Journal of Biomedical and Translational Research
pISSN 2508-1357 eISSN 2508-139X

Original Article
J Biomed Transl Res 2024;25(1):1-9
https://doi.org/10.12729/jbtr.2024.25.1.1

Ex vivo culture conditions for bioluminescence imaging of porcine skin tissue

Mina Kim[1], Hao Jin[2], Yong Jin Lee[1], Sang-Myoeng Lee[2*]

[1]*Department of R&D, Hulux, Seongnam13207, Korea*
[2]*College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea*

Received: Mar 8, 2024
Revised: Mar 13, 2024
Accepted: Mar 14, 2024

***Corresponding author**
Sang-Myoeng Lee
College of Veterinary Medicine,
Chungbuk National University,
Cheongju 28644, Korea
Tel: +82-43-261-3356
E-mail: smlee@chungbuk.ac.kr

ORCID
Mina Kim
https://orcid.org/0000-0002-1730-4276
Hao Jin
https://orcid.org/0009-0005-2369-2745
Yong Jin Lee
https://orcid.org/0009-0000-4567-0743
Sang-Myoeng Lee
https://orcid.org/0000-0002-3624-3392

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Acknowledgements
This work was supported by the Technology Innovation Program (20019452, Development of LNP free mRNA vaccine using new concept vaccination equipment based on Korean technology) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea)

Ethics Approval
This study was approved and oversighted by Endic's Internal Ethics Committee (P233001).

Abstract

In response to the expanding landscape of the biotechnology industry and the increasing demand for comprehensive drug development as well as the conduct of preclinical and clinical trials, there is a growing need for employment of diverse animal models, including both small and large animals. The focus of this study was on refining *ex vivo* culture techniques for bioluminescence imaging following administration of intradermal injections in large animals. To examine the feasibility of our approach, varying concentrations of the rFluc protein were administered to rats and live imaging was employed to validate the corresponding levels of expression. Subsequently, following administration of rFluc to mini-pigs, *ex vivo* analyses were performed on sample tissues to assess the levels of protein expression across different concentrations. In particular, optimal culturing conditions that facilitated the sustained expression of the protein in samples post-euthanasia were identified. Moreover, by employing small animal imaging devices, we were able to capture clear images of the sample plates, which provided evidence of the successful application of our experimental techniques. The findings from this research represent a significant effort toward refining bioluminescence imaging methods tailored for use with large animal models—an imperative facet of contemporary drug development and biomedical research.

Keywords: bioluminescence imaging; mini-pig; luciferase; injections, intradermal; multi-needle

INTRODUCTION

In the field of medicine, the use of animal models is essential for preclinical evaluation of drugs and therapies. Each of the various animal models offers its own advantages and disadvantages, and use of rodent models, such as mice, which are easy to breed and maintain, has been the preference. Increasing use of pigs, known for having anatomical, physiological, and biochemical characteristics most similar to humans, as animal models has been reported. In particular, mini-pigs have emerged as an important animal model for assessing toxicity and in conduct of pharmaceutical research.

Although drugs can be administered through various routes, interest in intradermal administration as an alternative to conventional subcutaneous, intramuscular, or intravenous methods is on the rise, owing to its minimal invasiveness, reduced pain, rapid pharmacokinetics, and the

presence of a high density of antigen-presenting cells and blood vessels. Successful intradermal administration is based on the presence of a small visible wheal on the surface of skin. Although commonly used for administration of a tuberculosis vaccination, intradermal administration, which can be technically challenging, is significantly influenced by the practitioner's experience and the biomechanical properties of the skin [1]. Devices that included pen injectors, microinjection equipment, and needle-free injection systems have been developed in recent years to enhance the accuracy and ease of intradermal administration [2]. Some have been commercialized, while others are still under development. Effective delivery of vaccines and drugs through intradermal administration requires optimization of the drug composition along with administration-related equipment. Ideally, this process of optimization should occur in animal models or *in vitro* skin models during the early stages of drug or device development.

