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Bioluminescence Applications in Preclinical Oncology Research

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1. Introduction

In vitro studies have offered vast insight into much of cancer biology, however, it is widely accepted that cell based assays are unable to provide a complete picture when attempting to understand the dynamic nature of cancer as it behaves in situ. Critical processes to cancer progression such as angiogenesis, metastasis and response to treatment, rely on complex interactions between tumor cells and their microenvironment. To overcome this challenge, xenografts have been widely used to study cancer biology within the context of a whole organism since as early as the 1950's. These models rely on use of murine (syngeneic) and human (allogeneic) tumor cell lines injected subcutaneously into a rodent host. The subcutaneous animal model has been a valuable tool in the study of cancer and has directly led to the validation of many of the anticancer agents which benefit patients today. Subcutaneous tumor models are easy to implement and monitor due to the accessibility of the tumor tissue. Evaluation of subcutaneous tumors involves calliper measurements of tumor size (width, length and/or height), which are then used to define tumor volume. However, like cell-based assays, subcutaneous tumor models have proven to be poor predictors of therapeutic activity in patients and this is likely due to the reliance on cell lines which when inoculated subcutaneously develop tumors that poorly mimic the biological behaviour of human disease. Cancers arise slowly and evolve into a heterogeneous structure both in terms of cellular composition (host cells and tumor cells) and microenvironment (vascularization and transient regions of hypoxia and nutrient stress). Subcutaneous xenografts are implanted in microenvironments that will be remarkably different from the tissue of origin. This means subcutaneous tumor cells do not receive the same signals from the stroma that influence immunity, angiogenesis and metastasis; all factors that impact tumor progression and response to therapeutic interventions. Although the initiators and drivers of tumors in humans remain poorly understood, it is generally accepted that following initiation, endogenous disease progresses into a primary tumor which in time can invade surrounding tissues. The latter process involves both extravasation and intravasation

of cancer cells. Thus if left untreated primary malignancies can evolve into a metastatic disease which ultimately engender systemic changes that are incompatible with life. Even this very simplistic description of cancer biology highlights the serious shortcoming of subcutaneous tumor models derived following injection of cultured tumor cell lines. The limitations are even more profound when considering changes in tumor biology that occur as a consequence of treatments.

In recent years orthotopic inoculation of tumor cells has been viewed as a reasonable alternative for initiation of model tumors. In these models, tumor cells are injected in a site that represents the tissue of origin and thus may be closer in characteristics to the original microenvironment. Orthotopic models can exhibit tumor growth rates, capacity for angiogenesis and an invasive potential that better mimic the evolution of cancers in situ. In several examples primary tumors arising following orthotopic injection of tumor cells have been shown to metastasize through lymphatic drainage and/or hematological spread in ways that are comparable to that seen in human disease. For this reason, some investigators believe that orthotopic tumors more accurately reflect human disease, and may serve to better predict therapeutic outcomes.

Further, in an effort to model systemic disease a variety of cell inoculation methods can be employed to promote hematological spread of tumor cells. These methods include, but are not limited to, tail vein and intracardiac injections. Many groups have used different inoculation methods to assess specific metastatic sites, such as intratibial inoculations of prostate cancer cells in order to study bone metastases, or intraperitoneal injections of ovarian cancer cells to study disease development/progression in the peritoneal cavity. Finally, transgenic animal models are now readily available, where oncogenes and tumor suppressor genes relevant to a specific cancer are knocked in or knocked out. These genetically engineered animals have been useful for studies exploring how genetic alterations are linked to carcinogenesis.

A very large obstacle in studies using orthotopic, systemic and transgenic animal models is monitoring disease burden. The tumour tissue is often inaccessible for visual inspection, localized in organs deep within the body. Assessment of such tumors requires termination of animals at various time-points following disease initiation or at a time when the animal experiences signs of distress/illness. Mice are then euthanized and organs removed for gross and histological assessment of primary and metastatic disease; a practice that frequently requires serial sacrifice and large numbers of animals for a single study. Additionally, in this type of study design, comparisons are made between different groups of animals that were sacrificed at different time-points. Due to animal to animal variations, comparisons are often difficult to interpret and conclusions may be over- or even understated.

The use of orthotopic and transgenic cancer models has fostered development of small-animal imaging methods to follow tumour development and progression in live animals. There are several modalities that are applicable to small animal imaging, including ultrasound (US), magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and single photon emission computed tomography (SPECT). Each of these imaging modalities have strengths and weaknesses as recently reviewed by Ray et al (Ray 2011). Over the last decade use of Bioluminescence Imaging (BLI) has become

increasingly popular in part because of the accessibility of imaging tools and because the method is extremely sensitive with a capability of detecting as few as 10 tumor cells in a live animal. BLI provides a non-invasive, semi-quantitative approach to localizing small tumors, following growth and metastasis and monitoring tumor response to treatment in the same animal longitudinally. This non-invasive determination of tumor burden over time reduces the numbers of animals required for experiments and provides information on the various stages of tumor development in the same animal as the disease progresses.

The first experiments using BLI for monitoring tumor phenotypes and response to therapy were performed by assessing luciferase activity as a measure of metabolism as described in section 6.0. Recently, more complex studies have been designed. For example, our group used BLI to track the development of an experimental metastatic model of breast cancer after an intracardiac injection of tumor cells. Further, we evaluated the use of an existing and clinically relevant drug to treat orthotopic, metastatic and ascities disease and correlated changes in tumor burden as measured by BLI to pharmacokinetic data in the same animals. The utility of BLI in assessing drug efficacy is multi-faceted in that it is able to address semi-quantitatively the issue of disease burden, and also to assess disease physiology. For example, as firefly luciferase (F-Luc) activity is dependent on the presence of oxygen and ATP, photons are only emitted from metabolically active cells. Thus, therapeutic effects involving changes in tumor metabolism can be readily assessed, where necrotic regions within a tumor can be identified and potentially act as a marker for a positive drug response. The high sensitivity of BLI also allows for the detection of small numbers of tumor cells very early in the development of primary or metastatic disease; cancer cells can be visualized using BLI before they can be visualized by other imaging methods. Over a very short period of time, studies involving BLI have demonstrated that the technique is highly sensitive, high throughput, and relatively easy to use. It is likely that the use of BLI over the next decade will continue to increase in its complexity and its elegance.

In this chapter, the information gleaned from oncology research using BLI is summarized. The strengths, weaknesses and major findings from the last twenty years are considered in an attempt to exemplify the utility of BLI as well as illustrate some of its limitations. Major problems associated with this imaging modality are recognized in order to assist in designing preclinical experiments for those using this imaging modality. Topics summarized below include the development of luciferase positive orthotopic, metastatic and genetically engineered models of human cancer as well as the use of BLI for the assessment of therapeutic activity of drug candidates, as a tool for monitoring gene delivery and gene expression *in vivo*, for assessment of processes such as angiogenesis and apoptosis, and, finally, for imaging of metastasis and minimal disease in cancer models.

2. Bioluminescence

Bioluminescence (BL) is defined as the production of light by a living organism. Many organisms such as bacteria, fungi, fish, marine invertebrates, and insects use BL for the purpose of mating, camouflage, repulsion, communication and illumination. The chemical reaction that produces BL requires a pigment known generally as luciferin and enzymes called luciferase (see Reaction 1). The reaction between luciferase and its substrate is an oxidation reaction which is sometimes mediated by cofactors such as calcium and may require energy in the form of ATP.



Luciferins are a family of light emitting proteins that act as substrates to luciferase. Luciferins evolved many times in various organisms, hence there are a variety of luciferins that are structurally and chemically distinct (Shimomura 2006). Table 1 provides a list of

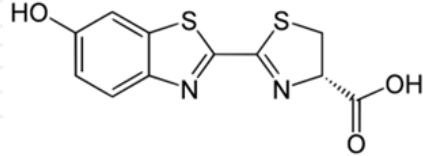
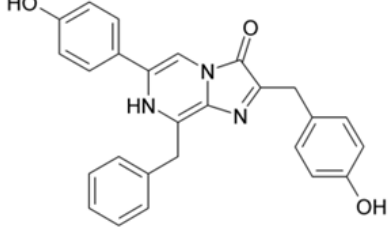
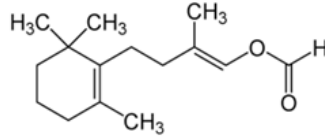
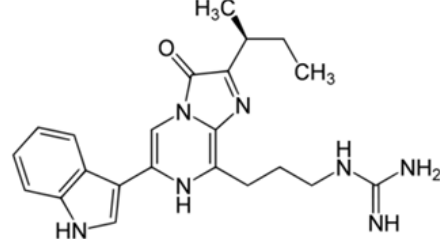
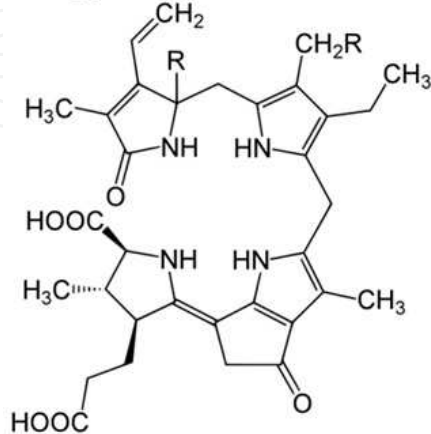
Organism	Luciferin structure	Co-factors	Luminescece
Firefly (Photinus pyralis) Clickbeetle (Pyrophorus plagiophthalmus)	D-Luciferin 	ATP Mg2+	560-615nm
Sea Pansy (Renilla reniformis) Marine copepod (Gaussia princeps)	coelenterazine 	Ca2+	480nm
Snail (Latia neritoides)	Latia 	O2	536
Marine Ostracod (Vargula Hilgendorfi)	Cypridina 	O2	450-460nm
Dinoflagellates	Tetrapyrrole 	O2	474nm

Table 1. The structure, luminescence wavelength and cofactors required by the luciferases and luciferins used in biological and chemical research (Shimomura 2006)

luciferins that are currently used in biological research. Luciferase, is the general term for a class of enzymes which catalyze oxidation reactions involving luciferins. The structure of luciferase seems to have an impact on the wavelength of photons emitted and color of light produced so that reactions may exhibit yellow-green to red light. Although many of the luciferase enzymes discussed in Table 1 have been applied to in vitro and in vivo work, firefly luciferase (F-Luc) is most commonly employed in oncology research. In the chemical reaction between F-Luc and its substrate D-luciferin, ATP and oxygen promote the formation of oxy-luciferin species. The emission spectrum of the F-Luc catalyzed reaction is broad with an emission peak at approximately 560 nm and a large component above 600 nm. Yellow-green to yellow-orange light is emitted following the relaxation of oxy-luciferin to its ground state (see reaction 2).

