Spying on cancer: Molecular imaging in vivo with genetically encoded reporters

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Genetically encoded imaging reporters introduced into cells and transgenic animals enable noninvasive, longitudinal studies of dynamic biological processes in vivo. The most common reporters include firefly luciferase (bioluminescence imaging), green fluorescence protein (fluorescence imaging), herpes simplex virus-1 thymidine kinase (positron emission tomography), and variants with enhanced spectral and kinetic properties. When cloned into promoter/enhancer sequences or engineered into fusion proteins, imaging reporters allow transcriptional regulation, signal transduction, protein-protein interactions, oncogenic transformation, cell trafficking, and targeted drug action to be spatiotemporally resolved in vivo. Spying on cancer with genetically encoded imaging reporters provides insight into cancer-specific molecular machinery within the context of the whole animal.

Introduction to reporter genes and molecular imaging

Novel medical therapies, diagnostics, and cures previously not imagined are anticipated to emerge from the refined map of the human genome. The application of genomics has provided new means for recognizing the molecular basis of numerous diseases and identifying novel drug targets for pharmacological intervention and diagnostics. Routinely used methodologies to study biological processes are based on destructive sampling of biological material, thus allowing the researcher to witness only a static snapshot taken at the respective experimental endpoint. Introduction of green fluorescent protein (GFP) and its analogs and the evolution of live-cell fluorescence microscopy revolutionized our understanding of many processes at the molecular and cellular levels. With the development of suitable probes and instrumentation for functional imaging in vivo, our ability to identify and measure biological processes in real time has progressively extended to the whole organism. This new set of molecular probes, detection technologies, and imaging strategies, collectively termed molecular imaging (Sharma et al., 2002; Weissleder, 2002; Blasberg and Tjuvajev, 2003; Contag and Bachmann, 2002; Gelovani Tjuvajev and Blasberg, 2003; Herschman, 2003; Massoud and Gambhir, 2003; Piwnica-Worms et al., 2004), is providing biologists with exciting new opportunities to perform noninvasive and longitudinal studies of dynamic biological processes in intact cells and living animals. In the last five years, a series of groundbreaking studies have demonstrated that molecular imaging is a powerful tool that enables visualization of gene expression, biochemical reactions, signal transduction, and regulatory pathways in whole organisms in vivo. The ability to image fundamental processes such as transcriptional regulation, signal transduction, protein-protein interactions, oncogenic transformation, cell trafficking, and targeted drug action provides ample reason to incorporate imaging reporters into cancer research.

Imaging reagents can comprise injectable radiopharmaceuticals and contrast agents, with or without activation strategies (Bremer et al., 2001; Louie et al., 2000; Ntziachristos et al., 2002; Piwnica-Worms et al., 2004), or genetically encoded reporters (Blasberg and Tjuvajev, 2003; Gelovani Tjuvajev and Blasberg, 2003; Sharma et al., 2002). Both types of reagents are useful in biological studies, but injectable agents have the potential to directly translate to the clinic. Except in the context of gene therapy, genetically encoded reporters are less likely to be used in humans, but possess a fundamental advantage in basic research, in that once validated, a genetically encoded reporter can theoretically be cloned into a variety of vectors, and a broad array of regulatory pathways can be interrogated with the same validated reporter. For radiopharmaceuticals, this eliminates constraints inherent to traditional routes of synthesizing, labeling, and validating a new and different radioligand for every new receptor or protein of interest. Genetically encoded imaging reporters, representing the focus of this review, also provide the potential for a stable source of signal enabling longitudinal studies in living organisms with high temporal and, in some cases, high spatial resolution.

Regardless of the examined process, any strategy for imaging genetically encoded reporters is comprised of three major components: (1) a reporter gene that generates an imagable signal, (2) a regulatory element governing the activity of the reporter gene and therefore generating contrast (e.g., a constitutive or inducible promoter, an upstream *cis*-regulatory sequence, or a polypeptide fused in frame with the reporter gene and thereby posttranscriptionally regulating its activation), and (3) a detection device able to noninvasively sense and quantify the signal produced by the reporter gene within the intact cell or organism. Genetically encoded reporters can produce signal (1) intrinsically by the reporter (e.g., fluorescent proteins), (2) through enzymatic activation of an inactive substrate by the reporter (e.g., firefly luciferase that catalyses the light-producing reaction from the substrate D luciferin in the presence of O_2 and Mg^{2+}-ATP), (3) by enzymatic modification of an active (e.g., radiolabeled) substrate producing selective retention in reporter cells (e.g., selective retention of [^{18}F]FHBG by herpes simplex virus 1 thymidine kinase [HSV1-TK]; Luker et al., 2002b), or (4) by direct binding or import of an active substrate (e.g., binding of radiolabeled somatostatin to somatostatin receptor type 2 [*SSTR2*]-expressing cells; Rogers et al., 2000). Table 1 lists common reporter genes used in molecular imaging.

Specialized detection devices for imaging small animals more or less coevolved during the late 1990s. The imaging

Table 1. Common reporter genes for molecular imaging

Imaging modality	Reporter gene	Mode of action	Substrate	Reference
Fluorescence imaging	Fluorescent proteins (GFP, RFP, mPlum, etc.)	Fluorescence	None	Shaner et al., 2004; Wang et al., 2004; Yang et al., 2004; Zhao et al., 2001
Bioluminescence imaging	Firefly luciferase (FLuc)	Enzymatically catalyzed oxidation of substrate to form a light emitting unstable intermediate	D luciferin	Contag and Ross, 2002; Contag et al., 1997; Contag and Bachmann, 2002
	Click beetle luciferases (CBR, CBG)		D luciferin	Zhao et al., 2004
	Renilla luciferase (RLuc)		Coelenterazine	Bhaumik and Gambhir, 2001; Pichler et al., 2004
	Bacterial *Lux* operon		Self-encoded by the C, D, and E genes of the *Lux* operon	Hardy et al., 2004
MR imaging and spectroscopy	Transferrin receptor (ETR)	Selective retention of iron	Transferrin-iron oxide	Moore et al., 2001; Weissleder et al., 2000
	yCD	Transformation of 5-fluorocytosine to 5-fluorouracil (5-FU)	5-FC	Stegman et al., 1999
Nuclear imaging (PET,SPECT)	HSV1-TK	Intracellular trapping of radiolabeled probe by phosphorylation	[^{18}F]FHBG, [^{124}I]/[^{131}I]FIAU, and analogs	Doubrovin et al., 2001; Jacobs et al., 2001; Luker et al., 2002b, Morin et al., 2004
	XPRT	Intracellular trapping	[^{14}C]/[^{11}C]xanthine	Doubrovin et al., 2003
	SSTR2, D2R	Binding of radiolabeled ligand	[^{111}In]somatostatin analogue, [^{18}F]FESP	Liang et al., 2002; Rogers et al., 2000
	hNIS	Selective retention of radiolabeled probe	[^{124}I]/[^{131}I]iodine or [^{99m}Tc]pertechnetate	Groot-Wassink et al., 2002

