Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

Creating bioluminescent indicators to visualise biological events in living cells and animals

Sung Bae Kim[a]; Takeaki Ozawa[bc]
[a] Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan [b] Department of Chemistry, School of Sciences, The University of Tokyo, PRESTO, Tokyo, Japan [c] Science and Technology Agency, Saitama, Japan

Online publication date: 23 June 2010

To cite this Article Kim, Sung Bae and Ozawa, Takeaki(2010) 'Creating bioluminescent indicators to visualise biological events in living cells and animals', Supramolecular Chemistry, 22: 7, 440 — 449
To link to this Article: DOI: 10.1080/10610278.2010.485251
URL: http://dx.doi.org/10.1080/10610278.2010.485251

Supramolecular Chemistry
Vol. 22, Nos. 7–8, July–August 2010, 440–449

Taylor & Francis
Taylor & Francis Group

Creating bioluminescent indicators to visualise biological events in living cells and animals

Sung Bae Kim[a] and Takeaki Ozawa[bc]*

[a]*Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba 305-8569, Japan;* [b]*Department of Chemistry, School of Sciences, The University of Tokyo, PRESTO, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan;* [c]*Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

(*Received 10 February 2010; final version received 9 April 2010*)

Dynamic molecular processes inside animal cells are controlled specifically by key proteins, the activities of which occur in the timescales from milliseconds to several hours. To understand such spatial and temporal processes, development of optical probes for analysing cellular events is a challenging task in recent studies. Complementation and reconstitution from split fragments of luminescent proteins present a useful technique for detection of intracellular signalling in living cells and animals. We specifically examine recent advances in protein-fragment complementation and reconstitution strategies for illuminating intracellular molecular events, using genetically encoded bioluminescent probes. The probes are introduced into specific tissues, cell types or subcellular compartments, thereby allowing elucidation of biological events in considerable detail.

Keywords: bioluminescence; protein–protein interactions; luciferase; imaging

1. Introduction

Eukaryotic cells comprise numerous proteins and lipids whose functions and molecular dynamics inside the cells are elaborate and highly specialised in every process in signalling cascades. To disclose molecular events, particular proteins governing the molecular events must be traced with high spatial resolution in real time. Nevertheless, it has remained nearly impossible to visualise target proteins of interest non-invasively in living animals and plants. In the past decade, fluorescent and bioluminescent proteins have revolutionised the field of biomolecular imaging.

In 1962, Dr Shimomura first discovered the green fluorescent protein (GFP) in a jellyfish, *Aequorea victoria* (*1*). Actually, GFP has become well established as a marker of gene expression and molecular events in intact cells and organisms (*2*). The chromophore of GFP is a *p*-hydroxybenzylidene-imidazolinone, which is formed from the residues of Ser65-Tyr66-Gly67 in the native protein (*3*, *4*). The chromophore is surrounded by 11 strands of β-barrels and two α-helixes, which form a can-like structure (Figure 1(A)). The optical character of the wild-type fluorescent protein was improved through mutagenesis to achieve brighter emission, faster chromophore maturation, temperature stability and a wavelength shift of the emission spectrum. Now, numerous fluorescent proteins, ranging in spectra from blue to far-red, are available for practical use as fluorescent proteins. Among many applications of fluorescent proteins, a technique of fluorescence resonance energy transfer (FRET) is an attractive method to visualise biological functions in living cells (*5*). The FRET techniques are based on changes in the fluorescence intensity or lifetimes of two fluorophores that are brought sufficiently close together. The FRET techniques using fluorescent proteins were started in 1997 (*6*) and are now widely used for imaging molecular events in real time.

Another fluorescence technique is fluorescent protein-fragment complementation and reconstitution analysis (*7*, *8*), which has emerged as a new tool for visualising molecular events, particularly those involving molecular interactions in living cells. The basic strategy of the complementation is to split a fluorescent protein into two non-fluorescent fragments that are fused to a pair of interacting proteins. Interactions between the two proteins bring two fragments of a fluorescent protein into proximity, allowing reconstitution of an intact fluorescent protein. The techniques have been applied not only for imaging protein–protein interactions but also for formation of enzyme–substrate complexes, localisation of multiple protein complexes (*9*), interactions involving post-translational modifications (*10*), screening of interacting proteins (*11*), protein folding and aggregation (*12*, *13*), localisation and dynamics of RNA (*14*), etc.

In addition to fluorescent proteins, bioluminescent proteins named luciferases have been applied to analyses of gene functions and biological events in living cells. Luciferases are a family of light-emitting proteins that can

