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BIOLUMINESCENCE IMAGING: PROGRESS AND APPLICATIONS

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Abstract

Application of bioluminescence imaging has grown tremendously in the past decade and has significantly contributed to the core conceptual advances in biomedical research. This technology provides valuable means for monitoring of different biological processes for immunology, oncology, virology and neuroscience. In this review, we will discuss current trends in bioluminescence and its application in different fields with emphasis on cancer research.

Some living organisms are capable of converting chemical energy into light. This natural phenomenon stemmed a research field defined as bioluminescence imaging (BLI). The technique simply relies on the detection of photons emitted from cells or tissues in a living organism. Unlike fluorescence, BLI does not require light absorption in order to emit light at a longer wavelength. Bioluminescence is a biological process that requires an enzyme known as luciferase, a substrate (luciferin) and oxygen. Some luciferases require other cofactors such as ATP and Mg^{2+} for full activity (Fig. 1).

Luciferases encompass a wide range of enzymes that catalyze light-producing chemical reactions in living organisms. Although many luminescent species exist in nature, generally in lower organisms (beetles, bacteria, algae, crustaceans, annelids, mollusks and coelenterates), three luciferases have been studied thoroughly and are used in biomedical research: the *Photinus pyralis* (firefly) luciferase (Fluc); the sea pansy *Renilla reniformis* luciferase (Rluc); and the marine copepod *Gaussia princeps* luciferase (Gluc). Since Gluc is naturally secreted and similar to Rluc does not require ATP for activity, it can report from the cell itself as well as its immediate environment. Further, Gluc can be detected in blood and urine of small animals thereby allowing *ex vivo* monitoring of biological processes [1,

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2]. On the other hand, cell-associated luciferases (Fluc and Rluc) yields higher light output when localized *in vivo*. Fluc and Rluc (or Gluc) use different substrates and therefore can be combined as dual reporters for bioluminescence imaging of two different processes sequentially in cultured cells as well as in the same living animal, with kinetics of light production (glow versus flash; Box1) being temporally distinct [3, 4]. The blue bioluminescence (480 nm peak) of Gluc and Rluc which has higher absorption by pigmented molecules (e.g. hemoglobin and melanin) and scattering by tissues make them less suited for *in vivo* imaging as compared to Fluc which emits green light (562 nm). The discovery of red-emitting luciferases from *Pyrophorus plagiophthalmus* (Jamaican click beetle) [5], *Photinus pyralis* (American firefly) [6], *Luciola italica* (Italian firefly) [7] and railroad worm (the only luciferase that naturally emits red light) [8] as well as novel chemical reactions leading to red-light output [6, 9–12] will greatly enhance the sensitivity of BLI in deep tissues.

In the past decade, bioluminescence imaging has become indispensable for non-invasive monitoring of biologic phenomena *in vivo*, providing fast and effective ways for validating in culture findings (Table 1). Here, we present recent advances in bioluminescence imaging and applications in different biomedical fields.

IMAGING OF GENE EXPRESSION

Bioluminescent reporters have been used to study gene expression at the transcriptional level. Cis-transcriptional reporter systems allow the analysis of gene expression and gene regulation. This is performed by either generating point mutations/deletions in a promoter region of a gene of interest or the use of different transcription factors binding sites linked to a minimal promoter to drive the expression of a luciferase. This approach is useful for reporting different events that affect transgene expression such as signal transduction, receptor activation and transcription factors activity, thus complementing conventional *in vitro* methods of molecular biology and biochemistry. Post-transcriptional events on the mRNA level such as RNA processing and splicing have been performed successfully using BLI reporter systems. For example, mRNA stability is imaged by fusing a luciferase reporter to the full-length 3' untranslated region (3' UTR) of a gene of interest [13]. RNA interference (RNAi) has recently emerged as a therapeutic strategy in many diseases. RNAi-mediated silencing of luciferase expression is a useful strategy to test the delivery and targeting efficiency of small-interfering RNAs (siRNAs) to specific tissues. The ability of nanoparticles to deliver siRNAs to the liver was tested using a luciferase-targeted siRNAs. Knock-down of luciferase gene expression was efficiently measured in the liver using BLI [14]. MicroRNAs (miRNAs; endogenous small non-coding RNAs) can also inhibit gene expression through translational repression or mRNA cleavage by binding to the 3' UTRs of the target mRNA. By fusing luciferase to a miRNA target site at the 3' UTR, miRNA function and activity could be imaged. In one application, miRNA124a-mediated repression of chromosome 14 open reading frame24 (c14orf24) during neuronal differentiation was monitored using similar reporters [15]. These reporter systems are useful in monitoring gene expression and regulation in different fields ranging from embryo development to disease progression.

IMAGING OF PROTEIN-PROTEIN INTERACTION

Understanding how proteins interact is crucial and highly sought in biological and medical applications. The most commonly used approaches for studying protein-protein interaction include the two-hybrid system and the split reporter system, each discussed below, as well as bioluminescence resonance energy transfer (BRET; recently reviewed [16]).

Two-hybrid system

Recent studies have identified different members of transcriptional activators in mammalian cells. These proteins contain two domains, a DNA binding domain and an activating domain which interacts with components of the transcriptional machinery. The most commonly used transcriptional activator system is based on a fusion between the DNA binding domain of the yeast activator GAL4 and the activator herpes simplex virus protein VP16 [17]. To study the interaction of two proteins of interest, X and Y, two vectors encoding fusions between GAL4-X and VP16-Y are co-expressed with a third vector encoding the reporter gene under the control of GAL4-responsive elements. Upon interaction of the X-Y proteins, the GAL4-X-Y-VP16 complex binds to the GAL4 responsive elements, and the transcriptional activator VP16 will then drive the expression of the luciferase reporter [18] (Fig. 2a). Using this system, Pichler *et al.* have recently generated transgenic mice where Fluc is cloned under the control of the GAL4 promoter [19]. These mice were used to image a well known interaction between the tumor suppressor p53 (fused to GAL4) and large T antigen (TAg, fused to VP16) (Fig. 2b).

The two-step transcriptional amplification (TSTA) method uses the same approach to image the activation of weak promoters. In one application, the prostate specific antigen promoter (PSA) was designed to drive the expression of a GAL4-VP16 fusion protein. The first step consists of activating this weak promoter and expression of GAL4-VP16 fusion. In the second step, the GAL4 binds to its responsive elements which induce the expression of a luciferase [20]. This approach leads to an amplified expression of the reporter protein therefore increasing the luminescence signal. The sensitivity of this amplification system allowed longitudinal imaging of therapeutic gene expression [21], T-cell effector function and proliferation [22] as well as cardiac specific transgene expression [23].

