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Optimizing live animal bioluminescence imaging prediction of tumor burden in human prostate cancer xenograft models in SCID-NSG mice

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Abstract

Background: Non-invasive live animal longitudinal monitoring of xenograft tumor growth and metastasis by bioluminescent imaging (BLI) has been widely reported in cancer biology and preclinical therapy literature, mainly in athymic nude mice. Our own experience at calibrating BLI readout with tumor weight/volume in human prostate cancer (PCa) xenograft models in haired SCID NSG mice through intra-prostatic (orthotopic) and subcutaneous (SC) inoculations revealed either non-existent or poor correlation (coefficient of determination r^2 ranged~ 0.01 - 0.3). The present work examined several technical and biological factors to improve BLI utility.

Methods: After ruling out promoter-luciferase (*luc*) specificity and *luc* gene loss in the cell inoculum with LNCaP-AR-*luc* cells expressing androgen receptor (AR) and tagged with AR responsive probasin promoter-*luc* gene, we evaluated different routes of D-luciferin administration, imaging time during the day, charge-coupled device (CCD) camera image acquisition settings, and hair removal methods to improve the imaging protocol. For most imaging sessions, BLI was carried out within the same day of tumor volume measurement. After necropsy, histological and immuno-histochemical (IHC) analyses were performed on the tumors to evaluate necrosis and expression of luciferase and AR, respectively.

Results: Injection of D-luciferin by SC route, robust image capture setting (30,000 counts and auto-exposure), imaging in the morning and thorough hair removal resulted in a substantial improvement of r^2 to ~ 0.6. Histological analyses confirmed lack of BLI signal in necrotic tumor masses consistent with luciferase-mediated light emission only in oxygenated ATP-producing

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viable cells. IHC staining detected heterogeneous expression of luciferase tracking generally with AR expression in non-necrotic tumor tissues.

Conclusions: Our body of work highlighted a framework to validate imaging protocols to ensure the acquisition of interpretable BLI data as an indicator of xenograft tumor burden. The vast tissue heterogeneity in prostate tumor xenografts and variable luciferase expression constrained this technology from achieving a high correlation.

Keywords

Prostate cancer; bioluminescence; IVIS instrumental setting; heterogeneous expression of luciferase; necrosis

Introduction

Mouse models are crucial for understanding the biology and cellular and molecular mechanisms of prostate cancer (PCa) genesis and progression, and for evaluating the efficacy of therapeutic regimens [1]. For studying human PCa cellular targets, xenograft models have often been established by subcutaneous (SC) or orthotopic intra-prostatic inoculation of human PCa cell lines (such as LNCaP, DU-145, or PC-3) into immunocompromised rodent (predominantly murine) hosts. The orthotopic xenograft models provide more suitable tissue condition and microenvironment milieu for the PCa cells to establish and express malignant features than the SC models [2]. Nevertheless, the SC tumor xenografts have been the most widely used models because the cancer cell inoculation technique is easy to perform and the tumor volume is conveniently monitored by caliper measurement [3]. When done consistently, tumor volume is highly correlated with and predictive of the tumor weight. Longitudinal monitoring of intra-prostatic PCa growth or metastasis to other organs is more challenging and can be facilitated by non-invasive imaging modalities [4,5].

Current available live animal imaging modalities include ultrasound, magnetic resonance imaging (MRI), micro-computed tomography (micro-CT) and molecular optical imaging techniques, especially bioluminescence imaging (BLI)[6]. BLI is capable of detecting and quantifying minute quantities of light emitted by luciferase-labeled cancer cells through centimeter-deep tissue in live animals [7]. The expressed luciferase catalyzes a reaction with D-luciferin substrate, oxygen and ATP for visible light emission, enabling the *in vivo* BLI to measure metabolically viable cells [8]. The ability to simultaneously image the entire body of up to 5 small rodents with short imaging times (acquisition times of 1 second to 5 minutes) also provides relatively high throughput [9]. This technique has been widely used in preclinical oncology research to measure the tumor burden and their responses to intervention as well as to track metastasis noninvasively in live hosts, mainly athymic nude mice. However, it was questionable whether all applications had tested and validated the reliability of BLI to predict the tumor burden.

Our own experience at calibrating BLI readout with tumor weight/volume revealed either non-existent or poor correlation (coefficient of determination r^2 range ~ 0.01 - 0.3) with human PCa xenograft models in haired SCID NSG mice through orthotopic and SC

inoculations with the androgen sensitive LNCaP-AR-luc (probasin promoter) and the androgen independent PC-3-luc2 (human ubiquitin C promoter) cells (Table 1). These experiments were carried out in Texas Tech University Health Sciences Center School of Pharmacy and denoted as TX studies. Under the same imaging conditions, we established a reasonable BLI correlation with SC tumor volume ($r^2 \sim 0.55$) in the MDA-MB-231-BR-luc2 human breast cancer xenograft model with human ubiquitin C promoter-*luc2* reporter that yielded an order of magnitude greater BLI output (Table 1) and could further improve the correlation by IV delivery of D-luciferin (Table 1).

