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Chapter 19

In Vivo Tracking of Bacterial Colonization in Different Murine Models Using Bioluminescence: The Example of *Salmonella*

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Abstract

Applications of bioluminescence for the in vivo study of pathogenic microorganisms are numerous, ranging from the quantification of virulence gene expression to measuring the effect of antimicrobial molecules on the colonization of tissues and organs by the pathogen. Most studies are performed in mice, but recent works demonstrate that this technique is applicable to larger animals like fish, guinea pigs, ferrets, and chickens. Here, we describe the construction and the utilization of a constitutively luminescent strain of *Salmonella* Typhimurium to monitor in vivo and ex vivo the colonization of mice in the gastroenteritis, typhoid fever, and asymptomatic carriage models of *Salmonella* infection.

Key words *Salmonella* Typhimurium, Murine models, Bioluminescence, In vivo imaging, Infection kinetics

1 Introduction

Bacterial infections are an important economic and public burden. Given the great diversity of potential hosts and their respective pathogens, the complexity of their interactions and the diversity of induced pathologies, numerous models were designed during the last decades to investigate these processes in vivo, sometimes focusing on one particular phenomenon. These experiments often aim to study how the pathogen colonizes and survives within the host. Usually, it involves the sacrifice of numerous animals and it is clear that bacterial infections might differ from one individual to another, even in the toughest experimental conditions.

Salmonella for example, is an enteric pathogen responsible for typhoid fever and gastroenteritis in a broad range of species,

depending on the infecting serovar. Sometimes an asymptomatic carrier state can also be observed. Three murine models are commonly used to study these phenomena in vivo [1, 2]. The first model involves Slc11a1$^{+/+}$ (Nramp1) mice (such as CBA/J or 129sv mice) which present an asymptomatic carrier state when infected with the pathogen. After oral inoculation, no inflammation is induced in the intestine, and postintestinal colonization is rigorously controlled by the host's immune system, thus limiting deep organs damaging. *Salmonella*-induced typhoid fever can be studied through oral infection of Slc11a1-deficient mice (e.g., BALB/c, C57BL/6) with *S.* Typhimurium. As the pathogen crosses the intestinal barrier, its replication is not repressed, leading to a systemic colonization of deeper organs. This model is used to study human infections with typhoidal serovars like *S.* Typhi or *S.* Paratyphi which are human restricted and are consequently not able to infect mice. The use of *S.* Typhimurium in mice simulates well typhoidal infections in humans. Finally, the third model is based on antibiotic pretreatment of mice which results in microbiota alteration, leading to a bloom of *S.* Typhimurium intestinal colonization, and the induction of inflammation responsible for the gastroenteritis phenomenon [3]. This model is thus more relevant to study pathogenesis following human or animal infection with non-typhoidal serovars (e.g., *S.* Typhimurium and *S.* Enteritidis).

As an alternative to theses burdensome protocols, in vivo bioluminescence and fluorescence imaging is increasingly being used to perform real-time monitoring of disease process, especially infectious diseases [4], and to analyze the effect of therapeutic molecules or vaccines against pathogens [5–7]. Luminescence monitoring is allowed by devices such as the IVIS Spectrum (PerkinElmer) or the PhotonIMAGER (Biospace Lab), engineered to detect low levels of light emitted from luciferase or fluorescent reporters on small living animals. One of the most used bioluminescence reporters is the *luxCDABE* operon of *Photorhabdus luminescens* [8]. Basically, the LuxAB (Luciferase) complex enzyme catalyzes the oxidation of reduced flavin mononucleotide (FMNH$_2$) and a long-chain fatty aldehyde to oxidized flavin and a long-chain fatty acid. On the other hand, the LuxCDE (fatty acid reductase) complex produces long chain fatty aldehyde by reducing the fatty acid previously produced. The excess energy for the reaction catalyzed by the LuxAB complex will result in the emission of blue/green light (490 nm).

Using the constitutive synthetic promoter described by Shivak et al. [9], termed sig70c35 and designed on the basis of the consensus binding sequence of the transcription factor σ70, we performed week-long monitoring of *Salmonella* colonization in the three main murine models of *Salmonella* infection described above. A strain harbouring the same construct, but depleted from any promoter, was also used in these experiments as a negative control.

