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## A New Diagnostic System in Cancer Research: Bioluminescent Imaging (BLI)

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EXPERIMENTAL / LABORATORY STUDIES

## A New Diagnostic System in Cancer Research: Bioluminescent Imaging (BLI)\*

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**Abstract:** Bioluminescent imaging (BLI) is particularly well suited for imaging small animals, and it can be readily used by scientists who are already routinely using the luciferase gene as a reporter in cell-based assays. This strategy relies on ATP and an O<sub>2</sub> dependent photochemical reaction between luciferin and luciferase, resulting in the release of photons from only live cells. In BLI, the intensity of the light detected by the device is proportional to the intensity of light emitted and the relationship is quantitative. This study was performed to determine the sensitivity of BLI for the imaging of lung metastasis and breast cancer models in addition to primary flank tumors. A549 lung cancer and MDA-MB 231 breast cancer cell lines were transfected with a lentiviral construct containing the luciferase gene and transfected cells were used as xenograft animal models. BLI was used to monitor tumor development and growth. Noninvasive bioluminescent imaging *in vivo* provides easy visualization of the tumor size and location so that imaging results can be used as an indicator of the treatment effectiveness, potentially accelerating the optimization of cancer treatment protocols.

**Key Words:** Cancer, bioluminescence, imaging

### Introduction

Molecular events promoting tumorigenesis and anticancer therapeutic strategies have been intensively studied in tumor cell culture models. In the past few years, bioluminescence imaging (BLI) has emerged as a powerful strategy for the validation of cell culture findings in animal models of cancer. Knowledge of the kinetics of the bioluminescent signals is required for the reliable quantification and comparison of signals during longitudinal studies (1-3). The objective of this study was

to analyze the time course of luminescent signals emitted from luciferase-expressing cells in flank tumors, lung metastasis and breast cancer models.

BLI is made possible through the use of charge-coupled (CCD) cameras along with the photon emitting properties of a particular enzyme-substrate reaction. Bioluminescence, the conversion of chemical energy into light in living organisms, is dependent on 2 principal components, an enzyme luciferase and the substrate D-luciferin (4,5).



\* This study was carried out in The University of Texas Southwestern Medical Center, Departments of Cell Biology, Radiology and Cancer Imaging Program.

The enzyme and substrate coupling causes photon-releasing chemical reactions, and the light produced has emission spectra ranging from 400 nm to 620 nm, which are detected by a CCD camera (Scientific Imaging Technologies Inc., Tigard, OR, USA (SiTe) SI-032AB CCD). Cooling of the CCD camera can significantly reduce background dark current signals. The low background of luminescence from normal tissue and the rapid turnover of the luciferase enzyme make this method ideally suited for temporal *in vivo* imaging of gene expression. Following treatment of tumor bearing mice with various anti-cancer drugs, the emitted light can be recorded quantitatively and the increase or decrease in tumor size can be easily monitored (6-12).

**Single head optical imaging system (Cyclops):** Cyclops is an optical imaging system based on a back-illuminated, high quantum efficiency CCD camera that has particularly high sensitivity (quantum efficiency 80%-85%) with a flat response from blue to near infrared and longer wavelengths. Light exposure is controlled using a mechanical shutter, which allows for very short exposure times (< 5 ms) and eliminates the smear effect observed in full frame CCD cameras without a shutter. This architecture provides a high dynamic range combined with very low readout noise. As the CCD camera is very sensitive to light, it is encapsulated in a light-tight enclosure and can be automatically positioned at the desired distance from the subject in order to adjust the field of view. The duration of the imaging varies on the intensity of the bioluminescent signal.

**Multi head optical imaging system (light emission tomography system):** Multiple high sensitivity CCD cameras simultaneously record views and a computer controlled rotation mechanism allows imaging at multiple angular positions, as required for three dimensional reconstructions. An optical imaging system with 5 cameras that surround the subject in the transverse plane was designed for permitting the simultaneous acquisition of images from 5 points of view 72° apart. A computerized system can rotate the cameras around the longitudinal axis to provide a more complete angular coverage (Figure 1).