Of the number of variables that might have contributed to the poor BLI correlation in the PCa xenograft models in the NSG mice, we investigated the impact of routes of D-luciferin administration, including IP, IV and SC, imaging time of the day (morning vs. afternoon), IVIS CCD camera settings (target photon counts and exposure time) and hair removal methods, achieving a substantial improvement of BLI correlation with tumor volume ($r^2 \sim 0.6$). Analyses of the tumor histology with H&E staining and immunohistochemistry (IHC) with luciferase as well as androgen receptor (AR) highlight vast tissue and molecular heterogeneity in the PCa xenograft tumors, constraining further BLI improvement.

Materials and Methods

Materials and reagents.

D-luciferin firefly potassium salt was purchased from Caliper Life Sciences (Hopkinton, MA). Matrigel was purchased from BD Biosciences (San Jose, CA).

Cell lines.

LNCaP-AR-luc cells were generously provided by Charles Sawyers, MD of Memorial Sloan Kettering Cancer Center, New York [10]. Wide-type AR cDNA was introduced into hormone-sensitive LNCaP cells by retroviral infection, and the threefold increase in AR abundance in the LNCaP-AR cells mimicked the expression difference observed in the hormone-refractory/hormone-sensitive pairs [10]. Stable transfection of the AR-dependent rat probasin-luciferase reporter (ARR2-Pb-luc) [10,11] into LNCaP-AR cells enable detection of molecular pharmacodynamic response to next-generation AR antagonists by BLI. The LNCaP-AR-luc cells were cultured in RPMI 1640 Medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) without any antibiotics. The cells were expanded and used for inoculation within 3-4 passages after thawing from liquid nitrogen storage.

Authentication of the LNCaP-AR-luc and LNCaP cell lines was accomplished by next-gen DNA whole genome sequencing (Macrogen Clinical Laboratory, Rockville, MD) and RNA sequencing (Penn State Hershey Genome Sciences Facility, Hershey, PA). At the genotype level, the cells display XY gender trait and the characteristic T877A mutation in the AR gene of the parental LNCaP cells. At the mRNA level, these cells express *KLK3* (PSA) at $\frac{1}{4}$ of parental LNCaP cells as expected from the Sawyers' original report [10]. Western Blot analysis confirmed the increased total AR protein and decreased PSA as compared to the LNCaP cells (data not shown).

The AR-negative androgen independent human PC-3-luc2-2E8 PCa cells were purchased from Xenogen/Caliper Life Sciences (now a subsidiary of Perkin Elmer). Human breast cancer MDA-MB-231-BR-luc2 cells were provided by the laboratory of Patricia S. Steeg, PhD, National Cancer Institute.

Flow cytometric analysis.

LNCaP-AR-luc cells were enzymatically dissociated into single cells by trypsin treatment and fixed in 2% paraformaldehyde for 20 min at room temperature. The cells were permeabilized in 0.5% Tween 20 for 20 min, blocked with 10% fetal bovine serum, incubated for 30 min. with a rabbit anti-luciferase antibody (ab21176, 1:500 dilution, Abcam) at room temperature. The cells were washed three times and incubated with a fluorochrome-labeled secondary antibody (1:500) (Fluorescent Goat Anti-Rabbit IgG Kit; FI-1200, Vector Labs, Burlingame, CA) in the dark for 30 min. The cells were washed three times, resuspended in ice cold PBS containing 0.5% Tween 20, and stored at 4°C in the dark. Fluorescence was measured using a BD LSRII flow cytometer (BD Biosciences) and data were analyzed with FACSDiva software (version 8.0.2; BD Biosciences).

Animal experiments.

The animal studies were approved by the respective Institutional Animal Care and Use Committee (IACUC) of Texas Tech University Health Sciences Center (TTUHSC) (TX studies) and Penn State College of Medicine (PA studies).

For TX studies, male SCID-NSG mice were purchased from the Jackson Laboratory (Bar Harbor, ME) (TX-011, TX-013, TX-017, 7-12 weeks of age) or obtained from the TTUHSC breeding colony (Lubbock, TX) (TX-019, TX-024, 18-20 weeks of age). The mice were acclimated for 1-2 weeks before xenograft cell inoculation. All mice were given water and irradiated rodent chow *ad libitum*. Orthotopic xenograft models were generated by inoculating 1×10^6 LNCaP-AR-luc cells (TX-011, n=16) or 1×10^6 PC-3-luc2-2E8 cells (TX-019, n=16) in 10 μ l serum-free medium intra-prostatically into the anterior lobes of mouse prostates under anesthesia. The SC xenograft models were established by subcutaneously inoculating 1×10^6 LNCaP-AR-luc cells into both sides of the lower flank of mice (TX-013, n=16), 1×10^4 LNCaP-AR-luc cells into right lower flank of mice (TX-017, n=12), or 1×10^6 PC-3-luc2-2E8 cells into right lower flank of mice (TX-024, n=11) in 100 μ l of serum-free medium containing 50% Matrigel. To test BLI of SC xenograft human cancer model of a different organ site, we used female NSG mice and inoculated with 1×10^6 MDA-MB-231-BR-luc2 human breast cancer cells in 100 μ l of serum-free medium containing 50% Matrigel (TX-023, n=9).

