

## Noninvasive In Vivo Imaging to Follow Bacteria Engaged in Cancer Therapy

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### Abstract

Non-invasive in vivo imaging represents a powerful tool to monitor cellular and molecular processes in the living animal. In the special case of bacteria-mediated cancer therapy using bioluminescent bacteria, it opens up the possibility to follow the course of the microorganisms into the tumor via the circulation. The mechanism by which bacteria elicit their anti-tumor potential is not completely understood. However, this knowledge is crucial to improve bacteria as an anti-cancer tool that can be introduced into the clinic. For the study of these aspects, in vivo imaging can be considered a key technology.

**Key words** In vivo imaging, Luminescent bacteria, Lux operon, Firefly luciferase, Chromosomal integration

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### 1 Introduction

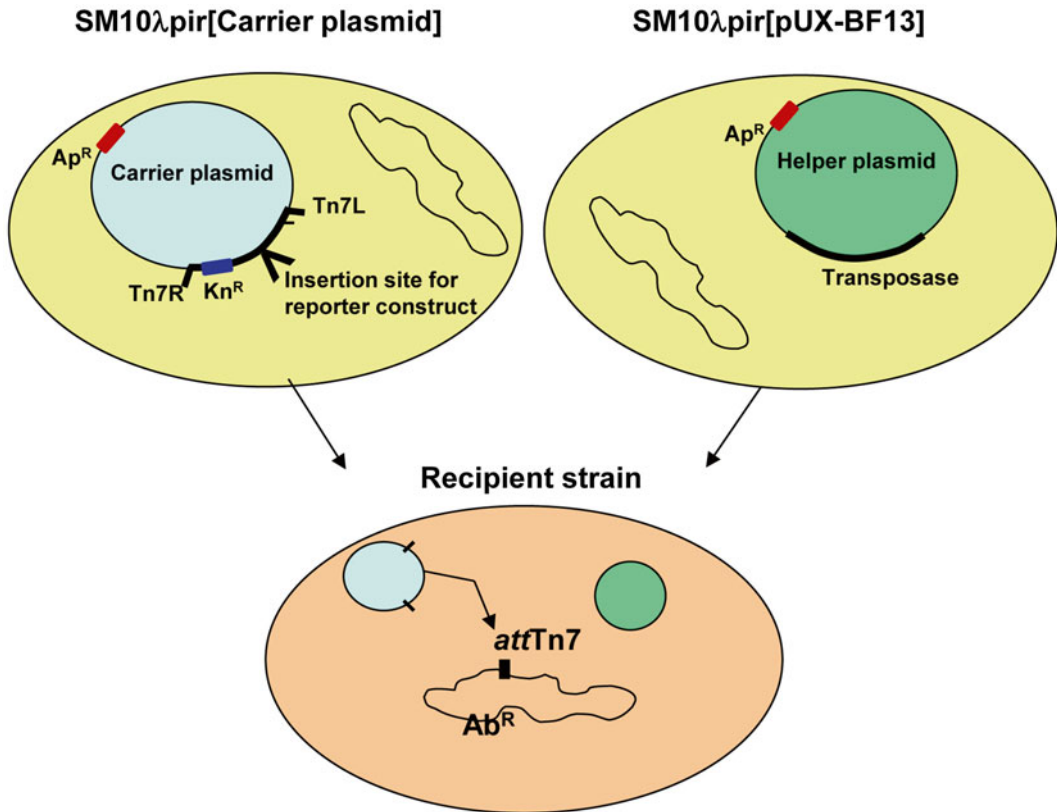
In vivo imaging with bioluminescent bacteria can be used to monitor infections in tumor-bearing mice [1]. This method has convincing advantages over conventional means to track bacterial locations in mice, such as plating of organ homogenates to determine colony-forming units (CFU). Concerning reliability and validity, in vivo imaging is outstanding as it allows following the infection process in individual mice at consecutive time points. Sacrificing the animals for analysis is obviously not required. As a consequence, fewer mice are needed for an experiment. First, this is generally desirable in order to reduce the number of experimental animals for animal welfare. Second, this renders studies more cost- and labor-efficient.

