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## Advances in Bioluminescence Imaging of Live Animal Models

Robin S. Dothager<sup>1,2</sup>, Kelly Flentie<sup>1,2</sup>, Britney Moss<sup>1,2</sup>, Mei-Hsiu Pan<sup>1,2</sup>, Aparna Kesarwala<sup>1,2</sup>, and David Piwnica-Worms<sup>1,2,\*</sup>

<sup>1</sup>Molecular Imaging Center, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO 63110, USA

<sup>2</sup>Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA

### Summary

Many of the obligate steps of physiology and disease are dynamic in time and space, and thus, end-point assays do not always provide a full understanding of these processes. Comprehensive understanding of the functional complexity of protein interactions and cell trafficking requires mapping of cellular and molecular function within complex systems over biologically relevant time scales. New approaches to bioluminescence imaging of cell migration, signaling pathways, drug action and interacting protein partners *in vivo* allow the study of biology and disease within the context of living animals.

### Introduction

Genetically-encoded imaging reporters introduced into cells and transgenic animals enable noninvasive, longitudinal studies of dynamic biological processes in intact cells and living animals [1]. These reporters can produce signal intrinsically (e.g., fluorescent proteins), through enzymatic activation of an inactive substrate (luciferases), by enzymatic modification of an imagable (e.g., optical) substrate with selective retention in reporter cells, or by direct binding or import of an active (e.g., radiolabeled) reporter substrate or probe. 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 and pre-clinical research in that once validated, a single genetically-encoded reporter can theoretically be cloned into a variety of vectors to interrogate a broad array of regulatory pathways. Compared to injectable radiopharmaceuticals, for example, 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.

The most common reporters include firefly luciferase (bioluminescence imaging), green fluorescence protein (fluorescence imaging), transferrin receptor (magnetic resonance imaging), Herpes Simplex Virus-1 thymidine kinase (positron emission tomography) and variants with enhanced spectral and kinetic properties optimized for use *in vivo*. When cloned into promoter/enhancer sequences or engineered into fusion proteins, imaging reporters enable

\*Correspondence: David Piwnica-Worms, M.D., Ph.D. Mallinckrodt Institute of Radiology, Washington University School of Medicine, 510 S. Kingshighway Blvd., Box 8225, St. Louis, MO 63110, Email: E-mail: piwnica-wormsd@mir.wustl.edu. Phone: (314) 362-9359; Fax: (314) 362-0152.

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fundamental processes such as transcriptional regulation, signal transduction cascades, protein-protein interactions, protein degradation, oncogenic transformation, cell trafficking and targeted drug action to be temporally and spatially registered *in vivo*. Ideally, the magnitude and time course of reporter gene activity should parallel the strength and duration of endogenous target gene expression. Genetically-encoded imaging reporters 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.

Bioluminescence imaging (BLI) 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, except in the case of bacterial *lux* operons, and in general, is usually represented as planar imaging datasets, therefore imposing some positional uncertainty on 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 plagiophthalmus*) luciferases and *Gaussia* luciferase [2-4]. 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. New mutants of *Renilla* luciferase were recently reported, and while favorably shifted approximately 66 nm, are still rather green for optimal use *in vivo* [5]. Moreover, the *Renilla* and *Gaussia* luciferase substrate, coelenterazine, has been shown to be transported by the multidrug resistance transporter P-glycoprotein [6] as well as to interact efficiently with superoxide anion and peroxynitrate in light-producing reactions [7], thereby complicating certain applications of *Renilla* and *Gaussia* luciferases *in vivo*. Nonetheless, the favorable attributes of luciferin-based imaging provide a versatile platform for studying biology *in vivo*.

Herein we will focus on regulatory and biochemical events in several of the major classes of diseases that can be interrogated with luciferases as the imaging reporter gene. Recent strategies to regulate genetically-encoded reporter activation and thereby detect and dynamically monitor various components of cell motility and cell machinery (transcriptional, post-transcriptional, translational and post-translational) in intact small animal models will be briefly highlighted in this review.

## Imaging Cancer

The most common use of BLI in cancer research has been to assess mass and location of xenografted cells constitutively expressing a luciferase gene, providing a robust strategy to monitor effectiveness of anti-tumor drugs *in vivo*. Galkin and co-workers utilized BLI to study small molecule (TAE684) inhibition of the anaplastic lymphoma kinase (NPM-ALK) fusion protein unique to anaplastic large-cell lymphomas (ALCL) [8]. They assessed the dose-response of constitutively bioluminescent Karpas-299 lymphoma cells in a mouse xenograft model, and found that TAE684 exhibited potent and specific inhibition of lymphoma cell growth.