Utilization of large animals is often required when performing these types of procedures. Culturing conditions that preserve protein expression in samples even after euthanasia were identified. Images of the sample plates were captured using small animal imaging machines. This research represents a significant contribution to the advancement of methods for bioluminescence imaging (BLI) in large animal models, which haven gained importance in the fields of drug development and biomedical research.

MATERIALS AND METHODS

Fluc protein expression detection in 96-well plates

QuantiLum® Recombinant Luciferase (Catalog #E1701, Promega, Madison, WI, USA), a custom-prepared imaging buffer (IB), and luciferase for detection of Fluc protein luminescence (Catalog #P1043, Promega) were mixed and added to 96-well plates, to a volume of 100 μL per well. The IB was composed of 30 mM HEPES pH 7.4, 1 mM β-mercaptoethanol, 1 mM adenosine triphosphate (ATP) pH 7.4, 1 mM potassium luciferin, 10 mM MgSO4, and 1 mg/mL bovine serum albumin; the concentration of luciferase was set at 21 μL/mL. After dispensing 100 μL of the mixture into each well, imaging was performed using imaging equipment acquired from DaVinci (Seoul, Korea) in ultra mode at 1-second intervals for 10 seconds. Analysis of the imaging was performed using custom software within the imaging equipment.

Rat Fluc protein expression (Hulux-2022-07-002, conducted in Hulux animal laboratory, two animals per cage)

Female SD rats aged six weeks (RaonBio, Yongin, Korea) were used; anesthesia was administered via inhalation using Isoflurane (DaonPharm, Seongnam, Korea) during administration and imaging. QuantiLum® Recombinant Luciferase (E1701, Promega) was prepared at concentrations of 0, 12.5, 25, 50, 100, 200, 400, 800, 1,600, and 3,200 ng with addition of 0.125 mg/mL Food green 3 (FCF) dye (F7252, Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) and mixed with a custom-made IB to a total volume of 50 μL,

https://doi.org/10.12729/jbtr.2024.25.1.1

and the mixture was incubated at 37°C for 30 minutes before intradermal administration. At 20 minutes post-administration, an intraperitoneal injection of luciferin (P1043, Promega) was administered at a concentration of 150 mg/kg, followed by performance of imaging 10 minutes later. Imaging was performed in fast mode at 30-second intervals for 2 minutes. Analysis of imaging was performed using custom software within the imaging equipment.

Mini-pig Fluc protein expression (approval and oversight by Endic's Internal Ethics Committee P233001)

Seven-month-old, 15 kg male mini-pigs (mini-pig, Endic, Gwangju, Korea; Aphrodite's micropig, Pyeongtaek, Korea) were used in the experiment. Fluc protein was administered at varying concentrations 0, 400, 1,600, and 6,400 with addition of 0.125 mg/mL FCF dye in PBS at a volume of 50 μL on the back region. For 1-site injections, the entire 50 μL was administered at one location, while for 4-site injections, the 50 μL volume was divided and administered across four locations. Immediately following administration, euthanasia was performed, and an 8 mm biopsy punch (Kai Medical, Seki, Japan) was performed; 1 mL of DP BS was added to an E-tube containing the sample, which was then transported to Hulux, while a temperature of 4°C was maintained, which took approximately 2 hours. A 12-well plate with a transparent membrane (pore diameter: 1 μm, Greiner Bio-One, Kremsmünster, Austria) was prepared, followed by addition of 1 mL of media and 0.1 mL of luciferin (3 mg/mL) to each well in anticipation of the samples. The 8 mm punch samples were placed in each well. Imaging was performed approximately 3 hours after administration of luciferase to allow for sufficient absorption. Imaging was performed in ultra mode; images were taken at 10-second intervals over a period of 2 minutes. Analysis of the imaging was performed using proprietary software within the imaging equipment.

