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Continuous and Real-Time In Vivo Autobioluminescent Imaging in a Mouse Model

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Abstract

In vivo small animal bioluminescent imaging has become an indispensable technique for interrogating the localization, health, and functionality of implanted cells within the complex environment of a living organism. However, this task can be daunting for even the most experienced researchers because it requires multiple animal handling steps and produces differential output signal characteristics in response to a number of experimental design variables. The recent emergence of autobioluminescent cells, which autonomously and continuously produce bioluminescent output signals without external stimulation, has the potential to simplify this process, reduce variability by removing human-induced error, and improve animal welfare by reducing the number of required needlesticks per procedure. This protocol details the implantation and imaging of autobioluminescent cells within a mouse model to demonstrate how cells implanted from a single injection can be imaged repeatedly across any post-implantation timescale without the need for further human–animal interaction or signal activation steps. This approach provides a facile means to continuously monitor implanted cellular output signals in real-time for extended time periods.

Keywords

Autobioluminescence; Bioimaging; Drug discovery; In vivo; *lux*; Preclinical

1 Introduction

In vivo bioluminescent imaging relies upon the combination of a luciferase and its corresponding luciferin substrate to generate an optical signal. Firefly luciferase serves as the prototypical example, wherein a D-luciferin substrate reacts with ATP to form AMP, CO₂, and an oxyluciferin that releases energy in the form of photons. The inherent disadvantage of many in vivo bioluminescent imaging procedures is that the luciferin substrate must be added exogenously to initiate the bioluminescent reaction. In an animal model, this typically requires subcutaneous, intraperitoneal, or intravenous injections of the luciferin substrate prior to each desired bioluminescent measurement. The route and timing of substrate injection and its biodistribution within and clearance from the animal all impact light emission kinetics [1, 2], which influences experimental outcomes and study conclusions if not carefully optimized and rigorously controlled for. Furthermore, the

prerequisite for repeated luciferin injections limits the amount of imaging data that can be obtained from each animal and adversely affects animal welfare.

To circumvent the need to exogenously inject animals with a chemical substrate, autoluminescent imaging instead uses a synthetic luciferase (derived from the *Photobacterium luminescens lux CDABE* operon) designed specifically to express in mammalian cells using endogenous substrate scavenging for luciferin generation [3, 4]. This eliminates exogenous substrate addition, enables the continuous and autonomous emission of bioluminescence under in vitro, ex vivo, and in vivo formats, and removes the associated potential introduction of error resulting from luciferin quality variation, inconsistencies in dosage, the biological effects of foreign chemical introduction, or changes to substrate clearance rate resulting from changes in age or health among subjects [5, 6]. When paired with whole-animal imaging systems like those manufactured by PerkinElmer, MI Labs, or Spectral Instruments Imaging, autoluminescent imaging allows small animal models to be noninvasively imaged at any point over their lifetime. This approach can therefore provide longitudinal real-time surveillance of biological processes. The techniques for working with autoluminescent cells differ little from that of conventional bioluminescent cells expressing firefly luciferase, apart from the elimination of the multiple luciferin injection steps. The following protocol provides the details necessary for performing autoluminescent in vivo imaging in small animal research models.

2 Materials

2.1 Autoluminescent Cell Line

1. Autoluminescent HEK293 human kidney cell line.

2.2 Animal Subjects

1. Five-month-old, female nu/nu (nude) immunocompromised mouse.

2.3 Cell Culture Medium

1. Dulbecco's modified Eagle's medium (DMEM).
2. Fetal bovine serum (FBS): 10% solution.
3. Penicillin/Streptomycin (PenStrep): 1% solution.
4. G418: 100 µg/mL.
5. Dulbecco's phosphate-buffered saline (DPBS).

2.4 Implantation

1. Dulbecco's phosphate-buffered saline (DPBS).
2. Trypsin: 0.05% solution.
3. Trypan Blue.
4. Isoflurane–oxygen anesthesia induction chamber.