It allowed us to quantify the basal signals emitted by mice and their organs (spleen, liver, mesenteric lymph nodes [MLN], gut), and to determine a threshold for bioluminescence detection. This work provides relevant information about tracking of pathogenic microorganisms in animal models and can be adapted to other fields of research, for example as a tool to assess the impact of potential antimicrobial molecules on pathogen colonization [10, 11] or gene expression measurement [12, 13]. The most popular animal model for these imaging studies remains the mouse, but some recent works have used larger animals. For example, ferrets and guinea pigs have been used for bioluminescent imaging of influenza virus infection or colonization by a luciferase-expressing *Brucella suis* vaccine strain, respectively [14, 15]. Furthermore, infection of rainbow trout by the fish pathogen *Vibrio anguillarum* was directly analyzed by bioluminescent imaging and a luminescent avian *E. coli* strain was imaged in chicken to follow its colonization of the gastrointestinal tract [16, 17].

2 Materials

2.1 Tn7 Chromosomal Integration of Reporter Fusions

1. Bacterial strain with a single *att*Tn7 insertion site, *S.* Typhimurium 14028 in our experiments (*see* **Note 1**).
2. TSB (Tryptic soy broth) medium.
3. TSA (Tryptic soy agar) medium.
4. Antibiotics: chloramphenicol (Cm) or kanamycin (Kan), and ampicillin (Amp) or carbenicillin (Cb).
5. pCS26-sig70c35-*luxCDABE* (Cm^R or Kan^R) [9].
6. pUC18R6K-miniTn7T-PacI (Amp^R) [9].
7. pHSG415-*tnsABCD* (Amp^R) (*see* **Note 2**) [9].
8. *Escherichia coli* CC118 λ*pir* (or any *E. coli* λ*pir* strain) [18] and MC1061 [19].
9. Restriction enzymes and their appropriate buffer: PacI, XhoI, and BamHI.
10. DNA Blunting kit (e.g., DNA Blunting kit, Takara).
11. Clean-Up PCR kit (e.g., Macherey Nagel™ Nucleospin ™ Gel and PCR Clean-Up kit).
12. Ligation kit (e.g., DNA Ligation kit v2.1, Takara).
13. Genomic DNA extraction kit (e.g., Macherey Nagel™ NucleoSpin® Tissue kit).
14. Primer glmS-For: 5′- AACCATCAGCGCGGAA CAACTGGTC-3′ (*see* **Note 3**).
15. Primer glmS-Rev: 5′- ATGCCGCATGTGGAAGAGGT CATTG-3′ (*see* **Note 3**).

2.2 Inoculum Preparation	1. 1 L Erlenmeyer.
	2. 1.8 mL cryogenic vials.
	3. 50% glycerol solution in saline water (0.85% NaCl).
2.3 Mouse Models	1. 6–8-week-old BALB/c (Slc11a1$^{-/-}$) or CBA/J (Slc11a1$^{+/+}$) female mice.
	2. 200 mg/mL streptomycin solution.
	3. Mouse clipper and depilatory cream.
	4. Appropriate syringe and gavage needles [a dosing curved cannula 25 mm × 20G (Harvard Apparatus) and a 1 mL syringe in our case].
2.4 In Vivo Experiment and Bacterial Numeration	1. Imaging instrument suitable for in vivo bioluminescence imaging equipped with a cooled −90 °C camera with large CCD area and low F-stop (e.g., IVIS Spectrum In Vivo Imaging System [PerkinElmer] or any equivalent imaging instrument).
	2. Anesthesia device coupled to the imaging instrument (e.g., XGI-8 Anesthesia system, PerkinElmer).
	3. Nose cones depending on the animal model and the in vivo imaging device. In our case, we used XNC-2 Mouse Nose Cones branded by PerkinElmer.
	4. Low luminescence matt black sheet.
	5. Dissection kit.
	6. Weighted blender bags (1/organ of interest/mouse).
	7. Petri dishes containing selective medium [Salmonella-Shigella (SS) agar supplemented with antibiotic, 30 μg/mL chloramphenicol in our case]. We recommend 150 mm diameter petri dishes when using an automatic plater.
	8. Bagmixer (e.g., MiniMix CC, Interscience).
	9. Automatic plater (e.g., easySpiral Pro, Interscience) or dilution tubes with 1.8 mL saline water.
	10. Automatic colonies counter (e.g., Scan 4000, Interscience).

