Chapter 12

Bioluminescent Monitoring of In Vivo Colonization and Clearance Dynamics by Light-Emitting Bacteria

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Abstract

Bioluminescence is an excellent reporter system for analysing bacterial colonization and clearance dynamics in vivo. Many bacterial species have been rendered bioluminescent, allowing the sensitive detection of bacterial burden and metabolic activity in real-time and in situ in living animals. In this chapter we describe the protocols for characterizing in vivo infection models using bioluminescent bacteria: from real-time imaging in living animals by bioluminescence imaging (BLI) to ex vivo BLI of harvested organs and tissues and, finally, to quantification of bacterial numbers in organ and tissue homogenates by luminometry and viable counts. While the *lux* operon from *Photorhabdus luminescens* is ideally suited for use in such models, there may be times when alternative luciferases, such as those from the firefly (*luc*) or marine copepods (*Gluc*), may be more appropriate. Here we describe the protocols required to monitor colonization and clearance dynamics using bioluminescent bacteria that are *lux*-, *luc*-, or *Gluc*-positive.

Key words: Bioluminescence imaging, bacteria, in vivo, infection model, colonization dynamics, luminometry, luciferase, luciferin, coelenterazine.

1. Introduction

Bioluminescent reporter genes have been used to investigate microbial associations in a myriad of biological systems (1, 2). In essence, bioluminescent reporters offer a method of labelling microorganisms that is innocuous and allows the sensitive detection of live, metabolically active cells. There are three main approaches by which colonization and infection processes can be monitored using bioluminescent microorganisms: (1) monitoring bacterial numbers and location, (2) monitoring bacterial viability

P.B. Rich, C. Douillet (eds.), *Bioluminescence,* Methods in Molecular Biology 574,
DOI 10.1007/978-1-60327-321-3_12,

(for example, in response to antibiotic treatment) and (3) monitoring bacterial gene expression (for example, those genes involved in colonization and/or virulence).

Bioluminescence arises from the oxidation of a substrate (a luciferin) by an enzyme (a luciferase), which usually requires energy and oxygen. Luciferin and luciferase are generic terms as none of the major classes share sequence homology. Luciferase systems include, among others, the bacterial *luxAB* genes of terrestrial *Photorhabdus luminescens* and marine *Vibrio* sp., as well as eukaryotic luciferase genes such as *luc* from the firefly (*Photinus* sp) and *Gluc* from the marine copepod *Gaussia princeps* (3–6).

The firefly luminescence reaction involves the oxidation of a benzothiazoyl-thiazole "luciferin" (commonly referred to as luciferin) and ATP resulting in the production of oxyluciferin, AMP, CO_2 and light at 560 nm (7), while the copepod reaction involves the oxidation of an imidazolopyrazine derivative called coelenterazine (the "luciferin") to produce CO_2, coelenteramide and light at 470 nm (8). The genes required for substrate production and recycling in these systems are not available; hence exogenous substrate must be added. In contrast, the bacterial luminescence reaction involves the oxidation of a long-chain aldehyde (the "luciferin") and reduced flavin mononucleotide ($FMNH_2$), resulting in the production of oxidised flavin (FMN), a long-chain fatty acid and light at 490 nm (9). A multi-enzyme complex (encoded by the genes *luxC, D* and *E*) is responsible for regeneration of the aldehyde substrate from the fatty acid produced by the reaction (10, 11).

To date, an abundance of bacterial species have been rendered either *lux*-, *luc*- or *Gluc*-positive (2, 12–19). The choice of reporter gene depends on a number of factors, including the bacterial species under investigation and whether the organism itself or gene expression is being monitored. A significant advantage of the bacterial luciferase system is the ability to express the biosynthetic enzymes for substrate synthesis without the exogenous addition of substrate. However, in many bacterial species harbouring the *lux* operon, bioluminescence declines when cells enter stationary phase during in vitro growth (13, 20) and is most likely due to a decrease in metabolic activity. In contrast, the bioluminescence of *Gluc*-expressing cells (which is dependent on the exogenous addition of substrate) appears to be independent of cofactors that become limited during stationary phase (19). In addition, eukaryotic luciferases catalyse the most efficient bioluminescent reaction known (that is, the amount of light generated in relation to the energy expended), with the firefly luciferase reaction found to be approximately 10-fold more efficient than the reaction catalysed by the bacterial luciferases (21). The luciferase encoded by *Gluc* has been found to exhibit enhanced stability during exposure to low pH, hydrogen peroxide and high

temperature (19). This luciferase may therefore be more appropriate if gene expression is being monitored in response to environmental conditions which would have a detrimental effect on the luciferases encoded by *luxAB* and *luc*.