## Materials and Methods

**Generation of luciferase expressing cell lines:** The A549 lung adenocarcinoma cell line and MDA-MB 231 breast carcinoma cells were obtained from ATCC. MDA-MB 231 breast carcinoma cells were grown in a 4:1 mixture of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% cosmic calf serum (HyClone), whereas A549 lung carcinoma cells were grown in F12 media (Gibco) supplemented with 10% FBS (Invitrogen). The A549-Luc and MDA-MB 231-Luc cell lines were established using a lentivirus encoding the luciferase gene driven by a Ubiquitin promoter. These lentiviral expression plasmids encode for the HIV-1-Gag-pol, HIV-1-Reverse transcriptase and HIV-1-VSV-G envelope proteins. The 293T cells, a packaging cell line for lentivirus, were grown in DMEM (10% FCS, Invitrogen) at 37 °C, 5% CO<sub>2</sub>. The lentiviral vectors (CW-GagPol, CMW-Rev and CMV-VSV-G) were co-transfected into 293T cells using Fugene 6 (Roche Biosciences) overnight (14-16 h) at 37 °C. Virus containing medium was collected and filtered (0.45 µm) after 24, 36 and 48 h. and replaced with fresh DMEM (10% FCS, Invitrogen) each time. The A549 and MDA-MB 231 cells were infected with the virus containing medium supplemented with 10 mg/ml of DEAE-Dextran. After infection, the virus containing medium was replaced with fresh medium to allow the cells to recover for 24 h. The cells were then plated at 3 x 10<sup>4</sup> per 10 cm dish and placed under selection of G418 at 500 µg/ml for 2 weeks.

**Xenograft animal models:** Immunodeficient mice (Nu/Nu; Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were maintained in pathogen-free conditions within the animal resources center (ARC) at the University of Texas Southwestern Medical Center. The mice were irradiated with 350 rad <sup>137</sup>Cs, 18-24 h before the injection of tumor cells.

A549-Luc cells were assessed for tumorigenicity in both an intravenous metastasis model and a subcutaneous flank tumor model, whereas MDA-MB 231-Luc cells were used for the breast cancer model. A549-Luc cells and MDA-MB 231-Luc cells were grown until about 70% confluence just 1 day before injection. After checking cell viability with trypan blue, A549-Luc cells were diluted in PBS at a final concentration of 1 x 10<sup>6</sup> cells/100 µl PBS for tail vein injection. For mammary fat pad injections,

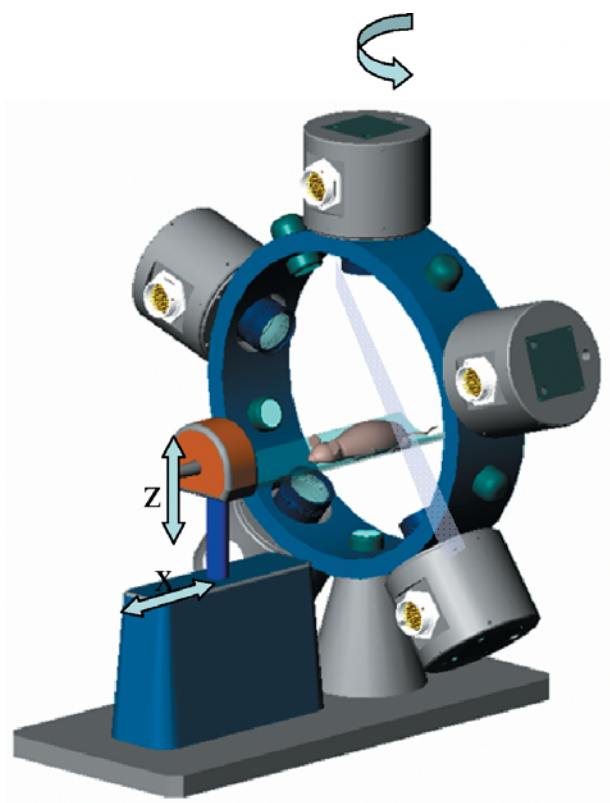


Figure 1. LETS (Light Emission Tomography System).