RESULTS

Enhancement of *ex vivo* rFluc luminescence by luciferase in an imaging buffer

The following conditions were used to evaluate *ex vivo* expression of rFluc: #1 = 0, #2-1, #2-2 = 0.61 μL/mL, #3 = 1.22 μL/mL, #4 = 2.44 μL/mL. Added to each well of a 96-well plate, experiments were performed according to the following specifications: concentrations of #1 = 0, #2-1, #2-2 = 0.61 μL/mL, #3 = 1.22 μL/mL, and #4 = 2.44 μL/mL. Following addition of 100 μL of PBS to each well of a 96-well plate, the specified concentrations of rFluc were added, followed by administration of luciferin at a concentration of 21 μL/mL imaging performed at 1-second intervals for 10 seconds showed that the highest concentration, #4, began to emit light after 5 seconds (Fig. 1A and B). We examined the question of whether the luminescence of rFluc could be enhanced by addition of a custom-made IB. When IB was included under the same conditions as outlined in Fig. 1A, luminescence was observable at all concentrations starting from the first second and was sustained for up to 10 seconds (Fig. 1C and D). Based on these experimental results, the establishment of IB buffer conditions not only facilitates performance of *ex vivo* luminescence experiments but also offers the potential

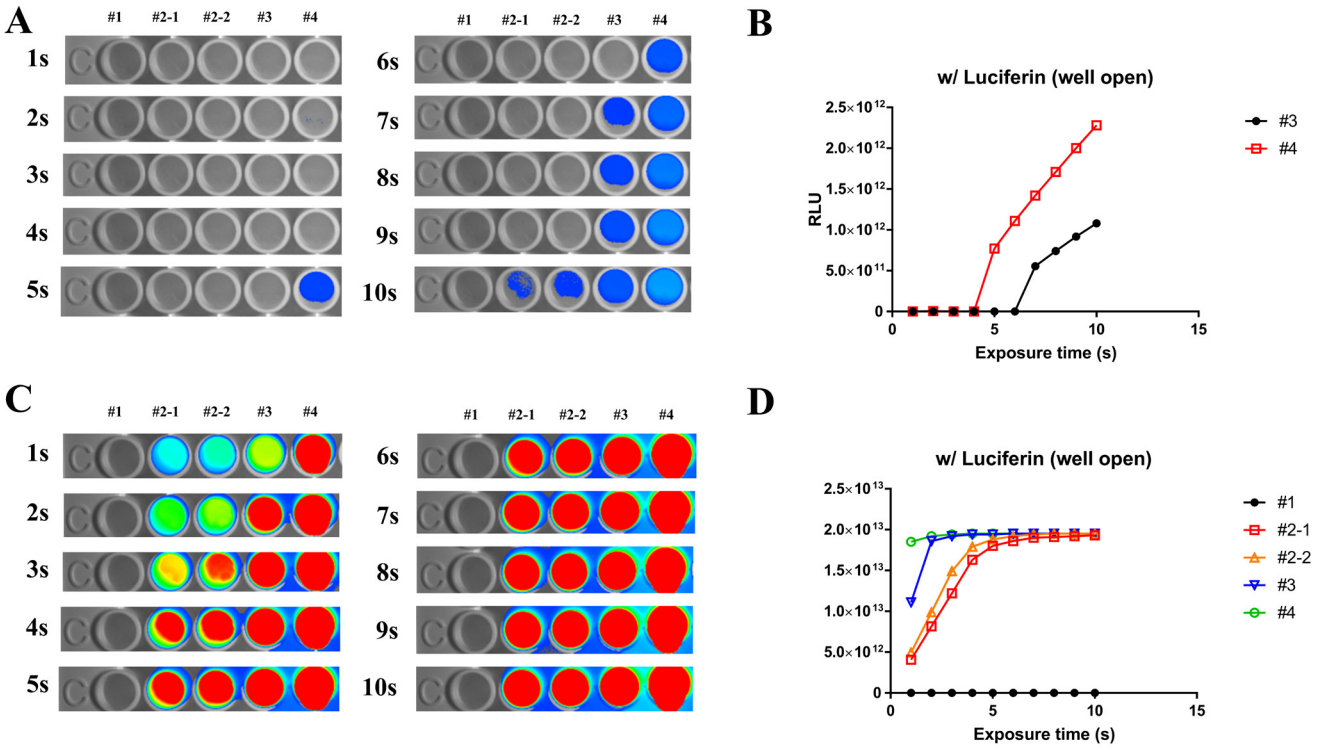

Fig. 1. Enhanced luminescence detection with and without an imaging buffer (IB). Time-lapse imaging of luminescence in a 96-well plate without IB, captured at one-second intervals for 10 seconds following addition of luciferase. Wells indicate increasing concentrations of rFluc: #1 = 0 (control), #2-1 and #2-2 = 0.61 μL/mL, #3 = 1.22 μL/mL, and #4 = 2.44 μL/mL (A). Quantification of luminescence as relative light units (RLU) plotted against exposure time for wells #3 and #4 from A (B). Luminescence visualization in a 96-well plate after addition of IB; images were taken at one-second intervals over 10 seconds. Well concentrations mirror those shown in A (C). RLU quantification corresponding to Fig. 1C, demonstrating an amplified luminescence signal with the addition of IB across all concentrations (D).