Split reporter system

Another widely used strategy to monitor protein-protein interaction is the split-reporter system also referred to as protein fragment complementation assay (PCA). The monomeric reporter in that case is split into N- and C-terminal fragments thereby disrupting its activity. Each of these fragments is then fused to a different protein of interest. Upon interaction of the two proteins, the N- and C-terminal fragments of the reporter are in close proximity and therefore the reporter is reactivated. This system allows monitoring of protein-protein interaction in real-time which can be detected even at the sub-cellular level in cultured cells and in small animals [24]. These systems are also useful in monitoring of protein-protein interaction in cell-free assays compatible with high-throughput screening [25]. In one application, an Rluc-based PCA was designed to study the G protein-coupled receptor (GPCR)-induced disassembly of protein kinase A (PKA) regulatory (R) and catalytic (C)

subunit [26]. GPCR represents a family of membrane proteins involved in many biological processes and are pivotal targets for therapeutics [27]. PKA activity depends on cellular cAMP levels, which are controlled directly by adenylyl cyclase (AC, production) and phosphodiesterase (PDE, degradation). Under high cAMP levels, the R and C subunits of PKA are dissociated resulting in a decrease in Rluc bioluminescence signal [26] (Fig. 2c–d). This sensor is amenable to high-throughput drug screening for new modulators of PKA dynamics and represents a reversible system for monitoring dissociation-association kinetics of PKA subunits. Similar study to monitor intracellular cAMP was published by Takeuchi *et al.* based on complementation of split luciferase fragments of two-color click beetle luciferase mutants from the Brazilian *Pyrearinus termitilluminans* luciferase (Emerald luc; Eluc; green), and the Jamaican *Pyrophorus plagiophthalmus* luciferase (CBluc; red) [5]. They showed that this reporter system which is based on the ratio of green to red bioluminescence intensities is more advantageous over the single split Fluc reporter, in which the absolute photon counts are affected substantially by ATP and *D*-luciferin concentrations, limiting temporal analysis in living cells. More advantageous multicolored heteroprotein complementation systems have been recently described [28, 29]. By pairing homologous and heterologous fragments of luciferases emitting at different light spectra (Fluc and the click beetle green and red luciferases), real-time assessment of multiple interacting proteins (each tagged with a different luciferase fragment) was achieved both in cultured cells and *in vivo*. This method presents a higher sensitivity paired with a low background activity.

IMAGING OF CELL-MEDIATED IMMUNITY AND INFECTIOUS DISEASES

The immune system embodies complex biological structures and processes including macrophages, natural killer cells and T-lymphocytes. An immune response can be initiated to mediate the killing of infectious pathogens or to impede tumorigenesis. Imaging of immune cells and infectious diseases can contribute significantly to the understanding of their physiology and the development of new therapeutic approaches. Some examples on imaging of T-cell in particular as well as infectious diseases are discussed in this section.

T-cell imaging

Hematopoietic cells, notably T-cells, have been extensively investigated using BLI due to their pivotal role in cell-mediated immunity. In several reports, luciferase-expressing T cells have been used to study the dynamics and function of these cells as well as their anti-tumor activity and capacity of infiltrating tumors. In one application, an enhanced version of Fluc was used to track as few as 10 T-cells after implantation in living animals [30]. In another report, a membrane-anchored form of Gluc (extGluc) was engineered and used to track T-cells implantation *in vivo* [31]. When these cells were genetically engineered to express a tumor-specific chimeric antigen receptor (CAR) as well as extGluc, BLI showed the accumulation of these cells in subcutaneous tumors (Fig. 3a). Dual BLI allowed concomitant imaging of extGluc-expressing T-cells in Fluc-expressing tumors [31]. Similarly, Patel *et al.* achieved longitudinal imaging of T-cells in living animals by expressing Fluc under the Granzyme B promoter, a prominent marker for T-cell activation [22].

Imaging of infectious diseases

In a pioneering report studying pathogens in living organisms, different strains of *Salmonella typhimurium* were engineered to express a bacterial luciferase and infected into experimental animals [32]. Visualization of infection site and infected tissues as well as the efficacy of an antibiotic in this model was monitored non-invasively. This approach provided the opportunity to study infectious diseases in the context of living subjects [33]. Recently, the glial fibrillary acidic protein (GFAP) was shown to be a sensitive marker for the detection of prions, infectious proteins that cause fatal neurodegenerative diseases [34]. A transgenic mouse expressing Fluc under the control of GFAP promoter showed an increase in bioluminescence signal upon prion inoculation proving that this imaging technique is a suitable surrogate for measuring prion infectivity which can be applied for many neurodegenerative illnesses.

IMAGING OF STEM CELLS

A major challenge in the stem cell field is the ability to track these cells, localize their niches and monitor their fate *in vivo*. These goals could be achieved with BLI of neural, cardiac, hematopoietic and mesenchymal stem cells which present vital tools for cell replacement therapies, regenerative medicine as well as tumor therapy. One of the main properties of stem cells is self-renewal. Recently, a single muscle stem cell transplanted in mice was shown to proliferate and generate muscle fibers [35]. BLI allowed monitoring of dynamics and behavior of these muscle cells *in vivo*. In another study, using a bioengineered substrate to recapitulate key biophysical and biochemical muscle stem cells niche features, Gilbert *et al.* used BLI to show that substrate elasticity is a potent regulator of muscle stem cells fate in culture. Unlike cells on rigid plastic dishes, muscle stem cells cultured on soft hydrogel substrates that mimic the elasticity of muscle, self-renew *in vitro* and contribute extensively to muscle regeneration upon transplantation into mice [36].

The therapeutic potential of mesenchymal stem cells (MSC) was exploited in a tibia fracture mouse model, where the migration of MSC expressing luciferase to the fracture site could be imaged over time [37]. Sher *et al.* monitored the survival, functional behavior, and stability of implanted neural stem cells (NSC)-derived oligodendrocyte precursor cells (OPCs) for potential application of NSC-based cell replacement therapy for demyelinating diseases [38]. Similarly, Takahashi *et al.* used luciferase-based imaging to compare different methods for administering neural stem/progenitor cells to treat spinal cord injury in mice [39]. Finally, Kammili *et al.* developed novel bioluminescent-based reporter to monitor differentiation of cardiac stem cells *in vivo* [40].