Reaction 2

D-Luciferin + ATP \rightarrow (reversible uses luciferase and Mg²⁺) luciferase luciferyl-AMP + PPi

Luciferyl-AMP + oxygen \rightarrow (uses luciferase and Mg²⁺) luciferase + oxyluciferin + AMP + CO₂ + h ν (de Wet, Wood et al. 1987)

3. Constructing bioluminescent mammalian cells and animal models

In the following sections the application of biological models expressing luciferase are described in detail. Mammalian systems do not naturally express luciferase and therefore these systems must be engineered to express the enzyme. A vector capable of constitutive expression of luciferase or vectors designed to achieve controlled expression of the enzyme can be used. Fusion genes can be used as reporter constructs to track gene delivery and integration. Expression of fusion proteins can be used to follow protein localization and protein-protein interactions. Cells with stable expression of luciferase can be used for in vitro assays or be injected into animals to examine metabolism, immunity, angiogenesis, or to establish disease that can be monitored using BLI. Additionally, transgenic animals may also be developed where reporter gene expression is introduced through the germline.

3.1 Engineering Luciferase expressing cells

Luciferase positive tumor cells used for generating animal models in cancer research are widely available in almost all histological types such as breast, cervical, colorectal, lung, prostate, ovarian cancer and melanoma. Developing a luciferase expressing mammary cell line can be accomplished in house by standard transfection or transduction methodology using reagents, such as plasmid vectors carrying the luciferase genes, which are commercially available.

Tang et al use a typical transfection procedure to create a luciferase positive neuroprogenitor cell line where the pGL3Basic plasmid (Promega, Madison, WI) carrying F-Luc was digested with HindIII and BamHI and the 1.9-kb cDNA fragment encoding F-Luc was isolated and cloned into a second vector (the pHGCX). The resulting pHGCX vector contained the F-Luc gene driven by the cytomegalovirus (CMV) promoter which enables constitutive expression of luciferase. The pHGCX vector also contained the gene for enhanced green fluorescent protein (eGFP) under the control of the viral immediate early

IE4/5 promoter, providing a method of fluorescence selection. The neuroprogenitor cell line (C17.2) were stably cotransfected with the engineered vector and pBabePuro, containing the gene for puromycin resistance, using Lipofectamine transfection reagent (Invitrogen Life Technologies, Carlsbad, CA) (Tang, Shah et al. 2003).

Stable luciferase expressing cells can also be generated through transduction strategies (Nyati, Symon et al. 2002; Kalra, Warburton et al. 2009; Kalra, Anantha et al. 2011; Yan, Xiao et al. 2011). Our own lab uses transduction procedures to co-express F-Luc and GFP in breast cancer cell lines. Briefly, the luciferase coding sequence was isolated from the pGL3Basic vector (Promega, Madison, WI) and cloned into the lentiviral vector, FG9, downstream of the CMV and UbiC promoters again to support constitutive activation of the F-Luc gene. The engineered vector was cotransfected with packaging constructs pRSVREV, pMDLg/pRRE and the VSV-G expression plasmid pHCMVG into a packaging cell line (HEK-293T) by a standard LipofectAMINE 2000 (Invitrogen, Burlington, ON Canada) transfection procedure. Conditioned medium containing Lentivirus-Luciferase (Lenti-Luc) particles was collected and cleared of debris by low speed centrifugation. A similar method was used to generate GFP-expressing lentivirus (Lenti-GFP). The Breast cancer cell line (MDA MB 435/LCC6 (LCC6)) was then infected with Lenti-Luc and Lenti-GFP creating LCC6-Luc/GFP cells. To enrich for luciferase positive cells, cells were sorted by FACS for GFP expression. Subsequently GFP-positive cells were re-plated in low concentrations into soft agar in the wells of a 96-well plate. Luciferin was added to each well and plates were imaged using IVIS to identify luciferase positive colonies (Kalra, Warburton et al. 2009; Kalra, Anantha et al. 2011).

3.2 Use of Luciferase positive cell lines

Once stable luciferase positive cell lines are generated, the cells should be assessed for growth rates and sensitivity to selected drugs; comparing engineered cells to parental cell lines. These assays are performed to ensure that the luciferase gene does not interfere with cell function. Many groups will also image a serial dilution of cells to associate BL produced with number of cells as shown in Figure 1. Figure 1A exhibits the CCD camera capture of a serial dilution of luciferase positive human breast cancer (LCC6-Luc) after luciferin substrate is added. BL data was quantified as photons emitted per second. The resulting graph (Figure 1B) shows that the photons emitted are proportional to the number of cells plated (Kalra, Anantha et al. 2011). These data can be used to estimate cell numbers from BL captured in an in vivo model. In this example the minimum number of cells detectable was approximately 10,000 cells, but the detection limit is dependent on multiple factors including luciferin concentration, imaging programs, parameters such as exposure time and the device used for image capture.

Once luciferase positive cells are adequately vetted in vitro, these cells can be used to establish an in vivo model of disease through multiple routes of inoculation as discussed in Section 6.

3.3 Luciferase expressing transgenic mice

A wide variety of transgenic reporter animals using luciferase-based technology have been engineered in order to visualize transgene expression in vivo. For example, in 2005 Hsieh et

al reported the development of a luciferase transgenic mouse model where F-Luc was placed under the control of the Prostate Specific Antigen (PSA) promoter to develop transgenic PSA-Luc mice. Figure 2A shows the BLI of representative PSA-Luc transgenic male and female animals, and non-transgenic littermate male mice. Figure 2B shows BLI of excised organs from a 12-week-old PSA-Luc male mouse. Hsieh et al demonstrate that the PSA-Luc mouse model has luciferase based BL restricted to the prostate gland (Hsieh, Xie et al. 2005). This mouse model has been used to monitor the prostate gland during development, tumorigenesis and in response to androgens or chemotherapy.