CBR/CBG, click beetle red/green luciferase; D2R, dopamine receptor type 2; [^{18}F]FESP, [^{18}F]3-2-(fluoroethyl)spiperone; [^{18}F]FHBG, [^{18}F] 9-[4-fluoro-3-(hydroxymethyl)butyl]guanine; Fluc, firefly luciferase; GFP/RFP, green/red fluorescent protein; hNIS, human Na/I symporter; HSV1-TK, herpes simplex virus-1 thymidine kinase; [^{124}I] or [^{131}I]FIAU, [^{124}I] or [^{131}I]5-iodo-2'-fluoro-2'deoxy-1-β-D-arabino-furanosyl-uracil; RLuc, *Renilla* luciferase; SSTR2, Somatostatin receptor type 2; yCD, yeast cytosine deaminase; XPRT, xanthine-phosphoribotransferase.

modalities can be divided into three major groups: (1) nuclear imaging (e.g., single photon emission computed tomography [SPECT] or positron emission tomography [PET]), (2) magnetic resonance imaging (MRI), and (3) optical imaging (e.g., fluorescence imaging and bioluminescence imaging). MicroPET, microSPECT, and cooled charge-coupled device (CCD) cameras for low light bioluminescent imaging of small animals are becoming widely available (Massoud and Gambhir, 2003; Piwnica-Worms et al., 2004). In addition, introduction of red-shifted fluorescence proteins (Campbell et al., 2002; Shaner et al., 2004; Wang et al., 2004) and injectable near-infrared (NIR) fluorescent probes (Bremer et al., 2001; Weissleder et al., 1999), progression in development of highly sensitive photon detection devices and in vivo microscopy (confocal and multiphoton; Zipfel et al., 2003), and advances in mathematical modeling of photon propagation in tissues (Weissleder and Ntziachristos, 2003) have yielded innovative macro- and microscopic fluorescence imaging modalities such as fluorescence tomography (Ntziachristos et al., 2002), spectrally resolved whole body fluorescence imaging (Levenson and Mansfield, 2004; Mansfield and Levenson, 2004), and intravital multiphoton imaging (Zipfel et al., 2003).

Although exciting advances are emerging, fluorescence imaging still suffers from pitfalls such as the inability to quantify photon output, high autofluorescence in the blue-green window resulting in low signal-to-noise ratios, fluorophore photo-bleaching, and high levels of photon attenuation and scattering in living tissues. However, imaging of fluorescence proteins (preferably monomeric red-shifted reporters) has an important advantage over other imaging modalities with genetically encoded reporters, i.e., no substrate is required, which uncouples readout from substrate pharmacokinetics and thereby enables true real-time imaging. In addition, subcellular localization is possible by correlative microscopic analysis.

Nuclear imaging (SPECT and PET) is highly sensitive (detecting fmole levels of probe), quantitative, and inherently tomographic. However, nuclear imaging demands sophisticated instrumentation, committed personnel, readily available in-house production of radiopharmaceuticals, and stringent dependency on tracer pharmacokinetics. Bioluminescence imaging of luciferase reporters provides a relatively simple, robust, cost-effective, and extremely sensitive means to image fundamental biological processes in vivo due to exceptionally high signal-to-noise levels. Nevertheless, bioluminescence remains dependent on substrate pharmacokinetics, and in general offers only planar imaging datasets, therefore imposing some positional uncertainty of the attained signal. There are many luciferases with matching substrates available. However, most are blue/green and therefore are less suitable for deep tissue imaging. The luciferases that have been found to be most useful for molecular imaging are firefly (*Photinus pyralis*) luciferase, *Renilla* luciferase, green or red click beetle (*Pyrophorus plagiophthalamus*) luciferases, and *Gaussia* luciferase (Contag and Bachmann, 2002; Tannous et al., 2004; Zhao et al., 2004). However, both *Renilla* and *Gaussia* luciferases emit blue light, which is highly attenuated in living tissue, and possess high bursting activity, therefore requiring care and precision in timing the readout. Moreover, their substrate, coelenterazine, has been shown to be transported by the multidrug resistance transporter Pgp (Pichler et al., 2004) as well as to

interact efficiently with superoxide anion and peroxynitrate in light-producing reactions (Tarpey et al., 1999), thereby complicating applications of *Renilla* and *Gaussia* luciferases in vivo.

As mentioned above, each of the imaging modalities (MR, nuclear, or optical) has its own strengths and weaknesses (i.e., tradeoffs of spatial and temporal resolution, depth of signal detection, acquisition time, cost, ease of operation, and the potential for clinical translation) and should therefore be selected primarily according to the examined biological process. This review is not intended for critical assessment or comparison of the technical merits of the various modalities, detection devices, or instruments, and therefore, the reader is referred to recent comprehensive reviews covering these topics (Bremer et al., 2003; Contag and Bachmann, 2002; Massoud and Gambhir, 2003; Rudin and Weissleder, 2003; Sharma et al., 2002). No single modality addresses all aspects of molecular imaging, and therefore, there is increasing interest in constructing fusion reporters that combine the positive attributes of different modalities (Doubrovin et al., 2003; Jacobs et al., 2003; Luker et al., 2002b; Ray et al., 2003, 2004). Herein we will focus on the regulatory and biochemical elements that govern activation of imaging reporter genes with an emphasis on cancer, regardless of their emission characteristics or the imaging modality used to detect their signal. Different strategies to regulate genetically encoded reporter activation and thereby detect and dynamically monitor various components of the cell machinery (transcriptional, posttranscriptional, translational, and posttranslational) in intact cells and small animal models are summarized in Table 2 and further discussed in the following sections.