3 Methods

3.1 Construction of a No Promoter (NoP) Reporter Transcriptional Fusion (See Note 4)	1. Bacterial cultures are always performed at 37 °C in TSB or TSA medium containing appropriate antibiotics unless otherwise stated (strains grown at 30 °C).
	2. Digest pCS26-sig70c35-*luxCDABE* (Cm^R or Kan^R) by XhoI and BamHI and purify the resulting linear DNA product.

3. Blunt the XhoI-BamHI restricted pCS26-sig70c35-*lux-CDABE* vector (using a DNA Blunting kit) and perform ligation on the resulting DNA molecule.

4. Transform the ligated product into competent *E. coli* MC1061 and plate the bacteria on TSA supplemented with the appropriate antibiotic (30 μg/mL Cm or 50 μg/mL Kan).

5. Successful clones can be easily selected using IVIS Spectrum (or any other imaging instrument suitable for bioluminescence imaging) by checking the loss of bioluminescence. Constructions are then checked by PCR and DNA sequencing.

3.2 Chromosomal Integration of Reporter Fusions (See Note 5)

1. Electroporate pHSG415-*tnsABCD* in competent bacteria of interest harbouring an *att*Tn7 site (*S.* Typhimurium ATCC 14028 in our case) and select on TSA containing 100 μg/mL Amp at 30 °C. Verify the introduction of the plasmid into the bacteria and freeze the strain in 25% glycerol at −80 °C.

2. Digest pCS26-sig70c35-*luxCDABE* (or the plasmid built in Subheading 3.1) and pUC18R6K-miniTn7T-PacI by PacI.

3. Purify the resulting linear DNA products (pCS26-sig70c35-*luxCDABE:* 8086 bp; pUC18R6K-miniTn7T-PacI: 3216 bp).

4. Ligate both purified DNA fragments using the DNA ligation kit, transform the ligated product into competent *E. coli* CC118 λ*pir* cells and select on TSA containing 100 μg/mL Amp supplemented with the appropriate antibiotic (30 μg/mL Cm or 50 μg/mL Kan). Verify the plasmid by restriction digestion and freeze the strain in 25% glycerol at −80 °C.

5. Cultivate the Amp^R/Cm^R or Amp^R/Kan^R clones and isolate the resulting plasmid (*see* **Note 6**). Electroporate it in pHSG415-*tnsABCD*-carrying bacteria cultivated at 30 °C with antibiotics. After resuspension, let the bacteria multiply for 2 h at 30 °C with agitation (190 rpm), before plating on TSA supplemented with the appropriate antibiotic (30 μg/mL Cm or 50 μg/mL Kan) at 37 °C to eliminate pHSG415-*tnsABCD*.

6. Removal of pHSG415-*tnsABCD* plasmid is checked by plating the clones on TSA containing 30 μg/mL Cm (or 50 μg/mL Kan) and TSA containing 100 μg/mL Amp. Only clones sensitive to 100 μg/mL Amp are kept.