Studying migrating tumor stem cells is highly desirable not only to understand cancer development and metastasis/invasion, but also for the potential of using normal stem cells as drug delivery vehicles. Luciferases were used to monitor trafficking of MSCs [41], neural precursor cells (NPC) [42], as well as embryonic stem cells [43] and to study their distribution, homing and differentiation into tumor-bearing mice. Our group has recently described a blood-based *ex vivo* approach based on the naturally secreted Gluc to monitor in real-time the viability and proliferation of circulating NPCs [2] (Fig. 3b). This assay has the

unique advantage in that the Gluc activity in blood reports from all viable circulating cells and not only from subpopulation of cells localized at a single site.

BLI IN NEUROSCIENCE

The secretory pathway is a critical index of the capacity of cells to incorporate proteins into cellular membranes and secrete proteins into the extracellular space. This pathway is disrupted in response to stress to the endoplasmic reticulum (ER) which can be induced by a variety of factors such as expression of mutant proteins and physiological stress. Activation of the ER stress response is critical in the etiology of a number of diseases, including neurodegeneration. We have recently developed a new assay to monitor the secretory pathway and ER stress using the secreted Gluc [44]. Upon interfering with the secretory pathway or induction of ER stress, the level of Gluc secreted in the conditioned medium is reduced significantly. The same approach was used by Suzuki *et al.*, to monitor protein trafficking in mammalian cells [45].

To circumvent time-consuming behavioral studies and postmortem neuropathological analysis in neurological diseases, Watts *et al.* generated a bioluminescent-based transgenic mouse model for Alzheimer disease in which Fluc is expressed under the control of GFAP promoter [46]. An age-dependent increase in luciferase signal correlated with disease progression and allowed a longitudinal follow up of this neuropathology. Similarly, Keller *et al.*, showed that glial cells, in particular activated astrocytes, can release factors that can directly kill motor neurons, a characteristic of the late-onset neurological disorder amyotrophic lateral sclerosis (ALS) [47]. Using ALS transgenic mice expressing GFAP-Fluc reporter, they showed that the disease in mice is initiated simultaneously in the spinal cord and in the peripheral nerves and is characterized by several cycles of GFAP upregulation. Further, the disease onset (90 days) was characterized by sharp and synchronized induction of GFAP in peripheral nerve Schwann cells suggesting that peripheral nerves pathology/denervation and associated Schwann cell stress may play an important role in the ALS pathogenesis.

The circadian rhythm in mammals is known to be controlled by key molecular regulators such as the Period family of genes [48]. Mice bearing an Fluc reporter for the Period circadian protein homologue 2 (Per2) were used to study oscillations in the circadian clock [49] and its implication on physiological processes such as jetlag [50]. Similarly, Yagita *et al.* monitored circadian rhythm during mouse embryonic stem cells development and differentiation and showed that this rhythm was undetectable in embryonic stem cells but was induced during differentiation [51].

Recently, Van der Perren *et al.* monitored the transduction efficiency of different adeno-associated viral (AAV) vectors serotypes in the adult Wistar rat substantia nigra (SN), an important target area in Parkinson disease[52]. In ischemic mouse or rat brains, the biodistribution of neural stem cells as potential therapeutics for stroke after intravascular delivery was also evaluated using BLI [53].

BLI IN CANCER

The high complexity of cancer, interaction of tumor cells with the surrounding tissues, and their invasion throughout the whole organism makes the specific tracking of tumor cells highly desirable. BLI has been used in assessing the molecular and cellular events leading to malignant transformation, tumor growth and behavior upon implantation in host animal models, and finally tracking of cancer stem cells or transformed adult cells. Further, this technology played an important role in identifying novel anti-tumor treatments and allowed testing of their efficacy in experimental animal models thereby breaching the gap between pre-clinical drug discovery and clinical trials. In this section, we will discuss current advances in BLI in the cancer field.

Imaging of tumorigenesis and tumor therapy

Luciferase expression correlates with the number of tumor cells both *in vitro* and *in vivo*, hence its use as a reporter for cell proliferation and cell death, compatible with high-throughput screening [54, 55]. Typically, tumor cells are engineered *ex vivo* to stably express luciferase under the control of a constitutively active, tissue-specific or gene/process-specific promoter (e.g. transcription activators, cancer stem cells/differentiation etc.). These cells are then implanted into experimental animal models and can be imaged to track tumor growth, metastasis, cancer stem cells differentiation or response to novel therapies [56, 57]. An *ex vivo* approach based on monitoring of secreted Gluc in blood was developed by our group [1, 2]. By measuring the Gluc activity in the blood, it was possible to monitor tumor growth and response to therapy in subcutaneous, intracranial as well as metastatic tumor models. In a more recent study, Bhang *et al.* used bioluminescence for tumor-specific imaging using progression elevated gene-3 (PEG-3) promoter driving the expression of firefly luciferase [58].

Dysregulation of different signaling pathways is a common trait in tumorigenesis. For instance, the nuclear factor kappa B (NFkB) signaling pathways are constitutively active in many cancer types. In one application, an NFkB transcriptional element driving the expression of Gluc was used to monitor NFkB activation and inhibition *in vitro* as well as in blood of mice *ex vivo* [59]. Other key regulators in oncogenesis such as p53 [60], p16(Ink4a) [61], c-myc [62] and many others have been recently studied using BLI. To recapitulate the *in vivo* interaction of tumor cells with its environment in an *in vitro* model, McMillin *et al.* devised a new tumor imaging platform in which luciferase expressing cancer cells are co-cultured in the presence of stromal cells and used it to screen for potential cancer therapeutics [63]. Such design allows the identification of new drugs which could potentially overcome stroma-mediated drug resistance.

Bioluminescent transgenic mouse models are useful tools for cancer research. To investigate the transcriptional regulation of the human multidrug-resistance 1 gene (*mdr1*) under different physiological conditions, Gu *et al.* developed a mouse model that allows noninvasive, real-time imaging of *mdr1* gene expression *in vivo* [64]. Overexpression of this gene activates the drug efflux from cells thereby conferring drug-resistance. By homologous recombination, the *Fluc* gene was inserted into the murine *mdr1a* locus so that *Fluc* expression is controlled by the *mdr1a* promoter. This strategy is useful in generating tissue-

specific gene expression in transgenic mice and can be extended to several other genes of interest.