As a prerequisite to study bacterial infections in an in vivo imaging system, a reporter strain has to be available. Depending on the demand of the experiment, a reporter strain can be simply purchased or acquired from colleagues, if possible. However, for specific needs such as in bacteria-mediated cancer therapy variants are required that are not commonly used. Thus, the strain might have to be newly constructed. Various expression plasmids can be used

for this purpose. However, high-copy-number plasmids, e.g., with a Puc origin of replication (ori), are not advisable. Although these vectors should give extremely bright signals, such plasmids have the tendency to be lost quickly from the transformants in vivo when antibiotic selection for plasmid maintenance is no longer possible. This is most likely due to the extreme metabolic burden such expression plasmids present for the bacteria. Low-copy-number plasmids such as pSC101 would result in more stable transformants. However, eventually, such reporter-encoding expression plasmids might also be lost. Since this is a random event, it is not predictable whether the loss occurs early during colonization or late. This renders a serious quantitative experiment difficult. A way around this problem would be to introduce balanced suicide plasmids where the plasmid complements a chromosomal deletion of a gene encoding an essential metabolic enzyme. However, to us the stable integration of the expression cassette into the bacterial chromosome appears to be the better alternative. For chromosomal integration, we targeted the transposon 7 (Tn7) attachment site, which can be found in *E. coli* and *S. typhimurium* as well as in other Gram-negative bacteria. This targeted integration ensures that we do not disrupt any essential gene. How this is achieved is the scope of the present article.

The principle of the Tn7 integration of constructs into the bacterial chromosome is as follows: Including the recipient bacterial strain, three strains are needed for this method (Fig. 1). The carrier strain contains a plasmid with the desired construct located between the two Tn7 recognition sites Tn7L and Tn7R. This plasmid has a *pir*-dependent ori and therefore has to be transformed into a strain supplying the *pir* product of the lysogenic bacteriophage  $\lambda$ , such as SM10 $\lambda$ *pir*. The same holds true for the helper strain which provides the transposase required to catalyze the integration step on a *pir*-dependent plasmid. Both plasmids contain mobilization elements in addition. Once all three strains are mixed and placed on a membrane filter, the helper and carrier strain will conjugate with the recipient and transfer their plasmids. As these plasmids are both *pir* dependent, they cannot be propagated in the recipient strain. However, the construct might integrate into the bacterial chromosome via homologous recombination of the Tn7 recognition sites catalyzed by the transposase. Successful integration events can be detected by antibiotic selection. Positive clones should now be resistant to kanamycin but no longer to ampicillin, since this marker is only carried by plasmids and will be lost.

Various bioluminescent reporter genes exist that can be used for noninvasive in vivo imaging. Fluorescent proteins (photoluminescence) as well as chemiluminescent enzymes such as bacterial lux or insect luciferases are available. In this chapter, the stable use of a lux operon and the firefly luciferase are introduced.



**Fig. 1** Chromosomal integration using the mini-Tn7 transposon. Successful integration of a specific construct requires three strains: SM10λpir containing the carrier plasmid with the construct flanked by the Tn7 recognition sites (Tn7L and Tn7R); a helper strain in which the plasmid supplies the transposase necessary to integrate into the chromosome of the third, namely, the recipient strain. Upon mixing all three strains, conjugation of carrier and helper strains takes place toward the recipient bacterium. Both plasmids possess mobilization elements which allow them to enter the recipient strain. Once inside the bacterial cell, the transposase catalyzes the integration of the transposon into the recipient's *attTn7* (chromosomal Tn7 attachment site)

Various bacteria produce light using the so-called *lux* operon (*luxCDABE*) such as *Photobacterium luminescens*, a pathogen of nematodes. In this system, *LuxCDE* encodes a fatty-acid reductase complex that is involved in synthesis of fatty aldehydes for the luminescence reaction. This reaction is catalyzed by the luciferase LuxA and LuxB subunits [2]. Therefore, all components that are required for the enzymatic reaction to produce light are already present in bacteria expressing the *lux* operon. This renders the addition of a substrate (such as luciferin in the case of the luciferase system) unnecessary which represents a big advantage concerning convenience of the experimental process and cost reduction. Using the firefly luciferase *luc*, introduced in this chapter as an example, requires luciferin injections into the mice prior to imaging [3]. As the substrate is degraded within the body of the mouse, additional

injections might be required for later time points of image acquisition. However, the advantage of the firefly luciferase systems is the superior strength of the signal compared to the signal from the *lux* operon. In a case of a weak expression or a low number of bacteria, this might determine whether a signal can be detected at all. Therefore, the choice of the reporter system has to be carefully made considering the special needs of the particular experiment.

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## 2 Materials

(Materials or methods marked bold are specific for firefly luciferase).

### 2.1 Construction of Reporter Strain via Chromosomal Integration

1. Recipient strain of choice that should be used for in vivo imaging (this strain should carry an antibiotic resistance gene for selection which must not be kanamycin or ampicillin—*see Note 1*).
2. SM10 $\lambda$ pir[pUX-BF5] (carrier strain) [4].
3. Plasmid containing the *lux* operon or the *luc* gene (e.g., plite201) under control of a constitutive or inducible promoter.
4. Chemically-competent SM10 $\lambda$ pir.
5. Suitable enzymes to clone the promoter *lux/luc* construct into the carrier plasmid.
6. LB medium/LB agar plates.
7. Antibiotics: kanamycin and ampicillin.
8. 0.45  $\mu$ m nitrocellulose membrane filter.