5. Hypodermic needle: 25-gauge.
6. Syringe: 1 mL.

2.5 Hardware

1. IVIS Lumina K preclinical benchtop imaging system (PerkinElmer).
2. Hemocytometer or automated cell counting device.
3. Benchtop centrifuge capable of holding 15 mL tubes.

2.6 Software

1. Living Image 4.5.2 software (PerkinElmer).

3 Methods

3.1 Preparation of Autobioluminescent Cells

This protocol uses an autobioluminescent HEK293 human kidney cell. This cell type is robust, easy to culture, fast growing, and maintains a strong autobioluminescent output signal under a variety of conditions. However, it is important to select an appropriate cell type to support the specific goals of your experiment. If an autobioluminescent version of your cell type of choice is not readily available, the cells can be made autobioluminescent as described in Note 1.

1. Using a tissue culture treated T₇₅ flask, culture the autobioluminescent HEK293 cell line in 15 mL of DMEM supplemented with 10% FBS, 1% PenStrep, and 100 µg/mL G418 (*see* Notes 2 and 3). A T₇₅ culture flask should be used to ensure a sufficient number of cells are available for injection (*see* Note 4).
2. Incubate the cells at 37 °C in a humidified, 5% CO₂ incubator and refresh the medium every 2–3 days until the cells reach ~80% confluency.
3. When refreshing medium, the spent culture medium should be aspirated, the cells should be washed once with DPBS, then 15 mL of fresh culture medium should be added.
4. Upon reaching 80% confluence, the cells are ready to be harvested and prepared for implantation as described below.

¹If an autobioluminescent version of the necessary cell type for your experimental design is not readily available, the autobioluminescent phenotype can be genetically encoded to your cell line of interest via transient or stable transfection as described in [7].

²The inclusion of 100 µg/mL G418 will ensure maintenance of the genetically encoded autobioluminescent phenotype. Depending on the source of the autobioluminescent cell line, this level of selective pressure may need to be increased or decreased. Always follow the supplier's recommended culture conditions or determine the ideal selective pressure empirically.

³If a cell type other than HEK293 is used, it may be necessary to use a different medium formulation.

⁴A T₇₅ flask provides a 75 cm² culture area. Alternative culture formats can be used to scale up or down, or multiple flasks can be used to obtain additional cells without necessitating deviation from the volumes listed in this protocol.

3.2 Implantation of Autobioluminescent Cells into the Mouse Subject

This protocol details the subcutaneous implantation of autobioluminescent cells into the dorsal flank of an immunocompromised mouse subject. However, alternative injection sites such as tail vein, intraperitoneal, or intraorganellar; or an alternative subject type can be used based on the ultimate objective of the study (*see* Note 5). All animal studies should be conducted in compliance with protocols approved by an appropriate institutional animal care and use committee and the highest ethical standards should be employed. This protocol was conducted in accordance and compliance with all relevant regulatory and institutional agencies, regulations and guidelines.

1. Harvest the prepared autobioluminescent cells by aspirating the culture medium, washing once with 5 mL of 1× DPBS, and disassociating with 3 mL of 0.05% trypsin.
2. Incubate the cells in trypsin for ~2 min, or until they have detached, at 37 °C in a humidified, 5% CO₂ incubator.
3. Once the cells have detached, recover them into a 15 mL tube by adding 7 mL of DMEM supplemented with 10% FBS and 1% PenStrep to bring the total volume to 10 mL and transferring the full volume to the 15 mL tube.
4. Count the cells using a hemocytometer or automated cell counting system (*see* Note 6).
5. Concurrent with the cell counting procedure, centrifuge the remainder of the harvested cells at 250 × *g* for 5 min.
6. Aspirate the supernatant from the centrifuged cells and resuspend at a concentration of 1 × 10⁷ cells/mL in DPBS (*see* Note 7).
7. Prior to implantation, anesthetize the mouse subject to be assayed by placing it into an anesthesia induction chamber and supplying 5% (v/v) inhaled isoflurane in 1 L/min of oxygen. Closely monitor the status of the subject inside the induction chamber. The isoflurane level may need to be adjusted accordingly to achieve desired anesthesia level. Be careful to not overanesthetize the subject.
8. Using a 25-gauge needle and a 1 mL syringe, draw up the resuspended cells from **step 6** into the syringe and inject 100 µL subcutaneously into the dorsal flank (*see* Note 8).