for application of these conditions in animal studies.

Rapid expression of rFluc in small animals *in vivo* facilitated by the imaging buffer

To confirm the luminescence of rFluc in live rats, a reaction time is required prior to administration of luciferin to ensure expression within the body. Before entry of protein into the cell, minimal necessary components including ATP must be supplied to initiate activity within a short time outside the cells [3–6]. Using the established IB shown in Fig. 1, luminescence was observable within 1 hour after administration (Fig. 2A). In 6-week-old female SD rats, varying concentrations of rFluc (0, 12.5, 25, 50, 100, 200, 400, 800, 1,600, and 3,200 ng with the addition of 0.125 mg/mL FCF dye) were mixed with the custom-made IB to a total volume of 50 μL, followed by incubation at 37 degrees Celsius for 30 minutes and then intradermal administration into the peritoneal cavity. Imaging performed in fast mode with a 5-minute exposure time showed that the luminescence of rFluc increased in a concentration-dependent manner (Fig. 2A and B).

Expression conditions for rFluc in large animal skin tissues

Luminescence of proteins can be rapidly confirmed *ex vivo* and in small animals *in vivo*, however, verifying luminescence using imaging equipment is challenging due the larger size

https://doi.org/10.12729/jbtr.2024.25.1.1

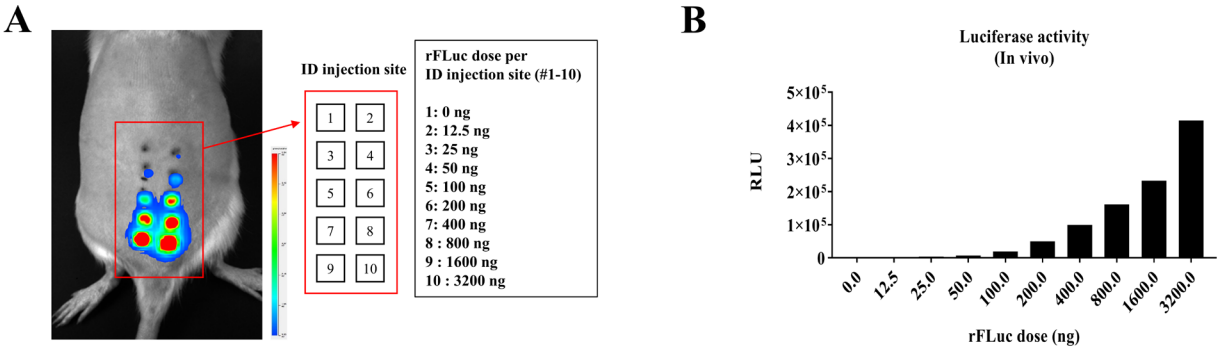

Fig. 2. Dose-dependent rFluc luminescence *in vivo*. *In vivo* bioluminescence imaging (BLI) of a rat following intradermal (ID) injection of varying doses of rFluc. Ten distinct ID injection sites are illustrated, with the administered doses ranging from 0 ng to 3,200 ng of rFluc (A). Graph showing the luciferase activity measured as RLU corresponding to the rFluc doses injected at each site shown in Fig. 1A (B).

