vitro and in mice. a) Overlay of fluorescence and differential interference contrast (DIC) images of QD655-Luc8-R9–labeled C6 glioma cells. Fluorescence image was collected with the following filter set (Chroma Technology): excitation, 420/40; emission, D660/40; dichroic, 475DCXR. Scale bar, 50 µm. b) Representative bioluminescence images of labeled cells acquired with a filter (575–650 nm) (left) and without any filter (right). c) Representative bioluminescence images of a nude mouse injected via tail vein with labeled cells, acquired with a filter (575–650 nm) (left) and without any filter (right).

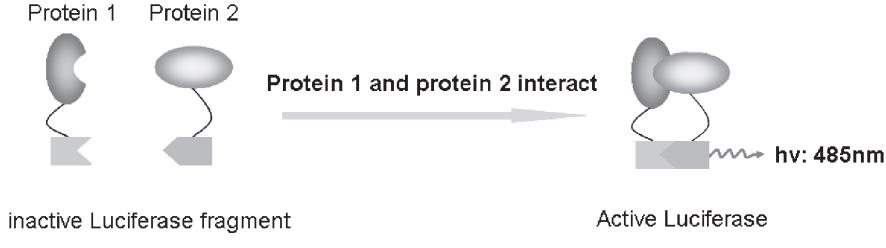

Figure 23. The scheme of luciferase complementation imaging (LCI)**.** In this method, the two proteins under study are tagged with complementary fragments of a luciferase, each inactive on its own, which combine to reconstitute luminescence activity when the two proteins associate.

truncation library yielding alternative complementary N-and C-terminal fragments of Fluc (nFluc residues 2-416 and cFluc residues 398-550). These fragments were used to monitor the rapamycin-mediated interaction of rapamycin-binding proteins FRB and FKBP12 (Luker *et al*., 2004).

β-lactamase (Bla)

β-lactamases (Blas) are well known as a family of bacterial enzymes that cleave penicillin and cephalosporin-based antibiotics with high catalytic efficiency and render these bacteria resistant to β-lactam antibiotics. β-lactamases can be classified into four molecular classes (A through D) on the basis of their primary structure or their substrate spectrum and response to inhibitors. Class A, C and D β-lactamases are the most common and all have a serine residue at the active site. Class B comprises the metallo-β-lactamase. Since these special enzymes exhibit the properties to resist the antibiotics in clinics, up to date, considerable effort has been expended in clinical studies to develop new antibacterial agents for circumventing the bacterial resistance caused by β-lactamase expression. On the other hand, β-lactamase has been exploited as a new biological reporter for biodetection and optical imaging of gene expression *in vitro* and *in vivo*.

A well-characterized plasmid encoded TEM-1 β-lactamase (Bla) from *Escherichia coli,* the monomeric isomer products of the ampicillin resistance gene (‘*amp’*), has proven to be an attractive biosensor for detecting biological processes and protein-protein interactions *in vitro* and *in vivo* because of its novel properties, such as being small size (29kD) to be easily expressed in eukaryotic cells without noticeable toxicity and with no interference from mammalian enzymes (Philippon *et al*., 1998; Campbell, 2004). Upon the reaction with the substrates which may generate the chromogenic and/or fluorescent signals, β-lactamases can serve as one sensitive reporter gene with distinctive advantages for a wide variety of specialized and general assays. Nitrocefin and PADAC are two commonly used chromogenic substrates that undergo distinctive color change from yellow to red as the amide bond in the β-lactam ring is hydrolyzed by β-lactamase. They are useful for detection of β-lactamases produced by gram-positive and gram-negative bacteria, for the detection of β-lactamase patterns from bacterial cell extracts (Day *et al*., 2004), and for reporting β-lactamase in tissue culture (*Figure 24*) (Livermore *et al*., 2001).

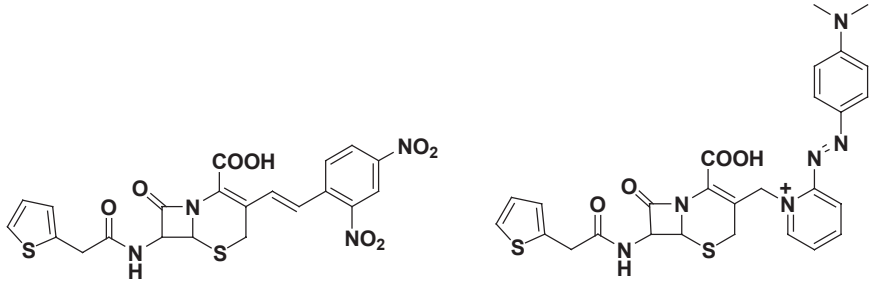

Figure 24. Structures of Nitrocefin and PADAC.