Transcriptional regulation of reporter activity

The simplest way to regulate activity of a reporter gene is by promoter-driven transcription. The earliest applications of genetically encoded molecular imaging reporters were intended for studying cell trafficking and engraftment, bacterial or viral distribution, transgene expression, or analyzing tumor burden and metastatic activity by expressing reporter genes under the control of constitutive promoters (e.g., viral promoters such as *p*CMV, *p*SV40, etc.). Typically, cells transfected ex vivo or transduced with engineered viruses enabled reporter monitoring of spatiotemporal changes in signal after implantation of the cells or vectors in an intact animal. For example, the ability to monitor primary tumor burden and response to therapy was studied by bioluminescence imaging and crossvalidated by MRI in an orthotopically implanted 9L glioma tumor, constitutively expressing firefly luciferase (Rehemtulla et al., 2000). In this study, excellent correlation was found between the two imaging modalities for the kinetics of both tumor growth and drug-induced cytotoxicity by 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU). Historically, in the case of internal orthotopic tumor models, animal survival was the dominant strategy for monitoring tumor growth and response to therapy prior to small animal imaging (Contag and Ross, 2002), and thus, imaging strategies provide more refined readouts of response. While MRI provides accurate three-dimensional measurements of tumor size, bioluminescence imaging of luciferase expression is potentially superior in reporting the quantity of viable cells.

Monitoring gene delivery and optimizing gene therapy protocols are other queries for application of simple constitutive promoters to regulate imaging reporter genes. Constitutive expression (by an immediate early CMV promoter) of two reporter genes (D2R and HSV1-TK) within a bicistronic (IRES) transcriptional unit was used to image adenoviral-mediated gene delivery by PET imaging for evaluation and optimization of gene therapy protocols (Liang et al., 2002). It was demonstrated in this study that the two genes coexpressed primarily in the liver (the main site for adenoviral infection in mice) over a 3 month period and over a 7- to 10-fold concentration range. In the case of cancer gene therapy, HSV1-TK is a particularly attractive reporter gene, since it has the advantage of being both a therapeutic and a reporter gene (by using ganciclovir treatment and the appropriate radiolabeled substrate, respectively) (Qiao et al., 2002; Tjuvajev et al., 1999). A variation of this strategy thereby enabled direct monitoring of inducible suicide gene therapy for controlling graft versus host disease after allogeneic bone marrow transplantation (Rettig et al., 2004). Tumoral accumulation of HSV-based oncolytic viruses also has been imaged with an HSV1-TK reporter (Bennett et al., 2001; Jacobs et al., 2001).

Reporter genes transfected in eukaryotic cells have been widely used to study *cis*-regulatory sequences or *trans*-acting factors that modulate the transcriptional activity of target promoters. By transducing cells in vivo with an expression cassette that contains a reporter gene under the control of a transcriptionally regulated sequence, it is now feasible to monitor transcriptional regulation in a living animal. As an example, a retroviral vector containing the dual reporter gene HSV1-TK-GFP regulated by an upstream p53 response element (p53→TK-GFP) was used to transduce U87 glioma ($p53^{+/+}$) and SaOS osteosarcoma ($p53^{-/-}$) cells that were implanted into rats to establish tumor xenografts (Doubrovin et al., 2001). Whole-body PET imaging and fluorescence microscopy demonstrated DNA damage-induced upregulation of TK-GFP in a p53-dependent manner, and this increase in activity correlated with upregulation of downstream p53-regulated genes as measured by independent assays.

Anatomical and temporal changes in transcriptional regulation have also been shown to be resolved by reporter gene imaging in transgenic mouse models. For instance, a transgenic mouse has been generated wherein firefly luciferase is expressed under the regulation of an NF-κB response element and used to study time- and organ-dependent changes in bioluminescence after administration of classical stressors such as tumor necrosis factor α (TNFα), interleukin-1α (IL-1α), or lipopolysaccharide (LPS), or after inducing genotoxic stress by UV irradiation (Carlsen et al., 2002). It was demonstrated in this study that in the absence of extrinsic stimuli, strong NF-kB activity was evident in cervical lymph nodes, thymus, and Peyer's patches. However, treatment with TNFα, IL-1α, or LPS increased the NF-kB-dependent bioluminescent signal in an organ-specific manner, with the strongest activity observed in skin, lungs, spleen, Peyer's patches, and the wall of the small intestines. It was further shown that induction of chronic inflammation resembling rheumatoid arthritis produced a strong signal in affected joints.

To analyze the dynamics of estrogen receptor (ER) transcriptional activity in vivo, a transgenic mouse model was generated (Ciana et al., 2003) wherein firefly luciferase is expressed under transcriptional control of the ER (ERE→FLuc). As expected, in reproductive organs and in the liver, luciferase activity paralleled circulating estrogen levels, peaking at proestrus. However, in non-reproductive organs such as bone and brain, peak transcriptional activity of estrogen receptors was observed in diestrus. It was further demonstrated that