of large animals, as opposed to small animals. In addition, after the death of an organism, the absence of essential components such as ATP, even after administering luciferin and waiting for the reaction time, can complicate the confirmation of rFluc expression and luminescence. Therefore, by integrating the findings shown in Figs. 1 and 2, conditions that enable measurement of rFluc luminescence *ex vivo* in large animals were identified. Using mini-pig skin, preparations were made in a 12-well plate under the conditions shown in Fig. 3A, and doses

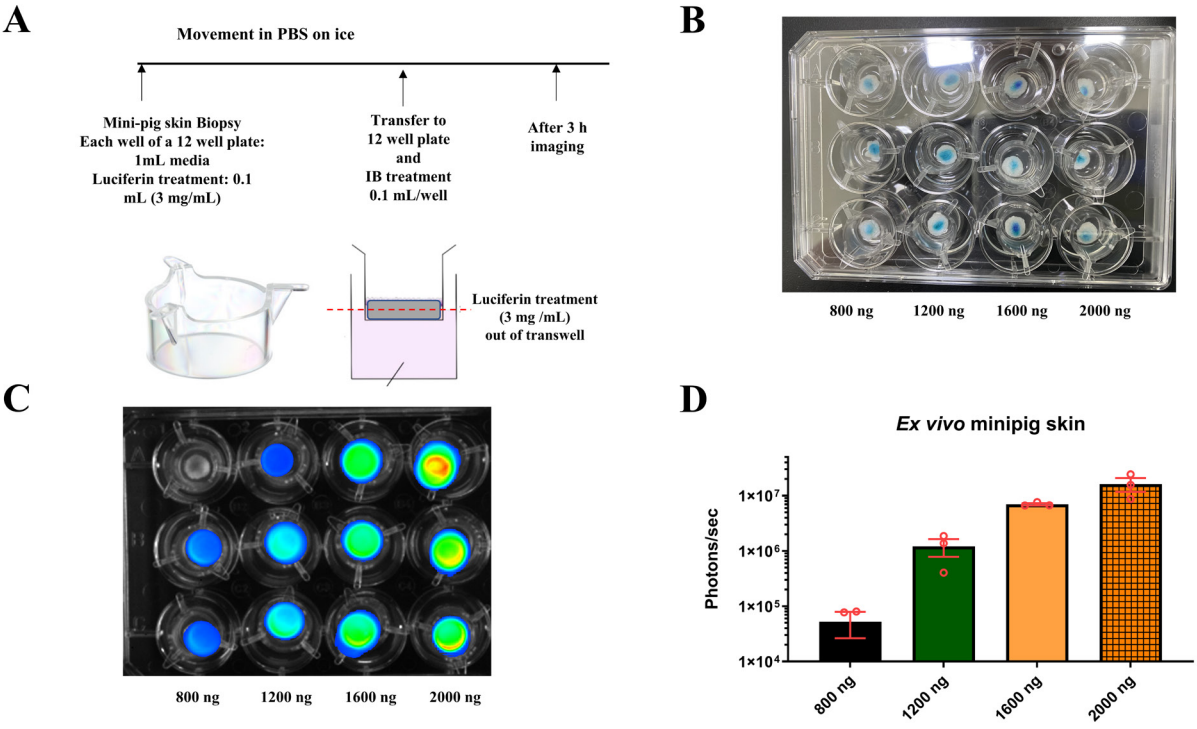

Fig. 3. Bioluminescence assessment of *ex vivo* mini-pig skin. Schematic representation of the experimental procedure for bioluminescence imaging (BLI) of minipig skin biopsies. Skin samples were first treated with luciferin (3 mg/mL) in media, followed by transfer to a 12-well plate with addition of 1 mL media and 0.1 mL of imaging buffer (IB) to each well (A). Photograph of the 12-well plate setup showing the arrangement of skin biopsies for BLI, with the indicated doses of rFluc (800 ng, 1,200 ng, 1,600 ng, 2,000 ng) (B). BLI results of minipig skin samples captured after a 3-hour incubation period post-IB treatment, depicting the luminescence emitted from each biopsy at different rFluc concentrations (C). Quantitative analysis of bioluminescence as photons/sec from mini-pig skin samples (D).

of 800, 1200, 1,600, and 2,000 ng of rFluc were mixed with the IB to a total volume of 50 μL prior to administration (Fig. 3B). Imaging was performed 3 hours after administration of luciferin, following the reaction time. *Ex vivo* administration of the protein into skin tissue confirmed luminescence at varying concentrations (Fig. 3C and D). Based on these experimental outcomes, the conditions for rFluc luminescence using large animal skin tissue were established.