*Corresponding author. Email: ozawa@chem.s.u-tokyo.ac.jp

ISSN 1061-0278 print/ISSN 1029-0478 online

DOI: 10.1080/10610278.2010.485251
http://www.informaworld.com

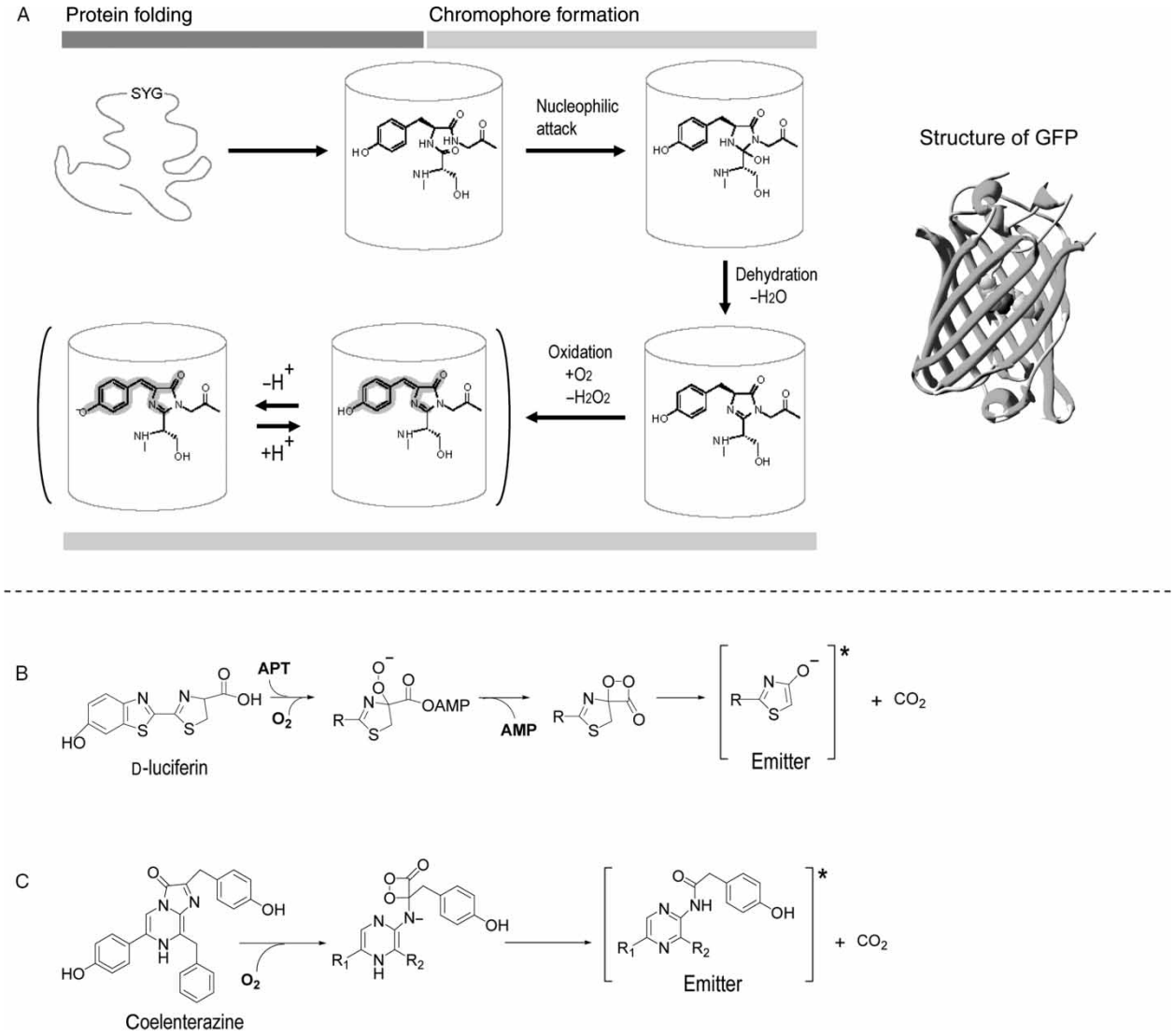

Figure 1. (A) Diagram representing folding and chromophore formation of *Aequorea* GFP. The right panels show the crystal structure of GFP. (B) Oxidation reaction of D-luciferin by luciferases to emit bioluminescence. (C) Oxidation reaction of coelenterazine by luciferases to emit bioluminescence.

be isolated from a large variety of insects, marine organisms and prokaryotes (*15*, *16*). The light emission occurs in many physiologically diverse groups, although the evolutionary origins of bioluminescent proteins remain obscure. A striking characteristic of bioluminescent proteins is the extremely high diversity of mechanisms, structures and functions that bioluminescent organisms have achieved. This high diversity suggests multiple independent origins of bioluminescence over the course of evolution (*17*). Unlike GFP or its spectral variants, luciferases are enzymes that catalyse the oxidation of a substrate named luciferin (Table 1). The emission wavelengths are 460–623 nm, depending on the luciferases and substrates.

The most well-known family of luciferases is that of beetles. Beetle luciferases including firefly luciferase and click beetle luciferase mediate oxidisation of D-luciferin in the presence of ATP and Mg^{2+} (Figure 1(B)). Beetle luciferases are classified into pH-sensitive and pH-insensitive luciferases. With decreasing pH or increasing temperature or concentration of heavy metal ions, pH-sensitive luciferases such as firefly luciferase undergo a red shift, although luciferases from the click beetle are insensitive to those conditions (*16*).

Marine luciferases, e.g. those derived from *Gaussia princeps* and *Metridia longa*, catalyse the oxidation of the small molecule coelenterazine to produce light. Unlike the beetle luciferase, these coelenterazine-utilising luciferases require no accessory molecules such as ATP and Mg^{2+} in enzymatic reactions (Figure 1(C)). The other luciferase from single-celled algae such as the tropical dinoflagellate *Pyrocystis fusiformis* emits short flashes of bioluminescence when disturbed. The chemical reaction occurs when

Table 1. Properties of representative luciferases.

Luciferase	Origin	Substrate	Required components	Molecular weight (kDa)	λ_{max} (nm)[a]	pH sensitivity	Reference
Firefly luciferase (FLuc)	*Photinus pyralis*	D-Luciferin	ATP, Mg^{2+}, O_2	61	562 nm	Sensitive	(*46*)
Renilla luciferase (RLuc)	*Renilla reniformis*	Coelenterazine	O_2	36	480 nm	Sensitive	(*47*)
Gaussia luciferase (GLuc)	*G. princeps*	Coelenterazine	O_2	20	480 nm	Sensitive	(*48*)
Click beetle luciferase (CBLuc)	*P. plagiophthalamus*	D-Luciferin	ATP, Mg^{2+}, O_2	65	540 nm (Green)	Relatively insensitive	(*49*)
Railroad worm luciferase (RWLuc)	*Phrixotrix viviani and Phrixotrix hirtus*	D-Luciferin	ATP, Mg^{2+}, O_2	61 (Green) 60 (Red)	546 nm (Green) 623 nm (Red)	Relatively insensitive	(*50*)
Metridia luciferase (MLuc)	*M. longa*	Coelenterazine	O_2	24	480 nm	Sensitive	(*51*)
Cypridina (*Vargula*) luciferase (VLuc)	*Vargula hilgendorfii*	Cypridina luciferin	O_2	62	460 nm	Sensitive	(*52*)
Oplophorus luciferase (OLuc)	*Oplophorus gracilirostris*	Coelenterazine	O_2	Complex of 35 and 19	454 nm	Sensitive	(*53*)

[a]Maximum wavelength of bioluminescence spectrum.

a compound of luciferin (a derivative of chlorophyll) is oxidised by the luciferase in the presence of molecular oxygen.