$2 \times 10^6$  MDA-MB 231-Luc cells/200  $\mu$ l PBS were injected on both sides subcutaneously. For flank tumors,  $4 \times 10^6$  cells/400  $\mu$ l PBS for the right flank and  $2 \times 10^6$  cells/200  $\mu$ l PBS for the left flank tumor model were used and imaged by BLI.

**Bioluminescent imaging:** The light-sensitive substrate, D-luciferin (Biosynth), was kept at 4 °C in darkness and given by intravenous, intraperitoneal or subcutaneous (150 mg/kg) injection. The mice were placed under the CCD camera and kept under isoflurane anesthesia (1.5%-2.0%) during imaging. The images were taken 8-10 min after the D-luciferin injection. Generally the test animal was exposed to the CCD for 8 min (Figure 2). With the use of computer image analyses software (Igor Pro), color images of the tumor overlaid upon a picture of the particular mouse were created. Relative intensities of emitted light were represented as a pseudocolor image ranging from blue (least intense) to red (most intense).

## Results

The kinetics of luciferase expression were analyzed after intraperitoneal luciferin (6 mg/kg) injection and the highest signal was obtained after 8-10 min. The signal decreased within 60 min (Figure 2). As the technique does not harm the animals, multiple sequential imaging studies in the same animal are possible. At the concentrations used for bioluminescence imaging, D-luciferin is nontoxic and nonimmunogenic, and so serial imaging examinations can be performed with the same mouse. D-luciferin crosses cell membranes and penetrates the intact blood-brain barrier in addition to placental barriers after injection in mice, allowing this reporter protein to be imaged in any anatomic site.

A549-Luc cells were assessed for tumorigenicity in both an intravenous metastasis model and a subcutaneous flank tumor model. After the injection of A549-Luc cells by tail vein, weekly serial *in vivo* images were taken from the animals. The lung metastases developed 3 weeks after the tail vein injection and continued to grow (Figure 3). Levels of bioluminescence from *in vivo* images corresponded to the frequency and size of metastatic lesions in lungs as well as flank and breast tumors as subsequently confirmed by histology (data not shown). After the subcutaneous injection of A549-Luc cells, usually 1-2 days is enough to get signal from flank tumors in nude mice. The image 1 week after flank injections is given in Figure 4;  $4 \times 10^6$  A549-Luc cells were injected into the left flank area and  $2 \times 10^6$  cells into the right flank.

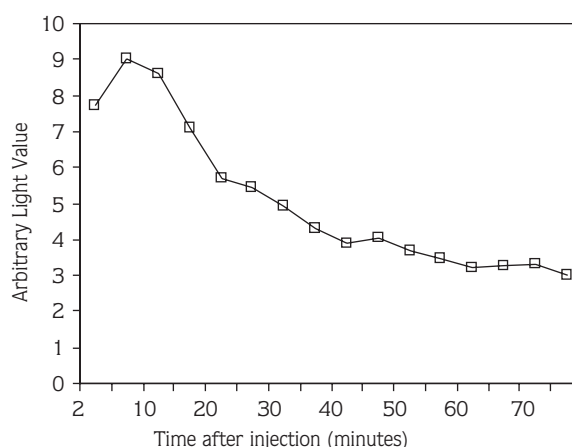


Figure 2. Kinetics of luciferase expression in A549-Luc cells after 6 mg/kg D-luciferin injection.