Using specialized equipment it is possible to visualize bioluminescence directly through the viscera, skin and fur of intact animals (a technique known as bioluminescence imaging [BLI]) (22, 23). BLI is a very powerful tool for implementation of the 3Rs (Replacement, Refinement and Reduction), one of the guiding principles surrounding the use of animals in scientific research. Using BLI, bioluminescent bacteria can be followed throughout the infection cycle as the bacteria expand and migrate to different tissues in the animal, simply by imaging the bioluminescent signal detected from infection sites within the animal. Repetitive study of the same animal over the course of an experiment reveals a dynamic and more meaningful picture of the progressive changes in microbial burden, yielding better-quality results from far fewer experimental animals. Importantly, luminescence is quantifiable and related to bacterial burden. In a number of infection models, death of the animal results from the rapid and uncontrolled expansion of the infecting bacteria. Using BLI the bioluminescent signal can be used to estimate whether an animal will survive or die and hence allows for humane euthanasia, perhaps even before the onset of clinical symptoms.

Characterization of an infection model involving bioluminescent bacteria is a three-stage process: (1) detection of bioluminescent bacteria in vivo in anaesthetized animals using BLI; (2) determination of the anatomical location of the bacteria by BLI using harvested tissue; and (3) quantification of bioluminescence (by luminometry) and viable bacterial counts (by plating onto selective media) from homogenized tissue and, where appropriate, organ contents (or stool, in the case of gastrointestinal pathogens). This chapter describes the protocols involved in this process. Importantly, the detection limits for a given bacterial strain will depend on numerous factors, including the level of light production and bacterial numbers present, the availability of cofactors for the bioluminescence reaction, the tissue tropism of the bacteria (and hence, the distance the photons must travel through tissue) and potential signal impedance (such as the absorption of light by oxyhaemoglobin and deoxyhaemoglobin, or by melanin within pigmented skin and fur). It is recommended that prior to embarking on the characterization process, serial dilutions of the bioluminescent bacterial strain grown in laboratory media are assessed by BLI and luminometry, and the relationship between bioluminescence and viable counts established. These results, coupled with an understanding of the in vivo model using the non-luminescent wild-type bacterial

strain, will allow an estimation of whether the bioluminescence of the engineered microorganism is bright enough to be detected in vivo. Here we describe the in vivo and in vitro protocols required to monitor colonization and clearance dynamics using bioluminescent bacteria that are *lux*-, *luc*- or *Gluc*-positive.

2. Materials

1. In vivo optical imaging equipment (with or without gaseous anaesthesia induction equipment) such as the IVIS range from Xenogen (Alameda, CA, USA) [now part of Caliper Life Sciences], the NightOWL II from Berthold Technologies (Germany) or the Photon Imager from Biospace Lab (Paris, France) (*see* **Note 1**).
2. Software programs: such as Living Image (Xenogen) and Igor (Wavemetrics, Seattle, USA)
3. Luminometer, such as the LB953 from Berthold Technologies (Germany), and appropriate vials/plates (*see* **Note 2**).
4. Hair-removal methods such as clippers or beard-trimmer (*see* **Note 3**).
5. Anaesthetic agents (*see* **Note 4**) such as ketamine hydrochloride (100 mg/ mL) combined with xylazine hydrochloride (2% 20 mg/mL) administered via intraperitoneal injection or inhalational isofluorane. A working solution of ketamine/xylazine comprises, for example, 0.5 mL (50 mg) ketamine and 0.25 mL (5 mg) xylazine in 4.25 mL of water. While the ketamine/xylazine mix can be stored at 4°C for up to 1 week, deterioration (lack of sedation) occurs after 48 h, so ideally it should be made up fresh and used immediately.
6. Sterile phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}: 50 m*M* Potassium phosphate, 150 m*M* NaCl pH7.2 sterilised by autoclaving. PBS may also be purchased from companies such as Invitrogen.
7. D-Luciferin, sodium salt (Gold BioTechnology, St. Louis, MO, USA) if the luciferase used is encoded by *luc*. For in vivo assays, make up a 50 m*M* working concentration in PBS without Mg^{2+} and Ca^{2+} (15 mg/mL) and filter sterilise through a 0.2-μm syringe filter. For in vitro assays a 100 m*M* (200x) stock solution is made in distilled water (30 mg/mL to give 150 μg/mL working solution). Alternatively, a number of luciferase assay systems are commercially available (*see* **Note 5**). Stock concentrations can be prepared in advance and aliquots stored at −20°C. Ideally, working concentrations should be

made up fresh and used immediately, but aliquots can be prepared and stored at –20°C for future use. Do not subject luciferin to repeated freeze-thaw cycles. Once in use, keep cool and protected from light.