Table 2. Strategies for regulating imaging reporter gene activity

Level of regulation	Examined process	Strategies	Examples	References
Transcriptional	Transcriptional regulation	The promoter of interest drives the reporter gene	ERE→FLuc	Ciana et al., 2003
			NF-κB→FLuc	Carlsen et al., 2002
			p53→HSV1-TK-EGFP	Doubrovin et al., 2001
			AP1→FLuc	Huang et al., 1997
			Grp78→HSV1-TK	Dong et al., 2004
			HRE→HSV1-TK-GFP	Serganova et al., 2004
		Transactivation	PSA→ Gal4BD-VP16 + Gal4→FLuc	Zhang et al., 2002a
Posttranscriptional and translational	RNA splicing	Spliceosome-mediated RNA trans-splicing	Trans-splicing-mediated reconstitution of hRLuc mRNA	Bhaumik et al., 2004
	Translational regulation	Fusing the reporter with a protein that regulates its own translation	LTR→DHFR-HSV1-TK	Mayer-Kuckuk et al., 2002, 2003
Posttranslational	Protein-protein interaction	Functional complementation of a split reporter (with or without intein-mediated reporter reconstitutuion)	CMV→FRB-NFLuc + CMV→CFLuc-FKBP	Luker and Piwnica-Worms, 2004; Luker et al., 2004
			NF-κB→NFLuc-ID + CMV→MyoD-CFLuc	Paulmurugan et al., 2002
			NF-κB→NFLuc-DnaEN-ID + CMV→MyoD-DnaEC-CFLuc	Paulmurugan et al., 2002
		Two-hybrid system	Gal4BD-p53←TetRE→VP16-TAg + 4xGal4→HSV1-TK-EGFP	Luker et al., 2002b, 2003b
		Energy transfer (FRET, BRET)	CMV→β-arrestin-RLuc + CMV→Ub-GFP	Jares-Erijman and Jovin, 2003; Perroy et al., 2004
	Proteasomal degradation (total)	N-terminal fusion of tetraubiquitin to the reporter	CMV→4xUb-FLuc	Gross and Piwnica-Worms, 2005; Luker et al., 2003a
			β-actin→4xUb-EGFP	Lindsten et al., 2003
	Proteasomal degradation (substrate-specific)	Fusion of the substrate of interest to the reporter	SV40→p27-FLuc	Zhang et al., 2004
			CMV→IκBα-FLuc	Gross and Piwnica-Worms, 2004, 2005
			CMV→p53-FLuc	Rehemtulla et al., 2004
	Protease activation	Introduction of a protease recognition motif that, when cut, activates the reporter	AMLP→ER-DEVD-FLuc-DEVD-ER	Laxman et al., 2002
	Nuclear transport	Intein-mediated reconstruction of nucleocytoplasmic separated reporter fragments	CMV→DnaEC-CRLuc-AR + CMV→FLAG-NRLuc-DnaEN- NLS	Kim et al., 2004
Cellular	Monitoring tumor burden, cell trafficking, stem cell engraftment, pathogen infection, multidrug resistance	Constitutive expression of the reporter gene in the target cells/viruses	CMV→FLuc in 9L glioma	Rehemtulla et al., 2000
			LTR→HSV1-TK in T cells	Koehne et al., 2003
			UL29→FLuc + UL30→RLuc in HSV	Luker et al., 2002a
			LTR→FLuc/GFP in αMBP-CD4$^+$T cells	Costa et al., 2001
			LP→lux in *L. Monocytogenes*	Hardy et al., 2004
			CMV→RLuc	Pichler et al., 2004
Combined	Conditional organ-specific reporter expression	Cre-loxP recombination	β-actin→lox-GFP-lox-FLuc	Lyons et al., 2003
			ROSA26→lox-stop-lox-FLuc	Safran et al., 2003
	Spontaneous tumorigenesis	Coupling reporter activation with transformation in a conditional and optionally organ-specific manner	POMC→Cre + POMC→FLuc crossed with a conditional lox-*Rb*-lox knockout	Vooijs et al., 2002
			E2F1→FLuc crossed with Nestin→ tv-a and infected with RCAS-PDGFB virus	Uhrbom et al., 2004

AMLP, adenoviral major late promoter; AP1, affector protein 1; AR, androgen receptor; Cre, Cre-recombinase; DEVD, asp-glu-val-asp; E2F1, E2F1 promoter; EGFP, enhanced green fluorescence protein; ERE or ER, estrogen receptor; FKBP, FK506-binding protein type 12; FRB, rapamycin-binding domain of mTOR; Gal4BD, Gal4 DNA binding domain; Grp78, glucose-regulated protein-78; HRE, hypoxia response element; HSV1-TK, herpes simplex virus-1 thymidine kinase; IκBα, inhibitor of nuclear factor κB type α; LP, listerial promoter; LTR, long terminal repeat; MBP, myelin binding protein; N/C FLuc, N- or C-terminal fragments of firefly luciferase; N/C RLuc, N- or C-terminal fragments of *Renilla* luciferase; NF-κB, nuclear factor κB; N-tv-a, nestin promoter driving TV-a avian virus receptor; NLS, nuclear localization signal; PDGFB, platelet-derived growth factor type B; POMC, lobe-specific pituitary promoter; PSA, prostate-specific antigen; Rb, retinoblastoma tumor suppressor gene; RCAS, replication-competent avian sarcoma and leucosis virus long terminal splice acceptor; ROSA26, ubiquitous ROSA26 promoter; TAg, simian virus large T antigen; TetRE, tetracycline-responsive element; Ub, ubiquitin; VP16, VP16 transactivator; →, promoter regulation; -, fusion gene; +, coexpression.

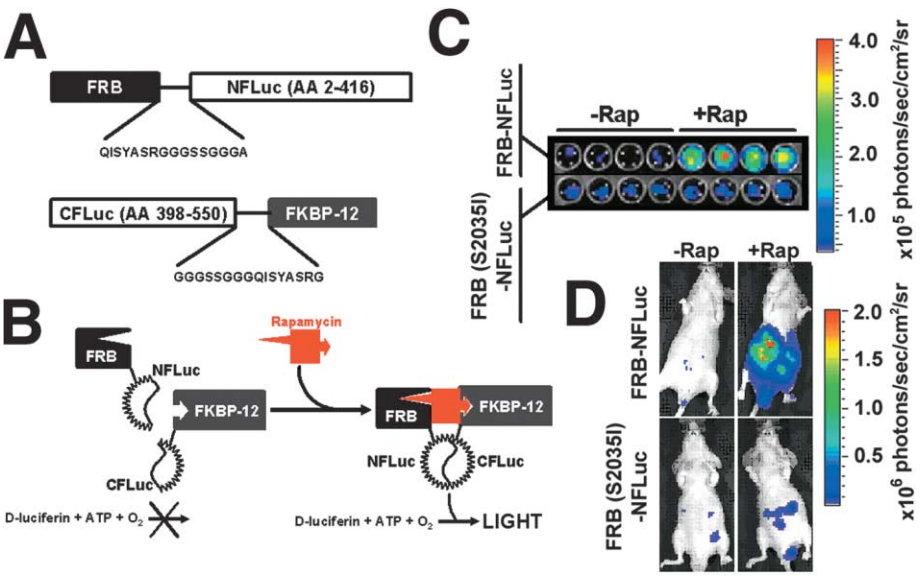

Figure 1. Optimization of firefly luciferase protein-fragment complementation imaging (LCI)

A: Schematic representation of the optimized N- and C-terminal fragments of luciferase (as revealed by screening of incremental truncation libraries), fused to FRB and FKBP-12, respectively.

B: Schematic of LCI. Rapamycin-induced association of proteins FRB and FKBP-12 brings inactive fragments of luciferase into close proximity, thereby producing bioluminescence activity.

C: Monitoring rapamycin-induced FRB/FKBP12 association in live cells. HEK-293 cells transfected with FRB-NFLuc + CFLuc-FKBP-12 (upper) or S2035I FRB-NFLuc + CFLuc-FKBP-12 (lower) were treated for 6 hr with 50 nM rapamycin. Note that the S2035I mutation of mTOR/FRB is known to abrogate the rapamycin-induced association of FRB and FKBP-12. A pseudocolor IVIS bioluminescence image of live cells in a 96-well plate is shown.