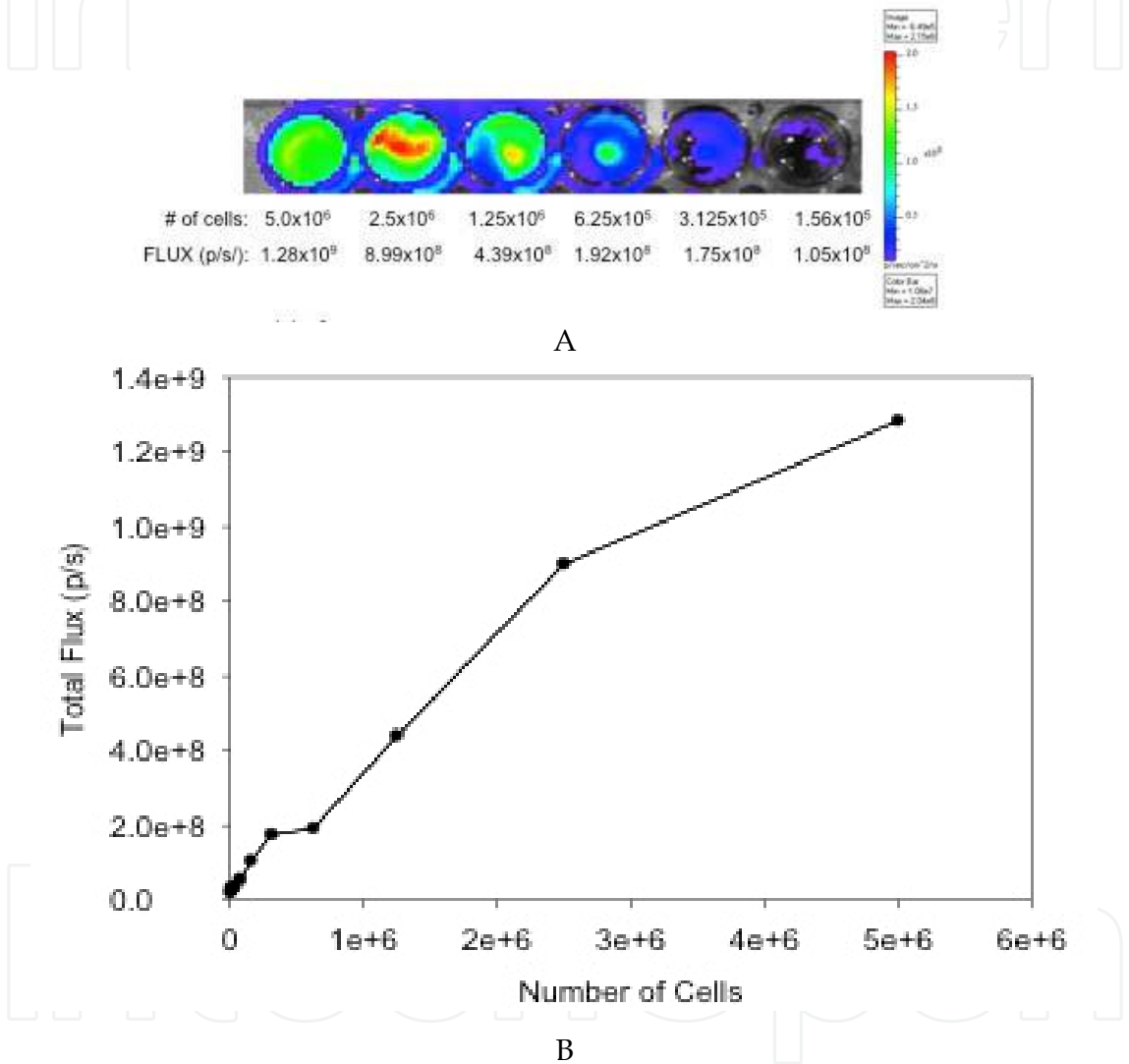


Fig. 1. Imaging luciferase positive breast cancer cells in vitro. LCC6-Luc cells were serially diluted and placed into wells of a 24 well plate. Luciferin was added and cells were immediately imaged using the IVIS 200 system to obtain BL measurements (A-representative images). These data were used to generate a plot comparing total light emission to cell number (B - graph). (Kalra, J., M. Anantha, et al. (2011). "Validating the use of a luciferase labeled breast cancer cell line, MDA435LCC6, as a means to monitor tumor progression and to assess the therapeutic activity of an established anticancer drug, docetaxel (Dt) alone or in combination with the ILK inhibitor, QLT0267." *Cancer Biol Ther* 11(9): 826-838. Reproduced by permission of author.)

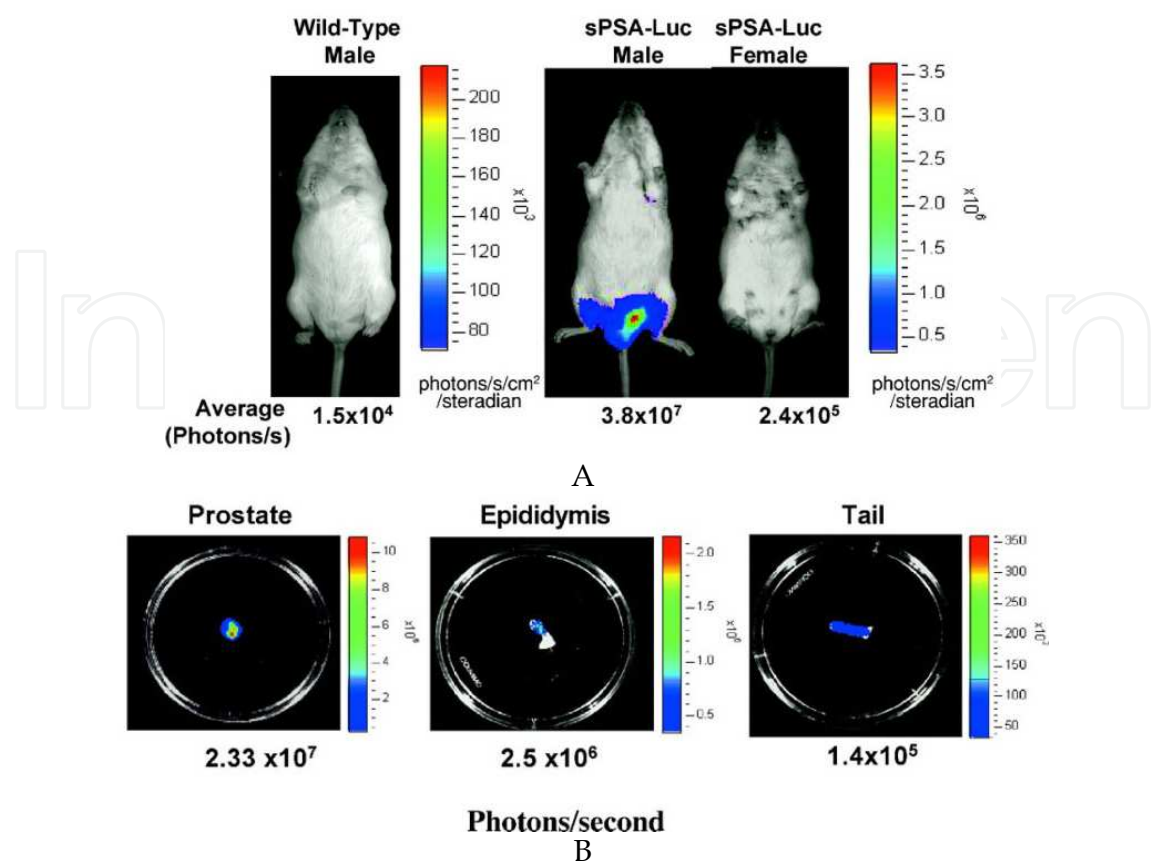


Fig. 2. Bioluminescence imaging of luciferase activity in PSA-Luc transgenic mice. BLI following injection of luciferin in control (A), PSA-Luc transgenic males (B), and PSA-Luc transgenic female (C) mice at 10–12 weeks of age. Isolated prostate, epididymis and tails from 12-week-old PSA-Luc male mice were imaged following luciferin administration (D). (Hsieh, C. L., Z. Xie, et al. (2005). "A luciferase transgenic mouse model: visualization of prostate development and its androgen responsiveness in live animals." *J Mol Endocrinol* 35(2): 293-304. Reproduced by permission.)

The PSA-Luc animal is just one example of a transgenic mouse models available for BLI. Table 2 summarizes a list of luciferase transgenic mouse models that have been established over the last 10 years and used recently in preclinical studies of cancer. Many of these strains are now commercially available. Goldman et al use the Jackson Laboratory ODD-Luc transgenic mouse strain to construct a spontaneous tumor model with a built in reporter that can be used to localize tumors. The ODD-Luc transgenic mouse expresses HIF-1 α oxygen-dependent degradation domain (ODD) fused to luciferase in all tissues, however, under hypoxic stress the ODD-Luc accumulates in hypoxic tissue and is readily observed by BLI. In the Goldman study, the ODD-Luc mice were crossed with Mouse Mammary Tumor Virus (MMTV) transgenic mice. MMTV can act as an insertional mutagen or induce transcription of nearby oncogenes post-insertion leading to the development malignant tumors in the mammary gland of infected mice. The MMTV transgenic mouse has been engineered to express the MMTV-LTR which predisposes the animal to develop multiple spontaneous mammary tumors (Taneja, Frazier et al. 2009). Since solid tumors often contain hypoxic centers, this group demonstrated that the ODD-Luc/MMTV transgenic mouse can be used to follow spontaneous tumor development, progression and response to treatment.

They were able to use BL to image the growth and development of spontaneous tumors and showed that in response to treatment using doxorubicin and prednisone, these tumors were able to regress.

Transgene	Research focus	Reference
Caspase cleavage sequence (ER-DEVD-Luc)	Apoptosis	Laxman 2002 (Laxman, Hall et al. 2002)
p53-Luc	Cell cycle, apoptosis	Rehemtulla 2004(Rehemtulla, Taneja et al. 2004), Briat 2008(Briat and Vassaux 2008)
Smad-responsive element (SBE-Luc)	TGFbeta/Smad signaling	Lin 2005(Lin, Luo et al. 2005), Luo 2009(Luo and Wyss-Coray 2009)
E2F1-Luc	Cell proliferation	Momota 2005(Momota and Holland 2005)
Inducible Nitric Oxide Synthase (FVB/N-Tg(iNOS-Luc)Xen)	Inflammation	Moriyama 2005(Moriyama, Moriyama et al. 2005)
Estrogen Receptor (ER-Luc)	Hormone receptor activity	Wu 2008(Wu, Xu et al. 2008)
Prostate Specific Antigen (PSA-Luc)	Hormone receptor activity	Hsieh 2005(Hsieh, Xie et al. 2005), Lyons 2006(Lyons, Lim et al. 2006), Iyer 2005(Iyer, Salazar et al. 2004; Iyer, Salazar et al. 2005)
Vascular Endothelial Growth Factor (VEGF-Luc)	Angiogenesis	Faley 2007(Faley, Takahashi et al. 2007)
NFkB-Luc	Signaling and inflammation	Vykhovanets 2008(Vykhovanets, Shukla et al. 2008), Robbins 2011(Robbins and Zhao 2011)
EL1-Luc/TAg	Spontaneous pancreatic tumors	Zhang 2009(Zhang, Lyons et al. 2009)
Cre/Lox Luc	Conditional glioblastoma multiforme model	Woolfenden 2009(Woolfenden, Zhu et al. 2009)
Survivin (Survivin -Luc)	Apoptosis	Li 2010(Li, Cheng et al. 2010)
RipTag-IRES-Luc	Pancreatic beta cell carcinogenesis	Zumsteg 2010(Zumsteg, Strittmatter et al. 2010)
Collagen1 alpha 1 (Col-Luc)	Bone metastasis	Lee 2010(Lee, Huang et al. 2010)
Mouse Period 1 (mPer1-luc)	Modulators of circadian rhythm and stromal signaling to tumors	Geusz 2010(Geusz, Blakely et al. 2010)
X-box binding protein 1 (XBP1-Luc)	A marker for stromal stress and reporter for endoplasmic reticulum	Spiotto 2010(Spiotto, Banh et al. 2010)