For PA studies, male NSG mice were obtained from a breeding colony maintained by the laboratory of David Claxton, MD and Arati Sharma, PhD (Hershey, PA) (PA-03 and PA-04, 2-3 months of age). One week after acclimation, the mice were subcutaneously inoculated with 1×10^6 LNCaP-AR-luc cells suspended in 100 μ l of serum-free medium containing 50% Matrigel into the right flank of PA-03 (n=13) and PA-04 (n=22) mice. Starting from 4 weeks after the inoculation, xenograft tumor size was measured by caliper.

Tumor bioluminescence imaging.

The mice were administered D-luciferin (150 mg/kg) by IP injection in most experiments, and by IV or SC injection for comparison. The mice under anesthesia using 1.5-2.5% isoflurane-oxygen mixture were placed onto a warmed stage inside the light-tight box of Xenogen IVIS Lumina Series III Imaging System (PerkinElmer, Waltham, MA). The IVIS camera image capture was set starting from 3 minutes after D-luciferin injection for peak BLI. Depending on experimental conditions, target max count (3,000 or 30,000 photon counts) and exposure time (10 sec. or auto-exposure with a maximum exposure time of 1 minute) were set before acquiring the images. Regions of interest (ROI) of the same size and shape were applied to all acquired images to measure total flux (photons per sec) in ROI. Image acquisition and analysis were performed with Living Image software packages (Version 4.5.5).

The tumor size was measured with a caliper on the same day of BLI and calculated by the formula of ellipsoid $\frac{1}{2}$ (Length $\times$ Width²) for tumor volume.

Histology and immunohistochemistry (IHC).

The mice were euthanized and tumor tissues collected after the last IVIS imaging. Tumor tissues were fixed overnight in 10% neutral buffered formalin. The tissues were transferred to and kept in 70% ethanol. The tumor blocks were sectioned to a 5 μ m thickness. The sections were stained with haematoxylin and eosin (H&E) for histological analysis. IHC staining was performed using mouse- (ab64259; Abcam, Cambridge, MA) and rabbit- (ab64264) specific HRP/DAB (ABC) detection IHC kits in accordance with the manufacturer's protocols. The following primary antibodies and conditions were used: AR (554225, 1:400 dilution; 3 min antigen retrieval by boiling in 0.01 M citrate buffer, pH 6; BD Biosciences) and luciferase (ab21176; 1:20,000 dilution; 3 min antigen retrieval by boiling in 0.01 M citrate buffer, pH 6; Abcam). Negative control sections, including a no primary antibody control for antibody specificity confirmation and the luciferase expression checked in no luciferase-expressing xenograft tumor, were run with each batch.

Statistical analysis.

Coefficient of determination for correlations (r^2) between tumor volume (mm³), tumor weight (mg or g) and bioluminescence intensity (total flux/sec) were analyzed by regression calculation. Statistical significance was set at a level of P less than 0.05. All statistical analyses were performed using SAS System for Windows release 9.4 (SAS Institute Inc., Cary, NC).

Results

Orthotopic PCa models showed no correlation between BLI readout and tumor weight.

Given the promise of BLI for non-invasive live animal imaging for *luc*-tagged prostate and other cancer cells in internal organs and metastases initially established in athymic nude mice [12–15], we applied BLI following “standard operating protocol” by IP delivery of D-luciferin substrate and IVIS default setting (3,000 counts, auto-exposure) on intra-prostatically-inoculated xenografts in white-haired SCID NSG mice with LNCaP-AR-luc

cells (Table 1, study TX-011). Expecting to calibrate BLI readout with tumor weight with an excellent linear equation, we found no correlation at all ($r^2 = 0.013$, $n=16$).

The type of promoter is known to influence the expression of the *luc* reporter gene [16] and so is the version of *luc* gene so that *luc2* yields more luciferase protein than regular *luc* due to less peroxisomal degradation [17,18]. In the LNCaP-AR-luc cells the AR target rat probasin promoter drives luciferase expression. In the AR-negative androgen independent PC-3-luc2-2E8 cells, the *luc2* gene is driven under human UbC promoter. However, there was no correlation for orthotopic xenografts established with PC-3-luc2-2E8 cells in the NSG mice ($r^2 = 0.080$, $n=16$) (Table 1, study TX-019). In addition, in both studies, abdominal hair was removed with a clipper only at time of surgery to inoculate cancer cells into the prostate. BLI was carried out weekly for 10 weeks without removing the regrown hair.