Technological advances using the luciferase began from analyses of gene function, named reporter gene assays. Luciferases are nearly ideal reporters for bio-analysis for several reasons. (i) The assay procedures are potentially simple and time efficient (merely mixing the reagents for measurements). (ii) Luciferase assays are generally very sensitive. (iii) Luciferase assays are broadly applicable to various organisms, from bacteria to living subjects. (iv) Reagents used for luciferase assays are not hazardous or radioactive. (v) Some marine luciferases contain a secretion signal, enabling the measurement of gene expression easily without destroying the cells or tissues. Consequently, luciferases are excellent reporter proteins showing great potential for use in various assay systems. Here, we specifically examine the potential application of the luciferase-based technologies for detecting complicated cellular processes in living cells and animals.

2. Technologies used for molecular imaging

2.1 Two-hybrid assays

Two-hybrid assays for detection of protein–protein interactions were established with yeast cells in 1989 (*18*), and a diverse series of mammalian two-hybrid technologies have emerged in the past few decades (*19*). The two-hybrid assays are based on reporter gene assay (Figure 2(A)): a transcription factor is dissected into two fragments, a DNA-binding domain and a transcriptional activation domain, each of which is linked with a pair of proteins of interest. Constitutively active interactions between a pair of target proteins trigger reassembly of the split transcription factor and the subsequent transcriptional activation of a reporter protein such as luciferase. The fundamental principle of mammalian two-hybrid assays is almost identical to that of the yeast two-hybrid ones, but an advantage of mammalian two-hybrid assays is that they allow the proteins of interest to undergo proper modifications in their native cellular context. Additionally, this native background provides the necessary adaptor proteins to bridge the association of indirectly interacting proteins. Furthermore, novel assay formats are available that enable high-throughput mammalian two-hybrid applications, facilitating their use in large-scale interactome mapping and drug discovery projects.

The two-hybrid assay formats, however, present some drawbacks. Monitoring temporal dynamics of target proteins is limited because it requires a long stimulation time for the reporter accumulation to attain a sufficient signal-to-background noise ratio. Additionally, it requires nuclear translocation of the target proteins or their localisation within the nucleus because their

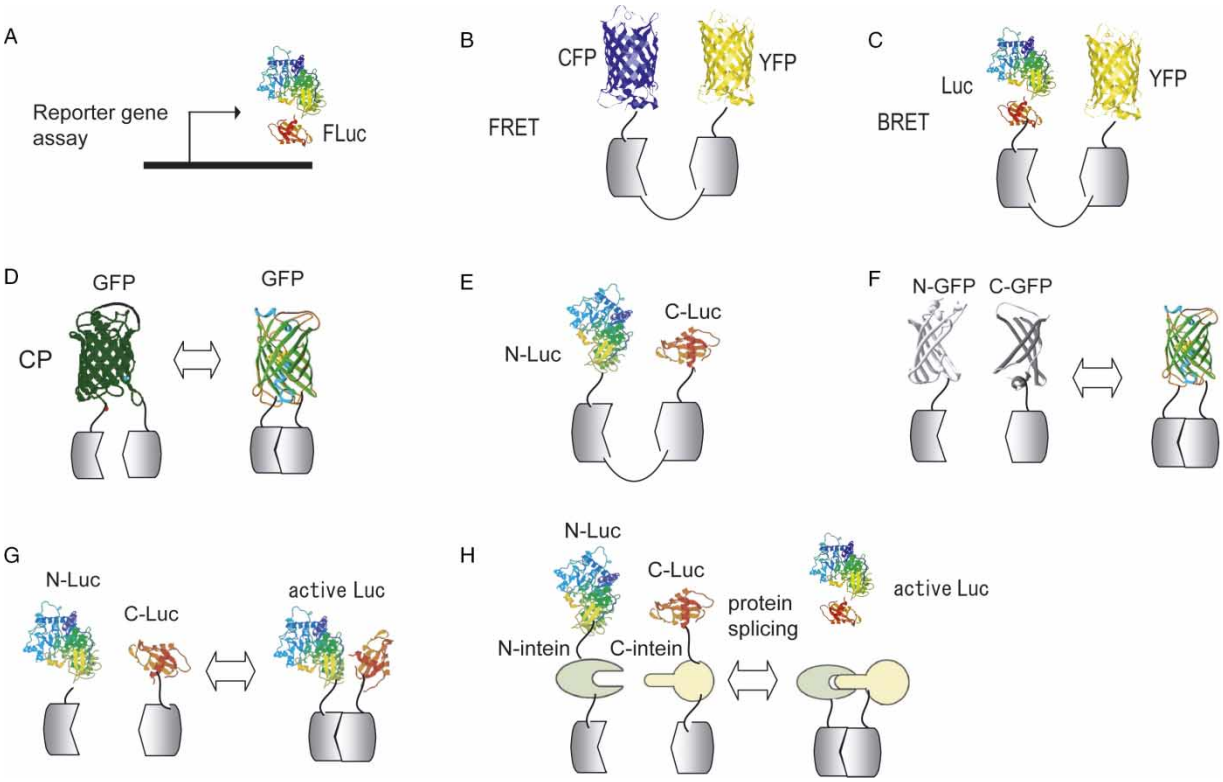

Figure 2. Major strategies for molecular imaging. (A) Reporter gene assays or mammalian two-hybrid assays. (B) Fluorescence resonance energy transfer (FRET). (C) Bioluminescence resonance energy transfer (BRET). (D) Circular permutation (CP). (E) Intramolecular protein-fragment complementation. (F) Intermolecular fluorescent protein-fragment complementation. (G) Intermolecular bioluminescent protein-fragment complementation. (H) Intermolecular bioluminescent protein-fragment reconstitution.

proximity to DNA and the transcriptional machinery is necessary for expression of the reporter proteins. For that reason, new techniques of mammalian protein–protein interaction trap (MAPPIT) for studying protein–protein interactions in the cytosol have been developed to complement conventional two-hybrid assays (*20*, *21*). In the MAPPIT, proteins of interest are linked to signalling-deficient cytokine receptor chimeras. Interaction of proteins and ligand stimulation restore intracellular signalling, which ultimately engenders the transcription of a reporter under control of a specific promoter. The MAPPIT has been used for identifying novel proteins from cDNA library and for making high-throughput interactome analysis in model organisms.