8. Coelenterazine (Prolume Ltd., Pinetop, AZ, USA) if the luciferase used is encoded by *Gluc*. Make up a 12 m*M* (5 × [5 mg/mL]) stock solution in acidified methanol (100% methanol with 20 µL/mL 3 *N* or 6 *N* HCl). For working concentration, dilute stock in PBS without Mg^{2+} and Ca^{2+}. Keep cool and protected from light. For in vitro use incubate at room temperature for 15–20 min before use. As coelenterazine spontaneously decays and is unstable for prolonged periods in aqueous solutions, it is best made up fresh. The 5 × stock solution can be stored at –20°C or colder for 1–3 weeks although there will be some loss of activity. Therefore, for accurate, reproducible and comparative data, freshly prepared coelenterazine is recommended (*see* **Note 6**).
9. 70% Ethanol
10. Dissection kit
11. Petri dish
12. 25–27 gauge needles (*see* **Note 7**).
13. Stomacher 80 Biomaster bags, Seward (Worthing, UK).
14. Appropriate media for determination of bacterial colony-forming units.

3. Methods

3.1. In vivo Bioluminescence Imaging

1. Anaesthetise mice (*see* **Note 8**). For inhalational agents, mice are placed into a clear plastic anaesthesia box that allows unimpeded visual monitoring of the animals. The commercially available imaging systems also have a gaseous anaesthesia manifold located inside the imaging chamber so that once sufficiently anaesthetised, animals can be transferred to the imaging chamber and anaesthesia maintained. Anaesthesia is induced within the box using a flow rate of 1 L/min 100% oxygen combined with 5% isoflurane. When the animals have lost their righting reflex, they are removed from the box and placed within the imaging chamber. Anaesthesia is maintained on 1.5–2% isoflurane with an oxygen flow rate of 0.4–0.5 L/min. If no gas anaesthesia equipment is available, mice can be injected via the intraperitoneal route (*see* **Note 9**) with a working concentration of ketamine/xylazine at 100 µL per 10 g bodyweight (24) (*see* **Note 10**).

2. If necessary, remove the fur from dark animals (*see* **Note 3**).
3. Administer substrate if required (if bacteria are expressing the whole *lux* operon, for example, from *Photorhabdus luminescens*, no substrate is required for light production and animals can be imaged as soon as they are anaesthetised).
   a) If bacteria are expressing the firefly luciferase (*luc*), administer luciferin to the mice 5–10 min prior to imaging. Common quantities and delivery routes are given in **Table 12.1** (*see* **Note 11**). The most common route of luciferin delivery is via the intraperitoneal route (*see* **Note 9**).

Table 12.1
Common routes and quantities for administration of luciferin

Route	Dose
Intravenous[a]	10 uL/g body weight of 15 mg/mL luciferin solution
Intraperitoneal[a]	10 uL/g body weight of 15 mg/mL luciferin solution
Intramuscular[b]	50 μL of 1–2 mg/mL luciferin solution
Intranasal[c]	50 μL of 3 mg/mL luciferin solution

[a] Using a 25–27 gauge needle.
[b] Using a 27-gauge needle.
[c] Using a pipette.

   b) If bacteria are expressing the copepod luciferase (*Gluc*), inject 100 μL of 1 × stock (1 mg/mL) coelenterazine intravenously into the tail vein using a 26-gauge needle (*see* **Note 12**) and image the mice immediately.
4. Place mice into the BLI equipment. Placement will be determined by the anatomical region the light signal is expected from, for example, for colonisation/infection of the visceral organs/lungs, mice are placed on their backs.
5. Image following manufacturer's instructions for the particular machine. The level of luciferase expression (bacterial numbers) will determine the amount of bioluminescence to be detected. This is turn will determine the period of time required to detect the signal, but usually ranges from 1 to 10 min.
6. After imaging, remove mice from the BLI equipment for recovery from anaesthetic (*see* **Note 13**).
7. Using this protocol, mice may be imaged repeatedly, especially if gaseous anaesthetic is used. Animals highly infected/colonised with *lux*-expressing bacteria (imaged in a

couple of minutes) can be monitored three to four times a day using isoflurane, although ideally this intensive monitoring regime should not be followed for more than 3 days. The limiting factor with monitoring *Gluc*- and *luc*-expressing bacteria is the clearance of substrate and the number of times animals can be subjected to substrate delivery (*see* **Table 12.2**). While coelenterazine rapidly disappears, mice should only be dosed intravenously twice on a given day. In contrast, mice can safely be dosed with luciferin by the intraperitoneal route up to three times per day. However, luciferin is cleared much more slowly than coelenterazine, requiring intervals of approximately 4 h between administrations.