In basic cancer research, BLI combined with genetic alteration (gene knock-out or over-expression) has increased understanding of the consequences of specific genes (or gene combinations) on tumor load and metastasis. For instance, using a breast cancer metastasis model, Podsypanina and co-workers investigated whether primary mammary cells can be induced to form metastases in the absence of oncogene-driven transformation at the primary site. They harvested mammary tissue from a transgenic mouse expressing a mammary-specific transactivator driving a doxycycline-inducible cassette comprised of middle T antigen and

luciferase separated by an IRES. Dissociated mammary cells from doxycycline-naive animals were injected via tail vein into recombination activation gene null (Rag<sup>-/-</sup>) mice maintained on a doxycycline diet. The presence of doxycycline was sufficient to cause tumor formation in the lungs, indicating that previously untransformed mammary cells can produce metastatic-like disease, once induced, without first undergoing transformation at a primary tumor site [9]. The authors further found that untransformed mammary cells could survive in the blood and lung for up to 17 weeks, however subsequent foci formation at a metastatic site required transformation.

Inhibition of angiogenesis is an increasingly important cancer treatment strategy since angiogenesis is a process that is critical to tumor growth. One study employed BLI to quantify angiogenesis, monitored on a long time-scale, after co-implantation of human umbilical vein endothelial cells (HUVEC) expressing firefly luciferase (FLuc) and bone marrow-derived human mesenchymal stem cells (HMSC) into immunodeficient mice [10]. HSMC+ implants demonstrated stable bioluminescent signal for up to 120 days; however, a marked decrease in photon emission was observed in the two weeks following treatment with a VEGFR tyrosine kinase inhibitor (SU5416), thus demonstrating the usefulness of BLI in ascertaining anti-angiogenic potential of therapeutic agents.

While chemotherapy using small molecules has been and remains the most effective method of systemic cancer treatment, future therapies may build on human genome sequence data coupled with safe and effective methods of delivering gene therapy. Toward this end, Xie and co-workers sought to find a highly specific and effective way to deliver a proapoptotic transgene that could be used in pancreatic cancer gene therapy [11]. They utilized BLI to aid generation and optimization of a gene delivery vector encoding the pancreatic-specific cholecystokinin type A receptor (CCKAR) promoter driving a Gal4-VP16 transactivator combined with a Gal4-response element driving a luciferase reporter. Addition of the post-transcriptional regulatory element from the woodchuck hepatitis virus (WPRE) further enhanced expression from this vector, while maintaining pancreatic-specificity, by comparison to a CMV-driven luciferase. Following luciferase-aided vector optimization, the authors replaced luciferase with the proapoptotic gene, Bcl-2-interacting killer (Bik), containing two phospho-mimetic mutations that activate Bik (BikDD). The expression vector bearing BikDD was then demonstrated to specifically and effectively reduce pancreatic tumors compared to a non-specific CMV-driven BikDD. Thus, the imaging vector facilitated the rapid design and implementation of an optimal therapeutic vector.

Beyond assessing the role that various genes have on tumor mass and location, BLI has furthered *in vivo* mechanistic studies especially through the ability to directly interrogate signal transduction processes. These studies typically involve luciferase reporter genes fused to promoter regions or gene chimera sequences of interest. For instance, Naik and Pivnicka-Worms recently described an *in vivo*  $\beta$ -catenin-FLuc reporter system that enables direct visualization of  $\beta$ -catenin stabilization, a process that is frequently upregulated in many cancers [12]. Furthermore, split-luciferase reporter strategies aid assessment of the initial events in pathway activation, and in specific cases, has allowed interrogation of post-translational modifications. For instance, using a previously described split-luciferase reporter system [13] incorporated into a single-chain biosensor consisting of N- and C-terminal luciferase fragments flanking an FHA2 domain linked to a peptide sequence recognized by Akt (Aktpep), Zhang and co-workers have monitored *in vivo* activation of the serine/threonine kinase Akt [14]. Under basal conditions, the single-chain bioluminescence Akt kinase reporter (BAR) is flexible enough to allow complementation of the luciferase fragments (Figure 1). However, upon Akt activation, the binding of the FHA2 domain to the newly phosphorylated Aktpep sterically hinders complementation, which in turn attenuates bioluminescent signals. While limitations exist for single-chain biosensors as equilibrium reporters [15], molecule-specific

pharmacodynamics of a kinase inhibitor were successfully monitored in living animals. Aberrant expression of epidermal growth factor receptor (EGFR), another kinase critical for growth of certain tumors, can be similarly monitored. Herein, the optimized split luciferase complementation system [13] was again used, now to monitor association of EGFR with its downstream binding partners Grb2 and Shc, by fusing each interacting protein to a luciferase fragment [16]. Upon association of interacting proteins, functional luciferase is reconstituted and can deliver accurate readout of radiotherapy-induced EGFR activity in the presence/absence of tyrosine kinase inhibitors in tumor models *in vivo*.