7. Cultivate Amp^S/Cm^R or Amp^S/Kan^R clones in standard conditions and extract total DNA.

8. Confirm the insertion of the reporting fusion by PCR using primers glmS-For and glmS-Rev (Fig. 1).
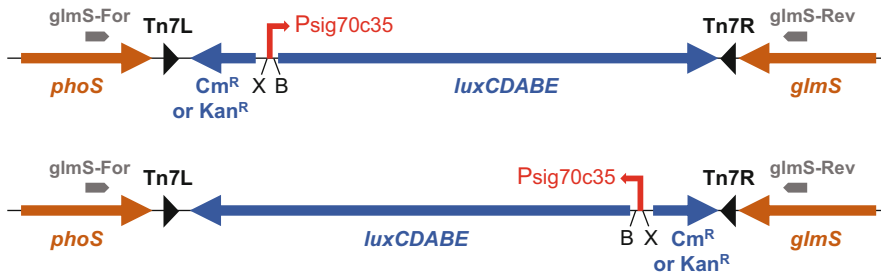
Fig. 1 Schematic representation of the insertion of the transcriptional fusion expressing constitutively the *lux* operon into the chromosome of *S.* Typhimurium 14028. Orange arrows represent genes carried by the *Salmonella* chromosome while the *lux* operon as well as the antibiotic resistance gene are indicated by blue arrows. The constitutive sig70c35 promoter is symbolized by the red broken arrow. The left and rights ends of Tn7 are indicated with black triangles. Restriction sites (X, XhoI; B, BamHI) are shown in black letters on both sides of the constitutive promoter. Deletion of the DNA sequence located between these two restriction sites in the pCS26-sig70c35-*luxCDABE* plasmid yielded the no promoter transcriptional fusion (NoP). Primers used to verify the chromosomal insertion of the transcriptional fusion are represented by grey arrows above *phoS* and *glmS* genes. The two possible insert orientations in the chromosome after Tn7 transposition are shown

9. After verification by DNA sequencing, freeze selected clones in 25% glycerol at −80 °C: two with the no promoter fusion (NoP) and two with the constitutive promoter fusion (Lux+).

3.3 Preparation of Inocula (See Note 7)

1. Grow the previously constructed bacteria in 10 mL of TSB supplemented with the appropriate antibiotic overnight at 37 °C with agitation (190 rpm). The next day, sow 400 mL of TSB supplemented with the appropriate antibiotic with 4 mL of preculture and incubate for 24 h at 37 °C with agitation (190 rpm).

2. Centrifuge (4800 × g; 20 min), and discard the supernatant. Resuspend the bacteria in 20 mL of saline water containing 50% glycerol. The volume of resuspension depends on the desired final concentration of the inoculum. In our case, this protocol generates inocula at a concentration of approximately 1×10^{11} CFU/mL.

3. Distribute 1.2 mL of the obtained solution within 1.8 mL cryogenics vials and store them at −80 °C.

4. The following day, thaw one inoculum and numerate it to help adjusting the bacterial concentration before inoculation. Numeration can be performed on the automatic plater (easy-Spiral Pro) or by serial dilutions in saline water.

3.4 Preparation of Animals

Animal experiments have to be performed with the approval of an Ethical Committee for Animal Research, in compliance with ethical guidelines respecting the "Replacement, Reduction and Refinement" rule as much as possible and they must be carried out in a certified animal facility.

1. It is strongly recommended prior to the experiment, especially in case of gastrointestinal imaging study, to perform quantification of bioluminescence on isolated food chip to check if it is signal-free.

2. A few days before inoculation, shave the fur in areas where signals will be looked for (e.g., ventral side and left side for the digestive tract and spleen for *Salmonella*) (*see* **Note 8**) and apply a depilatory cream (choose a cream without autoluminescent signal) on the mowed areas to remove the remaining hair. Leave to act for 3 min then rinse with lukewarm water (*see* **Note 9**). Dry the animals as much as possible by placing them in a cage under a heating lamp. Then place back the animals in their cages and increase the temperature of the room, where the animals are housed, at 24 °C.

3.5 Mice Infection, Anesthesia, and Imaging

1. For the gastroenteritis-induced *Salmonella* model, animals must be fasted 4 h prior to streptomycin inoculation. Orally inoculate the mice with streptomycin (20 mg/animal in 0.1 mL of saline water) 24 h prior to *Salmonella* inoculation.

2. Prior to *Salmonella* inoculation, animals must also be fasted for food and water (2 h prior inoculation for the typhoid fever and the asymptomatic carriage models; 4 h for the gastroenteritis model).

3. Before inoculation, unfreeze one (or more depending on the number of mice) inoculum and adjust the bacterial concentration to the desired concentration (2.5×10^9 CFU/mL in our case) in saline water.

4. Using a dosing curved cannula 25 mm $\times$ 20G (Harvard Apparatus) and a 1 mL syringe, orally inoculate 5×10^8 CFU of the reporter strains suspended in 0.2 mL of saline water per mice (*see* **Note 7**). Replace animals in boxes, provide water and food ad libitum.