SC-xenograft PCa models also showed poor correlation between BLI and tumor burden.

Because SC-inoculated xenograft PCa models could be easily monitored for tumor volume, we next attempted to demonstrate a good correlation for BLI and tumor volume or tumor weight using SC xenografts. In TX-013 Study, 2 sites per mouse were inoculated to allow intra-animal comparison of two tumors for BLI output consistency within the same host environment and D-luciferin delivery kinetics. As exemplified by Mouse #1 (Fig. 1A), BLI readout fluctuated for each tumor at weekly interval or shorter. There was a 2-fold difference in BLI output for the 2 tumors with the same final weight. With all the tumors included, there was no correlation between BLI readouts and tumor weights ($r^2 = 0.082$, $n=32$) (Fig. 1B and Table 1). When expressed on a unit weight basis (Fig. 1C), the BLI light output ranged from the minimum at (Mouse #3 left tumor) 1.1×10^6 flux/g tumor to the maximum (Mouse #8 left tumor) of 25.0×10^6 flux/g tumor. Six out of the 14 mice bearing dual tumors showed greater than 2 folds intra-animal tumor BLI differences (left vs. right, Fig. 1C). Hair over the tumor inoculation sites was not removed for BLI in these mice.

In another experiment TX-017 with LNCaP-AR-luc SC xenografts, the hair over the tumors was removed with a clipper prior to BLI. The correlation between BLI and final tumor weight was better ($r^2 = 0.295$, $n=12$). In contrast, tumor volume measurements highly predicted tumor weight for both experiments, $r^2 = 0.746$ (TX-013) and 0.766 (TX-017), respectively (Table 1).

In the SC xenografts established with PC-3-luc2-2E8 cells (Table 1, TX-024 study), there was no correlation of BLI readouts with tumor weights ($r^2 = 0.070$, $n=11$) (Table 1). Yet, tumor volume measurement again reliably predicted tumor weight ($r^2 = 0.874$, Table 1). Therefore, regardless of the anatomical location and size of the xenograft tumors, the AR status of the inoculated PCa cells and the promoters-*luc* reporters in these different PCa cell lines, the correlation of BLI with tumor volume or weight was rather poor or even non-existent.

A SC-xenograft breast cancer xenograft model showed better BLI correlation with tumor burden under same imaging condition.

Suspecting possible suboptimal substrate injection technique and/or IVIS instrument setting, we tested BLI of SC xenografts established with human breast cancer MDA-MB-231-BR-luc2 cells in female NSG mice under the same imaging conditions without hair removal (Table 1, TX-023). For IP delivery of D-luciferin, the BLI correlation with tumor volume was much greater ($r^2 = 0.554$, $n=9$) than in the PCa models. The BLI correlation was further improved with IV delivery of D-luciferin to $r^2 = 0.754$ with tumor volume and $r^2 = 0.791$ with tumor weight (Table 1, Supplemental Fig. S1F). The BLI output of the MDA-MB-231-BR-luc2 xenograft was an order of magnitude greater than in the two PCa xenograft models. These results suggested an inherent poor BLI performance with the PCa xenograft models and that D-luciferin delivery route might provide a possible means to improve correlation between BLI and tumor burden in the PCa models.

LNCaP-AR-luc cells retained and expressed luciferase before inoculation into mice.

Because the above studies suggested inherent variable BLI output for PCa xenografts, our investigation focused on the LNCaP-AR-luc SC xenograft model to improve BLI correlation with the tumor volume by different administration routes of D-luciferin, imaging time of the day, IVIS CCD camera image acquisition settings, and hair removal methods.

To exclude the possibility of *luc* gene loss prior to xenograft inoculation, we evaluated luciferase protein expression in LNCaP-AR-luc cells by flow cytometric analysis after staining with an antibody for luciferase. The data demonstrated 100% presence of luciferase in LNCaP-AR-luc cell inoculum (Fig. 2). However, the high-expressing cells out-performed the low-expressors by an order of magnitude for luciferase protein content (note log scale for x-axis in Fig. 2).

D-luciferin delivery by IV injection did not improve BLI correlation with LNCaP-AR-luc SC xenograft tumor volume.

Given the low yet real probability of IP injection failure (see example in Fig. 1D) and the better BLI correlation observed for IV luciferin injection in the MDA-MB-231-BR-luc2 model (Table 1), we compared the impact of IP and IV luciferin on BLI to predict tumor burden. Cognizant of the faster IV D-luciferin delivery kinetic and clearance, we performed BLI with IV luciferin in the morning (AM) and then BLI with IP luciferin in the afternoon (PM) (Table 2). The BLI signals in LNCaP-AR-luc SC xenograft tumors were kinetically followed every 3-4 minutes using default IVIS setting (3,000 photon counts, 10 second exposure). In our hands, the IV delivery resulted in BLI peak much earlier (~4 min) and declined more sharply (Fig. 3A) than IP delivery which peaked at 15 min (Fig. 3D). These BLI kinetic patterns were consistent with the MDA-MB-231-BR-luc2 xenograft model (Supplement Fig. S1) and literature reports [19,20]. The peak BLI signals were used for correlating with the tumor volume.