Table 12.2
Suggested maximum volumes and frequencies of administration of substances (in accordance with *(34)*)

	Intraperitoneal	Intramuscular	Subcutaneous	Oral gavage	Intravenous
Maximum number of doses	24	6	24	20	14
Maximum daily volume	20 mL/kg	500 μL	20 mL/kg	20 mL/kg	10 mL/kg
Number of daily doses <7 days	2–3	2	3	2	1–2[a]
Number of daily doses >7 days	1	1	2	1	<1[a]

[a]For intravenous administration, 1 dose per day should be administered for no more than 6 days, while 2 doses per day should be administered for no more than 2 days.

3.2. Ex Vivo Bioluminescence Imaging of Harvested Tissue

1. Cull mice by an appropriate humane method. Mice may be imaged intact at this point, but the availability of oxygen and cofactors may be a limiting factor (*see* **Fig. 12.2** and **Note 14**).
2. Spray mice with 70% ethanol before dissecting and remove any organs/tissues of interest. Wash in sterile PBS if appropriate, and place into Petri dish. Organs such as the stomach and gastrointestinal tract can be washed in PBS to determine numbers of organisms intracellularly, extracellularly but adhering or extracellular and non-adherent (*see* **Note 15**).
3. Bathe organs/tissue in substrate (luciferin/coelenterazine) as appropriate (*see* ***Subheading 3.1.*** **Step 3** and **Notes 5** and **6**).
4. Image following manufacturers instructions.

3.3. Tissue Homogenate Bioluminescence Assay

1. Cull mice by an appropriate humane method.
2. Dissect to remove organs/tissues of interest.
3. Wash in sterile PBS if appropriate. Do not discard the wash to determine numbers of organisms intracellularly, extracellularly but adhering or extracellular and non-adherent if appropriate (*see* **Note 15**).
4. Place organs in a plastic stomacher bag and determine weight of organ/tissue.
5. Mash organs to make a homogenate, being careful not to pierce the bag. Add an appropriate amount of PBS (usually 1–5 mL) to bag and resuspend organ/tissue (*see* **Note 16**).
6. Remove three replicate samples from each sample (*see* **Note 17**). Prepare serial dilutions of organ/tissue homogenate (*see* **Note 18**) in PBS or another appropriate buffer (*see* **Note 19**). The volumes required will depend on the luminometer used.
7. Place samples into luminometer, add or inject luciferin/coelenterazine if required (*see* **Notes 5** and **6**) and record the photon emission for an appropriate time, usually 1–10 s (*see* **Note 20**).
8. Plate serial dilutions of samples onto the appropriate selective agar for measurement of bacterial numbers (colony forming units).

3.4. Expected Results

The three-stage process of model characterization is illustrated in **Figs. 12.1–12.3** with the extracellular gastrointestinal pathogen *Citrobacter rodentium* (25). Laboratory-grown *C. rodentium*