Transgene	Research focus	Reference
A triple transgenic strain (MMTV-Cre, CAG-betageo-tTA, TetO-Luc)	Spontaneous mammary tumors	Zhang 2010(Zhang, Triplett et al. 2010)
A double transgenic strain (p38DN/ AP-1-Luc)	Cell Cycle and apoptosis	Dickinson 2011(Dickson, Hamner et al. 2007)
HIF-1 α oxygen-dependent degradation domain (ODD-Luc)	Hypoxic stress	Goldman 2011(Goldman, Chen et al. 2011)
Human Reverse Telomerase Transcriptase (hTERT-Luc)	Telomerase activity	Jia 2011(Jia, Wang et al. 2011)
Vascular Endothelial Growth Factor Receptor (VEGFR2-Luc or VEGFR2-Luc)	Angiogenesis	Angst 2011(Angst, Chen et al. 2010)
Matrix metalloproteinase 9 (MMP-9-Luc)	Invasion and metastasis	Biron-Pains 2011(Biron-Pain and St-Pierre 2011)
Early growth response 1 (Egr-1-Luc)	Growth factor signaling	Dussman 2011(Dussmann, Pagel et al. 2011)

Table 2. Luciferase based transgenic mouse models with applications in preclinical cancer research

4. Bioluminescent imaging (BLI) and sensitivity

As outlined in the previous section, cells and animal models can be engineered to express luciferase in a variety of ways. However, as implied in the examples above these cells must be exposed to a substrate in order for BL to be produced. Thus although BLI is non-invasive, in vivo luminescence is generated only following intraperitoneal (IP), subcutaneous (SQ), intratumoral (IT), oral (PO) or intravenous (IV) injections of the substrate luciferin. Following injection, up to 15 minutes are required for sufficient distribution of the substrate to sites where luciferase-expressing cells are located and to achieve optimal signal intensity prior to imaging. This step represents the most significant limitation of BLI in models of cancer.

It should also be noted that BL is a weak phenomenon producing low-intensity light that cannot be observed using conventional cameras. Therefore specially designed low light imaging cameras are required. Several commercially available systems are capable of detecting such low levels of light and are listed in Table 3. In general, the components of each system include a light-tight imaging chamber and a super-cooled charged coupled device (CCD) camera. Detected photons emitted from within the body of the animal are converted to a pseudo-colour image representing light intensity (from blue for least intense to red for most intense) and superimposed over the grayscale digital image as shown in Figure 3A. The spatial resolution of BLI, however, is relatively low (1-2 mm), when compared to CT, PET and SPECT. It has been suggested that the poor resolution of BLI is the result of scattering and diffraction of light due to changes in the refractive index at cell membranes and organelles. However, because BLI is associated with little to no background noise, the anatomical origin of photons can be determined, to approximately 1 mm (Edinger,

Cao et al. 2002). Using topographical scanning it is now possible to construct a three dimensional image of the animal at the same time BL data is being collected. This combined imaging may help to provide better resolution and signal localization. The IVIS 200 system is able to create a 3 dimensional image of the animal where a scanning laser positioned in the horizontal plane is used to make a measurement of surface topography as shown in Figure 3B. This image is converted into a digital reconstruction of the animal that can be superimposed onto an animal atlas and used to localize the depth of signal as seen in Figure 3C. Newer BL imaging systems such as the Spectrum CT by Caliper are able to simultaneously create a Computed Tomography scan for the purpose of constructing a three dimensional image of the animal (Figure 3D). Multi-modal imaging can aid in localization, and an assessment of the depth of signal within body cavities with more precision than BLI alone.

Company	System	Method used for supercooling
Caliper LifeSciences	IVIS	Thermoelectric cooling
Andor	iXon	Thermoelectric cooling
Berthold	NightOWL	Peltier cooling
Hamamatsu +	VIM camera	Intensified cooling
Improvision	Model C2400-47	Cryogenic cooling
Roper Scientific	ChemiPro	Cryogenic cooling
Biospace	PhotoImager	Intensified cooling
Kodak	In vivo FX	Thermoelectric cooling

Table 3. Commercially available BL imaging systems (Baert 2008)

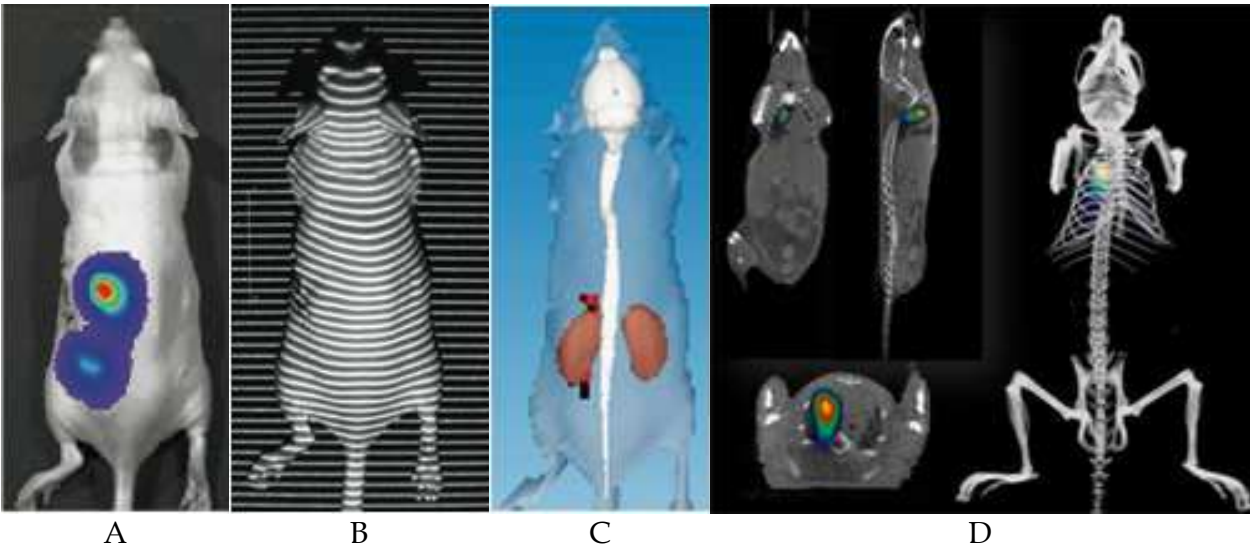


Fig. 3. 2D and 3D detection of F-Luc labeled cells using the IVIS system from Caliper Life Sciences. A grayscale digital image is taken at the same time as photon capture, subsequently BL is superimposed onto the digital image (A). Surface topography is constructed using horizontal laser scanning (B), and used to create 3D rendering of the animal which can be overlain on an organ atlas (C). Figure C shows red dots where photon intensity is highest, indicating the depth and localization of the signal. Newer systems incorporate a CT scan with BL (D) in order to gain higher precision in signal localization. (Reproduced by permission from Caliper Life Sciences, Hopkinton, MA, USA.)

Although some of the limitations of this imaging method are summarized above, these limitations are offset by the fact that BLI can detect low levels of gene expression as well as small numbers of cells in animal tumor models. This method can capture minimal disease and/or micro-metastases at distant sites before the appearance of palpable nodules or clinical symptoms. Indeed, signals can be readily visualized immediately following inoculation of tumor cells (Kalra, Anantha et al. 2011). To illustrate the sensitivity of BLI, Lipshutz et al used adenoviral vector carrying the luciferase gene to deliver luciferase to day 15 fetal mice through intraperitoneal delivery. This group was able to image a single luciferase positive liver cell in one million using BLI (Lipshutz, Titre et al. 2003). Sweeney et al were able to use BLI to detect as few as 100-2500 luciferase positive cervical cancer cells in the peritoneal cavity (Sweeney, Mailander et al. 1999). It is also worth noting that in addition to excellent sensitivity, BLI is amenable to medium throughput screening where 6 animals can be imaged at once. Once optimized, the image acquisition times can be less than 30 seconds, and the image acquisition and follow-up analysis are user-friendly.

There are additional advantages associated with BLI in comparison to other imaging methods. The equipment and reagent costs tend to be relatively low compared to PET and/or MRI. BLI is also a non-radioactive imaging modality, in contrast to other modalities such as PET and SPECT. Furthermore, BL light is emitted directly by the specimen without the need to add excitation light, which is required for fluorescence imaging. Also photobleaching and phototoxic effects are not a concern. Mammalian tissues and chemical agents such as chemotherapeutics do not normally emit BL, but can generate autofluorescence that can interfere with fluorescent based imaging methods. BLI has low background and any photon emission would be the result of engineered BL cells or genes. Finally, luciferase and its substrate, luciferin, are not toxic to mammalian cells, and no functional differences have been reported between cells expressing luciferase when compared with parental cell lines (Sweeney, Mailander et al. 1999; Edinger, Cao et al. 2002; Tiffen, Bailey et al. 2010; Kalra, Anantha et al. 2011).

When designing animal studies that employ BLI, it is important to consider some factors that can influence the detection of BL photons; factors that make this approach to imaging semi-quantitative at best. These factors include 1) the distance signals must travel through tissues, 2) the nature of overlying structures and 3) cell physiology. For example, luciferase positive cells located deep within the body will appear less bright than an equivalent number of cells located near the surface of the skin (El-Deiry, Sigman et al. 2006; O'Neill, Lyons et al. 2010); as tissues overlying the target cells can attenuate photon emission. Melanin is a pigment that is meant to scatter light for the purpose of protection against harmful radiation, and by a similar mechanism will attenuate light that arises from within the animal. Thus skin, fur and hair color may interfere with BL output and influence sensitivity of imaging. Studies have shown that the light emission from dark-colored mice such as the Rag2M strain, is significantly reduced when compared with white or hairless mice. For this reason, albino nude animals are often used in BL studies (Edinger, Cao et al. 2002). Curtis et al showed that even local depilation can cause pigment changes which interfere with BLI (Curtis, Calabro et al. 2010). Hemoglobin is another pigment that quenches light, thus highly vascularized organs tend to have lower levels of photon emission compared with less vascularized tissues. Finally, in the context of metastatic

cancers or gene expression studies, the detection of smaller signals will depend on the presence of larger signals located close by as signal intensity from one region can attenuate less intense signals from other regions.