Imaging of apoptosis

Apoptosis or programmed cell death plays a major role in embryogenesis, homeostasis, and pathogenesis. Drugs that induce apoptosis are highly sought for cancer therapies. Apoptosis is executed through the activation of cysteine aspartyl proteases (caspases). A main approach to monitor apoptosis uses an inactive luciferase reporter gene fused to a caspase-cleavage sequence. Upon caspase activation, this sequence is cleaved thereby restoring the luciferase function. Niers *et al.* fused GFP to Gluc (including its signal sequence) separated by a caspase-3/7 cleavage peptide (DEVD). This fusion protein resides in the cytoplasm in an inactive form. Upon caspase activation, DEVD is cleaved, freeing Gluc which can then enter the secretory pathway where it is folded properly and can be imaged with bioluminescence. Further, Gluc is released from tumor cells into circulation where it can be detected in blood of mice *ex vivo* [65]. Another approach consists of fusing DEVD to the Fluc substrate luciferin. When apoptosis is induced in cells expressing Fluc, DEVD is cleaved thereby releasing luciferin which would subsequently react with the luciferase thereby generating bioluminescence [66].

Imaging of tumor hypoxia and angiogenesis

Oxygen is needed for proper cellular metabolism. Oxygen deprivation, also known as hypoxia, occurs in cancer among other pathologies such as stroke, myocardial infarction and diabetes. Characterizing and monitoring of hypoxia during which different growth factors, transcription factors and cytokines are induced have implications in many pre-clinical as well as medical settings. One of the main transcription factors induced by low levels of O₂ is the Hypoxia inducing factor 1 (HIF1) which binds to the hypoxia response element (HRE) in order to promote transcriptional activation [67]. Reporter vectors based on HRE elements driving the expression of a luciferase have been designed for longitudinal imaging of hypoxia [68]. These reporters provide valuable information on hypoxia and the molecular pathways underlying this process and can lead to the development of HIF1-targeted therapies.

High levels of H₂O₂ leading to oxidative stress and inflammation is a critical aspect in several chronic diseases including cancer, diabetes, neurodegenerative, cardiovascular and pulmonary diseases [69]. Van de Bittner *et al.* devised a new bioluminescent H₂O₂ sensor, named Peroxy Caged Luciferin-1 (PCL-1; Fig 4a) [70]. This luciferin caging system is comparable to the DEVD-luciferin apoptosis reporter described above. Here, the luciferin is linked to an H₂O₂-sensitive aryl boronic acid. The H₂O₂ reaction with the aryl boronic acid will release the trapped luciferin allowing the luciferase reaction to occur. Basal levels, fluctuations and inhibition of H₂O₂ with antioxidants could be quantitatively detected in luciferase expressing mice using this PCL-1 sensor (Fig 4b–e).

Hypoxia also stimulates the secretion of the vascular endothelial growth factor (VEGF) and promotes angiogenesis to increase the oxygen availability for tissues through the capillaries [71]. Transgenic mice based on VEGF receptor 2 (VEGFR2) promoter driving the

expression of Fluc reporter can be used to monitor angiogenesis induced by tumors, skin wounding and contact hypersensitivity [72]. Further, many other luciferase-based reporters for angiogenesis imaging have been recently described [73, 74].

BLI in cancer gene and cell therapy

Despite several pitfalls, the gene therapy field has expanded significantly over the last decade. This could be noticed by the increasing number of clinical trials, the majority of which are directed towards cancer [75]. BLI provides a means to monitor neoplastic cells, evaluate the efficiency of the treatment, study gene activation and cellular mechanisms, and to image the expression of encoded toxins delivered to tumors and the silencing of tumor promoting genes. Gene therapy in general relies on two basic factors: the transfer and expression of the therapeutic gene. In addition to the spatio-temporal evaluation of gene therapy, BLI allows the evaluation of viral tropism and transduction as well as viral replication [76, 77]. To evaluate the therapeutic response to suicidal gene therapy in a subcutaneous tumor model expressing Fluc, the therapeutic gene cytosine deaminase fused to uracil phosphoribosyltransferase [converts the nontoxic compound 5-fluorocytosine (5FC) into the drug 5-fluorouracil] was linked with Gluc through an internal ribosomal entry site, all under the control of the tumor-specific NFkB promoter[78]. Upon gene transfer into tumors, Gluc allows monitoring of the duration and magnitude of transgene expression. Following treatment with the pro-drug 5FC, Fluc imaging is used to monitor tumor growth and response to this therapy which showed the effectiveness of this therapeutic strategy. In a similar study, Ahn *et al.* developed a Survivin-targeted amplifiable adenoviral vector in an orthotopic hepatoma rat model [79]. They used the TSA system described above to amplify the expression from a Survivin promoter driving the expression of a reporter gene (Fluc) as well as a therapeutic gene (tumor necrosis factor apoptosis-inducing ligand or TRAIL) and showed tumor-specific gene expression based on Fluc imaging.

Adoptive cell transfer in which the patient's own white blood cells are isolated, genetically engineered *ex vivo* to enhance their anti-tumor effect and re-injected back into the same patient is an attractive strategy for cancer immunotherapy. BLI has been indispensable for pre-clinical analysis of this therapeutic strategy by monitoring kinetic phases of distribution, tumor targeting and therapeutic efficacy of different tumor-killing cells including: lymphocytes engineered to express T cell receptor [80], Tumor-specific T cells expressing CXCR2 chemokine receptor [81], natural killer cells in conjunction with a proteasome inhibitor [82], and MSC engineered to express a bispecific alpha-carcinoembryonic antigen (alphaCEA)/alphaCD3 diabody and seeded in a synthetic extracellular matrix which leads to continuous release of diabody [83]. Manishkumar *et al.* extended the use of BLI and developed a noninvasive imaging strategy to monitor T-cell activation in living subjects by fusing the Fluc reporter to the Granzyme B promoter, known to be induced during T-cell activation [22]. Using engineered T-cells, they were able to image T-cell effector function longitudinally in response to tumor antigens in living animals. This method has the potential to accelerate the study of adoptive immunotherapy in preclinical cancer models.

The emerging of the stem cell field has introduced a new alternative for transgene delivery since these cells have the potential of being used as delivery vehicles. MSC in particular

have been shown to specifically target tumor cells [84]. These stem cells have been labeled with a luciferase to study their fate after *in vivo* engraftment and subsequently monitor transgene expression and/or the therapeutic outcome [85]. Transgene delivery could also be attained using lipid nanoparticles. The ability of these nanoparticles to deliver siRNAs to the liver was tested using luciferase-targeting siRNAs after systemic administration. The knock-down of luciferase gene expression could be measured in the liver by BLI [14].