Although colorimetric substrates for Bla have been utilized extensively, however, the sensitivity of the colorimetric assays is not sufficient compared to the fluorescent assays. The first application of Bla in the single living cells was realized by Tsien *et al* with one sensitive and membrane-permeable fluorogenic cephalosporin-type substrate, CCF2/AM (*Figure 25*) (Zlokarnik *et al*., 1998). The basic design of the substrate relies on the intramolecular Fluorescent Resonance Energy Transfer (FRET). The donor fluorophore in the 7'-position of cephalosporin in CCF2/AM is 7-hydroxycoumarin with a 6-chloro substituent. Fluorescein, as the acceptor, is attached at the 3'-position through a stable thioether linkage which minimizes the rate of spontaneous aqueous hydrolysis. After diffusion of non-polar CCF2/AM through the plasma membrane, four labile esters are hydrolyzed by non-specific intracellular esterases to generate CCF2. If the cell is not expressing the TEM-1 Bla, the intact molecule of CCF2 will emit green fluorescence at 520nm when excited at 409 nm, owing to FRET between the coumarin donor and the fluorescein acceptor. However, if Bla is present in the cytoplasma, CCF2 will be readily hydrolyzed, resulting in disrupt of the FRET and a dramatic increase in the blue coumarin fluorescence (447nm). CCF2 exhibits good catalytic properties and can sensitively detect 5fM of β-lactamase *in vitro*. Noninvasive imaging of blue and green fluorescence emission generates a ratiometric signal that is minimally influenced by varieties in cell lines and substrate uptake. CCF2/AM is able to examine the promoter/regulator activities in living mammalian tissue culture cells (Day *et al*., 2004) and to monitor constitute and inducible proteins interactions (Galarneau *et al*., 2002; wehrman *et al*., 2002; Spotts *et al*., 2002).

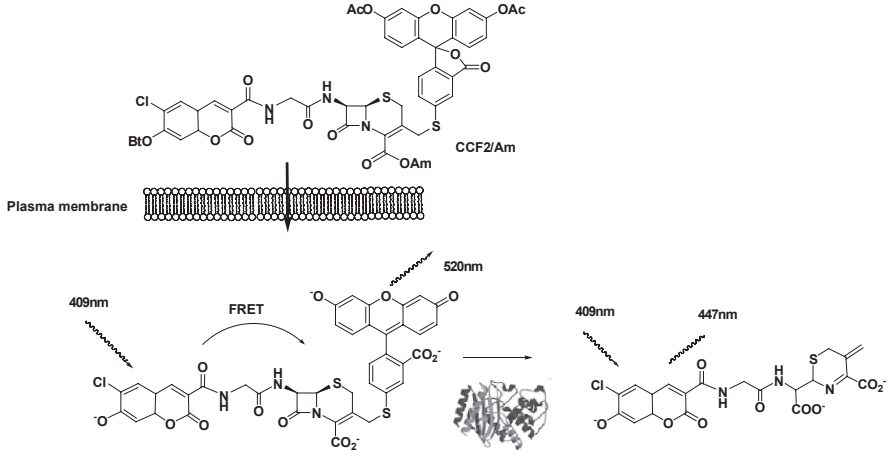

Figure 25. Mechanism of action in the FRET in CCF2/AM [Zlokarnik *et al.*, 1998]

Recently, Rao reported that CCF2/AM and Bla could image tetrahymena ribozyme splicing activity in living cells. This reporter system allowed high throughput screening of single living mammalian cells for a direct and facile selection of desired ribozyme variants *in vivo* (*Figure 26*) (Hasegawa *et al*., 2002; Hasegawa *et al*., 2004). Similarly, Zuck (Zuck *et al*., 2004) presented a cell-based reporter assay for the identification of inhibitors of hepatitis C virus replication by using CCF2/AM. Leppla and Bugge (Hobson *et al*., 2006) developed a simple, sensitive and noninvasive assay that used reengineered anthrax toxin-β-lactamase fusion proteins with altered proteases

cleavage specificity to visualize specific cell-surface proteolytic activities in single living cells. The assay could be used to specifically image endogenous cell-surface furin, urokinase plasminogen activator metallo-protease activity. Cunnningham *et al* explained that polystyrene beads could be successfully utilized for establishing fluorescent-activated cell sorting to sort cells with the Bla reporter gene by using the substrate CCF2 (Cunningham *et al*., 2005). Lippard demonstrated the efficient screening over 3600 reaction products of platinum based antitumor drugs for their ability to inhibit transcription of Bla in the BlaM HeLa cell line by monitoring the cleavage of CCF2/AM. Four species were identified among the reaction products from this screening, three of them are previously determined active cisplatin analogues and another one represents a new kind of antitumor drug candidate similar to ZD0473, a recently reported antitumor analogue (Ziegler *et al*., 2000).