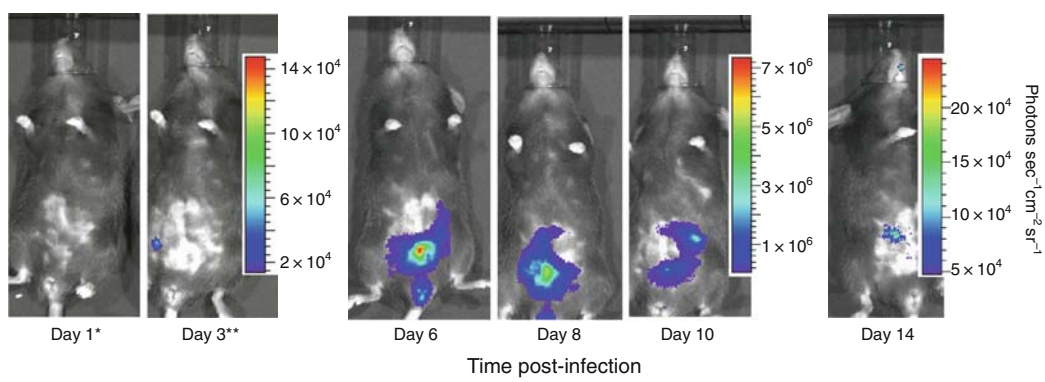

Fig. 12.1. In vivo colonisation and clearance dynamics of *Citrobacter rodentium* ICC180. BLI was performed at regular intervals over a 14-day period (representative images from the same animal are shown) using an IVIS50 system (Xenogen) after gaseous anaesthesia with isoflurane. Images are displayed as pseudocolour images of peak bioluminescence, with variations in colour representing light intensity at a given location. *Red* represents the most intense light emission, while *blue* corresponds to the weakest signal. The colour bar indicates relative signal intensity (as photons s^{-1} cm^{-2} sr^{-1} [where sr = steradian]). Mice were imaged with an integration time of 1 min at a binning of 4. If no luminescence was detected, a 5-min (indicated by **) or 10-min exposure (indicated by *) was used. The abdomen of each mouse was shaved with a beard-trimmer prior to imaging.

administered by oral gavage first become established within a specialized patch of lymphoid tissue in the caecum known as the caecal patch. A few days later, the bacteria migrate to colonize the colon. Mice were orally given 10^9 colony-forming units (cfu) of a constitutively bioluminescent *C. rodentium* derivative (strain ICC180 which expresses the *luxCDABE* operon from *P. luminescens*). In vivo BLI was performed at regular intervals over a 14-day period (representative images from the same animal are shown in **Fig. 12.1**) using an IVIS50 system after gaseous anaesthesia of mice with isoflurane. The sample shelf was set to position D (field of view 15 cm), and animals were imaged for 1–10 min at a binning of 4 (*see* **Note 21**) using the software program Living Image as an overlay on Igor. For anatomical localization, a pseudocolour image representing light intensity (blue, least intense to red, most intense) was generated using the Living Image software and superimposed over the grey-scale reference image. At various time points, animals were humanely culled and organs were harvested and imaged ex vivo using the IVIS50 system, with the sample shelf in position D and an imaging time of 1 min at a binning of 4. **Figure 12.2** shows the detection of light from an anaesthetised mouse (A), moments after death (B), after opening of the abdomen (with peritoneum still intact) (C), after introduction of air to gastrointestinal organs (D) and after harvesting and washing

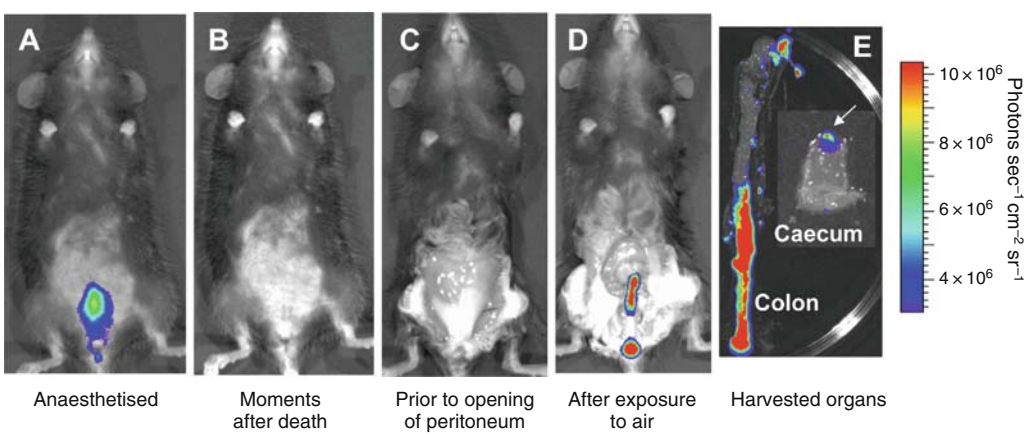

Fig. 12.2. Determining the organ/tissue localization of the signal during colonization/infection of mice with bioluminescent bacteria. At particular intervals post-infection, BLI was performed using an IVIS50 system (Xenogen) (the results for a representative animal 10 days post-infection is shown) while anaesthetised (**A**), moments after death (**B**), after opening of the abdomen (with peritoneum still intact) (**C**), after introduction of air to the gastrointestinal organs (**D**) and after harvesting and washing of colon and caecum (*arrow* indicates caecal patch) with PBS (**E**). Images are displayed as pseudocolour images of peak bioluminescence, with variations in colour representing light intensity at a given location. *Red* represents the most intense light emission, while *blue* corresponds to the weakest signal. The colour bar indicates relative signal intensity (as photons s^{-1} cm^{-2} sr^{-1}). Mice were imaged with an integration time of 1 min at a binning of 4.

with PBS (E). Harvested organs were then homogenized and samples assessed for bioluminescence by luminometry and viable counts by plating onto selective agar (**Fig. 12.3**).