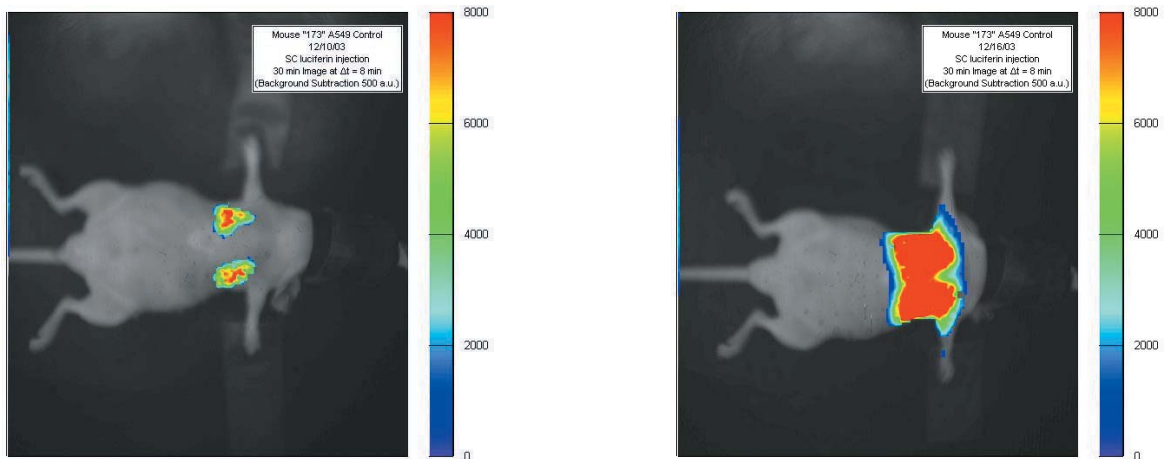


Figure 3. Lung metastases formed 3 weeks after tail vein injection of A549-Luc cells ( $1 \times 10^6$ ). The image on the left side is 3 weeks after injection, the right one is 4 weeks after injection.

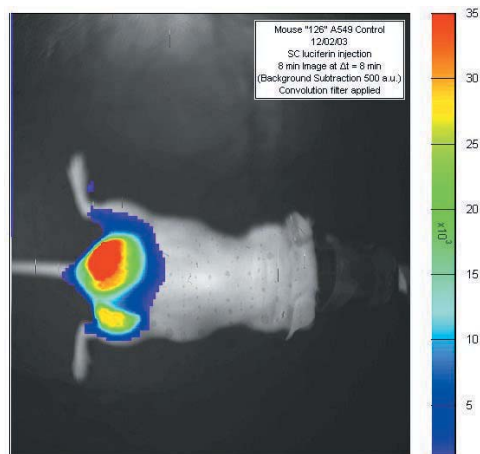


Figure 4. Flank tumors developed 1 week after subcutaneous injection of A 549-Luc cells ( $4 \times 10^6$  /left flank,  $2 \times 10^6$  / right flank).

MDA-MB 231-Luc cells were used for the mammary fat pad model. Following a 0.5-1 cm incision,  $2 \times 10^6$  MDA-MB 231-Luc cells were implanted on both fat pad areas. Visible tumors developed within 1-2 weeks on both sides (Figure 5).

The quantitative results of BLI are summarized in the Table.

### Discussion

In our study, we constituted xenograft animal models by using A549-Luc and MDA-MB 231-Luc cells and a bioluminescence imaging system for monitoring the tumors formed by luciferase expressing cancer cells. By using a single CCD, it was recognized that a problem appears in the quantification of the light emitted from a tumor or organ inside an animal because internal organs, muscles, and bones can partially or totally obscure the area of interest in the direction of imaging. To overcome this problem, the animals were imaged by light emission tomography system (LETS), a novel optical imaging system

Table. Quantitative results of bioluminescent imaging.

Lung metastasis model	$1.421 \times 10^7$ (3 weeks)	$8.619 \times 10^7$ (4 weeks)
Flank model	$1.985 \times 10^8$ (left side)	$3.346 \times 10^8$ (right side)
Breast cancer model	$6.695 \times 10^7$ (left side)	$4.986 \times 10^7$ (right side)