An alternative method to study *in vivo* protein-protein interactions is modeled after the two-hybrid transactivation system. Pichler and colleagues generated a transgenic *Gal4-FLuc* universal reporter mouse strain and tested its usefulness by characterizing the interaction between p53 and SV40 large T antigen (TAg) [17]. In this transactivation system, p53 was fused to a Gal4 DNA-binding domain and TAg was fused to the activator domain from VP16 (Figure 2). After hepatocellular somatic gene transfer of the interacting chimeras, only the reporter mice that were transfected with the interacting pair induced FLuc expression in the liver compared to a negative control. Beyond studies of signaling cascades in cancer, the future of this technique will likely find use in assessments of viral progression and preclinical experimental therapeutics.

## Immunology/Transplant Biology

For immunologists and transplant biologists, BLI has emerged as a valuable tool, not only to longitudinally track spatiotemporal cell fates *in vivo*, but also to probe signaling pathways involved during a variety of immune responses. BLI has proven especially useful in mouse models of graft versus host disease (GVHD) to non-invasively monitor the location and activity of engrafted T cells, replacing or augmenting techniques that previously relied upon cell labeling and flow cytometry end point assays. Nguyen and colleagues used luciferase positive murine T regulatory cells (Tregs) in an allogeneic bone marrow transplant model and evaluated the *in vivo* dynamics of Tregs over time; they observed early, robust Treg expansion in secondary lymphoid organs with sequential migration and localization to peripheral tissues, suggesting that T cell priming events occur in secondary lymphoid tissues [18]. Furthermore, by using gene-deficient donor or recipient mice, the effects of cytokines (IL-18, [19]) and their receptors (CD-30, [20]) upon the development and progression of GVHD were evaluated. Nervi and colleagues used retrovirally-transduced human T cells injected retro-orbitally into NOD/SCID- $\beta$ 2m null mice (xenogenic GVHD); BLI allowed them to validate this model and show that lethal GVHD was dependent upon initial retention and early expansion of human T cells in the retro-orbital sinus cavity [21].

Researchers have also utilized bioluminescent islet-specific auto-antigenic T cells (BDCs) to visualize trafficking patterns and proliferation in a model of Type 1 diabetes [22]. Non-CD4 splenocytes cotransferred with the BDCs were found to help induce diabetes by promoting invasive infiltration of BDCs into pancreatic islets without affecting the systemic trafficking patterns of the BDCs within the animals. Furthermore, bioluminescent pancreatic allografts have been used to monitor graft viability and to determine the appropriate timing of antilymphocyte serum therapy, which yielded prolonged graft survival and completely protected from graft loss [23].

BLI has also proven useful for *in vivo* study of nuclear factor-kappa b (NF- $\kappa$ B) pathway signaling, a key regulator of physiological immunity and pathological inflammation. Recently, a transgenic mouse expressing a luciferase reporter-driven by an NF- $\kappa$ B responsive promoter has been used to examine prostate NF- $\kappa$ B activity in response to acute and chronic cytokine exposure [24], an approach that may be amenable to the *in vivo* study of pharmacological NF-

$\kappa$ B modulators. Ma and colleagues used NF- $\kappa$ B-Luc mice to non-invasively detect NF- $\kappa$ B activation during cardiac allograft rejection and tissue ischemia-reperfusion (IRI) [25]. They showed elevated NF- $\kappa$ B activity in both the cardiac allografts and the IRI cardiac grafts, and demonstrated that CD154 monoclonal antibody therapy inhibited luciferase activity and resulted in long-term graft survival. Using a post-translational reporter strategy, Gross and colleagues demonstrated that an I $\kappa$ B $\alpha$ -FLuc reporter introduced into mouse liver can be used to study *in vivo* regulation of both ligand-induced Inhibitor of  $\kappa$ B Kinase (IKK) activation and the pharmacodynamics of IKK inhibition [26].

Immune cell infiltration and activation at sites of infection or inflammation can also be studied using BLI. Davies and coworkers imaged eosinopoiesis and eosinophil infiltration in a model of schistosome infection that utilized a transgenic mouse expressing luciferase under control of the eosinophil peroxidase promoter [27]. While schistosome egg deposition is the primary stimulus for eosinophil activation (a process that was easily followed using BLI), the sensitivity of BLI allowed the authors to demonstrate that, contrary to expectations, schistosome worms themselves induce significant intestinal eosinophilia and eosinopoiesis within the bone marrow at very early stages of infection.