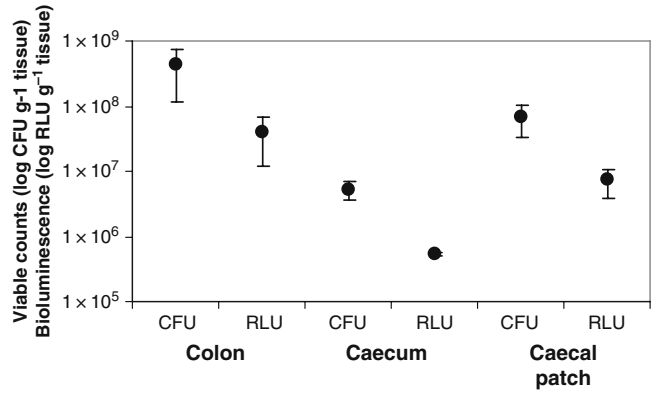

Fig. 12.3. Quantification of bioluminescence and viable counts in harvested organs. Organs were harvested from mice 10 days post-infection, homogenized in PBS and assessed for bioluminescence (by luminometry, expressed as relative light units [RLU]/g tissue) and viable counts (by plating onto selective agar, expressed as colony-forming units [CFU]/g tissue). Luminometry was performed over a 10-s period with a 1-s integration time using a Berthold Autolumat LB953. Error bars are standard deviations.

4. Notes

1. In vivo optical imaging machines comprise a charge-coupled device (CCD) camera mounted within a light-tight specimen chamber. The shelf of the imaging chamber is heated to enhance the well-being of the anaesthetised animals. Typically, a photographic reference image is acquired under weak illumination and then the bioluminescent signal is captured in complete darkness, which may take from seconds to minutes depending on the strength and location of the signal. The CCD camera spatially encodes the intensity of incident photons which are then displayed as a pseudocolour image superimposed on the grey-scale photographic image. Variations in colour within the pseudocolour image represent variations in light intensity at a given location, with red representing the most intense light emission and blue corresponding to the weakest.
2. Luminometers measure light emission using photomultiplier tubes, which convert photons into electrical pulses. Luminometers come in many different formats, from a simple manual one-tube luminometer to a computer-controlled micro-titre plate instrument. Some models also include an

injection system which allows the rapid and precise addition of reagents, and are highly recommended if using *luc*- and *Gluc*-encoded luciferases require the addition of exogenous substrate and typically cause a flash reaction (*see* **Note 5**). Detected photons are displayed as relative light units (RLU). However, this value is not the actual number of photons emitted but a correlate thereof. As a result, absolute values of light emission vary greatly from luminometer to luminometer for a given amount of luciferase protein. For this reason, samples from an experiment should be run on the same machine using the same protocol.

3. In our experience there is at least a 10-fold quenching of the bioluminescent signal from mice with black fur, such as C57Bl/6, in comparison with white mice, such as Balb/C. Depending on the region the bioluminescent signal comes from, it may be advisable to remove the fur from dark animals. We routinely do this using a cordless rechargeable beard trimmer, which is lighter than a pair of hair clippers and possesses a smaller blade. Alternatively, we used commercially available moisturising hair-removal cream applied according to the manufacturer's instructions.
4. Mice are anaesthetised for restraint purposes. The level of bioluminescence signal emitted by the bacteria will determine the period of time required for the animals to remain under anaesthesia, but will usually be in the range of 5–30 min in total, with imaging times of 1–10 min. It is preferable to anaesthetise mice using inhalational agents such as isoflurane. The advantage of maintaining animals on gas is the greater control of the level of anaesthesia. Inhaled agents are mainly eliminated by the lungs, whereas injectable agents need to be metabolised by the liver and excreted by the kidneys, a process which can be prolonged. Recovery is therefore more rapid from inhaled agents, which is important in regaining normal physiology, to control post-procedural hypothermia and fluid or electrolyte imbalance. Inhalational agents are also suitable for high-frequency anaesthesia studies, where animals are repeatedly imaged. Ketamine can cause muscle rigidity, so in certain situations the mice may appear to twitch. This is less than ideal, especially if the bioluminescent signal is located in the limbs. Where injectable agents are used, to ensure proper dosing each animal should be weighed and dosed according to its bodyweight.
5. The kinetics of the bioluminescence reaction catalysed by firefly luciferase can vary between a flash reaction and a glow reaction, depending on the buffer components. In a flash reaction, the bioluminescence signal rapidly decays to background levels, requiring measurements to be taken in the