Conclusions and perspectives

BLI provides valuable insight into biological processes in intact cells as well as small-animal models. Further, it helps expedite drug identification and development and subsequently functional assessment of new treatment regimens in animal models before translation into the clinic. Genomic research has come a long way during the last few years which led to better understanding of pathologies and identification of new treatment paradigms. A major challenge for BLI is to cope with these advances and help complement genomic studies while providing additional inputs in biology, pathology as well as pharmacology. The development of multiplexed compound screenings that combine genomic and BLI-based phenotypic read-outs is highly desirable. Development of new small-animals CCD cameras capable of three dimensional image reconstruction similar to fluorescence imaging and generation of more stable, red-shifted luciferases, and novel luciferases which utilize different substrates will further enhance the utility of BLI. While the field of bioluminescence imaging keeps expanding in a rapid pace, the challenge remains to translate this technique to the clinic currently unattainable due to the limited depth penetration of light (typically absorbed and scattered by mammalian tissues), potential toxicity of luciferin as well as potential immunogenicity of luciferases.

REFERENCES

1. Tannous BA. Gaussia luciferase reporter assay for monitoring biological processes in culture and in vivo. *Nat Protoc.* 2009; 4:582–591. [PubMed: 19373229]
2. Wurdinger T, et al. A secreted luciferase for ex vivo monitoring of in vivo processes. *Nat Methods.* 2008; 5:171–173. [PubMed: 18204457]
3. Bhaumik S, Gambhir SS. Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc Natl Acad Sci U S A.* 2002; 99:377–382. [PubMed: 11752410]
4. Shah K, et al. Real-time imaging of TRAIL-induced apoptosis of glioma tumors in vivo. *Oncogene.* 2003; 22:6865–6872. [PubMed: 14534533]
5. Takeuchi M, et al. Ratiometric bioluminescence indicators for monitoring cyclic adenosine 3',5'-monophosphate in live cells based on luciferase-fragment complementation. *Anal Chem.* 2010; 82:9306–9313. [PubMed: 20979393]
6. Caysa H, et al. A redshifted codon-optimized firefly luciferase is a sensitive reporter for bioluminescence imaging. *Photochem Photobiol Sci.* 2009; 8:52–56. [PubMed: 19247529]
7. Branchini BR, et al. Luciferase from the Italian firefly *Luciola italica*: molecular cloning and expression. *Comp Biochem Physiol B Biochem Mol Biol.* 2006; 145:159–167. [PubMed: 16916615]
8. Viviani VR, et al. Active-site properties of Phrixotrix railroad worm green and red bioluminescence-eliciting luciferases. *J Biochem.* 2006; 140:467–474. [PubMed: 16963787]
9. Branchini BR, et al. Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. *Anal Biochem.* 2007; 361:253–262. [PubMed: 17181991]

10. Du J, et al. Quantum-dot-decorated robust transductable bioluminescent nanocapsules. *J Am Chem Soc.* 2010; 132:12780–12781. [PubMed: 20795619]
11. Ma N, et al. Near-infrared light emitting luciferase via biomineralization. *J Am Chem Soc.* 2010; 132:6884–6885. [PubMed: 20441172]
12. Wu C, et al. In vivo far-red luminescence imaging of a biomarker based on BRET from Cypridina bioluminescence to an organic dye. *Proc Natl Acad Sci U S A.* 2009; 106:15599–15603. [PubMed: 19805215]
13. Subramaniam D, et al. Translation inhibition during cell cycle arrest and apoptosis: Mcl-1 is a novel target for RNA binding protein CUGBP2. *Am J Physiol Gastrointest Liver Physiol.* 2008; 294:G1025–G1032. [PubMed: 18292181]
14. Tao W, et al. Noninvasive imaging of lipid nanoparticle-mediated systemic delivery of small-interfering RNA to the liver. *Mol Ther.* 2010; 18:1657–1666. [PubMed: 20628357]
15. Ko HY, et al. Noninvasive imaging of microRNA124a-mediated repression of the chromosome 14 ORF 24 gene during neurogenesis. *FEBS J.* 2009; 276:4854–4865. [PubMed: 19663910]
16. Xia Z, Rao J. Biosensing and imaging based on bioluminescence resonance energy transfer. *Curr Opin Biotechnol.* 2009; 20:37–44. [PubMed: 19216068]
17. Sadowski I, et al. GAL4-VP16 is an unusually potent transcriptional activator. *Nature.* 1988; 335:563–564. [PubMed: 3047590]
18. Ray P, et al. Noninvasive quantitative imaging of protein-protein interactions in living subjects. *Proc Natl Acad Sci U S A.* 2002; 99:3105–3110. [PubMed: 11854471]
19. Pichler A, et al. Generation of a highly inducible Gal4-->Fluc universal reporter mouse for in vivo bioluminescence imaging. *Proc Natl Acad Sci U S A.* 2008; 105:15932–15937. [PubMed: 18843112]
20. Iyer M, et al. Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proc Natl Acad Sci U S A.* 2001; 98:14595–14600. [PubMed: 11734653]
21. Ray S, et al. Noninvasive imaging of therapeutic gene expression using a bidirectional transcriptional amplification strategy. *Mol Ther.* 2008; 16:1848–1856. [PubMed: 18766175]
22. Patel MR, et al. Longitudinal, noninvasive imaging of T-cell effector function and proliferation in living subjects. *Cancer Res.* 2010; 70:10141–10149. [PubMed: 21159636]
23. Chen IY, et al. Indirect imaging of cardiac-specific transgene expression using a bidirectional two-step transcriptional amplification strategy. *Gene Ther.* 2010; 17:827–838. [PubMed: 20237511]
24. Ozawa T, Umezawa Y. Detection of protein-protein interactions in vivo based on protein splicing. *Curr Opin Chem Biol.* 2001; 5:578–583. [PubMed: 11578933]
25. Porter JR, et al. A general and rapid cell-free approach for the interrogation of protein-protein, protein-DNA, and protein-RNA interactions and their antagonists utilizing split-protein reporters. *J Am Chem Soc.* 2008; 130:6488–6497. [PubMed: 18444624]
26. Stefan E, et al. Quantification of dynamic protein complexes using Renilla luciferase fragment complementation applied to protein kinase A activities in vivo. *Proc Natl Acad Sci U S A.* 2007; 104:16916–16921. [PubMed: 17942691]
27. Lappano R, Maggiolini M. G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov.* 2011; 10:47–60. [PubMed: 21193867]
28. Hida N, et al. High-sensitivity real-time imaging of dual protein-protein interactions in living subjects using multicolor luciferases. *PLoS One.* 2009; 4:e5868. [PubMed: 19536355]
29. Villalobos V, et al. Dual-color click beetle luciferase heteroprotein fragment complementation assays. *Chem Biol.* 2010; 17:1018–1029. [PubMed: 20851351]
30. Rabinovich BA, et al. Visualizing fewer than 10 mouse T cells with an enhanced firefly luciferase in immunocompetent mouse models of cancer. *Proc Natl Acad Sci U S A.* 2008; 105:14342–14346. [PubMed: 18794521]
31. Santos EB, et al. Sensitive in vivo imaging of T cells using a membrane-bound Gaussia princeps luciferase. *Nat Med.* 2009; 15:338–344. [PubMed: 19219023]
32. Contag CH, et al. Photonic detection of bacterial pathogens in living hosts. *Mol Microbiol.* 1995; 18:593–603. [PubMed: 8817482]