D: Luciferase complementation imaging of two representative *nu/nu* mice, one implanted with HEK-293 cells expressing FRB-NFLuc + CFLuc-FKBP (upper) and the other with cells expressing mutant S2035I FRB-NFLuc + CFLuc-FKBP-12 (lower). Images were taken 18 hr before treatment with rapamycin (left) and 2.5 hr after receiving a single dose of rapamycin (4.5 mg/kg, i.p., right).

these tissue-specific responses are masked when mice undergo conventional hormone treatment, and that estrogen receptors are transcriptionally active even in immature mice (before gonadal production of sex hormones) and in ovariectomized mice. Overall, this study emphasizes the importance of estrogen-independent activation of ER, especially in non-reproductive organs, and provides far-reaching implications for hormone replacement therapy and cancer risk.

Imaging posttranscriptional molecular events

Imaging posttranscriptional events, such as translational regulation, protein-protein interactions, protein processing, or protein degradation, is primarily performed by fusing the reporter gene, a partial reporter fragment, or an upstream transactivator to the protein of interest, thereby generating a molecular sensor that activates (or deactivates) the reporter in response to a given protein interaction or modification. Fundamentally, the detection of physical interaction among two or more proteins can be assisted if association between the interactive partners leads to the production of a readily observed biological or physical readout. At present, three general strategies are feasible for imaging interacting protein pairs in cellulo or in vivo: (1) protein-protein interaction-dependent reporter gene transactivation or repression (two hybrid system; recruitment of signal transduction cascades), (2) reporter complementation, achieved by fusing inactive reporter fragments to interacting proteins, thereby bringing the fragments into close proximity and restoring reporter activity (Luker et al., 2004; Paulmurugan et al., 2002, 2004), and (3) energy transfer techniques such as Forster resonance energy transfer (FRET) (Jares-Erijman and Jovin, 2003) and bioluminescence resonance energy transfer (BRET) (Perroy et al., 2004). However, energy transfer strategies have not yet been demonstrated in living animals.

Reporter transactivation

As an example of noninvasive molecular imaging of protein-protein interactions in vivo by PET and fluorescence imaging, a fusion reporter gene was engineered comprising a mutant HSV1-TK and EGFP (mNLS-sr39TK-EGFP) under regulation of a concatenated Gal4 promoter (Luker et al., 2002b, 2003b). The p53 tumor suppressor was fused to the Gal4 DNA binding domain (p53-Gal4BD), and simian virus-associated large T antigen (TAg) was fused to the transactivator VP16 (TAg-VP16). Expression of p53-Gal4BD and TAg-VP16 was regulated by a bidirectional, tetracycline-responsive promoter. Thus, upon treatment with doxycycline, transcription of the reporter gene was regulated by the known high-affinity interaction between p53 and TAg. Visualization of the reporter was accomplished with [^{18}F]FHBG and microPET imaging. Based on region-of-interest values from the microPET images, the uptake of [^{18}F]FHBG was 5.5-fold higher than in control xenografts expressing polyoma virus coat protein fused to VP16 (CP-VP16) instead of TAg-VP16.

The interaction of two other proteins, ID and MyoD, was also interrogated in vivo by a similar strategy applied to bioluminescence (Ray et al., 2002). To modulate the expression of these two proteins, the NF-κB promoter was used to regulate transcription of the ID-Gal4 and MyoD-VP16 hybrid proteins, while TNFα was used to induce activation of the NF-κB promoter. Firefly luciferase regulated by five repetitive Gal4 elements was used as the reporter gene. Thus, upon treatment with TNFα to induce expression of the interacting hybrids, bioluminescence was correlated with interaction of ID and MyoD and was transiently higher than when induced by a noninteracting protein pair (MyoD and p53).

Reporter fragment complementation and reconstitution

Limitations of transactivation strategies for studying protein-protein interactions are the requirement for protein translocation and stable protein interactions in the nucleus, as well as temporal delays inherent to transcriptional readouts. To circumvent these limitations, other techniques have been developed. A split reporter protein approach can be used through either complementation or reconstitution strategies. Complementation strategies do not require the formation of an intact protein from split fragments, as opposed to reconstitution strategies that attempt

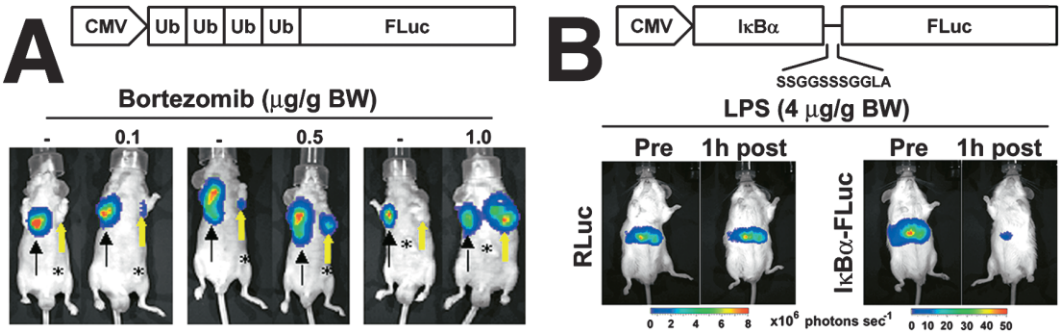

Figure 2. Imaging total- and substrate-specific proteasome activity

A: In vivo bioluminescence imaging of Ub-FLuc monitors total proteasome function and inhibition in living mice. Upper panel: schematic of the Ub-FLuc reporter. Lower panel: mice bearing size-matched tumors were imaged one day before (–) and four hours after tail vein injection of the indicated doses of bortezomib. Unfused FLuc, Ub-FLuc, and vector control tumors are denoted by black arrows, yellow arrows, and asterisks, respectively.
B: Real-time bioluminescence imaging of IκBα-specific proteasomal degradation in a somatic gene transfer mouse model. Upper panel: schematic of the IκBα-FLuc reporter. Lower panel: representative bioluminescence images of RLuc (left two panels) and IκBα-FLuc (right two panels) taken before or 1 hr after induction of acute liver inflammation by intravenous injection of LPS (4 μg/g BW). All images correspond to an individual mouse. Note that plasmids encoding RLuc and IκBα-FLuc were delivered to the liver by high-volume intravenous injection.

to reconstitute the mature reporter protein. Reporter complementation is based on the principle that reporter activity (e.g., enzymatic activity) is regained when its split fragments are brought into close proximity due to a specific protein-protein interaction. Protein-protein interactions can also drive reporter reconstitution by intein-mediated protein autosplicing.