region of seconds after substrate addition. In a glow reaction, the bioluminescence signal decays to background levels much more slowly, allowing measurements to be taken several minutes after substrate addition. A glow reaction is much more desirable if the luminometer available does not have an injection system, as the substrate can be added to all samples prior to measuring the luminescence. Alternatively, samples would have to be read immediately after the addition of substrate to each tube. Riska et al. (1999) described the use of 0.33 m*M* luciferin in 50 m*M* Sodium Citrate (pH 4.5) produced a flash reaction, while 0.2 m*M* luciferin in a buffer comprising 50 m*M* Sodium Citrate (pH 5.3), 8 m*M* dithiothreitol and 12 m*M* $MgSO_4$ produced a glow reaction when assaying luciferase expression in *Mycobacterium tuberculosis* (26). A number of luciferase assay systems are commercially available in which samples are resuspended in particular substrate/buffer combinations, such as Bright-Glo (E2610), which results in an approximate 30-min luciferase half-life, and Steady-Glo (E2510), which results in a 5-h luciferase half-life, from Promega Corporation.

6. Once mixed, coelenterazine solutions oxidize and gradually decay to their coelenteramide oxidation product. Dry coelenterazine compounds are only sparingly soluble in aqueous solutions, and must be dissolved in alcohols or propylene glycol prior to making aqueous buffer solutions. Alternatively, New England Biolabs Inc. (Ipswich, MA, USA) sell a *Gaussia* luciferase assay system (product number E3300) for use in vitro. Preparations of coelenterazine for use in vivo are also commercially available (Prolume Ltd., Pinetop, AZ, USA). Provided in sterile vials, the coelenterazine is made up in a 50/50 mixture of ethanol and propylene glycol, designed to rapidly solvate the coelenterazine, prolong its storage life and be far less inflammatory to small vessels. This preparation is recommended in the case of repeated in vivo studies. Furthermore, coelenterazine itself is chemiluminescent and so it is important to have appropriate controls in both in vivo (for example, an animal without the *Gluc*-expressing bacteria injected with coelenterazine) and in vitro experiments (for example, homogenized tissue with bacteria not expressing *Gluc*).
7. Ensure a sharp needle is always used. It is preferable to change needles between animals to avoid potential transfer of infection.
8. Anaesthetised animals must be monitored to ensure that they stay in the proper anaesthetic plane. The animals should not be too lightly anaesthetised that they regain consciousness, or

too deep that vital functions are compromised. Parameters that should be monitored include mucous membrane colour (should be pink not blue or grey) and respiratory rate and pattern (can be assessed by movement of the chest wall and observation of abdominal movements). The respiratory rate of a normal undisturbed mouse is approximately 180 breathes per minute. A slow rate drop of 50% is acceptable during anaesthesia. Breathing should be steady. If the animals' breathing becomes "jerky", too much anaesthetic is being applied and this will be fatal if maintained for long periods of time. If an animal appears too deeply anaesthetised, administer supplemental oxygen or alternatively administer reversal agents if available (*see* **Note 13**). For prolonged periods of anaesthesia (>30 min), an ophthalmic artificial tear ointment (e.g. Lacrilube, Allergan, Buckinghamshire, UK) should be applied to the eyes to prevent corneal drying and trauma.

9. Intraperitoneal administration involves injection through the abdominal wall and into the peritoneal cavity. It is important to note that intraperitoneal delivery is difficult to perform correctly as it is easy to misplace the dose into the intestine, gut, urinary bladder, muscle or other organs rather than into the peritoneal cavity. To avoid puncturing the abdominal viscera, introduce the needle rapidly at an angle of 30°, slightly left of the midline umbilicus, about halfway between the pubic symphysis and the xiphisternum (27). For mice, the technique may best be performed by holding the animals with the head tilted downwards. Note that with this technique withdrawal of the plunger will not usually be helpful as gut contents are too viscous to be drawn into the needle.
10. Ketamine/xylazine can also be administered by the intramuscular route. To ensure proper dosing when using injectable anaesthetics, each animal should be weighed and dosed according to its bodyweight.
11. Researchers have recently suggested direct injection of luciferin in situ when widespread distribution is not needed and utilised this delivery method for imaging of luciferase expression in muscle and the knee joint (28). Furthermore, intranasal administration is recommended if the signal is derived from the lungs (29). It is therefore recommended that the route of administration reflect the location of the bioluminescent signal. Alternative methods of luciferin delivery have been described in the literature and include the use of an osmotic pump for continuous delivery (30) or within food and water (31).