5. At each time point of interest, anesthetize first one negative control mouse (NoP) and second 4 tested mice (Lux+) in the induction chamber following institutional guidelines (e.g., 3% Vetflurane mixed 1.5 liter per minute O_2). Then transfer them on the black flat sheet placed on the 37 °C thermostated platform inside the imager. Put the nose of each mouse in one nose cone that delivers Vetflurane (3%) mixed oxygen (1.5 L/min) gas (*see* **Note 10**). The induction chamber is sanitized with 70% alcohol after the anesthesia of each group of mice.

6. Acquire a photographic image and the bioluminescence signal (Living Image 4.5.5 software from PerkinElmer allows data acquisition) first automatically and then manually if required (Fig. 2a). Exposure time depends on the intensity of the
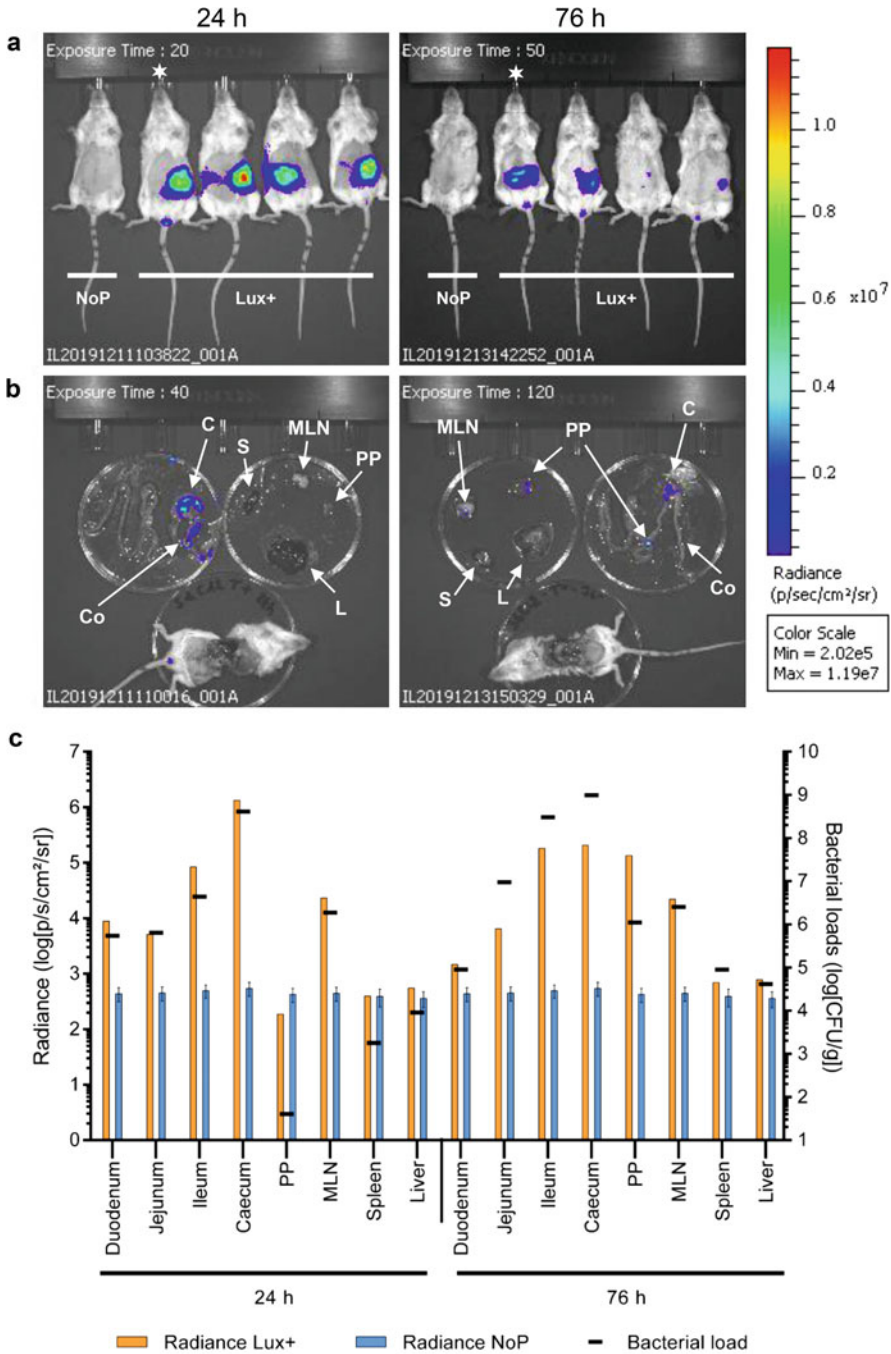
Fig. 2 In vivo imaging of infection with a *Salmonella* strain expressing constitutively the *lux* operon in a murine gastroenteritis model. BALB/c mice pretreated with streptomycin were orally infected with *S*. Typhimurium 14028 carrying the mock fusion (no promoter: NoP) or the sig70c35-*luxCDABE* transcriptional fusion (Lux+). (**a**) One control mouse (NoP) and four Lux+ mice were imaged at 24 h and 76 h postinfection with the IVIS Spectrum. The exposure time expressed in seconds is indicated in the upper left corner of each image. The color scale shows relative signal intensity or radiance (expressed in p/sec/cm²/sr), red being the most intense and blue the least intense. The minimal and maximal values measured are indicated in the box below the color scale. (**b**) Isolated organs from one Lux+ mouse (indicated by a white asterisk in the upper part of the figure)