The interaction of ID and MyoD was demonstrated in vitro and in vivo by firefly luciferase complementation and reconstitution (Paulmurugan et al., 2002). However, in this study, firefly luciferase fragments suffered from constitutive activity of the N-terminal fragment. Reporter complementation was also demonstrated using *Renilla* luciferase split fragments (Kim et al., 2004; Paulmurugan and Gambhir, 2003; Paulmurugan et al., 2004), though the blue-green emission spectrum of *Renilla* luciferase penetrates tissues poorly, thereby precluding general use. Furthermore, coelenterazine, the bioluminescent substrate for *Renilla* luciferase, was shown to be transported by the multidrug resistance transporter Pgp (see Pichler et al., 2004, and below), complicating applications of *Renilla* luciferase in vivo. Consequently, to facilitate the study of regulated protein-protein interactions in cells and living animals, an optimized firefly luciferase protein fragment complementation system was developed by screening incremental truncation libraries of N- and C-terminal fragments of firefly luciferase. The initial seeds for the screen were inactive firefly luciferase fragments fused to the rapamycin binding domain (FRB) of the kinase mammalian target of rapamycin (mTOR) and FK506 binding protein 12 (FKBP), respectively (Luker and Piwnica-Worms, 2004; Luker et al., 2004). The optimized FRB-NLuc/CLuc-FKBP pair (containing 2–416 N-terminal and 398–550 C-terminal overlapping luciferase fragments; Figure 1A) generated luciferase activity in cells upon single-site binding of rapamycin in an FK506-competitive manner (Figure 1B). The inducibility range of optimized luciferase complementation imaging (LCI) was robust, with drug-specific induction of bioluminescence reaching 1,200-fold over background, exceeding currently available systems (Figures 1C and 1D). This property enabled monitoring of lower affinity protein-protein interactions, such as homodimerization of nonphosphorylated STAT1 and the phosphorylation-dependent interaction between human Cdc25C with 14-3-3ε in vivo (Luker et al., 2004).

Imaging total- and substrate-specific proteasomal degradation

The ubiquitin-proteasome pathway is the central mediator of regulated proteolysis, an instrumental switch for a variety of signaling cascades. Deregulation of proteasomal activity or improper substrate recognition and processing by the ubiquitin-proteasome machinery may lead to cancer, stroke, chronic inflammation, and neurodegenerative diseases. To monitor total proteasomal activity, an ubiquitin-luciferase bioluminescence imaging reporter was developed by fusing the N terminus of firefly luciferase to four copies of a mutant ubiquitin (Ub^{G76V}, Figure 2A, upper panel) (Luker et al., 2003a). The tetraubiquitin fusion degradation motif has been shown to significantly destabilize heterologous proteins in cultured cells (Stack et al., 2000), while the glycine to valine substitution at the C terminus of ubiquitin limits cleavage by ubiquitin hydrolases (Johnson et al., 1992; Stack et al., 2000). Both in cultured cells and in tumor xenografts, the 4xUb-FLuc reporter was degraded rapidly under steady-state conditions and stabilized in a concentration- and time-dependent manner in response to various proteasome inhibitors (Figure 2A, lower panel). Bioluminescence imaging revealed that proteasome function in tumor xenografts was blocked as soon as 30 min after administration of a single dose of the chemotherapeutic proteasome inhibitor bortezomib and returned to nearly baseline by 46 hr. However, after a two-week regimen of bortezomib, imaging of target tumors showed significantly enhanced proteasome inhibition that no longer returned to baseline.

Similar to 4xUb-FLuc for imaging total proteasome activity, fusing a proteasomal substrate to the N terminus of FLuc generates a reporter responsive to degradation of that specific proteasomal substrate. For example, it was recently demonstrated that bioluminescence imaging of inhibitor of κB (IκBα)-specific proteasomal degradation using an IκBα-FLuc fusion reporter can monitor in real time nuclear factor-κB (NF-κB) activation and pharmacological modulation (Gross and Piwnica-Worms, 2004, 2005), independent of transcriptional or translational events. Reporter degradation was monitored both in cultured cells after treatment with tumor necrosis factor (TNFα) and in response to treatment with lipopolysaccharide (LPS) in vivo using a liver inflammation model, wherein the IκBα-FLuc

Table 3. Examples of transgenic mouse models expressing reporter genes suitable for molecular imaging applications

Reporter gene	Activity	Transgene	References
Firefly luciferase	AP-1-dependent transcription	AP-1→FLuc	Huang et al., 1997
	NF-κB-dependent transcription	κB→FLuc	Carlsen et al., 2002
	Activation of estrogen receptor (ER)	ERE→FLuc	Ciana et al., 2003
	Circadian gene expression	mPer1→FLuc	Wilsbacher et al., 2002
	Neuronal damage	GFAP→FLuc	Zhu et al., 2004
	Drug metabolism	CYP3A4→FLuc	Zhang et al., 2003
	Bilirubin synthesis	HO1→FLuc	Zhang et al., 2002b
	Bone repair and development	hOC→FLuc	Iris et al., 2003
	Cre-mediated activation	β-actin →lox-GFP-lox-FLuc; ROSA26→lox-stop-lox-FLuc	Lyons et al., 2003; Safran et al., 2003
	E2F1-dependent transcription	E2F1→FLuc	Uhrbom et al., 2004
	Pituitary spontaneous tumorigenesis	POMC→Cre + POMC→Fluc	Vooijs et al., 2002
	Angiogenesis	VEGFR2→FLuc	Zhang et al., 2004
Fluorescent proteins	Neuronal development	nestin→GFP	Yamaguchi et al., 2000
	Embyonic germ cell migration	Oct4→GFP	Anderson et al., 2000
	Ureteric bud development	Hoxb7→GFP	Srinivas et al., 1999
	Angiogenesis	VEGF→GFP	Fukumura et al., 1998
	Lymphocyte development	Gfi1→GFP	Yucel et al., 2004
	X chromosome inactivation	CMV→GFP in X chromosome	Hadjantonakis et al., 2003
	Synaptic formation and plasticity	Thy1→GFP, Thy1→YFP, Thy1→CFP, TetRE→PKCγ-GFP	Feng et al., 2000; Sakai et al., 2004; Trachtenberg et al., 2002
	Proteasome activity	β-actin→Ub-GFP	Lindsten et al., 2003
	Embryonic perfusion	ε-globin→GFP	Jones et al., 2002
	Ubiquitous GFP expression	β-actin→GFP	Yang et al., 2004
HSV1-TK	Endothelial damage	VE-cad→HSV1-TK	Dancer et al., 2003
	Liver targeting	Alb→Cre + CAG→lox-stop-lox-HSV1-TK adenovirus	Sundaresan et al., 2004
	Germ cell ablation	Inhα→HSV1-TK	Ahtiainen et al., 2004
	GVH disease control	CD2→ΔCD34-HSV1-TK	Rettig et al., 2004