12. For easier intravenous injection into the tail vein, warm the animals up in a "hot box" at 37°C for 5 min maximum, to dilate the veins. It is important not to overheat the mice. It is also important not to inject the substrate too rapidly as this can result in a bolus going straight to the central nervous system or other organs and can be fatal. In occasional circumstances (<1 in 100), an animal may suffer heat exhaustion. In mild cases, where the animal immediately revives on removal from the heat source, it will be possible to continue. In severe cases, where the animal does not immediately recover, the animal should be humanely culled. The site of injection may bleed after intravenous dosing. Apply gentle but firm pressure with a swab until the bleeding stops. Wipe traces of blood away to prevent excessive licking or gnawing at the injection site.
13. If an inhalation agent is used, this will take seconds to minutes. However, if a ketamine/xylazine mix is used, then animals should be injected with an appropriate reversal agent such as atipamezole (Antisedan, 5 mg/mL, Pfizer, Kent, UK), which can be administered at 1 mg/kg intraperitoneally or subcutaneously to speed recovery. Atipamezole should not be administered until at least 30 min after administration of the ketamine/xylazine mixture. If it is administered any earlier, the ketamine component of the mix will not have worn off and the animal will experience violent muscle tremors during the recovery phase. Complete recovery may still take 3–4 h. It is very important that animals are kept warm during this period, preferably by placing within a hotbox (28–30°C). Failure to do so will almost certainly be fatal. Animals may be returned to their holding area once they are awake, able to move about the recovery cage and appear to be making normal behavioural adjustments. An animal should not be placed in a group cage unless it is capable of protecting itself from cage mates.
14. Signals originating in the gastrointestinal tract and peritoneal cavity are lost almost immediately after cervical dislocation, while those in the lungs and nose remain for a period (32).
15. In mice infected with the extracellular gastrointestinal pathogen *Citrobacter rodentium*, despite the presence of both intimately attached organisms and those being shed from the caecum and colon, the bioluminescent signal detected in vivo was found to be slightly less than for the harvested organs containing only attached bacteria (2.20×10^8 compared to 2.75×10^8 photons/s) (33). This is likely due to the distance photons must travel through tissue and signal impedance by the melanin within pigmented skin and fur.

16. Tissue homogenates can be made using a "stomacher" machine such as the Stomacher 80 Biomaster from Seward (Worthing, UK) (120 s on medium power setting). Alternatively, tissue/organs can be pummelled using the lid of a 50-mL plastic tube or pushed through a sterile sieve.
17. Multiple samples are required to allow calculation of mean values and standard deviations. Within any set of three samples, readings should not be significantly different.
18. Light emission measurements can be quenched by high cell/tissue densities as well as dark/red tissue and organs (such as the liver and spleen). Measure the luminescence of several serial dilutions to determine if the values are linear. If the relationship between luminescence and dilution is not linear, the light is being quenched at low dilutions. In this case, use the readings taken from samples at higher dilutions where the effect of quenching is minimised.
19. Coelenterazine is itself chemiluminescent and can exhibit high background luminescence values depending on the buffer used. As a result, it is very important to determine the most appropriate buffer for performing in vitro assays. In our experience, different bacterial growth media result in very different effects on coelenterazine chemiluminescence, and even small traces carried over into the diluent can have an effect (19). An important control is samples containing the wild-type non-bioluminescent parent strain to determine the absolute levels of background chemiluminescence.
20. Very bright samples may result in spurious light readings from neighbouring wells/tubes in luminometers which process multiple samples. Separate samples using blank tubes/wells and consider potential cross-talk before assuming low values are significant if they came from wells neighbouring strongly bioluminescent ones.
21. When using in vivo optical imaging equipment, one way of increasing the sensitivity of photon detection is to increase the exposure time of the camera to the sample. In addition, some machines have a second method for increasing sensitivity known as "binning". With this technique, pixels are summed together to form super-pixels, that is, for binning a, $a \times a$ pixels are combined. The higher the value of a, the more sensitive the detection level. However, the trade-off of binning is the loss of spatial resolution, so conversely the higher the value of a, the lower the resolution.

Acknowledgments

The authors would like to thank the Wellcome Trust for supporting this work.

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