2.2 *Bioluminescence resonance energy transfer*

Bioluminescence resonance energy transfer (BRET) is a technique for determining protein–protein interactions based on the non-radiative transfer of energy between a bioluminescent donor, such as *Renilla* luciferase, and a fluorescent acceptor of fluorescent proteins (*13*, *22*, *23*) (Figure 2(C)). Therefore, BRET is useful to examine protein–protein interactions and protease activities because the BRET-permissive distance of less than 10 nm closely resembles the dimensions of biological macromolecular protein complexes. The first generation BRET assay, called '$BRET^1$', consisted of *Renilla* luciferase and an enhanced yellow fluorescent protein (EYFP) that yielded a spectral separation of nearly 50 nm (*24*). A new generation BRET, called '$BRET^2$', was developed to increase the spectral separation between *Renilla* luciferase and GFP up to about 100 nm using the proprietary substrate coelenterazine, DeepBlueC, with an emission maximum at 395 nm (*25*).

In spite of the distinctive merits of BRET technologies for observing biological events, its use demands caution; $BRET^2$ is conducted using coelenterazine or DeepBlueC as a substrate. Both are unstable in aqueous solutions or media containing serum and both are autoluminescent. The bioluminescence intensity of DeepBlueC is relatively low in comparison to that of coelenterazine, which may limit some applications to living animals. In addition, the enzyme reaction of *Renilla* luciferase produces mono-anions as the product that absorbs light, causing modification of the emission spectra. Recently, Hoshino et al. have presented a new BRET system with an excellent efficiency in energy transfer, named BRET-based

autoilluminated fluorescent protein on EYFP (BAF-Y), emitting largely enhanced fluorescence (*25*). Their BRET system not only induces a red shift of the emission peak but also extremely enhances the integrated luminescence intensity. The BAF-Y system can be used for the imaging of protein–protein interactions in single cells. Future studies of BRET should be directed to find an optimal structure of the probes allowing efficient resonance energy transfer, and to find an efficient pair of luciferase and fluorophore that enables us to monitor molecular interactions *in vivo*.

2.3 Protein-fragment complementation

Protein-fragment complementation is a useful strategy for investigating protein–protein interactions in living subjects because this system shares several characteristics that distinguish it from the methods described above. We first demonstrated the basic concept using split firefly luciferase fragments for detection of protein–protein interactions (*26*); since then, applicability of the luciferase fragments has been demonstrated successively in applications such as imaging of a particular protein–protein interaction in living mice (*26–28*). Protein-fragment complementation methods are based on the fusion of complementary fragments of a reporter protein to two proteins of interest: a monomeric luciferase is dissected into two fragments, producing a temporally inactive form. A pair of proteins of interest is fused genetically to the split N-terminal and C-terminal luciferase fragments. Successively, an interaction between the fused proteins triggers an approximation of the adjacent fragments of the luciferase and subsequent recovery of the enzyme activity (Figure 2(E)–(H)). One challenge for the development of complementation strategies is the determination of the optimal dissection sites of luciferase for visualising protein–protein interactions. The split sites in luciferase are of critical importance for guaranteeing temporal inactivation and conditional reconstitution of the luciferase activity. After split fragments of firefly luciferase were reported, several pairs of luciferase fragments were identified such as split *Renilla* luciferases (*29*) and split *Gaussia* luciferase (*30*), which are suitable for studying protein–protein interactions in living cells and animals. Recently, we have developed novel luciferase fragments from click beetles in green (Brazilian *Pyrearinus termitilluminans*; ELuc) and in red (Caribbean *Pyrophorus plagiophthalamus*; CBR) to visualise dynamic protein–protein interactions in living *Xenopus* embryos (*31*). The use of click beetle luciferase presents important benefits in terms of brightness of the bioluminescence; the photon count of click beetle luciferase is estimated as being more than 10-fold higher than that of firefly luciferase. The optimal dissection sites of luciferases of many kinds are presented in Table 2.

Luciferase complementation strategies provide distinct merits for determining protein–protein interactions compared to other methods. The rapid association and moderate dissociation rates in general enable us to detect reversible kinetics of protein–protein interactions within minutes (*31*). In addition, complementation assays provide general applicability in protein interactions with considerable spatial and temporal resolution in opaque or strongly autofluorescent living subjects. In fact, Hida et al. (*31*) demonstrated the utility of luciferase fragments in spatio-temporal characterisation of Smad1–Smad4 and Smad2–Smad4 interactions in early developing stages of a single living *Xenopus laevis* embryo (Figure 3). The report included a description of a novel luciferase fragment that enables cross complementation between luciferase fragments from different species such as an N-terminal fragment from firefly luciferase and a C-terminal fragment from the click beetle luciferase. Such inter-cross complementation is particularly useful for imaging competitive protein interactions with a third shared protein.

Most complementation analyses are based on intermolecular complementation between two independent fragments. Actually, analyses of these types are validated on the premise that the two component proteins of the system should be expressed equally beforehand. To address these shortcomings, Kim et al. (*32*, *33*) have recently demonstrated a single-chain probe called ‘an integrated molecule-format (IMF) bioluminescent probe’. The IMF probes enable us to determine intramolecular protein–protein interactions in a single-chain probe. In this strategy, all components for ligand sensing and light emission were integrated in a single molecule. The probe efficiently completes all probing actions in a single molecule. Thereby, it sustains its ligand sensitivity even in cell-free conditions, such as those which exist in a paper strip or a test tube.

2.4 Circular permutation

Another practical approach to molecular imaging is to exploit the rearrangement of the protein sequence, known as circular permutation (CP). In fact, the CP of GFP was first conducted using a fluorescent protein to trace the intracellular dynamics of Ca^{2+} (Figure 1(D)) (*34*). For the construction of CP indicators, the original N-terminal and C-terminal of GFP were linked with a short peptide linker, and a new N-terminal and C-terminal were created at random. The new ends of GFP were extended, respectively, with a pair of interacting proteins, calmodulin (CaM) and its target peptide of M13. This insertion of peptides at β-sheet linkers of GFP temporarily disrupts fluorescence because of the solvent penetration within the protein core. The inhibitory action is relieved by the

Table 2. Optimal dissection sites of luciferases for their split fragment reconstitution.