luminescent signal, ranging from 0.75 to 600 s. Exposing for a too long time may generate saturated signals, but exposing for too short time may not allow the detection of weak signals. Reiterate on all relevant sides of the mouse body. After imaging, it is important to ensure that the animals wake up well (*see* **Note 11**).

7. Euthanize an animal for each required time point (*see* **Note 12**), remove aseptically each organ of interest (e.g., gut, spleen, liver) and place them in a nonreflective sterile petri dish inside the imaging device. Acquire the bioluminescence signal first automatically and then manually if required (Fig. 2b). This step will allow to identify the positive organ(s) and to quantify the signal in this organ.

8. Collect imaged organs in preweighted blender bag with filter and weigh the bag to estimate organ's weight. Small organs such as the ileal Peyer's patches can be collected in preweighted tubes, and ground with sterile glass stirring rod.

9. Depending on the size of the organ, add aseptically 1 mL (e.g., Peyer's patches), 3 mL (e.g., MLN, spleen) or 5 mL (e.g., liver, gut) of TSB and homogenize using a Bagmixer (in our case, with the MiniMix CC set at speed 4 for 2 min).

10. Plate 50 μL of the homogenized solution on one 150 mm petri dish containing SS medium with 30 μg/mL Cm or with 50 μg/mL Kan using an automatic plater (e.g., easySpiral Pro, Interscience) set on exponential mode. Depending on the colonization level of the organ and on the dilution apparatus used, it could be necessary to dilute the sample (10^{-2} or 10^{-4}) and to plate it on a second petri dish using the automatic plater on exponential mode (*see* **Note 13**).

11. Incubate overnight at 37 °C and count the colonies using a colony counter after adjusting dilution parameters (*see* **Note 14**).

3.6 Bioluminescence Quantification

Living Image 4.5.5 software (PerkinElmer) allows data analysis. After acquisition, each image is displayed and is the result of the coregistration of a photographic image and a luminescent image.

Fig. 2 (continued) were imaged at both time points. Organs on the images are indicated with white arrows. *C* cecum, *Co* colon, *L* liver, *MLN* mesenteric lymph nodes, *PP* Peyer's patches, *S* spleen. (**c**) Quantification of luminescence (calculated from the ROI of each organ) and bacterial loads on the different isolated organs. Orange and blue bars represent the radiance of the different organs from Lux+ and NoP mice, respectively. For NoP mice, average values (±standard error of the mean) of radiance were calculated based on all euthanized control mice. For both time points, note that some organs which appeared negative on the images in (**b**) were really positive when the radiance was quantified (for example, MLN at 24 h postinfection). Black dashes correspond to bacterial loads of the organs from the Lux+ mouse

1. Visualize each image of interest in pseudocolors that represent intensity, for example violet for the smallest number on the color scale, red for the largest number, and all of the spectral colors of the rainbow for the values in between (Fig. 2a, b). Pixels reporting less intense signals might not be displayed at the selected scale.