For IV injection of D-luciferin, the BLI outputs were on average 4-5 fold higher than IP route but the correlation between peak BLI and tumor volume was not detectable ($r^2 = 0.125$, $P = 0.11$ for the first measurement; $r^2 = 0.0132$, $P = 0.63$ for the repeat measurement

6 days later) (Fig. 3B and 3C). In IP injected mice (Fig. 3E and 3F), correlations were modest between BLI and the tumor volume ($r^2 = 0.302$, $P < 0.01$ for the first measurement; $r^2 = 0.243$, $P < 0.05$ for repeat measurement 6 days later), and better than those obtained by BLI with IV luciferin. The IV injection was more technically challenging and BLI readout peaked and declined too rapidly to allow margin of timing error if multiple mice were to be imaged. Tail vein injection site damage could also compromise repeated BLI in a longitudinal study.

Morning BLI and SC D-luciferin injection improved BLI correlation with LNCaP-AR-luc tumor volume.

The poor correlation performance of morning BLI with IV luciferin vs. afternoon BLI with IP luciferin raised a question of imaging timing during the day. PerkinElmer's technical bulletin noted that the presence of white fur, as on NSG mice, resulted in a less than 50% BLI output compared with the signal acquired after hair removal [21]. Throughout our earlier imaging studies in NSG mice, hair was either not removed (TX studies) or was roughly removed from near the tumor areas (PA-4 study, tumor range 0-670 mm³, Table 2) by an electrical clipper. In addition, a consultation with IVIS technical service prompted us to use a more robust image capture setting with 30,000 photon counts and auto-exposure up to 1 minute for the next series of imaging sessions.

We compared BLI in the morning vs. in the afternoon to assess the intra-day imaging reproducibility by IP D-luciferin delivery after thorough hair removal by the electrical clipper. The BLI signal correlated with the tumor volume better for morning measurement ($r^2 = 0.407$, $P < 0.005$) than for afternoon measurement ($r^2 = 0.251$, $P < 0.05$) (Fig. 4A and 4B) (Table 2).

Literature review suggested that SC injection of D-luciferin offered similar BLI flux output kinetics as IP route in other cancer models in athymic nude mice, yet less likely for injection failure [19]. In the NSG mice, SC delivery of D-luciferin led to the same BLI peak time for morning and afternoon and the same as the IP route, 15 min (data not shown). The trend for morning BLI superiority for correlation to tumor volume also held true for BLI with SC luciferin. The BLI correlation with tumor volume was numerically better for morning BLI session ($r^2 = 0.593$, $P < 0.0005$) than BLI in the afternoon ($r^2 = 0.469$, $P < 0.005$) (Fig. 4D and 4E).

Comparing BLI with SC luciferin to that with IP luciferin, superior correlations were observed for SC regardless of morning or afternoon imaging (Table 2). For the BLI reproducibility within the same day, the IP luciferin route, in spite of one injection failure in the afternoon (Fig. 4C), resulted in better correlation outcome than the SC luciferin route (Fig. 4F). Nevertheless, we chose to use the SC route for the remainder of the BLI testing sessions due to its greater predictive power for tumor volume than IP luciferin (Fig. 4D and 4E).

Impact of Instrument Setting on BLI correlation with tumor volume.

To de-construct the merit of instrument setting recommended by PerkinElmer's IVIS technical service (30,000 photon counts and auto-exposure 1 min max), we next compared

BLI with prior default settings (3,000 photon counts with either auto-exposure (TX studies) or 10s exposure (PA-03 and -04) using SC delivery of D-luciferin. All BLI peak times of each condition were same, 15 min (data not shown). The correlation for BLI with the tumor volume for 30,000 counts and auto-exposure setting ($r^2 = 0.535$, $P < 0.001$, measured in morning) was better than that for 3,000 counts and auto-exposure ($r^2 = 0.254$, $P < 0.05$, measured in afternoon). When repeated 3 days later, BLI correlation with the tumor volume for 30,000 counts and auto-exposure ($r^2 = 0.518$, $P < 0.005$, measured in morning) again outperformed that at 3,000 counts and 10s exposure ($r^2 = 0.272$, $P < 0.05$, measured in afternoon) (Table 2). However, we could not rule out a bias against the 3,000 count default settings due to performance of BLI for these settings in the afternoons.

Chemical depilation slightly improved the BLI correlation with tumor volume but caused skin pigmentation that impeded further BLI.