Three main aspects of cell physiology have been shown to affect BLI. First, Czupryna et al suggested that F-Luc activity can be substantially altered in studies where reactive oxygen species are elevated (Czupryna and Tsourkas 2011). This poses a very relevant problem in studies of tumor biology as oxidative stress can occur within a tumor or as a result of therapy. Second, hypoxic regions within tumors may also affect signal intensity. BL is dependent on oxygen and a number of studies have found that the amount of light emitted from luciferase-labeled cells is reduced as the oxygen concentration decreases (Cecic, Chan et al. 2007) (Moriyama, Niedre et al. 2008). Lastly, the expression level of ABC transporters can affect BLI intensity. Huang et al did a comparative study looking at the effects of different ATP-binding cassette (ABC) transporters on BLI readout when Click Beetle, Firefly, Renilla or Gaussia substrates were used in vitro. They show that ABCG2/BCRP is able to pump D-luciferin out of cells. Some groups have begun looking into increasing the stability of luciferin in vivo. For example, to improve the stability of and provide a continuous and prolonged delivery of the substrate D-luciferin for BLI, Kheirloom et al created a liposomal formulation of luciferin which had a prolonged release over 24 hours compared to the free form (Kheirloom, Kruse et al. 2010).

5. Use of BLI in oncology research

In oncology research, BL was first used in vitro and in vivo to assess cellular metabolism and indirectly to measure viability in an experiment known as the ATP assay. In this assay, luciferin and luciferase are added to media and in the presence of ATP, luciferin is oxidized giving off light that can be measured using a luminometer or a CCD camera. These assays are a direct measure of cell viability and can be used to assess cell proliferation and cytotoxicity in vitro (Garewal, Ahmann et al. 1986; Kuzmits, Aiginger et al. 1986; Kuzmits, Rumpold et al. 1986; Ahmann, Garewal et al. 1987; Sevin, Peng et al. 1988; Petru, Sevin et al. 1990; Crouch, Kozlowski et al. 1993). Using the ATP assay Garewal et al were able to distinguish between cytostatic and cytotoxic effects of therapeutics on a colon cancer cell line in vitro (Garewal, Ahmann et al. 1986).

In the late 80s and early 90's Mueller-Klieser and Walenta et al began mapping metabolites in excised tumor tissues using similar principles to the ATP assay. Their method allowed for the assessment of glucose, lactate, and ATP distributions in sections of tumors and normal tissue from cryobiopsies. Briefly, the procedure involves application of a solution containing gelatin and enzymes linked to luciferase. Upon tissue thawing, the enzyme solution diffuses into the tissue section initiating the BL reaction wherever the substrate of interest is found. The photons emitted are visualized in tumor sections using a CCD equipped microscope (Mueller-Klieser, Walenta et al. 1988; Mueller-Klieser, Kroeger et al. 1991; Walenta, Schroeder et al. 2002). Figure 4 shows a bright-field image (A) and BLI of ATP (B) from a cryosection of melanoma from the Syrian golden hamster. The BLI clearly indicates high concentrations of ATP in viable cell regions of the periphery. Furthermore, the studies show that high levels of glucose are found in the tumor periphery while necrotic tumor centers exhibited high lactate levels (Walenta, Dellian et al. 1992).

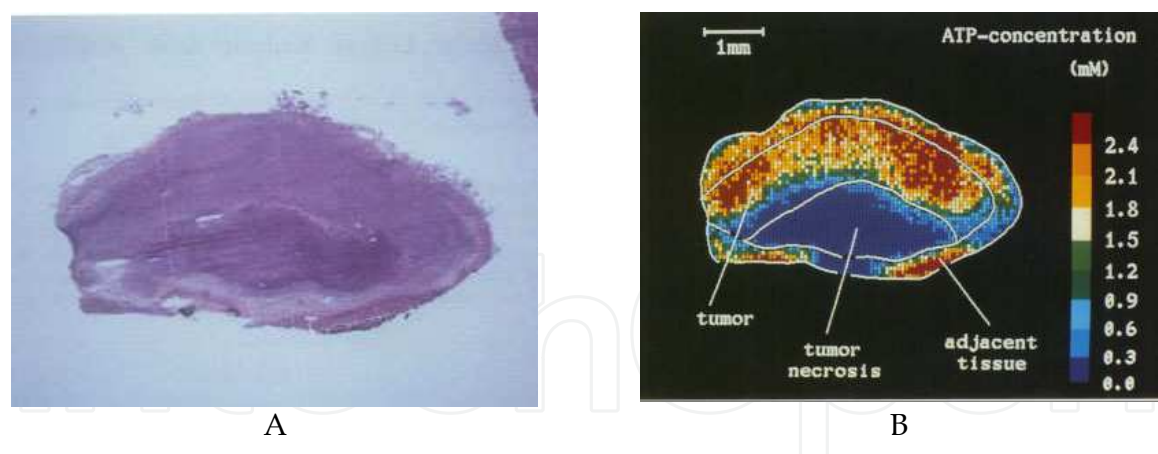


Fig. 4. Mapping ATP in melanoma of the Syrian Hamster using BLI. Cryosection stained with hematoxylin and eosin (A) shows necrosis in the center of the tumor, Colour-coded intensity image of bioluminescence (B) illustrates the local distrubiton of ATP concentrations. (Walenta, S., M. Dellian, et al. (1992). "Pixel-to-pixel correlation between images of absolute ATP concentrations and blood flow in tumors." *Br J Cancer* 66(6): 1099-1102. Reproduced by permission.)

The metabolic switch to aerobic glycolysis and enhanced lactate production is characteristic for aggressive tumor cells and a factor for tumor response and treatment outcome. Thus, BL mapping of metabolites can be used as an early marker for treatment response. Broggini-Tenzer et al use BL metabolite mapping strategies in mice carrying tumor xenografts derived from A549 lung cancer cells to show that metabolite levels are influenced by treatment with the microtubule stabilizing agent patupilone, ionizing radiation or a combination of the two modalities (Broggini-Tenzer, Vuong et al. 2011). Their results are shown in Figure 5. The BLI of tumor sections indicated that lactate levels were significantly reduced and glucose levels drastically increased in treated tumors compare to the untreated tumors. However, ATP levels did not change significantly with any of the treatments used.

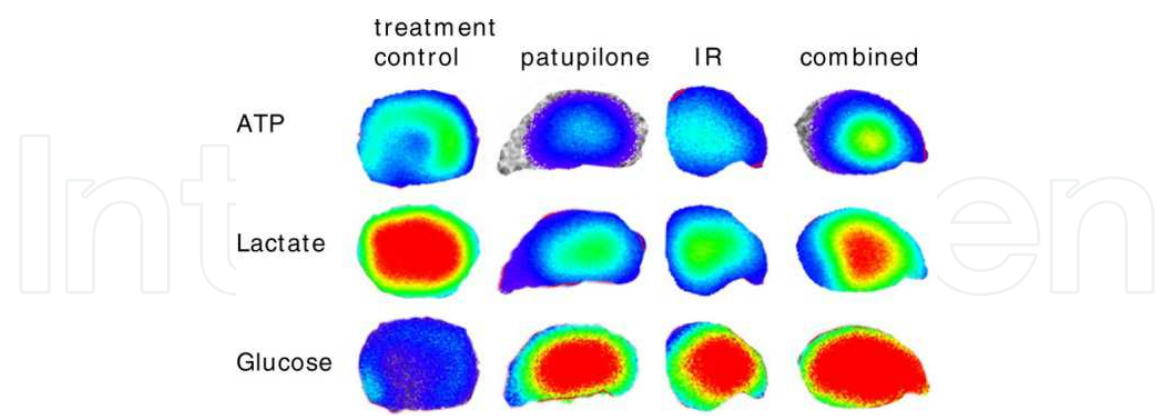


Fig. 5. Distribution of metabolites within lung tumor sections after treatment. Representative tumors are shown for each treatment group and consecutive sections were stained for ATP, lactate and glucose. Treatment with patupilone or irradiation exhibit decreased ATP levels and Lactate levels but increased glucose levels within the tumor core. (Broggini-Tenzer, A., V. Vuong, et al. (2011). "Metabolism of tumors under treatment: mapping of metabolites with quantitative bioluminescence." *Radiother Oncol* 99(3): 398-403. Reproduced by permission.)