## Infectious Disease

Studies of microorganisms *in vivo* are uniquely suited to BLI. This is evident especially when imaging bacterial infection models, since use of a bacterial *lux* operon allows for BLI in real time, without requiring administration of exogenous substrate. Disson and colleagues developed a new *lux* operon-based fetoplacental listeriosis model in gerbils [28], using bioluminescent *Listeria monocytogenes*, a bacterium associated with pregnancy-related fetal infections. The authors imaged fetoplacental infection in pregnant gerbils in real time and placentas and fetuses *ex vivo* post-mortem. Using isogenic deletion mutants, they also proved the necessity of *Listeria* internalin proteins InlA and InlB in the fetoplacental infection process.

Furthermore, traditional *in vivo* infection models have required host sacrifice and enumeration of microorganisms from individual host organs to determine extent and kinetics of dissemination during infection. BLI provides a unique opportunity to serially monitor infection in a single host over time, often resulting in identification of new sites of replication and persistence within an infected animal. For example, Hwang and colleagues studied *in vivo* infection by gamma-herpesviruses expressing firefly luciferase [29]. They discovered novel sites of viral replication, including the salivary glands and thymus. Importantly, imaging facilitated this discovery as typical assays involving gamma-herpesvirus replication focused only on lung and spleen. In another study, Sjolinder and colleagues used bioluminescent *Neisseria meningitidis* to monitor bacterial sepsis in CD46 transgenic mice in real time [30]. The researchers showed three different disease outcomes in CD46 mice: a “sepsis-like” disease, a “meningitis-like” disease, and a mild disease pattern. In addition to the three different manifestations of disease, the mice exhibited waves of replication followed by clearance, followed by re-initiation of infection, making real time BLI an ideal method for longitudinal study of meningococcal disease *in vivo*.

BLI can also be used to visualize noteworthy events during the course of an infection, such as spore germination and parasite stage conversion. Glomski and colleagues used *Bacillus anthracis* containing the bacterial *luxCDABE* operon [31]; dormant anthrax bacilli form spores and do not emit bioluminescence, but once the spores germinate in a mouse, bioluminescence will indicate sites of active bacterial infection (Figure 3). This model was used to demonstrate that bacterial spores germinate and replicate at the initial site of infection following both inhalational and cutaneous inoculation, and exhibited localization within Peyer's patches during an intestinal model of anthrax. This contrasts with prevailing dogma that spores travel

to the draining lymph nodes before germination. In another study using *Toxoplasma gondii*, a parasite that exists in two developmental stages *in vivo*, Saeij and colleagues used firefly luciferase linked to a stage-specific promoter to show stage conversion *in vivo* for the first time [32]. Furthermore, they also demonstrated a role for stage-specific surface proteins in reactivation of infection.

Imaging also facilitates studies of host response to microbial infection. In a recent study, Hutchens and colleagues used mice with inactive toll-like receptor-4 (TLR4) and bioluminescent vaccinia to demonstrate a protective role of TLR4 in infection by vaccinia, especially following a high initial dose of virus [33]. This paper confirms other recent reports demonstrating a new role for TLR4 in immunity to viral infection, in addition to the traditionally acknowledged role in response to bacterial lipopolysaccharide (LPS).

Finally, both bacteria and viruses have been investigated as potential diagnostic and treatment tools for cancer and use of BLI has often facilitated these studies. Two recent papers employed carrier cells to deliver bioluminescent oncolytic viruses to tumors *in vivo*. Thorne and colleagues used cytokine-induced killer (CIK) cells loaded with attenuated, luciferase-expressing vaccinia viruses to target and kill tumor cells [34]. Power and colleagues used *Renilla* luciferase-expressing carrier cells and firefly luciferase-expressing vesicular stomatitis virus in order to image carrier cell dispersion and viral delivery to tumors [35]. In each case, researchers demonstrated by BLI that carrier cells improved viral delivery to tumors and tumor growth was attenuated. In other work by Cheng and colleagues, *Escherichia coli* expressing bacterial luciferase and prodrug-activating  $\beta$ -glucuronidase were used to target tumors and activate a glucuronide prodrug (9ACG) [36]. BLI was used to determine bacterial replication in the tumors and to demonstrate that drug treatment resulted in delayed tumor growth.