33. Engelsman AF, et al. Real time noninvasive monitoring of contaminating bacteria in a soft tissue implant infection model. *J Biomed Mater Res B Appl Biomater.* 2009; 88:123–129. [PubMed: 18618733]
34. Tamguney G, et al. Measuring prions by bioluminescence imaging. *Proc Natl Acad Sci U S A.* 2009; 106:15002–15006. [PubMed: 19706444]
35. Sacco A, et al. Self-renewal and expansion of single transplanted muscle stem cells. *Nature.* 2008; 456:502–506. [PubMed: 18806774]
36. Gilbert PM, et al. Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. *Science.* 2010; 329:1078–1081. [PubMed: 20647425]
37. Granero-Molto F, et al. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells.* 2009; 27:1887–1898. [PubMed: 19544445]
38. Sher F, et al. Bioluminescence imaging of Olig2-neural stem cells reveals improved engraftment in a demyelination mouse model. *Stem Cells.* 2009; 27:1582–1591. [PubMed: 19544465]
39. Takahashi Y, et al. Comparative study of methods for administering neural stem/progenitor cells to treat spinal cord injury in mice. *Cell Transplant.* 2010
40. Kammili RK, et al. Generation of novel reporter stem cells and their application for molecular imaging of cardiac-differentiated stem cells in vivo. *Stem Cells Dev.* 2010; 19:1437–1448. [PubMed: 20109065]
41. Wang H, et al. Trafficking mesenchymal stem cell engraftment and differentiation in tumor-bearing mice by bioluminescence imaging. *Stem Cells.* 2009; 27:1548–1558. [PubMed: 19544460]
42. Waerzeggers Y, et al. Multimodal imaging of neural progenitor cell fate in rodents. *Mol Imaging.* 2008; 7:77–91. [PubMed: 18706290]
43. Sun N, et al. Long term non-invasive imaging of embryonic stem cells using reporter genes. *Nat Protoc.* 2009; 4:1192–1201. [PubMed: 19617890]
44. Badr CE, et al. A highly sensitive assay for monitoring the secretory pathway and ER stress. *PLoS ONE.* 2007; 2:e571. [PubMed: 17593970]
45. Suzuki T, et al. Real-time bioluminescence imaging of a protein secretory pathway in living mammalian cells using Gaussia luciferase. *FEBS Lett.* 2007; 581:4551–4556. [PubMed: 17825828]
46. Watts JC, et al. Bioluminescence imaging of Abeta deposition in bigenic mouse models of Alzheimer's disease. *Proc Natl Acad Sci U S A.* 2011; 108:2528–2533. [PubMed: 21262831]
47. Keller AF, et al. Live imaging of amyotrophic lateral sclerosis pathogenesis: disease onset is characterized by marked induction of GFAP in Schwann cells. *Glia.* 2009; 57:1130–1142. [PubMed: 19115383]
48. Dunlap JC. Molecular bases for circadian clocks. *Cell.* 1999; 96:271–290. [PubMed: 9988221]
49. Guilding C, et al. Circadian oscillators in the epithalamus. *Neuroscience.* 2010; 169:1630–1639. [PubMed: 20547209]
50. Davidson AJ, et al. Visualizing jet lag in the mouse suprachiasmatic nucleus and peripheral circadian timing system. *Eur J Neurosci.* 2009; 29:171–180. [PubMed: 19032592]
51. Yagita K, et al. Development of the circadian oscillator during differentiation of mouse embryonic stem cells in vitro. *Proc Natl Acad Sci U S A.* 2010; 107:3846–3851. [PubMed: 20133594]
52. Van der Perren A, et al. Efficient and stable transduction of dopaminergic neurons in rat substantia nigra by rAAV 2/1, 2/2, 2/5, 2/6.2, 2/7, 2/8 and 2/9. *Gene Ther.* 2011; 18:517–527. [PubMed: 21326331]
53. Pendharkar AV, et al. Biodistribution of neural stem cells after intravascular therapy for hypoxic-ischemia. *Stroke.* 2010; 41:2064–2070. [PubMed: 20616329]
54. Badr CE, et al. Functional drug screening assay reveals potential glioma therapeutics. *Assay Drug Dev Technol.* 2010 in press.
55. Feng Y, et al. Multi-parameter phenotypic profiling: using cellular effects to characterize small-molecule compounds. *Nat Rev Drug Discov.* 2009; 8:567–578. [PubMed: 19568283]
56. Prasad G, et al. Inhibition of PI3K/mTOR pathways in glioblastoma and implications for combination therapy with temozolomide. *Neuro Oncol.* 2011; 13:384–392. [PubMed: 21317208]