The non-invasive utility of BLI as a small-animal imaging modality has led to the development of a wide range of luciferase positive orthotopic and metastatic animal models. Orthotopic models for breast cancer (Garcia, Jackson et al. 2008; Shan, Wang et al. 2008; Kalra, Warburton et al. 2009; Kalra, Anantha et al. 2011), bladder cancer (Mugabe, Matsui et al. 2011; van der Horst, van Asten et al. 2011), hepatocellular carcinoma (Frampas, Maurel et al. 2011), ovarian cancer (Cordero, Kwon et al. 2010; Bevis, McNally et al. 2011), head and neck SCC (Sano, Matsumoto et al. 2011), multiple myeloma (Runnels, Carlson et al. 2011), pancreatic cancer (Angst, Chen et al. 2010; McNally, Welch et al. 2010; Muniz, Barnes et al. 2011), glioma (Prasad, Sottero et al. 2011), lung cancer (Madero-Visbal, Colon et al. 2010; Li, Torossian et al. 2011; Yan, Xiao et al. 2011), prostate cancer (Svensson, Haverkamp et al. 2011), mesothelioma (Feng, Zhang et al. 2011), neuroblastoma (Teitz, Stanke et al. 2011; Tivnan, Tracey et al. 2011), rectal cancer (Huerta, Gao et al. 2011), renal cancer (Karam, Mason et al. 2003), sarcoma (Vikis, Jackson et al. 2010) have been established in vivo. Furthermore, several of these models have been used to develop systemic disease. For example, Mishra et al use intracardiac and intratibial inoculation of luciferase positive prostate cancer cells to investigate the effect of inhibiting TGF β on osteoblastic tumor growth and incidence in vivo (Mishra, Tang et al. 2011). As seen below, our group used a luciferase positive human breast cancer cell line (LCC6-Luc) to establish orthotopic disease via mammary fatpad injections, ascitic disease via intraperitoneal injection and metastatic disease via intracardiac inoculation (Kalra, Anantha et al. 2011).

5.1 Capturing minimal disease as well as quantifying tumor development and growth

Luciferase expressing cells can be used in vivo to monitor tumor growth. In an animal model, it is possible to observe small numbers of luciferase positive cells following luciferin administration. Indeed, post-inoculation, luciferase activity can be detected allowing for the confirmation of cell injection. Because small numbers of cells are readily detected, quantitative measurements of disease burden can be done earlier and for the identification of metastatic spread. A study done in our laboratory used LCC6-Luc cells to inoculate animals orthotopically, intracardiac, or intraperitoneal, to establish mammary tumors, systemic disease and ascities disease respectively. The results are summarized in Figure 6. This study demonstrated that the growth of orthotopic, systemic, and ascities disease can be monitored from day zero upon tumor cell inoculation, through to day 28 where there is established disease. The BLI data was quantified and used to create growth curves for each model. Further, the information provided in Figure 1 was used to estimate the number of cells detected at day 7, 14, 21 and 28. These data, in turn, could be used to generate a tumor growth curve (Kalra, Anantha et al. 2011).

As suggested by the above example, a major advantage of using BLI is the sensitivity of capturing photon emissions from small numbers of cells and monitoring the onset of disease, tumor growth, primary tumor dynamics and progression to metastasis. Using BLI it is possible to quantify the kinetics of tumor growth since photon emissions increase in proportion to the number of cells and thus disease burden. Light measurements can be made from whole body scans or from a selected region of interest and are most commonly quantified as total photon counts (photons/s). One of the first in vivo experiments performed using BLI of luciferase labeled cells was done by Edinger et al in 1999. This group

used a Luciferase positive human cervical carcinoma cancer cell line (HeLa-Luc) to inoculate animals via subcutaneous, intraperitoneal and intravenous injection. They were able to visualize cells immediately following inoculation using all of the injection routes. According to this study, 1×10^3 cells could be detected in the peritoneal cavity, 1×10^4 at subcutaneous sites, and 1×10^6 circulating cells following injection (Edinger, Sweeney et al. 1999).

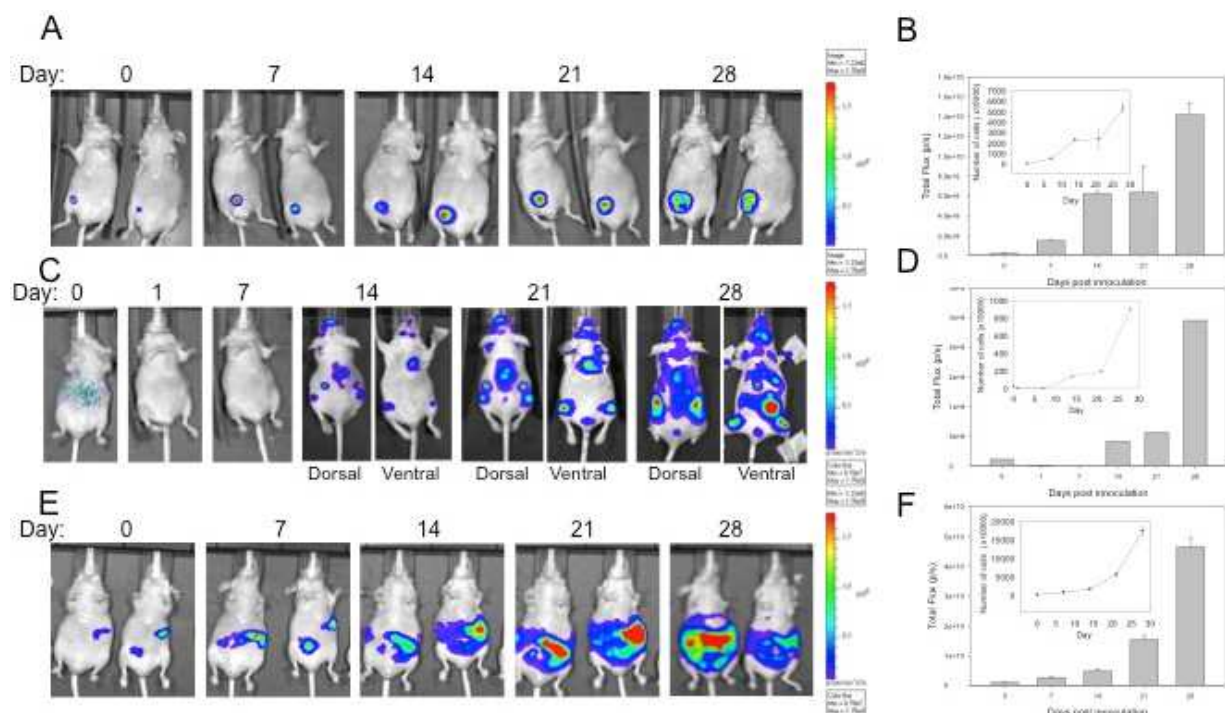


Fig. 6. Using Luciferase positive to establish orthotopic, systemic and ascitic tumors in animals. LCC6^{WT}-Luc cells were inoculated orthotopically (A & B), via intracardiac injection (C & D), or intraperitoneally (E & F). BLI was used to monitor tumor growth. Images shown were acquired on days 0, 1, 7, 14, 21, and 28. Photon emissions were measured using whole body scans for each animal (representative images shown in A, C and E). BL data was quantified to generate growth curves (B, D and F). The inset graphs represent BLI data as it correlates to the number of cells. (Kalra, J., M. Anantha, et al. (2011). "Validating the use of a luciferase labeled breast cancer cell line, MDA435LCC6, as a means to monitor tumor progression and to assess the therapeutic activity of an established anticancer drug, docetaxel (Dt) alone or in combination with the ILK inhibitor, QLT0267." *Cancer Biol Ther* 11(9): 826-838. Reproduced by permission from the author.)

In 2003 Jenkins et al developed a luciferase positive prostate cancer, lung cancer and colon cancer model to study tumor growth in vivo. Bioluminescent PC-3M-Luc-C6 human prostate cancer cells were implanted subcutaneously into mice and were monitored for tumor growth using BLI. They show that BLI data correlated to standard external caliper measurements of tumor volume, but BLI permitted earlier detection of tumor cells. In the lung colonization cancer model, bioluminescent A549-Luc-C8 human lung cancer cells were injected intravenously and lung metastases were successfully monitored in vivo by whole animal imaging. Bioluminescent HT-29-Luc-D6 human colon cancer cells implanted subcutaneously produced metastases to lung and lymph nodes. Both primary tumors and micrometastases were detected by BLI in vivo (Jenkins, Oei et al. 2003).

More recently in 2011, van der Horst et al used a re-engineered firefly luciferase (Luciferase 2) in which a transcription-factor binding sites that compromised luciferase expression was removed. A Luciferase 2-positive human transitional carcinoma cell line (UM-UC-3-Luc) was used to inoculate either the bladder of mice to produce an orthotopic model with systemic metastases or in the left cardiac ventricle to develop a model that simulates bone metastasis. This group was able to detect 100 cells three hours after subcutaneous inoculation. They were also able to detect micrometastases from both the orthotopic and metastatic tumors (van der Horst, van Asten et al. 2011).

In 2002, Bhaumik et al show that D-luciferin (the substrate for F-Luc) does not serve as a substrate for Renilla Luciferase (R-Luc), and coelenterazine (the substrate for R-Luc) does not serve as a substrate for F-Luc in cell culture or in living mice. This group made stable transfections of a rat glioma cell line (C6) with R-Luc or F-Luc. Mice were inoculated with C6-R-Luc in the left forearm, and C6-F-Luc in the right forearm. Once tumors had established, animals were subject to BLI using D-luciferin or coelenterazine. As shown in Figure 7, D-luciferin delivery was associated with photons emitted from the tumor cells in the right forearm, while coelenterazine was associated with photons emitted from the left forearm. Elegantly, Bhaumik et al were able to demonstrate that both R-Luc and F-Luc expression can be imaged in the same living mouse. This pivotal finding adds an extra layer of complexity to the BLI modality in that different luciferins can be used to track at least two separate 1) molecular events, 2) cell populations such as stem cells versus tumor cells, 3) gene therapy vectors, or 4) endogenous genes through the use of two reporter luciferase genes (Bhaumik and Gambhir 2002).