## Cardiovascular

BLI has also been widely utilized in cardiovascular research and has provided insights into possible therapies for cardiac ischemia. Firefly luciferase-expressing murine embryonic stem (ES) cells were injected into mouse hearts following left anterior descending (LAD) artery ligation, a common model of myocardial infarction [37]. BLI was used to track surviving ES cells for up to 8 weeks following injection, and increased bioluminescent signal correlated with improved neovascularization and cardiac function as evaluated by histology and echocardiogram, respectively. Further investigation into cell-based therapy was made by comparison of the bioluminescent signal from four different cell types - bone marrow mononuclear cells (MN), mesenchymal stem cells (MSC), skeletal myoblasts (SkMb), and fibroblasts (Fibro) - each expressing firefly luciferase, and injected into mouse hearts following LAD artery ligation [38]. The strongest signal and most favorable cardiac tissue survival was seen in those hearts injected with MN cells. Improvements in both neovascularization and cardiac function post-LAD artery ligation were also observed with shRNA inhibition of the prolyl hydroxylase-2 (PHD2) enzyme [39]. In this system, the source of the bioluminescent signal was a hypoxia response element-incorporated promoter driving firefly luciferase; HIF-1 alpha promotes angiogenesis, so the inhibition of PHD2 indirectly increases neovascularization.

## Central Nervous System Disorders

*In vivo* BLI has also been employed in studies of the central nervous system, including investigation of circadian rhythms mediated by *Period1*. Measuring the fluctuations in bioluminescent output from the main olfactory bulbs (OBs) of *Period1-FLuc* transgenic mice whose suprachiasmatic nuclei (SCN) were either intact or lesioned revealed that the OB contains a self-sustained circadian oscillator [40]. Additional insight into SCN intercellular coupling was obtained by crossing circadian clock gene knockout mice with the mPer2-

luciferase fusion knock-in reporter line [41]. Through measurement of bioluminescence in tissue explants, the authors concluded that Per1 and Cry1 are required for sustained rhythms in peripheral tissues and cells and SCN-dissociated neurons, but Per1 or Cry1 deficiency can be overcome by oscillator network interactions in the SCN.

Other exciting work in the CNS involves the potential for monitoring neuronal excitation and degeneration via the transforming growth factor-beta (TGF- $\beta$ ) pathway; activation of TGF- $\beta$  receptors results in the translocation of Smad proteins to the nucleus and activation of the pathway. Using Smad-binding element (SBE)-luciferase transgenic mice, Luo and colleagues were able to localize much of the Smad activity in the CNS to the pyramidal neurons of the hippocampus [42]. Increased bioluminescence, representing increased Smad activity following kainic acid (KA)-induced degeneration, correlated with pathological neurodegeneration. Both bioluminescent signal and pathology were reduced following treatment with a glutamate receptor antagonist (MK-801), indicating that *in vivo* BLI models can play a role in identifying potential treatments for neurodegenerative diseases.

## Conclusions

Animal models of human diseases greatly enhance our knowledge of the mechanisms of pathogenesis by placing target genes and processes in the appropriate physiological setting. Much of our current knowledge has been obtained by monitoring obvious phenotypic changes or by performing destructive analyses at defined end-points. However, many of the obligate steps toward development of disease are dynamic in time and space, and thus, end-point assays are not amenable to gaining a complete understanding of disease pathogenesis. Advances in small animal imaging instrumentation, molecular genetics and reporter gene design have yielded the ability to integrate “imagable” reporters into transgenic murine models of human diseases. Moreover, the successes obtained using BLI strategies to study diseases will continue paving the way for basic, preclinical, and translational research. In the future, we anticipate further incorporation of these non-invasive imaging strategies for use with *in vivo* high-throughput screening, multiplexed imaging of multiple luciferases simultaneously, and imaging multiple nodes within or across signal transduction pathways.