Luciferase	Optimal dissection sites (N-terminal/C-terminal)	Pair of interacting proteins	Methods	Reference
Firefly luciferase	1–415/416–550	AR LBD and FQNLF motif	Intramolecular complementation	(*32*)
	1–437/438–454	IRS-1 and SH2 domain of PI3 kinase	Intein-mediated protein splicing	(*26*)
	2–416/398–550	FRB and FKBP12	Intermolecular complementation	(*28*)
	1–437/438–550	FRB and FKBP12	Intermolecular complementation	(*30*)
	1–445/446–550			
	1–475/245–550			
	1–475/265–550			
	1–475/300–550			
	1–416/417–550	AR and Src	Intermolecular complementation	(*54*)
Click beetle luciferase	1–439/440–542	AR LBD and LXXLL motif	Intramolecular complementation	(*33*)
	1–439/443–542			
	1–439/437–542			
	1–414/395–542	Smad1–Smad4;		
	1–413/394–542	Smad2–Smad4	Intermolecular complementation	(*31*)
Renilla luciferase	1–229/230–311	Full-length AR	Intein-mediated protein splicing	(*40*)
	1–91/92–311	Dimerisation between ERK2 and ERK2	Intramolecular complementation	(*55*)
	1–229/230–311	MyoD and Id	Intermolecular complementation	(*56*)
Gaussia luciferase	18–109/110–185	FRB and FKBP12	Intermolecular complementation	(*30*)
	18–105/106–185	CaM and M13; AR LBD and LXXLL motif; ER LBD and Src SH2 domain	Intramolecular complementation	(*57*)

Notes: EGF, epidermal growth factor; IRS-1, phosphorylated insulin receptor substrate 1; FKBP12, mammalian target of rapamycin and FK506-binding protein 12; FRB, rapamycin-binding domain; AR LBD, ligand binding domain of androgen receptor; CaM, calmodulin.

interaction between the inserted CaM and M13 in the presence of Ca^{2+}.

Although the fundamental concept of CP was originally introduced with GFP variants, it can also be fabricated in luciferases. For instance, Kim et al. (*35*, *36*) have recently represented a new strategy for molecular imaging of bioactive small molecules using circularly permutated luciferases (cpLuc), derived from *G. princeps* (GLuc), *Photinus pyralis* (FLuc) and *P. termitilluminans* (CBLuc). The luciferases were first dissected into two fragments; the original N-terminal and C-terminal were linked with a peptide linker and the new termini were created in an appropriate site, each of which was linked correspondingly with proteins of interest. The most highly anticipated benefit of CP is the decrease in the background intensities and the subsequent improvement of a signal-to-background ratio. Using these approaches, probes with circularly permutated enzymes might be generally designed for tracing the molecular dynamics of target proteins and intracellular protein signalling.

2.5 Intein-mediated protein-splicing probes

Protein splicing is a naturally occurring, post-translational processing event involving precise excision of an internal protein segment, the intein, from a primary translation product with concomitant ligation of the flanking sequences, the exteins (external protein) (*37*). Since the discovery of protein splicing, more than 170 putative inteins have been identified in eubacteria, archaeal and eukaryotic unicellular organisms (*38*). A typical intein segment includes 400–500 amino acid residues and four conserved protein-splicing motifs – A, B, F and G – as well as a homing endonuclease sequence embedded between motifs B and F (Figure 4(A)). Although VDE (a VMA1-derived endonuclease originally discovered in *Saccharomyces cerevisiae*) and most inteins comprise a single polypeptide chain, a pair of functional and naturally split intein-coding sequences were found from the split *dnaE* genes in the genome of *Synechocystis* sp. PCC6803 (*39*). In this case, the DnaE intein can mediate a *trans*-splicing

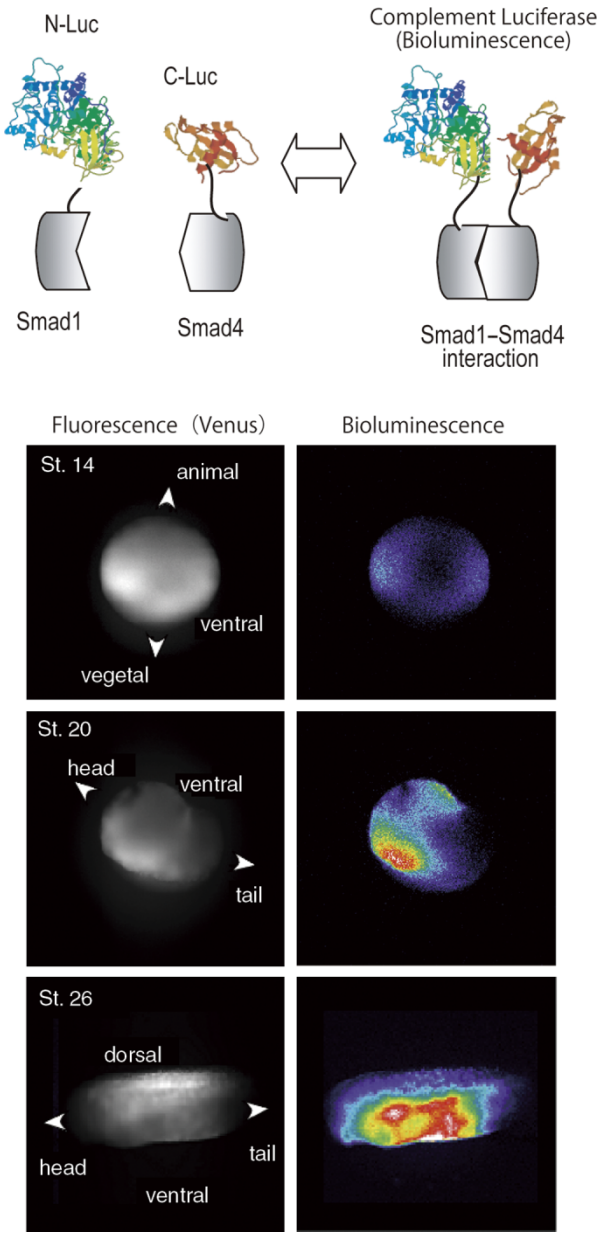

Figure 3. Bioluminescence analysis of Smad1–Smad4 interaction. (Upper) Schematic diagram of the bioluminescent probe used for imaging of the Smad1–Smad4 interactions. (Low) Real-time bioluminescence images of Smad1–Smad4 interactions in a single living *Xenopus* embryo. The embryo was injected with RNAs encoding Smad probes and Venus (fluorescence protein) at a two-cell stage. Images of fluorescence and bioluminescence were acquired with EM-CCD camera at 14, 20 and 26 stages (St. 14, 20 and 26).