Alb, albumin promoter; AP1, affector protein 1; CAG, cytomegalovirus immediate early gene 1 enhancer; CD2, T cell locus promoter; CFP, cyan fluorescent protein; CMV, cytomegalovirus promoter; CYP3A4, cytochrome P450-3A promoter; Cre, Cre-recombinase; E2F1, E2F1 promoter; ERE, estrogen receptor; FLuc, firefly luciferase; GFAP, glial fibrillary acidic protein promoter; Gfi1, growth factor independence promoter; GFP, green fluorescent protein; GVH, graft versus host; HO1, heme-oxygenase 1; hOC, human osteocalcin promoter; Hoxb7, homeobox B7 promoter; HSV1-TK, herpes simplex virus-1 thymidine kinase; Inhα, inhibin-alpha promoter; κB, κB response element; mPer1, mammalian period-1 promoter; Oct4, POU5F1 promoter; PKCγ, protein kinase Cγ; POMC, lobe-specific pituitary promoter; TetRE, tetracycline-responsive element; Thy-1, a mature neuronal marker promoter; Ub, ubiquitin; VE-cad, vascular endothelial cadherin promoter; VEGF, vascular endothelial growth factor promoter; VEGFR2, VEGF receptor type 2 promoter; YFP, yellow fluorescent protein; →, promoter regulation; –, fusion gene; +, coexpression.

reporter was delivered to liver hepatocytes by hydrodynamic somatic gene transfer (Figure 2B). Moreover, pretreatment with proteasome inhibitors or inhibitors of IκB-kinase (IKK) was shown to abrogate ligand-induced reporter degradation.

A similar strategy was used to monitor cell cycle-regulated degradation of the cyclin-dependent kinase 2 (cdk2) inhibitor p27 using a p27-FLuc fusion reporter (Zhang et al., 2004). It was shown in this study that reporter activity is regulated by its E3-ligase Skp2 in a cell cycle-dependent manner. Blockade of cdk2 activity by drugs, inhibitory proteins, peptides, or small interfering RNA (siRNA) induced reporter accumulation and increases in bioluminescence. Elevation in reporter activity due to pharmacological modulation of cdk2 was also documented in vivo in human tumor xenografts.

Imaging caspase-3 activation

The cysteine protease caspase-3 is an effector caspase activated during apoptotic cell death by upstream initiator caspases (i.e., caspases 8, 9, 10, and 12). Once activated, caspase-3 executes apoptosis by cleaving cellular proteins at a specific DEVD consensus motif. To enable noninvasive and repetitive imaging of apoptosis in living animals, a reporter was engineered (Laxman et al., 2002) for bioluminescence imaging wherein the estrogen receptor regulatory domain (ER) was fused to FLuc, thereby sterically silencing FLuc catalytic activity. Inclusion of a DEVD sequence between these two moieties allowed for caspase-3-mediated restoration of luciferase activity, enabling real-time monitoring of apoptotic activation. Using this reporter, the investigators demonstrated activation of caspase-3 in intact cells and living animals in response to treatment with TNFα-related apoptosis-inducing ligand (TRAIL). Furthermore, ZVAD-fmk, a general caspase inhibitor, was shown to abrogate TRAIL-induced reporter activation, thus confirming the role of caspases for regulating activity of this reporter.

Imaging multidrug resistance

Coelenterazine, the bioluminescent substrate of the reporter gene *Renilla* luciferase, is a substrate for the multidrug transporter Pgp (Pichler et al., 2004). In cultured living cells, stably

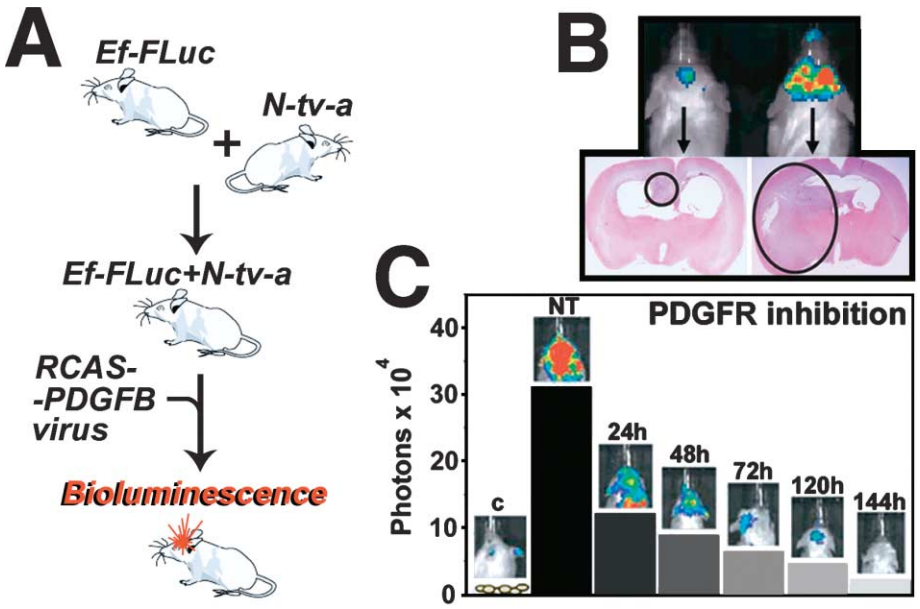

Figure 3. Imaging tumor burden and response to therapy in spontaneous glioma tumorigenesis modal

A: Generation of Ef-FLuc + N-tv-a double transgenic mice (see text for further details).
B: Approximate correlation between BLI output and tumor size. Upper panel: luciferase activity in tumor-bearing Ef-FLuc + N-tv-a transgenic mice. Lower panel: Whole-mount histologic analysis of the brains from the same mice as imaged in the upper panel. Note that tumor size correlates with the amount of emitted light.
C: Longitudinal imaging of one tumor-bearing mouse treated with the PDGFR inhibitor PTK787/ZK222584 daily for 6 days. NT, bioluminescence before drug treatment. Modified from Uhrbom et al. (2004) with permission from Nature Medicine (http://www.nature.com/nm).