Unlike athymic nude mice, the remaining hair after thorough clipping of NSG mice could still affect BLI signal output. The commercial chemical depilatory agent Nair is marketed for removing unwanted hair at or just below the surface of the skin. Hence, we applied Nair to the clipped skin for BLI in the afternoon and compared with morning BLI fresh after hair clipping. All BLI peak times were the same of 15 min for the uses of electric clipper and Nair (data not shown). In spite of BLI in the afternoon, Nair numerically improved BLI correlation with tumor volume ($r^2 = 0.634$, $P < 0.0001$) over clipping alone ($r^2 = 0.593$, $P < 0.0005$) (Table 2).

However, one mouse developed skin pigmentation from the one-time Nair application (Supplemental Fig. 2) and decreased BLI readout at termination of the experiment compared to the penultimate value. Because skin pigmentation is known to affect BLI signal [22], we excluded that tumor from the final BLI analyses. The final BLI correlated with tumor volume ($r^2 = 0.412$, $P < 0.005$) and tumor weight ($r^2 = 0.299$, $P < 0.05$). There was a much stronger correlation between tumor volume and weight ($r^2 = 0.926$, $P < 0.0001$).

Necrosis and heterogeneous expression of luciferase tracked with variable BLI output.

The dissected tumors showed variable hemorrhage and lobes of different shapes and sizes (Supplement Fig. 3). H&E staining showed that the tumors with the lobed or fragmented BLI output contours (Fig. 5A) contained large necrotic masses (Fig. 5B). In addition, some regions of the solid, non-necrotic tumor showed heterogeneous expression of luciferase (Fig. 5C, b,c&e vs. a&d), suggesting diminished expression or loss of the *luc* gene during tumor take and development after inoculation. The pattern of the luciferase expression (Fig 5C) tracked with BLI output contour within a tumor (Fig. 5A).

The rat probasin promoter-*luc* gene construct introduced into LNCaP-AR cells to drive luciferase expression is sensitive to AR complex binding to its androgen-response element [23]. The expression pattern of AR (Fig. 5D) was in general agreement with that of luciferase (Fig 5C).

Discussion

Live animal BLI would be deemed most beneficial for detecting tumor burden and metastases in internal organs, which would otherwise be difficult to non-invasively measure without sacrificing the hosts. Some studies have demonstrated strong correlations between BLI and tumor volume or weight mainly in xenograft models established in athymic nude mice [7,24–27]. Our initial BLI studies with xenografts established in white-haired NSG mice with LNCaP-AR-luc and PC-3-luc2-2E8 PCa cell lines by orthotopic intra-prostatic inoculation as documented in this communication showed surprisingly no correlation of BLI with tumor weights (Table 1). Moving into the simpler SC models to study the factors affecting BLI in order to improve its utility as intended, we encountered extremely poor performance of BLI to predict SC PCa xenograft tumor volumes and weights (Table 1). For many users, the IVIS instrument default settings were chosen for BLI without questioning the adequacy. Such a practice probably worked for BLI applications that were less challenging, as we documented for the SC MDA-MB-231-BR-luc2 breast cancer model, which emitted an order of magnitude stronger light output than the same size tumors in LNCaP-AR-luc or PC-3-luc2 models. Our team was able to obtain a much better correlation of BLI with tumor volume ($r^2 = 0.55$) under the sub-optimal imaging conditions used for TX studies (Table 1).

In retrospect, we suspected the extremely poor performance of BLI in our initial PCa xenograft studies could have resulted from many factors including D-luciferin delivery skill, IVIS instrument settings, variable length and/or density of hair cover over tumor region, *luc* gene expression status, and tumor biology and heterogeneity. With respect to optimizing D-luciferin administration for BLI, we confirmed that IV delivery resulted in a shorter time to peak BLI and 4-5 fold higher peak photon output than SC and IP which led to a gradual uptake of the substrate into the systemic circulation. In contrast to the breast cancer xenograft model in which IV delivery improved the already reasonable BLI correlation to tumor volume and weight (Table 1, Supplemental Fig. 1), we did not observe any improvement in the LNCaP-AR-luc xenograft model by IV luciferin, in fact, the correlation outcome was worse than by IP luciferin (Fig. 3E and 3F). The IV injection was more technically challenging in that the sharp BLI peak did not allow much margin of timing error, especially when multiple mice were to be imaged. Therefore, we affirmed SC and IP as more practical and preferable over IV. In addition, injection failure was previously reported at 3-10% for IP injection (15). In our hands, SC injection of luciferin was slightly more advantageous than IP in terms of avoiding outright injection failure and improved BLI correlation to tumor volume.

During our efforts to compare the routes of luciferin delivery, we noted a superior BLI correlation with volume when the imaging was in the morning vs. in the afternoon. We did not examine the causes of the difference. It is possible that because the mice are nocturnal and eat less during the day time, differences in their gastrointestinal contents, circulation and energy metabolism between the morning and afternoon could have resulted in more variable tumor ATP levels in the afternoon to account for the poorer BLI correlation outcome. Nevertheless, we recommend BLI be carried out in the morning whenever practical.