Fig. 7. BLI of F-Luc and R-Luc activity in the same animal. Both C6-F-Luc (A) and C6-R-Luc (B) cells were implanted subcutaneously at the right or left forearm sites respectively in the same mouse. Injection of D-luciferin via tail-vein in the mouse in figure 4A shows bioluminescence from site A and minimal signal from site B. Injection of coelenterazine via tail-vein in the mouse in Figure 3B exhibit bioluminescence from site B but minimal signal from site A. (Bhaumik, S. and S. S. Gambhir (2002). "Optical imaging of Renilla luciferase reporter gene expression in living mice." 99 (1): 377-382. Copyright (2002) National Academy of Sciences, U.S.A. Reproduced by permission.)

Indeed using Bhaumik's findings, Wang et al separately labeled murine breast cancer cells (4T1) with an R-Luc-monomeric red fluorescence protein (R-Luc-mRFP) reporter vector and mesenchymal stem cells (MSC) with a F-Luc-enhanced green fluorescence protein (F-Luc-

eGFP) reporter vector in order to study how MSC traffic and differentiate in either subcutaneous or metastatic animal models. Wang et al were successfully able to monitor tumor growth by R-Luc BLI and the MSC's by F-Luc BLI in the same animal (Wang, Cao et al. 2009).

As already indicated, F-Luc requires ATP in order to produce light, thus only metabolically active and oxygen rich cells contribute to the signal observed in BLI. A decrease in signal intensity occurs as cells undergo apoptosis or necrosis. In one of our own studies, we used the highly aggressive breast cancer cell line MDA MB 435/LCC6 to make a BL orthotopic mouse model. This cell line is known to rapidly develop tumors with necrotic cores. Using BLI it was noted that 28 days post tumor inoculation, the center of the tumor no longer emitted BL photons suggestive of a metabolically inactive tumor core. Dead or necrotic regions within a tumor, would still contribute to its volume, therefore traditional caliper measurements would have provided inaccurate results.

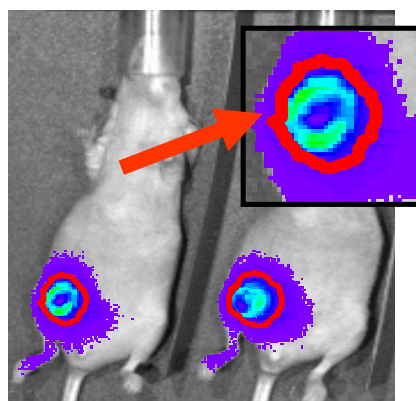


Fig. 8. Orthotopic LCC6-Luc tumors have necrotic cores that can be visualized using BLI. LCC6-Luc cells were inoculated into the mammary fat pad of female mice. Tumors were monitored using BLI. On day 28 post tumor cell inoculation, non-luminescing regions within the tumor was visualized after BLI suggestive of necrotic centers. (Kalra, J., C. Warburton, et al. (2009). "QLT0267, a small molecule inhibitor targeting integrin-linked kinase (ILK), and docetaxel can combine to produce synergistic interactions linked to enhanced cytotoxicity, reductions in P-AKT levels, altered F-actin architecture and improved treatment outcomes in an orthotopic breast cancer model." *Breast Cancer Res* 11(3): R25. Reproduced by permission.)

Since only metabolically active cells may produce BL, BLI can be used to indicate a positive drug response. BLI can be used to measure cell death with treatment using cytotoxic agents or even reduced proliferation post treatment with cytostatic agents. Many studies have been published to date describing the use of BLI to determine the efficacy of a variety of drugs currently being used in clinic and to look for valuable new drugs in the development pipeline. Because BLI is able to capture minimal disease, treatment success and cure of animals can be determined at earlier stages of, at metastasis and to monitor relapse before any clinical signs of disease are detectable. Further, since animals are monitored non-invasively, serial sacrifice of groups of animals at intermediate time-points is not necessary. This saves on the number of animals used per study and also means that multiple parameters of drug efficacy and dosing can be studied simultaneously. Li et al used luciferase-expressing A549 cancer cells injected into the mediastinum of athymic nude mice

to determine whether the luciferase positive model would allow for monitoring of response to therapeutic interventions. Animals were treated with paclitaxel or irradiated, and tumor burden was monitored using BLI. They noted that tumors responded to paclitaxel or radiation as shown by decreased tumor BL which also correlated to improved overall survival (Li, Torossian et al. 2011). Mugabe et al, use BLI to determine if a mucoadhesive nanoparticulate form of docetaxel is able to improve treatment of a bladder cancer by increasing the dwell time and uptake of the intravesical drug (Mugabe, Matsui et al. 2011). In 2009, Graeser et al use BLI to show that a liposomal formulation of gemcitabine has improved anti-tumor and anti-metastatic effects in an orthotopic model of pancreatic cancer when compared to the free drug (Graeser, Bornmann et al. 2009). As most agents are used as part of combination regimens, BLI is an ideal technique to study combination effects against single agent therapies and to elucidate combinatorial ratios and scheduling, as these experiments require multiple study arms and large numbers of animals. Prasad et al show that in a luciferase positive glioma model, combining a cytostatic agent (PI3K/mTOR dual inhibitor (XL765)) with a clinically relevant agent, temozolomide (TMZ), resulted in an additive reduction in tumor BL compared with control. The BLI data correlated to improvement in median survival time in the combination treated group (Prasad, Sottero et al. 2011). Finally, using a novel approach to study pharmacodynamics, Pensel et al use two imaging modalities to study probe accumulation at the site of tumor tissue. This group used a luciferase positive human leukemia model (HL-60-Luc) and a radiolabeled probe (spermine), imaged with BLI and SPECT respectively, to demonstrate that the spermine conjugate accumulates in tumor cells (Pesnel, Guminski et al. 2011).

5.2 Investigating key cancer processes in vivo

In addition to monitoring tumor initiation and growth as indicated above, BLI has been used in a variety of mechanistic studies. For example, in 2002 Shuetz et al were able to track transcription of luciferase reporter genes in an in vivo model of liver cancer before and after treatment (Schuetz, Lan et al. 2002). In 2003 Luker et al used a ubiquitin luciferase reporter to follow proteasomal function in vivo before and after treatment with proteasome inhibitors (Luker, Pica et al. 2003). In oncology research it is now possible to devise reporter strategies to assess key cancer processes such as dysregulated signaling, induction of apoptosis and angiogenesis in vivo.

5.2.1 Imaging apoptosis

Imaging apoptosis in vivo using a non-invasive modality would be a valuable method to evaluate drugs that induce programmed cell death. To this end, Laxman et al constructed an apoptosis biosensor by fusing the estrogen regulatory (ER) domain to F-Luc. The ER domain is able to silence the enzymatic activity of luciferase. The construct was further engineered so that the luciferase protein was flanked by the protease cleavage site for caspase-3. This cleavage site consists of aspartic acid (D), glutamic acid (E), valine (V), and aspartic acid (D) and is known as DEVD. If the DEVD site is cleaved by caspase-3, luciferase would be released from the construct, and the silencing effect from ER would be ablated. Stable human glioma cells line expressing this luciferase construct were generated and inoculated into animals subcutaneously. Animals were treated with tumor necrosis factor α -related apoptosis-inducing ligand (TRAIL), which induces apoptosis. With activation of caspase-3

the DEVD sites were cleaved, luciferase was able to fold appropriately and upon exposure to luciferin, BL photons were produced. Therefore, apoptosis was successfully imaged non-invasively using BLI (Laxman, Hall et al. 2002). Using another methodology, Niers et al engineered the naturally secreted G-Luc so that it is separated by the DEVD sequence. They showed that this fusion protein was retained in the cytoplasm of transfected cells in an inactive form. Upon induction of apoptosis, the DEVD peptide was cleaved in response to caspase-3 activation, freeing G-Luc, which then entered the secretory pathway where it was folded properly and released from the cells. The G-Luc can be detected in the conditioned medium in culture or in blood from live animals (Niers, Kerami et al. 2011). Scabini et al 2011 use a similar approach however in this case a formulated Z-DEVD-aminoluciferin is delivered intraperitoneal to mice carrying human colon cancer or human glioblastoma cell lines engineered to express luciferase. Upon induction of apoptosis Z-DEVD-aminoluciferin is cleaved by caspase 3/7 releasing aminoluciferin that is now free to react with luciferase to generate measurable BL. This group was able to show that after camptothecin and temozolomide treatment of xenograft mouse models of colon cancer and glioblastoma respectively, the treated mice showed higher induction of Z-DEVD-aminoluciferin luminescent signal when compared to the untreated group. Combining D-luciferin that measures the total tumor burden, with Z-DEVD-aminoluciferin that assesses apoptosis induction via caspase activation, they were able to relate inhibition of tumor growth with induction of apoptosis after treatment in the same animal over time (Scabini, Stellari et al. 2011). Hickson et al use the same methodology in a luciferase positive ovarian cancer and breast cancer model. In these experiments, tumor cells were inoculated and allowed to establish, subsequently animals were treated with docetaxel. Animals were injected with the Z-DEVD-aminoluciferin before BL images were acquired. This group shows that more light was detected in the docetaxel-treated group compared with the untreated group (Hickson, Ackler et al. 2010).

5.2.2 Imaging tumor hypoxia and angiogenesis

Oxygen is needed for proper cellular metabolism, thus hypoxia, which is common in proliferating cancers, can significantly alter tumor biology on a molecular level. Monitoring hypoxia in vivo can provide important information on tumor biology and response to treatment. The transcription factor Hypoxia-inducing factor 1 (HIF1), is induced under conditions of hypoxia and specifically binds to the hypoxia response element (HRE) to promote transcriptional activation. Reporter vectors based on HRE elements driving luciferase expression have been designed for longitudinal imaging of hypoxia. For example, Viola et al inoculated mice with breast carcinoma cells transfected with an HIF-1 α luciferase reporter construct and treated these animals using cyclophosphamide or paclitaxel. They showed that cyclophosphamide significantly inhibited tumor growth and caused an increase in HIF-1 α protein levels as quantified using BLI (Viola, Provenzale et al. 2008). As discussed above, a transgenic mouse model was generated in which a chimeric protein consisting of HIF-1 α oxygen-dependent degradation domain (ODD) is fused to luciferase. Hypoxic stress lead to the accumulation of ODD-luciferase which could then be identified by non-invasive BL measurement (Goldman, Chen et al. 2011).