transfected with a codon-humanized *Renilla* luciferase, it was shown that low baseline coelenterazine-mediated bioluminescence could be fully enhanced (reversed) to non-Pgp matched control levels with potent and selective Pgp inhibitors. Therefore, using coelenterazine and noninvasive bioluminescence imaging in vivo, tumor-specific Pgp transport activity and inhibition could be monitored in living mice. This study emphasizes the role of coelenterazine as a Pgp substrate, but at the same time raises concerns regarding the indiscriminative use of *Renilla* luciferase and aequorin as reporters in intact cells and transgenic animals, since Pgp-mediated alterations in coelenterazine permeability may impact results.

Imaging oncogenic transformation and spontaneous tumorigenesis

The use of transgenic animal models of human diseases promises to extend our understanding of the mechanisms of pathogenesis by placing target genes and processes in the appropriate physiological milieu. However, until recently, analysis of these animal models was limited by the ability to monitor only obvious phenotypic changes or perform destructive analyses at defined time points. In cancer research, this pitfall becomes a major drawback, because almost all aspects of tumorigenesis, tumor growth, invasion, metastatic potential, and response to therapy are dynamic in time and space. Moreover, while it is clear that the closest approximation of human cancers is attained by spontaneous transformation models, the stochastic nature of spontaneous tumors complicates and thereby severely limits the application of these models. Consequently, recent advances in small animal imaging instrumentation, molecular genetics, and reporter gene design have yielded the ability to integrate an imagable reporter capacity into transgenic models of human diseases. Such aptitude not only refines the data by allowing each animal to serve as its own control, but also permits in vivo high-throughput analyses of drugs for preclinical trials. Consecutive analysis of the same animal means that fewer animals are needed for each study and experimental uncertainties arising from inter-animal variations are greatly reduced (Herschman, 2003). Table 3 provides detailed information on available transgenic mouse models expressing such imagable reporters.

Of particular interest is a transgenic mouse model (Vooijs et al., 2002) wherein both firefly luciferase and Cre recombinase were expressed solely in the pituitary gland under the control of the intermediate lobe-specific POMC promoter. These mice were crossed with mice carrying conditional *lox-Rb-lox* alleles, thereby coupling luciferase activation to deletion of *Rb* and development of pituitary-specific melanotrophic tumors. This sophisticated model allowed the researchers to monitor, by bioluminescence imaging, tumor onset, progression, and response to antineoplastic therapy, thereby generating temporally resolved, statistically significant data from a relatively small cohort of animals.

There is no doubt that conditional activation or deletion of an oncogene or a tumor suppressor gene, coupled with reporter gene expression (e.g., by using Cre/loxP conditional recombination technology) is indispensable for longitudal studies of the role of a specific transformation event for tumorigenesis. However, to limit the need to generate, optimize, and validate novel independent transgenic luciferase mouse strains for each conditional transformation model, a ubiquitously expressing conditional luciferase reporter mouse was developed that can be used to render a wide range of Cre/loxP mouse tumor models for bioluminescence imaging (Lyons et al., 2003). Herein, a β-actin promoter drives FLuc in a Cre-dependent manner. To illustrate the usefulness of this model, the investigators coupled luciferase activation with lung tumorigenesis, induced by $Kras2^{v12}$ (a constitutively active ras mutant) in a preexisting mouse model of non-small cell lung cancer, and followed onset and progression of the spontaneously generated lung tumors. An improved version of a conditional loxP-luciferase mouse was recently described (Safran et al., 2003), where a true knockin was generated by introducing a lox-stop-lox cassette upstream to firefly luciferase cDNA under the control of the ubiquitous ROSA26 promoter. Another advantage of this model over previous attempts (Lyons et al., 2003) is the use of codon-optimized firefly luciferase, thereby increasing its activity by two orders of magnitude.

In the studies mentioned above (Lyons et al., 2003; Vooijs et al., 2002), luciferase activation was regionally coupled to Cre-mediated Rb knockout (Vooijs et al., 2002) or Cre-mediated $Kras2^{v12}$ expression (Lyons et al., 2003), but not biochemically dependent on the molecular transformation event; i.e., luciferase did not serve as a downstream target reporter of *Rb*

or *K-ras* function in Vooijs et al. (2002) and Lyons et al. (2003), respectively. In contrast, a recent transgenic mouse model to study gliomagenesis by bioluminescence imaging was reported (Uhrbom et al., 2004), wherein luciferase activation is not only coupled, but also dependent upon platelet-derived growth factor (PDGF)-induced loss of *Rb*. This mouse (Ef-FLuc) expresses luciferase under the control of the E2F1 promoter, which is negatively regulated by *Rb* under normal conditions, and thus luciferase activity increases upon loss of *Rb* in tumors, regardless of mitotic status. These mice were crossed with N-tv-a mice that express the viral receptor tv-a from the nestin promoter, thereby restricting retroviral transactivation of E2F1→FLuc to glial progenitor cells using viral PDGF-RCAS vectors (Figure 3A) (Holland, 2001). This strategy enables spontaneous gliomagenesis and tumor progression to be followed noninvasively and repetitively over time (Figure 3B). Furthermore, the bioluminescent signal correlates in this model to both tumor cell number and loss of *Rb* control, thereby enabling analyses of the potency and pharmacodynamics of drugs that interfere with tumor maintenance and proliferation (i.e., PDGFR and mTOR inhibitors) as well as cytotoxic drugs (see Figure 3C for a PDGFR inhibitor).

Concluding remarks

Integration of genetically encoded imaging reporters into intact cells and small animal models of disease has provided powerful tools to monitor cancer-associated molecular, biochemical, and cellular pathways in vivo. These types of studies are gaining widespread acceptance within the scientific community and therefore could be considered in some cases to be the method of choice for deciphering complex biological responses in a living animal. We predict that more researchers will continue to take advantage of these new capabilities, thus allowing them to noninvasively "spy" on cancer-specific molecular and regulatory cascades in the intact animal.

Acknowledgments

The authors would like to thank colleagues in the Washington University Molecular Imaging Center for their insightful discussions contributing to this review. Supported by NIH P50 CA94056.

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