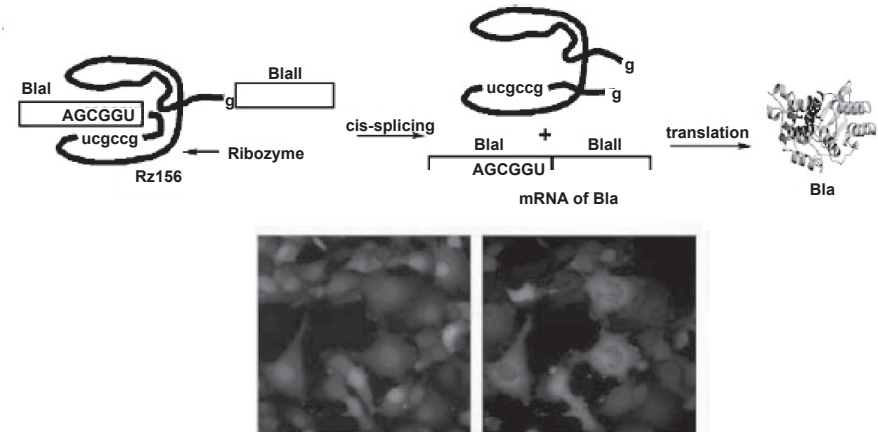

Figure 26. Scheme presentation of splicing-dependent β-lactamases (Bla) reporter gene strategy. The ribozyme reporter Rz-156 consists of the *tetrahymena* intron and a broken Bla ORF (BlaI and BlaII). Ribozyme self-splicing produces uninterrupted mRNA of β-lactamases, which is translated into the reporter enzyme: β-lactamases. And the fluorescent imaging of the COS-1 cells transfected with Rz156.

Rao and coworkers (Gao *et al*., 2003) have also developed a new class of small fluorogenic substrates to expand the application of Bla as a biosensor. These small fluorogenic substrates work by releasing a phenolic fluorescent dye from a vinylogous cephalosporin. In their design, 7'-hydroxy of umbelliferone or resorufin were connected to the 3'-position of cephalosporin through an allylic ether bond. The parent substrates become essentially non-fluorescence due to alkylation of the phenolic group of the fluorescent dyes. Treatment by β-lactamase leads to spontaneous release of fluorescent molecules and results in the production of fluorescent signals. These types of substrates exhibit fast kinetics toward Bla. The stability of substrates in the absence of enzyme can be further improved by oxidation of sulfide in the six-membered ring of the cephalosporin nucleus to sulfoxide counterparts. Based on this design, a membrane-permeable acetoxymethyl ester of the resorufin substrate, CR2 (CR2/AM) was able to image the Bla gene expression in Bla-stably transfected C6 Glioma cancer cells as shown in *Figure 27*.

These small molecular weight β-lactam fluorogenic substrates work well with imaging β-lactamase *in vitro* and in cell cultures, but not in intact tissues or whole organisms

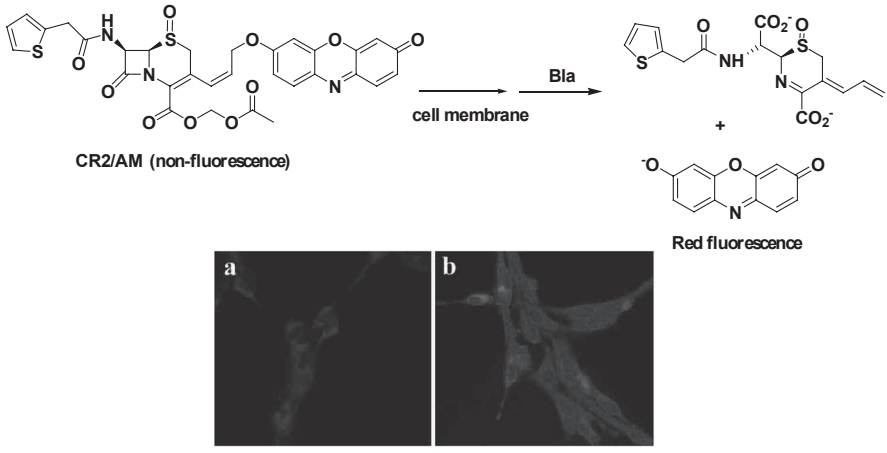

Figure 27. Fluorescent images of wild-type (a) and Bla-stably transfected (b) C6 glioma cells incubated with CR2/AM (Gao *et al.*, 2003)