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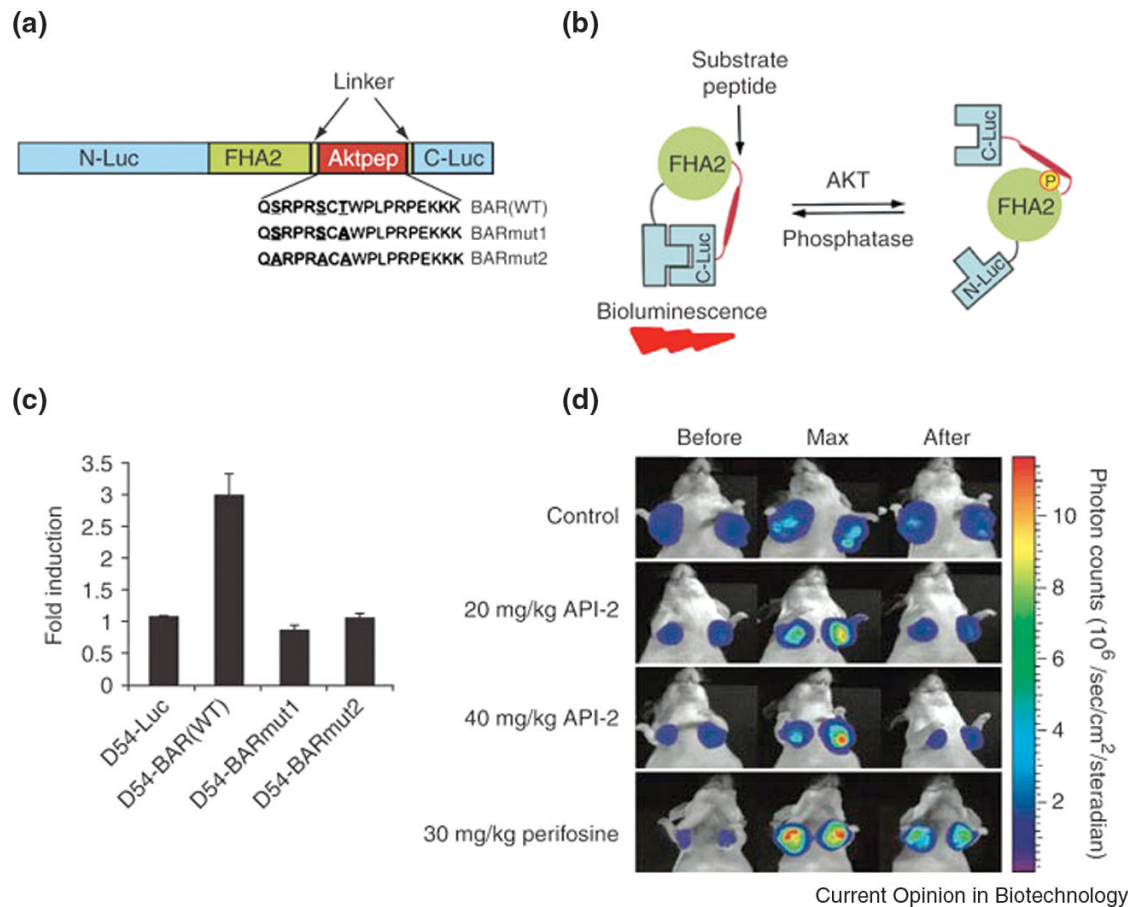
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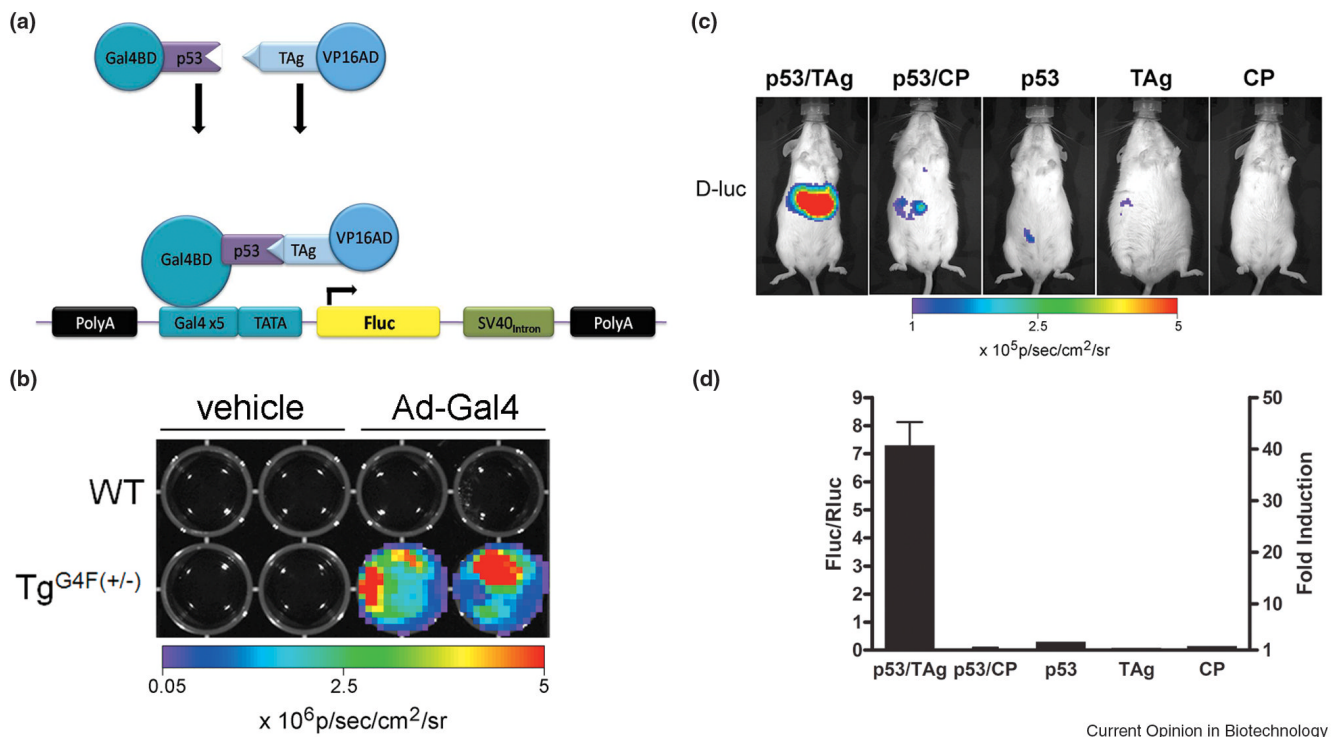
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**Figure 1.**