57. Sloan EK, et al. The sympathetic nervous system induces a metastatic switch in primary breast cancer. *Cancer Res.* 2010; 70:7042–7052. [PubMed: 20823155]
58. Bhang HE, et al. Tumor-specific imaging through progression elevated gene-3 promoter-driven gene expression. *Nat Med.* 2011; 17:123–129. [PubMed: 21151140]
59. Badr CE, et al. Real-time monitoring of nuclear factor kappaB activity in cultured cells and in animal models. *Mol Imaging.* 2009; 8:278–290. [PubMed: 19796605]
60. Hamstra DA, et al. Real-time evaluation of p53 oscillatory behavior in vivo using bioluminescent imaging. *Cancer Res.* 2006; 66:7482–7489. [PubMed: 16885345]
61. Yamakoshi K, et al. Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. *J Cell Biol.* 2009; 186:393–407. [PubMed: 19667129]
62. Fan-Minogue H, et al. Noninvasive molecular imaging of c-Myc activation in living mice. *Proc Natl Acad Sci U S A.* 2010; 107:15892–15897. [PubMed: 20713710]
63. McMillin DW, et al. Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. *Nat Med.* 2010; 16:483–489. [PubMed: 20228816]
64. Gu L, et al. A new model for studying tissue-specific mdr1a gene expression in vivo by live imaging. *Proc Natl Acad Sci U S A.* 2009; 106:5394–5399. [PubMed: 19282474]
65. Niers JM, et al. Multimodal In Vivo Imaging and Blood Monitoring of Intrinsic and Extrinsic Apoptosis. *Mol Ther.* 2011
66. Scabini M, et al. In vivo imaging of early stage apoptosis by measuring real-time caspase-3/7 activation. *Apoptosis.* 2011; 16:198–207. [PubMed: 21082356]
67. Semenza GL, et al. Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. *Proc Natl Acad Sci U S A.* 1991; 88:5680–5684. [PubMed: 2062846]
68. Lehmann S, et al. Longitudinal and multimodal in vivo imaging of tumor hypoxia and its downstream molecular events. *Proc Natl Acad Sci U S A.* 2009; 106:14004–14009. [PubMed: 19666490]
69. Reuter S, et al. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med.* 2010; 49:1603–1616. [PubMed: 20840865]
70. Van de Bittner GC, et al. In vivo imaging of hydrogen peroxide production in a murine tumor model with a chemoselective bioluminescent reporter. *Proc Natl Acad Sci U S A.* 2010; 107:21316–21321. [PubMed: 21115844]
71. Ryan HE, et al. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *Embo J.* 1998; 17:3005–3015. [PubMed: 9606183]
72. Zhang N, et al. Tracking angiogenesis induced by skin wounding and contact hypersensitivity using a Vegfr2-luciferase transgenic mouse. *Blood.* 2004; 103:617–626. [PubMed: 14512298]
73. Edwards WB, et al. Multimodal imaging of integrin receptor-positive tumors by bioluminescence, fluorescence, gamma scintigraphy, and single-photon emission computed tomography using a cyclic RGD peptide labeled with a near-infrared fluorescent dye and a radionuclide. *Mol Imaging.* 2009; 8:101–110. [PubMed: 19397855]
74. Sanz L, et al. Long-term in vivo imaging of human angiogenesis: critical role of bone marrow-derived mesenchymal stem cells for the generation of durable blood vessels. *Microvasc Res.* 2008; 75:308–314. [PubMed: 18252255]
75. Edelstein ML, et al. Gene therapy clinical trials worldwide to 2007--an update. *J Gene Med.* 2007; 9:833–842. [PubMed: 17721874]
76. Carlon M, et al. Efficient gene transfer into the mouse lung by fetal intratracheal injection of rAAV2/6.2. *Mol Ther.* 2010; 18:2130–2138. [PubMed: 20664525]
77. Gallaher SD, et al. Robust in vivo transduction of a genetically stable Epstein-Barr virus episome to hepatocytes in mice by a hybrid viral vector. *J Virol.* 2009; 83:3249–3257. [PubMed: 19158239]
78. Badr CE, et al. Suicidal gene therapy in an NF-kappaB-controlled tumor environment as monitored by a secreted blood reporter. *Gene Ther.* 2011; 18:445–451. [PubMed: 21150937]
79. Ahn BC, et al. Potent, tumor-specific gene expression in an orthotopic hepatoma rat model using a Survivin-targeted, amplifiable adenoviral vector. *Gene Ther.* 2011

80. Koya RC, et al. Kinetic phases of distribution and tumor targeting by T cell receptor engineered lymphocytes inducing robust antitumor responses. *Proc Natl Acad Sci U S A*. 2010; 107:14286–14291. [PubMed: 20624956]
81. Peng W, et al. Transduction of tumor-specific T cells with CXCR2 chemokine receptor improves migration to tumor and antitumor immune responses. *Clin Cancer Res*. 2010; 16:5458–5468. [PubMed: 20889916]
82. Lundqvist A, et al. Bortezomib treatment and regulatory T-cell depletion enhance the antitumor effects of adoptively infused NK cells. *Blood*. 2009; 113:6120–6127. [PubMed: 19202127]
83. Compte M, et al. Tumor immunotherapy using gene-modified human mesenchymal stem cells loaded into synthetic extracellular matrix scaffolds. *Stem Cells*. 2009; 27:753–760. [PubMed: 19096041]
84. Nakamizo A, et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res*. 2005; 65:3307–3318. [PubMed: 15833864]
85. Cao F, et al. Noninvasive de novo imaging of human embryonic stem cell-derived teratoma formation. *Cancer Res*. 2009; 69:2709–2713. [PubMed: 19318556]

Box1: Special considerations in BLI

Promoter driving the luciferase expression

Some commonly used promoters [cytomegalovirus (CMV) promoter for example] are subject to non-specific regulation by several stimuli, which could lead to false or non-specific read-outs. For example, the transcription factor nuclear factor kappa B (NFkB) can bind to a sequence motif on the enhancer region of the CMV promoter thereby increasing its activity. Many cancer treatments strongly induce NFkB. In cells expressing a CMV-driven luciferase, such treatments could lead to promoter-dependent increase in luciferase signal, resulting in erroneous quantification of cell viability by BLI.

Luciferase reaction kinetics

in vitro: Some luciferases (Gluc and Rluc) catalyze flash-type bioluminescence reaction in which the signal drops to background levels seconds after the substrate addition, therefore, a luminometer with a built-in injector should be used to acquire the signal immediately after substrate injection. On the other hand, for luciferases emitting a glow-type bioluminescence reaction in which the signal generated is stable for a longer period of time (e.g. Fluc), the substrate can be added to all wells simultaneously. *in vivo*: Depending on the administration route of substrate [intraperitoneal (IP) versus intravenous (IV)] and its biodistribution, as well as the location and type of tissue to be imaged, signal acquisition should be performed at optimal time. Since IP injection (typically performed for Fluc imaging with *D*-luciferin) will cause a slow absorption of the substrate, the signal peaks around 10 min after injection and remains constant for 30 min. Imaging should be performed 10–30 min post-injection. On the other hand, IV injection (typically performed for coelenterazine to image Gluc/Rluc) yield a much faster absorption with peak values attained within seconds post-injection of substrate. Imaging should be performed immediately after substrate administration.

Substrate Solubility

Coelenterazine is not soluble in aqueous solutions, 100% alcohol is often used to dissolve this substrate. Special consideration should be taken when diluting coelenterazine since it can come out of solution and form a precipitate which could yield to animal death upon IV injection. Dilution of coelenterazine should be performed immediately before injection. Two modified coelenterazine (Viviren and Enduren from Promega Co.) are available for *in vivo* BLI. These substrates present higher stability (Enduren) or sensitivity (Viviren) compared to the native substrate.