because of the poor tissue penetration, absorbance by hemoglobin and scattering of light with short excitation and emission wavelength. Over the past decade, infrared and/or near-infrared spectroscopy has attracted a great deal of attention in molecular imaging studies for living subjects because its long wavelength may lead to less photo damage to cells, produce less autofluorescent background and provide better sensitivity and tissue penetration (Weissleder, 2001). Rao *et al* (Xing *et al*., 2005) developed a new class of small cell-permeable near-infrared (NIR) fluorogenic β-lactam substrates for β-lactamase detection and their application in imaging gene expression in living mammalian cells. On the basis of their design, one carbocyanine dye Cy5 with maximum emission at 670nm and another quenching group Qsy21 with maximum absorbance at 660nm was chosen as a FRET pair (*Figure 28a*). Cy5 was tethered to the 7'-amino of the cephalosporin through a glycyl linkage, Qsy21 was connected to the 3'-position through a linker of amino thiophenol and cysteine residue. As a good leaving group, the amino thiophenol at the 3'-position would facilitate the fragmentation after enzyme hydrolysis. Introduction of one fully acetylated D-glucosamine in the cephalosporin nucleus (CNIR4) was found to improve the staining of the Bla stably transfected C6 glioma cells presumably with involvement of endocytosis in the uptake (*Figure 28b*). All the NIR flurogenic β-lactam substrates were stable in aqueous buffer and exhibited reasonable catalytic efficiency toward enzyme treatment. More recently, Rao *et al*. (Yao *et al*., 2007) reported the first bioluminogenic substrate (Bluco) for β-lactamase activity in the living animals through the coupling of D-Luciferin, one commonly used substrate for the bioluminescent enzyme firefly luciferase (fLuc), to the 3'-position of cephalosporin via ether bond. Their *in vivo* imaging results indicated that about 15-25 folds signal contrast could be identified after administration of Bluco via tail vein injection into mice with implanted tumors on the left and right rear thigh with the Bla and fLuc cotransfected or fLuc transfected COS7 cell line respectively (*Figure 29*). The maximum emission could be detected at about 30 min post-injection. As the first developed bioluminogenic substrate, Bluco has proven useful for *in vivo* optical imaging of the β-lactamase expression.

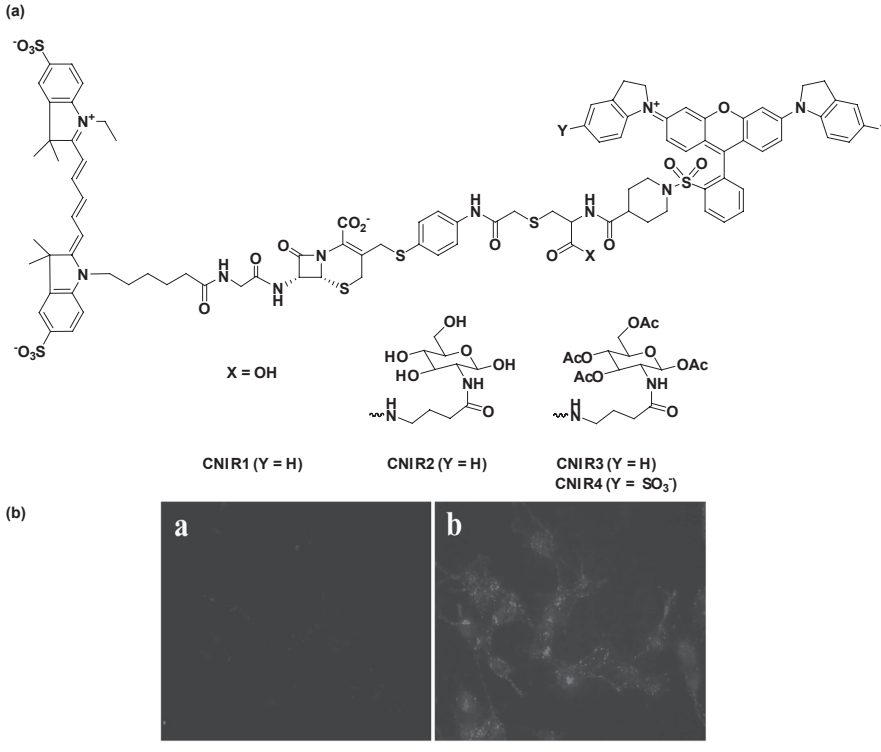

Figure 28. A). Structures of CNIR1, CNIR2, CNIR3 and CNIR4; B). Fluorescent imaging of wild-type (a) and Bla stably transfected (b) C6 Glioma cells staining with CNIR4 (Xing et al., 2005).