As far as CCD imaging acquisition was concerned, raising the target max count to 30,000 counts, auto-exposure up to 1 minute from 3,000 counts improved the BLI correlation to tumor volume substantially, perhaps due to the better image accuracy. We recommend the higher max count setting and auto-exposure up to 5 minutes be chosen for BLI as long as it is practical.

Because most BLI work had been done with athymic nude mice, the issue of hair on other strains of mice has likely been overlooked. In the BALB/c mice with white fur that is the same color as that of NSG mice, greater than 2 fold gain of BLI signal was obtained after hair was removed by Nair, per technical note of the instrument vendor PerkinElmer [21]. Mouse hair absorbs and scatters light in both the visible and the near-infrared spectrum regardless of the fur color [21]. We observed that Nair treatment slightly improved the BLI correlation with tumor volume compared with the electric clipper only. However, the longitudinal use of the chemical depilation in mice can cause skin pigmentation by disrupting normal hair growth cycle [28]. We observed that even a one-time application of Nair caused skin pigmentation in one mouse that led to a significant drop of subsequent BLI output (Supplemental Fig. 2). Others have documented highly pigmented skin significantly attenuated the BLI signal [22]. Hence, we do not recommend Nair or other chemical hair removal methods during repeated longitudinal imaging of haired NSG mice.

In spite of the efforts at optimizing imaging conditions as elaborated above, the vast heterogeneity in the PCa xenograft tumors (Supplemental Fig. 3) presented a major challenge to further improve BLI correlation with tumor volume. The tumor vascularization is crucial to provide the D-luciferin substrate and oxygen for luciferase to emit light for BLI. Tumor hypoxia is a pathogenic condition due to abnormal or absent vasculature in the tumor microenvironment [29]. Hypoxia drives tumor cell death and necrosis and contributes to tumor heterogeneity [30]. Given no ATP production in metabolically dead cells, necrosis is a major cause of reduction and/or loss of BLI signal [31]. H&E staining of the LNCaP-AR-luc tumor tissues demonstrated large necrotic masses in the tumors with weak or fragmented/lobed BLI contours (Fig. 5B). Within the non-necrotic solid regions of the LNCaP-AR-luc tumors, we detected variable expression of luciferase by IHC, with negative staining in some streaks/lobes (Fig. 5C). Specific to the LNCaP-AR-luc cell line, the *luc* transcription is regulated by AR and androgen due to the AR-dependent probasin-luciferase reporter (ARR2-Pb-luc) [10,11] which exhibits strong AR-specific regulation [32]. Consistent with this promoter feature, our IHC detection showed AR roughly tracking with luciferase in the tumor tissue sections (Fig. 5D). Because we found that the LNCaP-AR-luc cells all contained luciferase protein before inoculation (Fig. 2), albeit with a wide range of expression (an order of magnitude between min and max), the vast focal/regional luciferase protein expression differences within the tumors could result from extensive clonal evolution and selection during tumor take and growth.

A limitation of this study is that the comparison of imaging setups and conditions was carried out with the same cohort of tumor bearing mice sequentially over many weeks. In that time frame, their tumors had grown significantly over the course of the study. Additional studies with planned comparisons could have helped to more precisely determine the contribution of each of the variables examined.

Conclusion and recommendations

We have documented the poor performance of sub-optimal BLI protocols for predicting tumor burden in PCa xenograft models in haired NSG mice. Our work highlighted the critical need to optimize and validate BLI protocol to ensure the acquisition of interpretable data. The biology and heterogeneity of the tumor models and the imaging protocol will impact how reliably BLI can predict tumor burden. We provided a technical framework to improve the imaging protocol. In spite of the limitation of our study, we recommend 1) use robust IVIS imaging acquisition setting (30,000 counts, auto-exposure), 2) use SC or IP delivery of D-luciferin rather than IV route, 3) use clipper, but not chemical agents, to thoroughly remove hair in haired animals, and 4) perform imaging in the morning when practical to improve the BLI correlation to tumor burden.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

(A) Example of weekly BLI of a mouse for intra-animal comparison of BLI readouts between tumors inoculated subcutaneously on the left and right flank (TX-013 study). IP luciferin injection. (B) Correlation plot between BLI flux outputs and final tumor weights. (C) Plot of BLI flux normalized by tumor weight to compare left and right tumors in mice bearing dual tumors. (D) BLI images of a mouse (PA-04 study) measured in the morning (upper panel) and in the afternoon with IP injection failure (bottom left panel) and the repeat injection for BLI detection correction (bottom right panel).

Fig 2.

Flow cytometric analyses for luciferase expression in LNCaP-AR-luc cells. (A) Negative control (no antibody). (B) Luciferase antibody alone. (C) Secondary antibody alone. (D) Luciferase antibody plus 2nd antibody. Note log scale for x-axis. All cells stained positive for luciferase protein with a range of an order of magnitude apart from low to high expressors.