Short-term expression of rFluc according to dose in large animals

The established conditions for rFluc luminescence were further validated *ex vivo* in large animal models. Specifically, the rFluc protein was administered at concentrations of 0, 400, 1,600, and 6,400 ng in a 50 μL volume to the dorsal region of 7-month-old, 15 kg male mini-pigs (Fig. 4A and C). The strategy for administration was differentiated based on the use of two approaches: a single-site (1 site) treatment, where the entire dose of 50 μL was injected at one specific location (Fig. 4B), and a multiple-site (4 site) treatment, where the 50 μL dose was divided equally and administered across four distinct locations (Fig. 4D). This outcome not only corroborates the effectiveness of the custom-made IB in enhancing luminescence but also demonstrates the feasibility of applying these optimized conditions for precise and controlled evaluation of protein expression in large animal skin tissues, as previously established through conduct of *ex vivo* experiment using mini-pig skin (Fig. 3). Use of both experimental

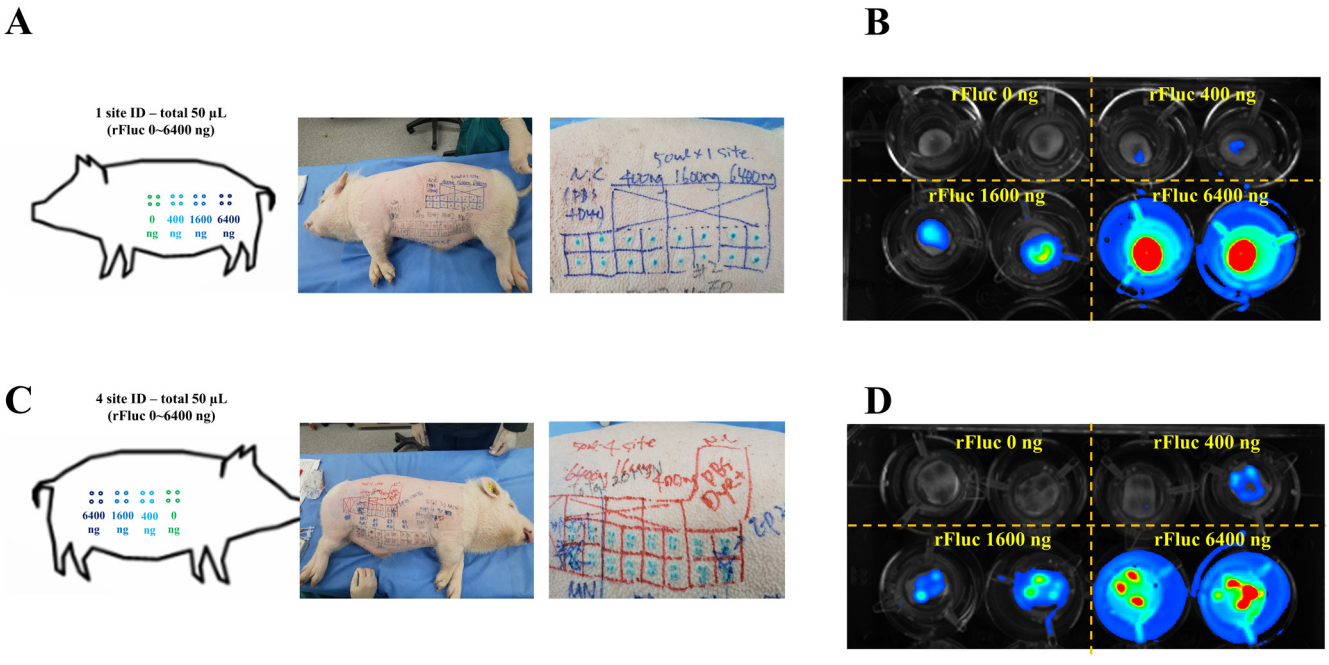

Fig. 4. Dose-dependent rFluc protein expression in mini-pig skin assessed by *ex vivo* imaging. Schematic and photographic depiction of a single-site intradermal (ID) administration of rFluc doses ranging from 0 to 6,400 ng in a total volume of 50 μL on a mini-pig's dorsum (A). Bioluminescence imaging (BLI) of the single-site ID injections on a mini-pig, showing dose-dependent luminescence intensity corresponding to the administered concentration of rFluc (B). Schematic and photographic depiction of a four-site ID administration strategy where the total 50 μL volume of rFluc is divided and administered across four locations on the dorsum of the mini-pig (C). BLI of the four-site ID injections, showing dose-dependent luminescence intensity corresponding to the administered concentrations of rFluc (D).

https://doi.org/10.12729/jbtr.2024.25.1.1

setups resulted in a concentration-dependent increase in rFluc luminescence. The findings from use of this integrated approach emphasize the potential for use of our methodology for broad applications in biomedical research involving large animal models.

DISCUSSION

In current biological research, application of *in vivo* BLI as a method has shown a steady increase [7]. As a result of advancements in BLI techniques, there is significant potential for the refinement of drug development processes and evaluation of preclinical models [8]. Due to its cost-effectiveness, high throughput, and ease of operation for visualizing a broad range of *in vivo* cellular events, researchers from diverse disciplines are adopting the use of whole-animal BLI [9]. Implementation of BLI is easy and can facilitate continuous monitoring during the progression of the disease, enabling localization and serial quantification of biological processes without sacrificing the experimental animal [10, 11]. Introduction of a custom-made IB has resulted in enhanced luminescence of rFluc, enabling precise and rapid validation of the levels of protein expression both *ex vivo* and *in vivo* across different animal models [12].