reaction with higher efficiency when fused to foreign proteins. An important general feature of protein splicing is a self-catalysed excision of the intein and ligation of the flanking exteins with no exogenous cofactor or energy source such as ATP or GTP. This feature encourages the eventual use of reconstitution of split luciferases and their applications to molecular imaging and bioanalysis.

Luciferase reconstitution by protein splicing was applied to a technique for monitoring protein movement across the nuclear membrane (*40*). In general, analysis of a protein's movements depends on the use of immunocytochemistry or optical imaging with genetically tagged GFP or its variants. These analyses are effective for imaging spatial and temporal dynamics of proteins of interest under fluorescence microscopes. However, image acquisition with immunocytochemistry is slow; the results obtained using the fluorescent protein are qualitative rather than quantitative. Furthermore, immunocytochemical analyses of the protein localisation in living animals necessitate complex assay procedures such as extraction of an organ and its division into sliced sections, which hamper temporal and quantitative analyses. We developed a bioluminescent reporter for detecting protein transport into the nucleus using split *Renilla* luciferase reconstitution (*40*). *Renilla* luciferase has desirable features for a monomeric protein: small size (36 kDa), strong luminescence and lack of necessity of ATP. Additionally, its substrate, coelenterazine, penetrates through cell membranes, which is suitable for a live-cell assay and *in vivo* imaging. The principle is that luciferase is split into N-terminal and C-terminal fragments that are connected, respectively, with N-terminal and C-terminal fragments of DnaEs. The C-terminal fragment is permanently located in the nucleus, although the N-terminal fragment connected with a test protein is in the cytosol. If the test protein translocates into the nucleus, the N-terminal and C-terminal fragments of *Renilla* luciferase coexist in the nucleus, and full-length *Renilla* luciferase is reconstituted by protein splicing (Figure 4(B)). The usefulness of the reporter is demonstrated with a ligand-binding type of nuclear receptor, androgen receptor (AR). Upon binding to 5α-dihydrotestosterone (DHT), AR is translocated from the cytosol into the nucleus. The luminescence signals increased concomitantly with increasing concentration of DHT, being sufficiently strong to discriminate them from background luminescence. The advantage of the split *Renilla* luciferase reporter is that the number of cells once analysed was about 10^4 cells, which was sufficient to evaluate the extent of AR translocation precisely into the nucleus. Therefore, this split reporter enabled precise and sensitive detection, which is of great advantage to evaluate the extent of protein nuclear transport quantitatively in a high-throughput manner. Further extension of the split *Renilla* luciferase reconstitution analysis was demonstrated in the case in which protein translocation is triggered by proteolysis, phosphorylation and stress (*41*, *42*).

2.6 *Luciferase cyclisation by protein splicing*

Unlike the split fragment reconstitution, an innovative design of reconstitution methods, named cyclic luciferase, has recently been developed (*43*,*44*) (Figure 4(C)). Two

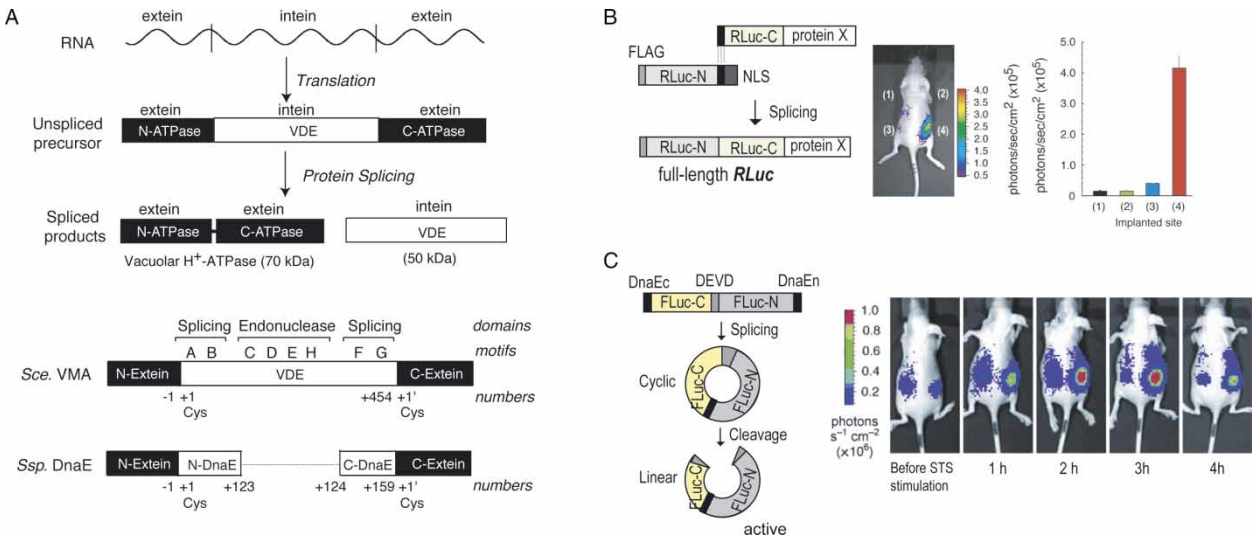

Figure 4. Intein-mediated protein splicing and design of the imaging probes. (A) Basic mechanism of protein-splicing reaction. (B) Intein-mediated reconstitution of *Renilla* luciferase (RLuc) triggered by nuclear trafficking of androgen receptor (AR). Bioluminescence displays the reconstitution of RLuc in the implanted site of the mouse. (C) Determination of caspase-3 activities based on the reconstitution of firefly luciferase (FLuc) through a protein splicing and cleavage of a four amino acid sequence (DEVD) by caspase-3.

fragments of DnaE inteins are fused to neighbouring N-terminal and C-terminal ends of firefly luciferase, connected with a substrate sequence of protease. After translation into a single polypeptide, the N-terminal and C-terminal ends of luciferase are ligated by protein splicing, which produces a closed circular polypeptide chain. Because the structure of the cyclic luciferase is distorted, the luciferase loses its bioluminescence activity. The luciferase changes into an active form and restores its activity if the substrate sequence is digested using a protease. The usefulness of the cyclic luciferase has been demonstrated as a method for quantitative sensing of caspase-3 activities in living cells upon extracellular stimuli and for non-invasive imaging of the time-dependent caspase-3 activities in living mice.