⁵Subcutaneous implantation deposits the autobioluminescent cells relatively near the surface of the subject, which limits the effects of absorption and dispersion on the bioluminescent output signal. If performing deep tissue implantation, it may be necessary to increase the number of implanted cells or the signal acquisition time to achieve similar levels of detection.

⁶It is helpful to prepare any necessary supplies for counting the cells before beginning the harvesting procedure. Most often, this entails the preparation of a 10 µL aliquot of Trypan Blue into a 1.5 mL microcentrifuge tube. Doing so will allow for 10 µL of the cell resuspension to be rapidly transferred to this preprepared aliquot and mixed 1:1 for live/dead counting.

⁷Resuspension of the cells at a concentration of 1 × 10⁷ cells/mL will provide for 1 × 10⁶ cells to be implanted in a 100 µL volume. This number can be adjusted based on the desired delivery volume or total number of implanted cells that are needed for a particular experimental design but represents a good starting point for evaluating the performance of most autobioluminescent cell lines.

⁸Care should be taken to ensure the injection is not made too deep. It is highly recommended that the practitioner work under the supervision of an experienced technician until they are confident in their ability to handle the anesthetized subject and perform the injection efficiently.

3.3 Imaging Autobioluminescent Output Signals

This protocol details the imaging of a single mouse subject in the IVIS Lumina K imaging system over a period of 30 min. Depending on the brand or model of imaging equipment being used, it may be possible to image multiple subjects simultaneously. Fortunately, if this is the case the same steps can be performed in parallel on each individual subject.

Alternatively, the steps can be iterated across multiple subjects in series to achieve the same result.

1. Using the Living Image software, initialize the IVIS Lumina K and set the stage temperature to 37 °C.
2. Manually adjust the in-chamber anesthesia system to deliver 1.5% (v/v) inhaled isoflurane in 1 L/min of oxygen (*see* Note 9).
3. Place the anesthetized subject harboring the injected autobioluminescent cells into the imaging chamber of the IVIS Lumina K. Be sure to position the subject so that the area harboring the injected cells is facing toward the camera and so that the subject's nose fits within the nose cone. Plug any unused nose cones with rubber stoppers to prevent the loss of anesthesia.
4. Close the door to the imaging system and ensure the interior lights are turned off.
5. Set the stage height setting to field of view C (*see* Note 10) so that the subject is centered in the field of view with minimal superfluous area visible.
6. Set the subject height to 1.50 cm and choose “use subject height” for focus.
7. Acquire a photograph by selecting the “Photograph” setting from the list of illumination settings and clicking the “Acquire” button. This photograph will show the position of the subject and allow you to determine if any changes in positioning need to be made prior to autobioluminescent signal acquisition. If repositioning is required, this step should be repeated to ensure the proper orientation has been achieved to provide an appropriate final image.
8. Once the subject has been appropriately positioned, select the “Luminescent” setting (both the “Photograph” and “Luminescent” settings should now be checked) from the list of illumination settings and input the following imaging acquisition settings (Fig. 1, *see* Note 11):

Exposure Time: 10 min.

Binning: Medium.

F Stop: 1.

⁹Slight adjustment of the isoflurane level may be necessary to keep the subject anesthetized throughout the entire imaging period depending on subject weight and system calibration.

¹⁰There are four fields of view ranging from A (the closest in length to the camera) to D (the farthest). It may be necessary to select an alternate field of view if using multiple subjects, if an alternative subject type is used, or depending on the size and orientation of the subject being used.