Hypoxia stimulates secretion of vascular endothelial growth factor (VEGF) which in turn promotes angiogenesis. Transgenic mice have been engineered to express the VEGF receptor

2 (VEGFR2) promoter that drives F-Luc expression. This mouse model can be used to monitor angiogenesis induced by tumors. Angst et al sought to investigate pancreatic cancer angiogenesis and thus employed the VEGFR2-Luc mouse. After orthotopic inoculation of pancreatic cells, light emission corresponding to VEGFR activity began at day 4, which this group suggests is likely due to wound healing, and continued throughout the experimental period during tumor growth suggesting angiogenesis was occurring. The BL results were confirmed using immunohistochemical staining for CD31 (Angst, Chen et al. 2010). In 2007, Faley et al generated a transgenic reporter mouse, VEGF-GFP/Luc, in which an enhanced green fluorescent protein-luciferase fusion protein is expressed under the control of a human VEGF-A promoter. The VEGF-GFP/Luc animals exhibited intense BL throughout the body at 1 week of age, but the signals declined as the mice grew so that the adult VEGF-GFP/Luc mouse showed BL only in areas undergoing active wound healing. However, in VEGF-GFP/Luc/MMTV mice, BL is observed in spontaneous tumors indicative of active angiogenesis (Faley, Takahashi et al. 2007).

5.2.3 Imaging Protein – Protein interactions and cell signalling

In order to have a mechanistic understanding of tumor biology and response to therapy, oncology research focuses on molecular alterations in the tumor or microenvironment. Under many circumstances up-regulation of oncogenes results in changes in protein-protein interactions, alterations in kinase activity and associated changes in important signalling pathways that promote tumour cell survival and proliferation. Much work has been accomplished to study these signalling cascades in vitro and in ex vivo tissue samples and as a result many therapies have been developed to target these dysregulated pathways. For these reasons there has been a great deal of interest in developing methods to visualize molecular changes in live animals.

Three general methods are currently available for imaging protein-protein interactions in living subjects using reporter genes: a modified mammalian two-hybrid system, a bioluminescence resonance energy transfer (BRET) system, and split reporter protein complementation and reconstitution strategies, these methods were reviewed by Massoud et al in 2007 (Massoud, Paulmurugan et al. 2007). Paulmurugan developed the split reporter system in vivo using very strongly interacting proteins MyoD and Id (Paulmurugan, Umezawa et al. 2002). In 2004 this same group used split synthetic R-Luc protein to evaluate heterodimerization of FRB and FKBP12 mediated by rapamycin. The rapamycin-mediated dimerization of FRB and FKBP12 was studied in living mice by locating, quantifying, and timing the R-Luc BL. Their work demonstrates that the split reporter system can be used to screen small molecule drugs that impact protein-protein interactions in living animals (Paulmurugan, Massoud et al. 2004).

It is also possible to use BLI for the evaluation of enzymatic activity such as kinase activity, in vivo. Khan et al established a luciferase-based reporter to image EGFR kinase activity in an in vivo model of squamous cell carcinoma (SCC). The EGFR Kinase reporter (EKR) is a multidomain chimeric reporter where BL can be used as a marker for EGFR kinase activity. The reporter is phosphorylated in the presence of active EGFR which interferes with luciferase activity, if the substrate is not phosphorylated BL is available for imaging. This reporter can therefore be used as an indicator for EGFR inhibition. Khan et al demonstrated

that a small molecule inhibitor of EGFR kinase activity (erlotinib) was able to inhibit kinase activity in the SSC tumor model using BLI (Khan, Contessa et al. 2011).

BLI has also been used to monitor cell cycle signaling. In vivo BLI can be used to visualize the accumulation of p27-Luc in human tumor cells after the administration of Cdk2 inhibitory drugs (Zhang and Kaelin 2005). Briat et al have generated luciferase-based p53-reporter animals to monitor p53 activation. They showed that in response to doxorubicin induced DNA damage, female animals had weak p53 luciferase activity in the oral cavity while in males, the signal increased in the lower abdominal region (Briat and Vassaux 2008). A reporter molecule has also been developed to measure Akt activity in animals via BLI. The reporter comprises of an engineered luciferase molecule that undergoes a conformational change and gains functionality in response to phosphorylation by Akt (Zhang, Lee et al. 2007).

6. BLI in the study of gene activity, delivery and silencing

BLI provides a means to study gene delivery, activation using inducible systems, or silencing of tumor promoting genes using RNA interference (RNAi). Delivery of genes can be accomplished using multiple strategies, such as bacterial or viral vector delivery systems, immune cell and stem cell based delivery systems or encapsulation using special nanoparticle formulations such as liposomes or glucosylated polyethyleneimine. Monitoring gene delivery using BLI has also been accomplished. For example Hu et al were able to monitor TGF β receptor gene therapy efficacy in luciferase positive breast cancer metastases simply by monitoring metastases development after gene delivery (Hu, Gerseny et al. 2011). BLI also enables the evaluation of delivery itself. For example, Badr et al have made a construct that comprises of 1) G-Luc, 2) the therapeutic gene cytosine deaminase and 3) uracil phosphoribosyltransferase which converts the nontoxic compound 5-fluorocytosine (5FC) into the drug 5-fluorouracil. A glioma cell line was engineered to express F-Luc. When the constructed gene transfers into tumors, G-Luc allows monitoring of the duration and magnitude of transgene expression while F-Luc imaging was used to monitor tumor growth and response to therapy with the pro-drug 5FC (Badr, Niers et al. 2011). Ahn et al made an adenoviral vector construct where the Survivin promoter (pSurv) amplifies the expression of both the reporter gene F-Luc and therapeutic gene TRAIL. In an orthotopic hepatocellular carcinoma (HCC) rat model, they showed that after systemic administration of the vector, BLI revealed increased F-Luc activity within the tumor compared with the liver indicating that the vector shows tumor-specific transgene expression (Ahn, Ronald et al. 2011). From a gene silencing standpoint, use of luciferase-targeting siRNAs has been studied to define the proof of principle that lipid based systemic administration of luciferase targeting siRNA is able to silence luciferase gene expression in glioma (Ofek, Fischer et al. 2010) and bone metastases (Takeshita, Hokaiwado et al. 2009).

7. Conclusion

BLI is a well-established tool in cancer research that can provide valuable insight into biological processes in intact cells, excised tissues as well as in animal models of cancer. It can facilitate medium-throughput assessments, it is very sensitive, and reasonably non-invasive. The utility of BLI surpasses simple surveying of tumor growth. More specifically, BLI can be used in the development of sophisticated animal models that examine minimal or metastatic disease, therapeutic efficacy, disease relapse, mechanistic assessments of new

treatment regimens, protein-protein interactions, and to gain a better understanding of basic cancer biology. BLI facilitates visualization of processes such as metastasis, angiogenesis, apoptosis and cell signaling in vivo. As noted by Badr et al, the sensitivity of BLI allows for the early detection of tumors and therefore can be useful in the design of preclinical studies assessing prevention strategies (Badr and Tannous 2011). As the BLI modality becomes more popular, work is being done to improve the technology in order to optimize the sensitivity and detection of BL photons. For example, IVIS by Caliper has introduced a system where CT scans and BLI can be used simultaneously to generate three-dimensional images of animals and their disease. Other groups are working on engineering novel luciferases and luciferins to enhance their stability and pharmacokinetics in vivo. As indicated, it is recognized that BLI faces some challenges (distribution and absorption of the substrate as well as scattering issues effecting quantification), however continued use of BLI and proper preclinical study design can overcome most of the problems associated with this modality. BLI as a small animal imaging modality will be an integral part of the future of pre-clinical oncology research and its applications are being refined to achieve an understanding of disease development and response to therapy that was not previously possible.

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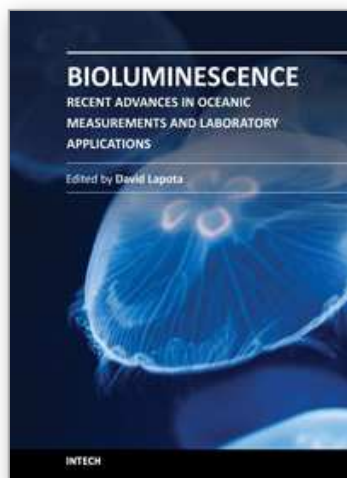
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We now find ourselves utilizing luciferase - luciferin proteins, ATP, genes and the whole complex of these interactions to observe and follow the progress or inhibition of tumors in animal models by measuring bioluminescence intensity, spatially and temporally using highly sophisticated camera systems. This book describes applications in preclinical oncology research by bioluminescence imaging (BLI) with a variety of applications. Chapters describe current methodologies for rapid detection of contaminants using the Milliflex system, and the use of bioluminescence resonance energy transfer (BRET) technology for monitoring physical interactions between proteins in living cells. Others are using bioluminescent proteins for high sensitive optical reporters imaging in living animals, developing pH-tolerant luciferase for brighter in vivo imaging, and oscillation characteristics in bacterial bioluminescence. The book also contains descriptions of the long-term seasonal characteristics of oceanic bioluminescence and the responsible planktonic species producing bioluminescence. Such studies are few and rare.

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