2.7 Molecular tension probe

A unique non-transcriptional assay system was demonstrated based on molecular tension of a luciferase artificially appended by protein–protein binding (*45*). Their results showed that an artificially appended molecular tension to a full-length luciferase diversifies the enzymatic activity through modification of the active site. Regarding the basic probe design, a full-length luciferase was sandwiched between two component proteins of interest. The flexible linker length between the components was minimised to exert an efficient molecular tension to the sandwiched luciferase. When the N-terminal and C-terminal ends of *Renilla* luciferase 8 were flanked by the ligand-binding domain of human oestrogen receptor α and SH2 domain of Src, this simple probe was surprisingly sensitive to oestrogens.

3. Conclusions

In conclusion, split reporter reconstitution methods have been applied to advance our understanding of many important molecular and cellular functions in living cells and animals. A strong advantage of the use of luciferase is a real-time analysis of protein–protein interactions and enzyme activities in living cells and animals. Split fragments of luciferases are in general reversible. Dissociation process of firefly luciferase, *Gaussia* luciferase and click beetle luciferases has been clearly demonstrated with a pair of mammalian target of rapamycin and FK506-binding protein and rapamycin-binding domain or their analogues. Although the timescale needed for detection ranges several seconds to minutes, it would be improved if much brighter luciferases were generated. In contrast, fluorescent protein-fragment complementation and reconstitution are irreversible; the fragments of fluorescent proteins cannot dissociate spontaneously each other. Also, chromophore formation of fluorescent proteins occurs taking a time from several minutes to hours. These facts limit the analysis of temporal interactions of proteins of interest. The spatial resolution of the bioluminescence does not reach the standard optical resolution of the fluorescence one. Modern fluorescence

microscopy techniques visualise a position of individual fluorescent molecules with nanometre precision. Therefore, it is necessary to use both fluorescent and bioluminescent proteins according to the experimental design in terms of spatial and/or temporal resolution.

Novel techniques and related applications of the methods are being developed and reported constantly. At present, the development of the split reporter constructs is rather semi-rational and laborious. This would be improved if systematic design and high-throughput screening of the probes were possible. Future biological analyses will investigate methods of non-invasive, quantitative, specific, signal-enhanced and real-time investigation of intracellular molecular events. The probes are strongly supported by advanced properties of 'lighting' proteins. Improvement of the properties of luminescent proteins is connected directly to advances in molecular recognition probes. Another pivotal ingredient in the advance of imaging technologies is the improvement of the instrumentation. The development is expected to be directed to the production of truly quantitative, highly sensitive and comprehensive probes. Such major advances in biological analytical methods will be achieved from ideas inspired by nature.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science (JSPS), JST PREST programme, New Energy Industrial Technology Development Organization (NEDO) and grants (S0801035) from MEXT, Japan.