¹¹These settings are optimized for the detection of low levels of autobioluminescent production and work well for acquiring autobioluminescent output signals from most applications without necessitating more total time per image than would be required for substrate-requiring luciferase reporter systems. It is often possible to reduce the exposure time significantly below the listed 10 min interval, which can reduce the overall time required per image.

Emission Filter: Open.

9. After confirming the settings are correct, click the “Sequence Set Up” button to open the sequence editor dialog.
10. Click the “Add” button to import your acquisition settings into the sequence editor dialog.
11. Check the “Number of Segments” box and adjust the corresponding number to 3. This will take three 10 min exposure images for a total of 30 continuous minutes of observation (Fig. 2).
12. After the imaging process completes, it can be repeated to obtain further data, or the subject can be returned to its cage and monitored until fully recovered from anesthetic. This recovery usually occurs within 5 min (*see* Note 12).

3.4 Processing Autobioluminescent Output Data

This protocol details the interrogation of a single implantation location from a single subject at one time point. The steps listed in this section can be duplicated for additional regions of interest, subjects, or time points as needed.

1. Double click the first image in the series to open that image for processing (Fig. 3).
2. Open the “ROI Tools” section of the Tool Palette in the Living Image software.
3. Select the circular ROI tool from the menu and use the cursor to draw a circle around the region of interest (ROI) in the pseudocolor image (Fig. 4, *see* Notes 13 and 14).
4. After the ROI has been drawn, click the “Measure ROI’s” button and a dialog box will open displaying the output signal level within the ROI (Fig. 5, *see* Note 15).
5. Click the “Select All” button to select the displayed data.
6. Click the “Copy” button to copy the information to the clipboard.
7. Paste the information into Microsoft Excel, or your preferred program for data analysis and storage.

¹²Because the autobioluminescent signal is continuous and does not exhaust a finite supply of externally supplied luciferin like bioluminescent reporter systems do, the subject can be imaged at any desired timepoint, or as often as needed for repeated image acquisition, without the introduction of error due to changes in the output signal strength of the autobioluminescent cells.

¹³The circular ROI tool is commonly used because it often fits neatly around the mass of implanted cells. However, any of the alternative ROI drawing tools may be used if they are deemed more appropriate.

¹⁴To apply this same ROI at the same position to all images within the sequence, right click the ROI and select “Copy ROI,” then right click and select “Paste ROI” in each additional image. This will allow you to make direct comparisons of the same region among all acquired images.

¹⁵Note that clicking the “Configure” button in this dialog box will allow you to include image metadata detailing a wealth of different data points. It is advisable to review these options prior to completing the next steps to ensure you are capturing sufficient metadata for downstream analysis.

8. Amend the file with any pertinent metadata that was not available for direct import from Living Image, save the file, and proceed with data analysis as appropriate.

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Fig. 1.

An example of the image acquisition settings for capture of autoluminescent signal from subcutaneously implanted cells. Some of these settings, such as the binning, can be adjusted following image acquisition, but most cannot. It is important to ensure that the Photograph and Overlay checkboxes are selected in addition to the Luminescent checkbox when acquiring bioluminescent output signals. Depending on the autoluminescent cell type used, the number of cells implanted, and the health of the implanted cells, the exposure time can be reduced down to as little as 1 s while still acquiring sufficient flux for pseudocolor localization

Fig. 2.

Example of an image capture sequence that will interrogate the subject continuously for half an hour using three 10-min exposures. The Add button and the check box for adjusting the number of segments in the sequence are circled

Fig. 3.
Example of a pseudocolor image showing the location of subcutaneously implanted autoluminescent cells within a nude mouse model. The location of the injected cells should be easily visible from the autoluminescent signal

Fig. 4.
A region of interest (ROI) drawn around the location of the implanted autoluminescent cells allows total signal output to be quantified

Fig. 5.

An example of the ROI measurement dialog box. These data show the quantification of autoluminescent signal from within the ROI and can be used to make comparisons between different locations, time points, or treatments. Note the Configure button in the bottom left of the box. This will allow you to select additional metadata beyond the minimal default data (shown)