Deep tissues yield lower bioluminescence signal

Since light is absorbed by mammalian tissues, lower signal is expected when imaging deep tissues. This problem could be overcome by using luciferases which emit red to near-infrared light.

Tumor size might affect substrate uptake and signals

Bigger tumors usually have higher uptake of substrate which could lead to higher signal and false data interpretation. Further, over saturation of signal when using CCD cameras

with lower linear range, as well as necrotic tumors can also generate erroneous data. Pharmacokinetic studies or reducing acquisition time should be considered.

Figure 1. Bioluminescence reaction of Fluc, Rluc and Gluc. The luciferin chemical structure is shown as well as the chemical reaction and the peak light emission ($\lambda_{\max}$) for each luciferase.

Figure 2. Imaging of protein-protein interaction. (a–b) Two-hybrid system

(a) Two vectors driving the expression of GAL4-protein X and VP16-protein Y fusions are co-expressed. Once protein X interacts with protein Y, the GAL4-X-Y-VP16 complex will subsequently bind to the GAL4 responsive elements in a third vector to drive the expression of luciferase, thereby re-activating its bioluminescence reaction. (b) Transgenic mice expressing the transgene in (B) were transfected *in vivo* with different plasmids: pGal4BDp53 combined with pVP16-TAg or pGal4BDp53 combined with off-target pVP16-CP, or each plasmid separately. A plasmid expressing Rluc was used for normalization. Mice were imaged 24 hours after transfection for Fluc expression (*D*-luciferin substrate) and Rluc expression (coelenterazine substrate). BD, binding domain; CP, polyomavirus coat. Adapted with permission from Pichler *et al.*, 2008. (c–d) **Split reporter system or PCA.** (c) Schematic overview of the Rluc-PCA system to study the interaction of the PKA subunits. Low levels of cAMP cause the regulatory (R) and catalytic (C) subunits to form a heterodimer and induce Rluc activity. Under high cAMP levels, both subunits are dissociated and the Rluc activity is decreased. (d) BLI of PKA subunits-interaction *in vitro*. HEK293T human fibroblast cells were transfected with full-length Rluc (upper panel) or with Rluc-PCA (middle and lower panel). Images were detected using a CCD camera after addition of coelenterazine. Forskolin which increases the cAMP levels was used to treat

cells co-expressing PKA C and R subunits. This cAMP induction disrupted the Rluc activity. DAPI was used for nuclear staining. Scale bar, 5 μ m. Adapted with permission from Stefan *et al.*, 2007.

Figure 3. Imaging of circulating hematopoietic and neuronal precursor cells

(a) Human T cells expressing extGluc and CAR accumulate in subcutaneous tumors. BLI of mice with subcutaneous CD19⁺ tumors at day 1 and 2 after systematic injection with human T-cells co-expressing CAR targeting CD19⁺ cells (19z1) and extGLuc or non-specific CAR (Pz1) and extGLuc. Adapted with permission from Santos *et al.*, 2009. **(b)** Monitoring of neuronal precursor cells (NPC) using the Gluc-blood reporter. Mice were injected with NPC cells expressing Gluc or PBS (control). Gluc activity was monitored in 5 μ l blood over time after the addition of coelenterazine using a luminometer. Adapted with permission from Wurdinger *et al.*, 2007.

Figure 4. Imaging of hydrogen peroxide production

(a) The PCL-1 sensor. H_2O_2 mediates the release of luciferin thereby generating bioluminescence. (b–e) *In vivo* BLI of H_2O_2 . Mice ubiquitously expressing Fluc were injected with PCL-1 followed by injection with different doses of H_2O_2 . A dose-dependent increase in BLI expressed as total photon flux was observed few minutes after injection of H_2O_2 (b–c). Injection of the antioxidant *N*-acetylcysteine (NAC) resulted in decrease in the BLI signal further confirming the specificity on PCL-1 as an *in vivo* reporter of H_2O_2 (d–e). Adapted with permission from Van de Bittner *et al*, 2010.

Table 1Summary of commonly used BLI approaches^a

Level of Regulation	Process	Strategy	Applications	Refs.
Nuclear	mRNA stability	Fusing a luciferase reporter to the full-length 3'UTR of a gene of interest	Study of post-transcriptional regulation; factors that control mRNA stability	[13]
	Gene regulation: siRNA miRNA transcription factors	siRNA-mediated silencing of luciferase expression. Fusing luciferase to microRNA target site at the 3'UTR. Binding sites for transcription factor driving expression of luciferase.	Evaluating siRNA delivery to specific tissues Monitor expression / activity of miRNAs Study of signaling pathways Inducible reporter systems	[14] [15] [68]
	Protein splicing ^b	Reconstitution of split luciferase after protein splicing.	Study of post-translational protein modification	[24]
Cytoplasmic	Protein-protein interaction	Gal4:VP16: fused to proteins of interest will act as a transcriptional activator for luciferase. PCA: luciferase split into C and N-terminal fragment, each fused to a protein of interest. BRET: luciferase and a FP are each fused to a protein of interest. Energy transfer from luciferase emission is absorbed by FP generating fluorescence	Study interaction of 2 proteins of interest for cell signaling, physiological processes, therapeutic targeting	[19]
	Protein folding	Chaperone-mediated protein folding	Monitor regulation, kinetics, and effect of external factors/ diseases on protein folding	[45]
	Protein secretion	Using of secreted luciferases	Monitoring the secretory pathway and ER stress	[44]
	Proteolytic enzyme activity	Split of full length luciferase linked to proteolytic cleavage site	Studying activity of caspases, ubiquitin, etc.	[66]
Cellular	luciferase expression	Constitutive promoter driving the expression of luciferase	Cell viability, proliferation, tumor burden Viral tropism, infectious organisms	[56]
	Tissue-specific promoters	Luciferase expression is tissue-dependent	Specific/localized expression, targeting strategies	[58]
	Gene/process-specific promoters	Luciferase expression depends on transcription factors/gene promoters	Stem cell/ differentiation markers, cell signaling pathway	[78]
	Conditional reporters	Using Cre/Lox recombination or Tetracycline-controlled transcriptional activation to induce luciferase expression	Bioluminescent transgenic mice, imaging tumorigenesis, conditional gene expression in transgenic animals	[64]
Cell-Free	Enzyme quantification	BLI signal correlates with enzyme concentration	Measuring of ATP, monoamine oxidase, etc.	[25]

^a Abbreviations: UTR: untranslated region; PCA: protein complementation assay; BRET: bioluminescence resonance energy transfer; FP: fluorescent protein; ER: endoplasmic reticulum

^b Protein splicing could also take place in the cytoplasm.