2. Define one or more regions of interest (ROI) in the optical image, for example measurement on abdominal area from the whole-body animal (Fig. 3a) or subject ROI on isolated organs such as spleen, liver, mesenteric lymph nodes, Peyer's patches and gut defined regions (Fig. 3b). The Living Image 4.5.5 software (PerkinElmer) records information about the ROIs. Display the ROI measurements table and export as an Excel format.

3. Analyze data. Quantification could be expressed in counts/second, or using the calibrated measurement units of the photon emission from the subject in photons/second/cm^2/steradian, that is, radiance. The radiance unit is the number of photons per second that leave a square centimeter of tissue into a solid angle of one steradian (Fig. 2c). Working with image data in radiance mode allows the quantitative comparison of images or ROI data regardless of the acquisition settings and the imaging device used.

4   Notes

1. Tn7 transposition was shown to be very effective in several enterobacterial species including *E. coli*, numerous *S. enterica* serovars, *Klebsiella pneumoniae*, *Cronobacter sakazakii*, and *Citrobacter rodentium* [20] and in *Pseudomonas aeruginosa* [21]. Since almost all bacterial genomes contain a single *att*Tn7 site, this technique should be applicable to many other bacterial species [22].

2. pHSG415-*tnsABCD* is a thermosensitive plasmid. Bacterial strain carrying such a plasmid should not be cultivated over 30 °C except for the curing step.

3. Primers glmS-For and glmS-Rev were designed for *S.* Typhimurium ATCC 14028 genome (GenBank accession number CP001363). They hybridize with the 3′ end of the genes *glmS* and *phoS* respectively (Fig. 1). Other primers hybridizing on both sides of the insertion site should be designed if chromosomal integration is performed on any other bacterial genomes.

4. Construction of a mock reporting transcriptional fusion (no promoter fusion) will allow determining the background
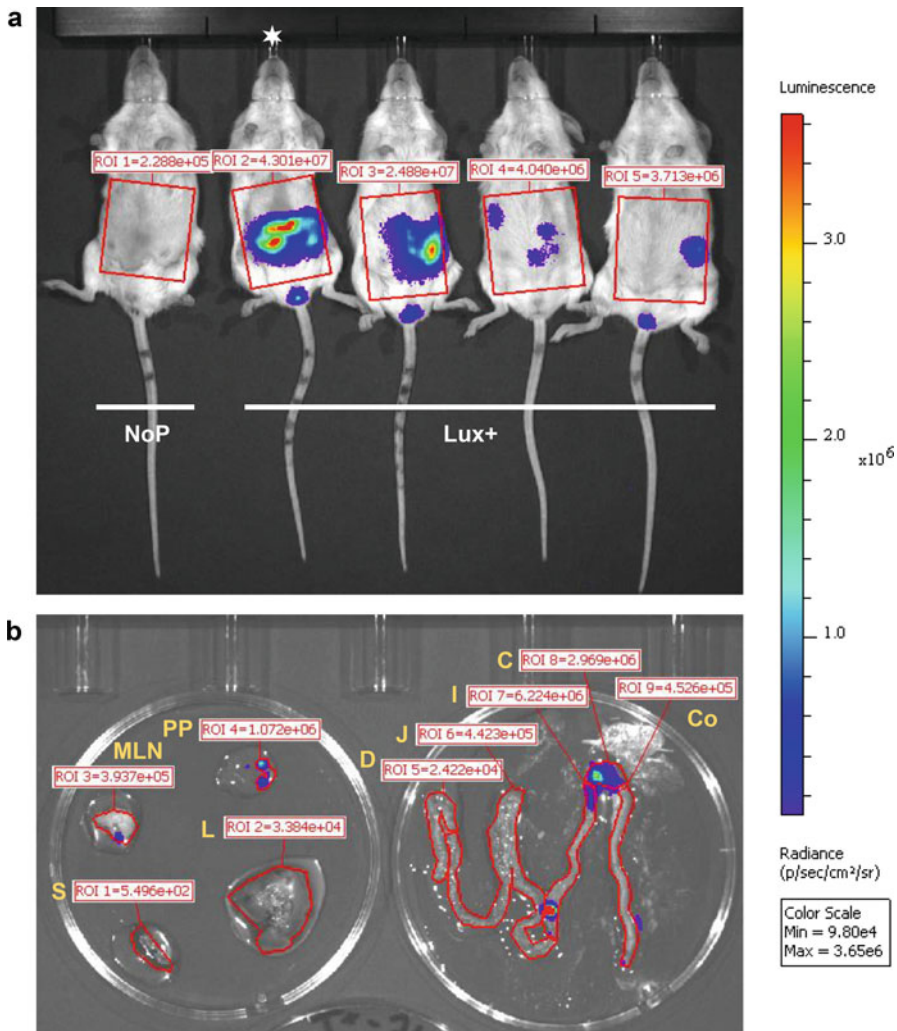
Fig. 3 Examples of ROI (regions of interest) measurements on mice orally infected with *S.* Typhimurium 14028 in the murine gastroenteritis model. (**a**) One control mouse (NoP) and four Lux+ mice were imaged at 76 h postinfection with the IVIS Spectrum. The color scale shows relative signal intensity or radiance (expressed in p/sec/cm^2/sr), red being the most intense and blue the least intense. The ROIs were defined manually and are expressed in p/sec/cm^2/sr. The ROI measurements of the 4 Lux+ mice were 16 to 188 times higher than that of the NoP mouse which corresponds to the background. (**b**) Isolated organs from the Lux+ mouse indicated by a white asterisk in the upper part of the figure were imaged and the ROIs were carefully defined in order to take into account the entire organs. In accordance with the bacterial loads of the different organs shown in Fig. 2c at 76 h postinfection, the cecum and the ileum exhibit the highest ROI measurements. *C* cecum, *Co* colon, *D* duodenum, *I* ileum, *J* jejunum, *L* liver, *MLN* mesenteric lymph nodes, *PP* Peyer's patches, *S* spleen