Fig 3.

Comparison of BLI by IV luciferin delivery in the morning vs. BLI in the afternoon with IP luciferin delivery for correlation with tumor volume (Instrument setting: 3,000 counts and 10 sec. exposure). For IV luciferin delivery, (A) BLI kinetics and correlations for (B) first and (C) second measurements were plotted. For IP luciferin delivery, (D) BLI kinetics and correlations for (E) first and (F) second measurements were plotted. Note the first and second measurements were done in separate sessions 6 days apart.

Fig 4.

Comparison of correlation and intra-day reproducibility for BLI after IP or SC luciferin administration (Instrument setting: 30,000 counts and auto-exposure). For IP luciferin delivery, correlations for (A) morning and (B) afternoon measurements were plotted. For SC luciferin delivery, correlations for (D) morning and (E) afternoon measurements were plotted. Correlations between morning and afternoon measurements after (C) IP or (F) SC injection were plotted. Note a failed IP injection for afternoon BLI in Fig. 4C.

Fig 5.

An example of tumor heterogeneity at gross appearance and histology level. (A) The magnified bioluminescence image of the tumor with arrows specifying where histopathological images were captured. Digital scans of (B) H&E of cross section of the tumor (scale bar, 3 mm) and IHC staining for (C) luciferase (scale bar, 3 mm) and (D) AR (scale bar, 4 mm). 'a-e' are higher magnification photomicrographs of specified regions of interests (scale bar, 100 μ m).

Table 1.

Correlation between BLI and tumor weight or volume in human prostate and breast cancer xenograft models in NSG mice

Study	Cell inoculation method	Cell type	Number of Tumors, n	Tumor volume vs. weight (R ²)	P-value	BLI vs. tumor weight or volume (R ²)	P-value
TX-011	Orthotopic	LNCaP-AR-luc	16	-		0.013 (weight) IP luciferin	0.68
TX-019	Orthotopic	PC-3-luc2-2E8	16	-		0.080 (weight) IP luciferin	0.28
TX-013	Subcutaneous	LNCaP-AR-luc	32	0.746	<0.0001	0.082 (weight) IP luciferin	0.11
TX-017	Subcutaneous	LNCaP-AR-luc	12	0.766	0.0002	0.295 (weight) IP luciferin	0.07
TX-024	Subcutaneous	PC-3-luc2-2E8	11	0.874	<0.0001	0.070 (weight) IP luciferin	0.43
TX-023	Subcutaneous	MDA-MB-231BR-luc2	9	0.899	0.0005	0.554 (volume), IP luciferin 0.754 (volume), IV luciferin 0.791 (weight) IV luciferin	0.02 <0.01 <0.01
PA-03	Subcutaneous	LNCaP-AR-luc	13	0.712	0.0003	0.035 (volume) 0.008 (weight) IP luciferin	0.54 0.77

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Table 2.

Optimization for BLI for SC-LNCaP/AR-luc xenografts in NSG mice (PA-04)

Tumor volume range	D-luciferin injection route, hair status, BLI time of day	CCD camera settings	BLI vs. tumor volume, (r ²)	P-value
0-338 mm ³ (n=22)	IV injection, hair roughly clipped, BLI AM	3,000 counts, 10 seconds	0.125	0.11
	IP injection, hair roughly clipped, BLI PM	3,000 counts, 10 seconds	0.302	<0.01
0-670 mm ³ (n=20)	IV injection, hair roughly clipped, BLI AM	3,000 counts, 10 seconds	0.0132	0.63
	IP injection, hair roughly clipped, BLI PM	3,000 counts, 10 seconds	0.243	<0.05
42-2524 mm ³ (n=18)	IP injection, hair clipped, BLI AM	30,000 counts, auto expo (1 min max)	0.407	<0.005
	IP injection, hair clipped, BLI PM	30,000 counts, auto expo (1 min max)	0.251	<0.05
	SC injection, hair clipped, BLI AM	30,000 counts, auto expo (1 min max)	0.593	<0.0001
	SC injection, hair clipped, BLI PM	30,000 counts, auto expo (1 min max)	0.469	<0.005
101-3805 mm ³ (n=17)	SC injection, hair clipped, BLI AM	30,000 counts, auto expo (1 min max)	0.535	<0.001
	SC injection, hair clipped, BLI PM	3,000 counts, 10 seconds	0.254	<0.05
	SC injection, hair clipped, BLI AM	30,000 counts, auto expo (1 min max)	0.518	<0.005
	SC injection, hair clipped, BLI PM	3,000 counts, auto expo (1 min max)	0.272	<0.05
175-3911 mm ³ (N=17)	SC injection, hair clipped freshly just before BLI, BLI AM	30,000 counts, auto expo (1 min max)	0.593	<0.0005
	SC injection, Nair applied on clipped skin, BLI PM (same day)	30,000 counts, auto expo (1 min max)	0.634	<0.0001