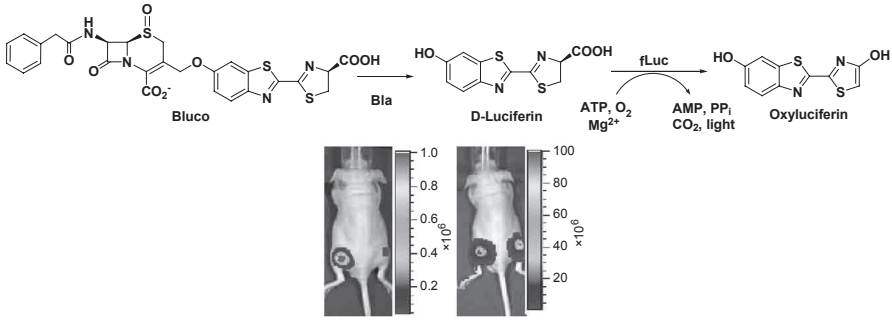

Figure 29. Structure of Bluco and bioluminogenic imaging of Bla expression in living mice (Yao et al., 2007).

Conclusions

Precisely and selectively deciphering the biological specificities often requires the development of simple, specific and sensitive approaches for noninvasive visualization of gene expression and signal transduction at the single cell or organismal level.

The biological reporter technology is such an ideal approach, which has been proven effective and already plays a key role in gene expression regulation studies. Among the most commonly used reporter genes, chloramphenicol acetyltransferase (CAT) is the first one used for detection of reporter expression. CAT is very stable and does not have an endogenous expression in mammalian cells. However, the assay must rely on radiochemicals, which are not broadly used compared to other reporters. Alkaline phosphatase (AP) has the advantage that the assay can make the detection straightforward by sampling culture medium directly. But AP expression can be detected in virtually all mammalian cell types, the use of AP as a reporter is limited due to high background levels of endogenous protein or enzyme activities. β-galactosidase (β-gal) is a well-characterized enzyme and has been one of the most widely used reporter genes in molecular biology, particularly in the study of transfection efficiency. This reporter system, nevertheless, requires more efficient cell permeabilization; the endogenous activity in mammalian cells can also be detected. GFPs have been widely used for noninvasive monitoring of gene expression in living tissues, specifically with the advent of GFP gene cloning and the availability of several GFP mutants. One major disadvantage of GFP labeling, however, is the size of the fused protein. It is problematic to append a protein of large size to another protein without influencing the desired functions. In addition, the fluorescent protein and its variants are very susceptible to external factors such as temperature or oxygen concentration etc. The fluorescence generated from fluorescent proteins is likewise relatively insensitive due to the lack of enzymatic amplification. The firefly luciferase has been one of the most popular reporter genes because of its high sensitivity, broad linear range (up to 7-8 orders of magnitude) and no endogenous activity in mammalian cells. One major limitation for the luciferase reporter system is that the assay typically lacks of single cell resolution. β-lactamase has demonstrated promising applications in optical imaging for detection of gene expression. Quite a few β-lactam derivatives have already been prepared and provide high sensitivity for the single cell analysis. However, the existed fluorescent or luminescent substrates for imaging β-lactamases are difficult to prepare, and have other drawbacks such as high molecular weight, poor cell permeability, and limited imaging contrast. The small but powerful probes will still be highly desirable.

Even with the potential limitations that have been outlined for these biological reporters, the advent of reporter gene methodology has nevertheless greatly enhanced our ability to evaluate gene expression in living cells and animals. As simpler, more rapid, and more sensitive assays continue to evolve, and new reporter genes and more sophisticated vectors are designed and marked, combined with advanced detection methods, reporter gene technology will continue to remain as one of the most useful methods for understanding intracellular signaling transduction, molecular basis of diseases, and therapeutic evaluation. Furthermore, such technology can also provide both novel targets and high throughput screening platforms for the discovery of new therapeutics in gene therapy and drug development.

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