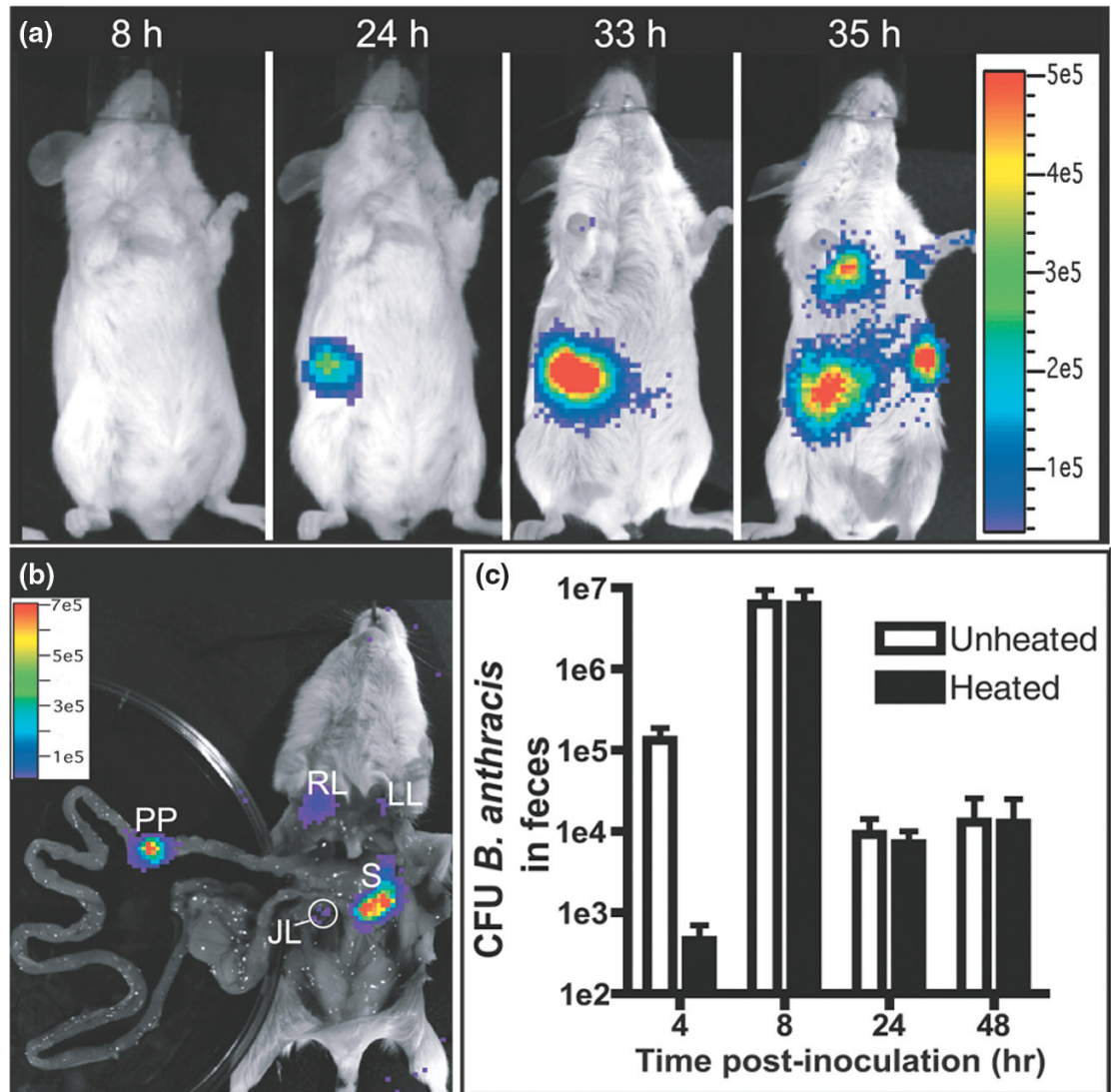
Bioluminescence imaging of Akt kinase activity *in vivo*. (A) Domain structure of the single-chain bioluminescence Akt kinase reporter (BAR). Three versions of the reporter were developed: the wild-type (BAR<sub>WT</sub>) molecule, which contains the wild-type Aktpep sequence; the BAR<sub>mut1</sub> molecule, which contains a threonine-to-alanine substitution at the primary phosphorylation site; and the BAR<sub>mut2</sub> molecule, which has all serine and threonine residues in the substrate sequence mutated to alanine. (B) The proposed mechanism of action for the BAR reporter involves Akt-dependent phosphorylation of the Aktpep domain (thick line), which results in its interaction with the FHA2 domain (right). In this form, the reporter is constrained and has minimal bioluminescence activity. In the absence of Akt activity, the N-Luc and C-Luc domains complement each other, restoring bioluminescence activity (left). (C) Stable cell lines expressing each of the reporters were treated with API-2 (40  $\mu$ M), an Akt inhibitor, for 1 h. The change in bioluminescence activity compared with pretreatment values was plotted as fold induction. Data were derived from a minimum of five experiments. (D) Molecular imaging of Akt activity. Tumor-bearing mice were treated with vehicle control (20% DMSO in PBS), API-2 (20 mg/kg or 40 mg/kg) or perifosine (30 mg/kg), a phosphoinositide 3-kinase inhibitor. Images of representative mice are shown before treatment, during maximal luciferase signal upon treatment (Max), and after treatment. Adapted by permission from Macmillan Publishers Ltd: Zhang et al., *Nature Medicine*, **13**:1114-1119, copyright 2007.