The successful application of these techniques in small animals, particularly rats, highlights the potential for use of BLI in conduct of in-depth pharmacokinetic and pharmacodynamic studies [13]. These types of studies are critical in the effort to understand the efficacy and safety profiles of new therapeutic agents before progression to human clinical trials. In addition, in our work we have extended the use of these applications to large animals, including mini-pigs, who share closer physiological and anatomical similarities with humans [14]. Thus, they are considered invaluable for conduct of translational research, where findings from animal studies can be applied more directly to human medicine [14].

Extrapolating findings from studies using small animals to the human situation, which may not accurately reflect human physiology and/or dimensional conditions, can be challenging [15]. Conversely, the dimensions of large mammals hinder performance of *in vivo* BLI due to the limited penetration depth of BLI. Utilization of BLI in *ex vivo* organ culture systems may be considered as a potential solution in the effort to address this issue [16, 17].

The technical challenges of using BLI in large animals, primarily due to their size and the post-mortem absence of essential components such as ATP [18], were addressed by adapting the conditions confirmed in our rat model studies. The key components of BLI include light-generating luciferase enzymes, including firefly luciferase, Renilla luciferase, Gaussia luciferase, Metridia luciferase, Vargula luciferase, or bacterial luciferase [19–22]. A buffer formulation for *ex vivo* BLI was developed after a review of literature for experiments in large animals. This adaptation facilitated assessment of rFluc luminescence in large animal skin tissues, offering a novel approach for evaluating protein expression and drug efficacy in models that are more representative of human patients.

In conclusion, our findings contribute to the growing body of research supporting expanded use of BLI in conduct of both small and large animal studies. Establishing optimized conditions for rFluc luminescence and demonstrating the feasibility of these techniques in large

animal models can enable performance of more accurate and efficient preclinical evaluations. Future research should focus on further refining these techniques, exploring their applications in other large animal models, and development of standardized protocols for their use in drug development and biomedical research.