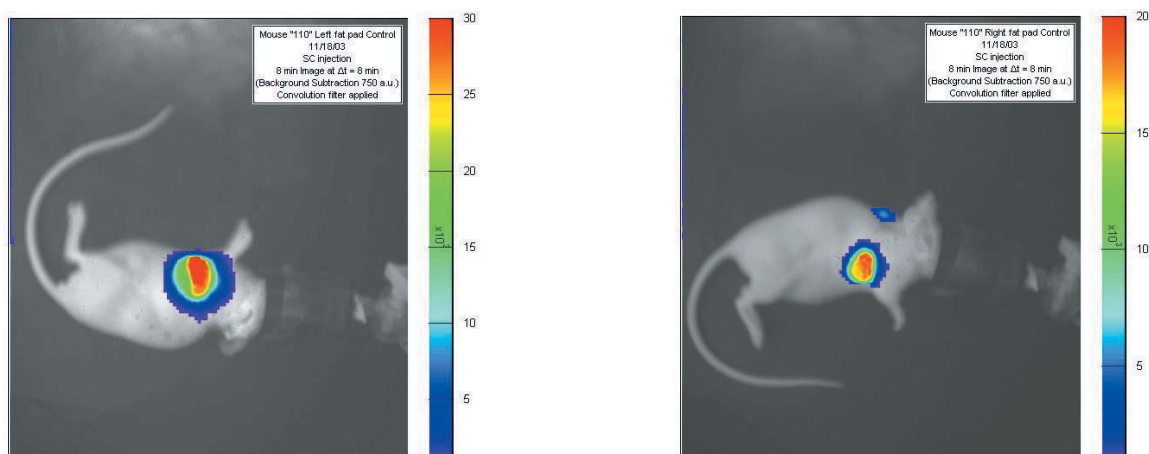


Figure 5. Breast tumours formed by MDA-MB 231-Luc cells ( $2 \times 10^6$ /each fat pad). The image on the left side is left fat pad and on the right side is right fat pad.

that combines the features of a radiologic tomography unit and multiple CCD cameras, being designed to measure quantitatively the light escaping the body over a complete set of angles (9,10).

This is the first study in which 3 dimensional LETS has been used for xenograft animal experiments. It is clear that additional cameras provide increased sensitivity and provide better time resolution. We have shown the efficiency of LETS, especially in detecting micrometastases within 3 weeks of tail vein injection, breast tumors within 1-2 weeks of implantation and flank tumors 1-2 days after subcutaneous injection.

These xenograft animal models in combination with a non-invasive *in vivo* imaging system may be useful for devising new therapies. The therapeutic effects of the drug can be assessed without having to sacrifice the mice since Luc-expressing cells can be monitored frequently, quantitatively and non-invasively with ease in real time. Statistically significant results can be achieved by using a small number of mice since multiple longitudinal measurements can be obtained over time (9,10). The conventional methods used to date to test the effect of novel therapies are time and labor-consuming. In addition, BLI instruments are considerably cheaper than those necessary for other modalities such as CT, MRI, PET and SPECT and the technology does not require the use of radionuclides, with their associated hazards (11,12).

In principle, fluorescent imaging of GFP should be more sensitive than BLI. However, fluorescence imaging is confounded by intense autofluorescence and in practice the 2 techniques show similar sensitivity. In some cases, BLI has been shown to be more sensitive than fluorescent GFP imaging. Luciferase-expressing tumors were detected as early as 1 day after tumor cell inoculation, whereas GFP-expressing tumors were not detected until 7 days later. Compared to bioluminescent imaging, fluorescent imaging does not require the injection of a substrate and may be appropriate for applications where sensitivity is not critical (13). In addition, another imaging group suggested that GFP-transfected cells may be useful for imaging studies of superficial tumors where both excitation and emission wavelengths are able to penetrate tissues, whereas luciferase-transfected cells appear superior for imaging studies of primary and metastatic tumors in distant sites and deep tissues (14).

Animal experiments are an important step providing insight for planning phase I clinical trials. *In vivo* imaging of luciferase - expressing tumor cells is a useful tool to investigate the dynamics of tumor growth and metastasis as well as the efficacy of anticancer treatment in animal models (10). Its application to traditional oncology animal models offers quantitative and sensitive analysis of tumor growth and metastasis (15). BLI can also be used to either track the course of an infection (viral, bacterial) or monitor the efficacy of antimicrobial therapies. By combining molecular biology with molecular *in vivo*

imaging modalities, it is possible to get functional information about disease processes in living systems (16). The future use of this technology may help in survival studies of the luciferase - expressing cells after implantation (stem cells, myoblasts, hematopoietic cells), the response of the host to the infections and disease processes that cannot be detected by conventional imaging.

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