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**Figure 2.**

Use of a universal *Gal4-Fluc* transgenic reporter mouse to image protein-protein interactions *in vivo*. (A) Schematic representation of the transgene vector (G4F) that was injected into pronuclei of FVB oocytes, and schematic diagram indicating a two-protein interaction system transactivating expression of FLuc. (B) Mouse embryonic fibroblasts (MEF) of a negative embryo (WT) and a positive embryo ( $Tg^{G4F(+/-)}$ ) were treated with either vehicle or  $4 \times 10^7$  pfu of Ad5Gal4BD-VP16 (Ad-Gal4). Cells were imaged 24 h after infection. MEFs from the positive embryo were induced 3-log fold when compared with the uninducible negative MEFs. (C) Imaging protein-protein interactions *in vivo* using a *Gal4-Fluc* reporter mouse. In this transactivation system, p53 was fused to a Gal4 DNA binding-domain and TAG was fused to the activator domain from VP16. Hepatocytes of  $Tg^{G4F(+/-)}$  mice were transfected *in vivo* by hydrodynamic somatic gene transfer with different plasmid combinations of *pGal4BDp53* together with *pVP16-TAg* or off-target *pVP16-CP*, or each plasmid separately as indicated. A plasmid for *Renilla* luciferase was used as transfection control (data not shown). Mice were imaged before (pretreatment) and 24 h after transfection for expression of firefly luciferase (FLuc with D-luciferin) as well as *Renilla* luciferase (RLuc with coelenterazine). Images of representative mice are shown. CP; polyomavirus coat protein. (D) Data are plotted for the different plasmid combinations as controlled for transfection efficiencies (FLuc/RLuc; left axis) and as fold-induction (right axis) 24 h after transfection. Data are presented as mean  $\pm$  range for plasmid combinations ( $n=2$ ), and without error bars for single plasmids ( $n=1$ ). Four independent experiments showed similar results. Adapted by permission from the National Academy of Science: Pichler et al., *Proc Nat'l Acad Sci USA*, **105**, 15932-15937, copyright 2008.



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**Figure 3.**

Bioluminescence imaging to map germination of *B. anthracis* in gastrointestinal infection. *B. anthracis* spores ( $1 \times 10^8$ ) were inoculated using a flexible plastic tube. (A) Infection of the lower digestive tract at the indicated times. Image series is representative of 23 mice. (B) Dissection of the mouse in (A) to confirm sources of luminescence. JL, jejunal lymph node; LL, left lung; PP, Peyer's patch; RL, right lung; S, spleen. (C) Total CFU of *B. anthracis* (unheated) or spores only (heated) were enumerated at the indicated times from the feces of mice inoculated intragastrically (mean  $\pm$  standard error of the mean,  $n = 4$ ). Vegetative bacteria were isolated from feces at four hours post-infection, indicating spore germination in the gastrointestinal tract. Adapted by permission from Public Library of Science: Glomski et al., *PLoS Pathogens*, **3**:0699-0708, copyright 2007.