REFERENCES

1. Dileo J, Miller TE Jr, Chesnoy S, Huang L. Gene transfer to subdermal tissues via a new gene gun design. Hum Gene Ther 2003;14:79-87.
2. Leoni G, Lyness A, Ginty P, Schutte R, Pillai G, Sharma G, Kemp P, Mount N, Sharpe M. Preclinical development of an automated injection device for intradermal delivery of a cell-based therapy. Drug Deliv Transl Res 2017;7:695-708.
3. Feeney KA, Putker M, Brancaccio M, O'Neill JS. In-depth characterization of firefly luciferase as a reporter of circadian gene expression in mammalian cells. J Biol Rhythms 2016;31:540-550.
4. Greiner JV, Glonek T. Intracellular ATP concentration and implication for cellular evolution. Biology 2021;10:1166.
5. Nguyen LT, Zajíčková M, Mašátová E, Matoušková P, Skálová L. The ATP bioluminescence assay: a new application and optimization for viability testing in the parasitic nematode *Haemonchus contortus*. Vet Res 2021;52:124.
6. McMorrow R, Zambito G, Nigg A, Lila K, van den Bosch TPP, Lowik CWGM, Mezzanotte L. Whole-body bioluminescence imaging of T-cell response in PDAC models. Front Immunol 2023;14:1207533.
7. Close DM, Xu T, Sayler GS, Ripp S. *In vivo* bioluminescent imaging (BLI): noninvasive visualization and interrogation of biological processes in living animals. Sensors 2011;11:180-206.
8. Mofford DM, Reddy GR, Miller SC. Latent luciferase activity in the fruit fly revealed by a synthetic luciferin. Proc Natl Acad Sci U S A 2014;111:4443-4448.
9. Baker M. Whole-animal imaging: the whole picture. Nature 2010;463:977-980.
10. Love AC, Prescher JA. Seeing (and using) the light: recent developments in bioluminescence technology. Cell Chem Biol 2020;27:904-920.
11. Liu S, Su Y, Lin MZ, Ronald JA. Brightening up biology: advances in luciferase systems for *in vivo* imaging. ACS Chem Biol 2021;16:2707-2718.
12. Thorne N, Inglese J, Auld DS. Illuminating insights into firefly luciferase and other bioluminescent reporters used in chemical biology. Chem Biol 2010;17:646-657.
13. Dothager RS, Flentie K, Moss B, Pan MH, Kesarwala A, Piwnica-Worms D. Advances in bioluminescence imaging of live animal models. Curr Opin Biotechnol 2009;20:45-53.
14. Sanchez MM, Morgan JT. Generation of self-assembled vascularized human skin equivalents. J Vis Exp 2021;168:e62125.
15. Alini M, Eisenstein SM, Ito K, Little C, Kettler AA, Masuda K, Melrose J, Ralphs J, Stokes I, Wilke HJ. Are animal models useful for studying human disc disorders/degeneration. Eur Spine J 2008;17:2-19.

https://doi.org/10.12729/jbtr.2024.25.1.1

16. Markova SV, Golz S, Frank LA, Kalthof B, Vysotski ES. Cloning and expression of cDNA for a luciferase from the marine copepod *Metridia longa*. A novel secreted bioluminescent reporter enzyme. J Biol Chem 2004;279:3212-3217.
17. Sadikot RT, Blackwell TS. Bioluminescence imaging. Proc Am Thorac Soc 2005;2:537-540.
18. Kimura S, Tsuchiya A, Ogawa M, Ono M, Suda N, Sekimoto K, Takeo M, Tsuji T. Tissue-scale tensional homeostasis in skin regulates structure and physiological function. Commun Biol 2020;3:637.
19. Wood KV, Lam YA, Seliger HH, McElroy WD. Complementary DNA coding click beetle luciferases can elicit bioluminescence of different colors. Science 1989;244:700-702.
20. Meighen EA. Molecular biology of bacterial bioluminescence. Microbiol Rev 1991;55:123-142.
21. Tsals I, Jarrahian C, Snyder FE, Saganic L, Saxon E, Zehrung D, Zimmerman G, Papania M, Klaff L. Clinical performance and safety of adapters for intradermal delivery with conventional and autodisable syringes. Vaccine 2015;33:4705-4711.
22. Vilela CAP, Souza LEB, Siqueira RC, Calado RT, Covas DT, Paula JS. *Ex vivo* evaluation of intravitreal mesenchymal stromal cell viability using bioluminescence imaging. Stem Cell Res Ther 2018;9:155.