bioluminescence emitted by both living animals and isolated organs.

5. In vivo studies can also be performed using luminescent transcriptional fusions carried by plasmids. The advantage is an increase of the sensitivity due to the higher copy number

compared to the chromosomal fusion. A potential drawback is a possible instability and therefore loss of the plasmid for experiments spanning several days. This is the case for pCS26-derived constructions which were starting to be lost in our murine models as early as 48 h postinoculation.

6. Insertion of pCS26-*luxCDABE* restriction products can result in two possible orientations within pUC18R6K-miniTn7T-PacI vector and therefore in the bacterial strains after Tn7 transposition (Fig. 1). In order to homogenize multiple reporting strains, it is strongly recommended to check the orientation of the insert before electroporation. However, it should be noted that, in agreement with previous results [9], both orientations exhibited only a weak difference of *lux* expression in liquid culture.

7. Depending on the *Salmonella* serovar and strain used, it is recommended to adapt the inoculum according to the virulence properties of the strain. For other pathogens of interest, please refer to literature in order to prepare the inoculum in the recommended conditions.

8. If an animal is accidently hurt during this step, it is not recommended to apply depilatory cream as it could irritate the animal.

9. For the comfort of the animals during the experiment, it is recommended to watch over them after application of the cream to avoid spread of the depilatory cream on their face (especially the eyes and the mouth).

10. It is not recommended to anesthetize an animal more than three times a day, especially during the later stage of infection due to a certain fragility of infected mice at these times. Moreover, in order not to leave the mice anesthetized for a too long period, the negative control mouse (NoP) should be changed every 20–30 min.

11. According to the kinetics of infection and the severity of the disease, animals can be imaged one or more times a day (up to three times). The first week, for all our *Salmonella* models, mice were imaged three times the first day and two times the other days. During the second week of the asymptomatic carriage experiment, mice were imaged once a day.

12. Cervical elongation is the simplest method and does not requires any special equipment. It can be performed on anesthetized animals. Other methods of euthanasia can be applied depending on the sensitivity of the experimenter toward this method (CO_2, etc.). Be careful to use a euthanizing method allowed by your institutional ethic committee regulations.

13. Alternatively, each sample can be serially diluted and plated on petri dishes. The use of an automatically diluter allows to save time and petri dishes as maximum two dishes are required for an organ.

14. When studying *Salmonella* and using SS plates, it is not recommended to note the petri dish with any other color than green as it could be interpreted by the apparatus as a colony.

Acknowledgments

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