References

(1) Shimomura, O.; Johnson, F.H.; Saiga, Y. *J. Cell Comp. Physiol.* **1962**, *59*, 223–239.
(2) Tsien, R.Y. *Annu. Rev. Biochem.* **1998**, *67*, 509–544.
(3) Heim, R.; Prasher, D.C.; Tsien, R.Y. *Proc. Natl Acad. Sci. USA* **1994**, *91*, 12501–12504.
(4) Reid, B.G.; Flynn, G.C. *Biochemistry* **1997**, *36*, 6786–6791.
(5) Miyawaki, A. *Dev. Cell* **2003**, *4*, 295–305.
(6) Miyawaki, A.; Llopis, J.; Heim, R.; McCaffery, J.M.; Adams, J.A.; Ikura, M.; Tsien, R.Y. *Nature* **1997**, *388*, 882–887.
(7) Ghosh, I.; Hamilton, A.D.; Regan, L. *J. Am. Chem. Soc.* **2000**, *122*, 5658–5659.
(8) Ozawa, T.; Nogami, S.; Sato, M.; Ohya, Y.; Umezawa, Y. *Anal. Chem.* **2000**, *72*, 5151–5157.
(9) Hu, C.D.; Kerppola, T.K. *Nat. Biotechnol.* **2003**, *21*, 539–545.
(10) Fang, D.; Kerppola, T.K. *Proc. Natl Acad. Sci. USA* **2004**, *101*, 14782–14787.
(11) Remy, I.; Michnick, S.W. *Methods* **2004**, *32*, 381–388.
(12) Cabantous, S.; Waldo, G.S. *Nat. Methods* **2006**, *3*, 845–854.
(13) Pfleger, K.D.; Eidne, K.A. *Nat. Methods* **2006**, *3*, 165–174.
(14) Ozawa, T.; Natori, Y.; Sato, M.; Umezawa, Y. *Nat. Methods* **2007**, *4*, 413–419.
(15) Hastings, J.W. *Gene* **1996**, *173*, 5–11.
(16) Viviani, V.R.; Uchida, A.; Viviani, W.; Ohmiya, Y. *Photochem. Photobiol.* **2002**, *76*, 538–544.
(17) Hastings, J.W. *J. Mol. Evol.* **1983**, *19*, 309–321.
(18) Fields, S.; Song, O. *Nature* **1989**, *340*, 245–246.
(19) Fearon, E.R.; Finkel, T.; Gillison, M.L.; Kennedy, S.P.; Casella, J.F.; Tomaselli, G.F.; Morrow, J.S.; Van Dang, C. *Proc. Natl Acad. Sci. USA* **1992**, *89*, 7958–7962.
(20) Eyckerman, S.; Lemmens, I.; Catteeuw, D.; Verhee, A.; Vandekerckhove, J.; Lievens, S.; Tavernier, J. *Nat. Methods* **2005**, *2*, 427–433.
(21) Eyckerman, S.; Verhee, A.; der Heyden, J.V.; Lemmens, I.; Ostade, X.V.; Vandekerckhove, J.; Tavernier, J. *Nat. Cell Biol.* **2001**, *3*, 1114–1119.
(22) De, A.; Gambhir, S.S. *FASEB J.* **2005**, *19*, 2017–2019.
(23) Prinz, A.; Diskar, M.; Herberg, F.W. *Chembiochem.* **2006**, *7*, 1007–1012.
(24) Xu, Y.; Piston, D.W.; Johnson, C.H. *Proc. Natl Acad. Sci. USA* **1999**, *96*, 151–156.
(25) Hoshino, H.; Nakajima, Y.; Ohmiya, Y. *Nat. Methods* **2007**, *4*, 637–639.
(26) Ozawa, T.; Kaihara, A.; Sato, M.; Tachihara, K.; Umezawa, Y. *Anal. Chem.* **2001**, *73*, 2516–2521.
(27) Paulmurugan, R.; Umezawa, Y.; Gambhir, S.S. *Proc. Natl Acad. Sci. USA* **2002**, *99*, 15608–15613.
(28) Luker, K.E.; Smith, M.C.; Luker, G.D.; Gammon, S.T.; Piwnica-Worms, H.; Piwnica-Worms, D. *Proc. Natl Acad. Sci. USA* **2004**, *101*, 12288–12293.
(29) Kaihara, A.; Kawai, Y.; Sato, M.; Ozawa, T.; Umezawa, Y. *Anal. Chem.* **2003**, *75*, 4176–4181.
(30) Remy, I.; Michnick, S.W. *Nat. Methods* **2006**, *3*, 977–979.
(31) Hida, N.; Awais, M.; Takeuchi, M.; Ueno, N.; Tashiro, M.; Takagi, C.; Singh, T.; Hayashi, M.; Ohmiya, Y.; Ozawa, T. *PLoS One* **2009**, *4*, e5868.
(32) Kim, S.B.; Awais, M.; Sato, M.; Umezawa, Y.; Tao, H. *Anal. Chem.* **2007**, *79*, 1874–1880.
(33) Kim, S.B.; Otani, Y.; Umezawa, Y.; Tao, H. *Anal. Chem.* **2007**, *79*, 4820–4826.
(34) Baird, G.S.; Zacharias, D.A.; Tsien, R.Y. *Proc. Natl Acad. Sci. USA* **1999**, *96*, 11241–11246.
(35) Kim, S.B.; Sato, M.; Tao, H. *Bioconjug. Chem.* **2008**, *19*, 2480–2486.
(36) Kim, S.B.; Sato, M.; Tao, H. *Anal. Chem.* **2009**, *81*, 3760–3768.
(37) Gimble, F.S. *Chem. Biol.* **1998**, *5*, R251–R256.
(38) Perler, F.B. *Nucleic. Acids Res.* **2002**, *30*, 383–384.
(39) Wu, H.; Hu, Z.; Liu, X.Q. *Proc. Natl Acad. Sci. USA* **1998**, *95*, 9226–9231.
(40) Kim, S.B.; Ozawa, T.; Watanabe, S.; Umezawa, Y. *Proc. Natl Acad. Sci. USA* **2004**, *101*, 11542–11547.
(41) Kim, S.B.; Ozawa, T.; Umezawa, Y. *Anal. Biochem.* **2005**, *347*, 213–220.
(42) Kim, S.B.; Takao, R.; Ozawa, T.; Umezawa, Y. *Anal. Chem.* **2005**, *77*, 6928–6934.
(43) Kanno, A.; Umezawa, Y.; Ozawa, T. *Methods Mol. Biol.* **2009**, *574*, 105–114.
(44) Kanno, A.; Yamanaka, Y.; Hirano, H.; Umezawa, Y.; Ozawa, T. *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 7595–7599.
(45) Kim, S.B.; Sato, M.; Tao, H. *Bioconjug. Chem.* **2009**, *20*, 2324–2330.
(46) de Wet, J.R.; Wood, K.V.; Helinski, D.R.; DeLuca, M. *Proc. Natl Acad. Sci. USA* **1985**, *82*, 7870–7873.
(47) Lorenz, W.W.; McCann, R.O.; Longiaru, M.; Cormier, M.J. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 4438–4442.

(48) Verhaegent, M.; Christopoulos, T.K. *Anal. Chem.* **2002**, *74*, 4378–4385.
(49) Wood, K.V.; Lam, Y.A.; Seliger, H.H.; McElroy, W.D. *Science* **1989**, *244*, 700–702.
(50) Viviani, V.R.; Arnoldi, F.G.C.; Venkatesh, B.; Neto, A.J.S.; Ogawa, F.G.T.; Oehlmeyer, A.T.L.; Ohmiya, Y. *J. Biochem.* **2006**, *140*, 467–474.
(51) Markova, S.V.; Golz, S.; Frank, L.A.; Kalthof, B.; Vysotski, E.S. *J. Biol. Chem.* **2004**, *279*, 3212–3217.
(52) Thompson, E.M.; Nagata, S.; Tsuji, F.I. *Proc. Natl Acad. Sci. USA* **1989**, *86*, 6567–6571.
(53) Inouye, S.; Watanabe, K.; Nakamura, H.; Shimomura, O. *FEBS Lett.* **2000**, *481*, 19–25.
(54) Kim, S.B.; Kanno, A.; Ozawa, T.; Tao, H.; Umezawa, Y. *ACS Chem. Biol.* **2007**, *2*, 484–492.
(55) Kaihara, A.; Umezawa, Y. *Chem. Asian J.* **2008**, *3*, 38–45.
(56) Paulmurugan, R.; Gambhir, S.S. *Anal. Chem.* **2003**, *75*, 1584–1589.
(57) Kim, S.B.; Sato, M.; Tao, H. *Anal. Chem.* **2009**, *81*, 67–74.