### 2.2 Cancer-Cell Inoculation

1. Cancer cell line of choice.
2. Cell culture medium.
3. TE (trypsin: 0.5 g/ml, EDTA in PBS: 0.2 g/ml).
4. PBS.
5. Insulin syringes.
6. Appropriate recipient mouse strain syngeneic with the cancer cell line.

### 2.3 Preparation of the Inoculum

1. Reporter strain.
2. LB medium supplemented with the appropriate antibiotics.
3. PBS.

### 2.4 In Vivo Imaging

1. In vivo imaging system such as IVIS 200 from PerkinElmer.
2. Inoculum of the reporter strain.
3. Tumor-bearing mouse.
4. Insulin syringe.

5. Isoflurane or other anesthesia.
6. Luciferin (150 mg/kg from Synchem or PerkinElmer).

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### 3 Methods

#### 3.1 Construction of Reporter Strain via Chromosomal Integration

1. Carrier plasmid containing construct of choice using the backbone of plasmid pUX-BF5 [4]. The reporter construct has to be placed in between the two transposon attachment sites Tn7R and Tn7L without interfering with the kanamycin-resistance gene (Fig. 1).
2. Once the carrier plasmid has been constructed and propagated in SM10 $\lambda$ pir, prepare overnight cultures of all required strains (recipient strain, carrier, and helper strain) in LB medium supplemented with the appropriate antibiotics. Note that SM10 $\lambda$ pir strains have to be incubated at 28 °C in order to prevent induction of the bacteriophage (*see Note 2*).
3. Grow bacterial cultures to an OD<sub>600</sub> of approximately 1.0.
4. Mix 500  $\mu$ l of each culture in a reaction tube and centrifuge at 3500  $\times g$  for 5 min.
5. Discard supernatant and resuspend the pellet in 1 ml LB without antibiotics.
6. Centrifuge again at 3500  $\times g$  for 5 min.
7. Discard supernatant and resuspend the pellet in 20  $\mu$ l LB medium without antibiotics.
8. Sterilely place a 0.45  $\mu$ m nitrocellulose membrane filter on an LB plate without antibiotics.
9. Carefully pipet the resuspended culture mixture on the filter and allow to dry for 5 min.
10. Incubate the plate inverted at 28 °C for 24–30 h.
11. Take the filter from the plate with a sterile forceps, place it in a reaction tube, and add 1 ml LB medium.
12. Vortex gently for 10 s.
13. Plate 100  $\mu$ l on one and the residual 900  $\mu$ l on another LB plate containing 30  $\mu$ g/ml kanamycin and the antibiotic that the recipient strain carries as a selection marker and incubate overnight at 37 °C.
14. Pick single colonies and streak them out on LB plates containing 100  $\mu$ g/ml ampicillin as well as on LB plates containing 30  $\mu$ g/ml kanamycin and the antibiotic the recipient is resistant to.
15. After overnight incubation at 37 °C, clones that have successfully integrated the reporter construct should grow on kanamycin-, but not on ampicillin-containing plates.

### 3.2 Cancer Cell Inoculation

1. Grow cancer cells at 37 °C and 5 % CO<sub>2</sub> in a humidified atmosphere.
2. Detach adherent cells by incubating with TE for 5 min at 37 °C or use a cell scraper.
3. Centrifuge the cell suspension for 5 min at 250 × *g* at 4 °C.
4. Discard the supernatant and resuspend the pellet in ice-cold PBS.
5. Adjust cell suspension to an appropriate cell number per ml.
6. Inject 100 µl of this cell suspension subcutaneously into the recipient mouse.
7. Allow the tumor to grow to a volume of approximately 200 mm<sup>3</sup>.

### 3.3 Preparation of the Inoculum

1. Prepare an overnight LB culture with appropriate antibiotics for the reporter strain to grow at 37 °C while shaking.
2. Dilute the overnight culture 1:300 in fresh medium and incubate at 37 °C, with shaking to achieve an OD<sub>600</sub> of 0.2–0.4.
3. Centrifuge the culture at 3500 × *g* for 5 min at room temperature.
4. Discard the supernatant and resuspend the pellet in PBS.
5. Repeat this washing step twice.
6. Adjust the OD<sub>600</sub> of the washed culture to an appropriate inoculation dose.

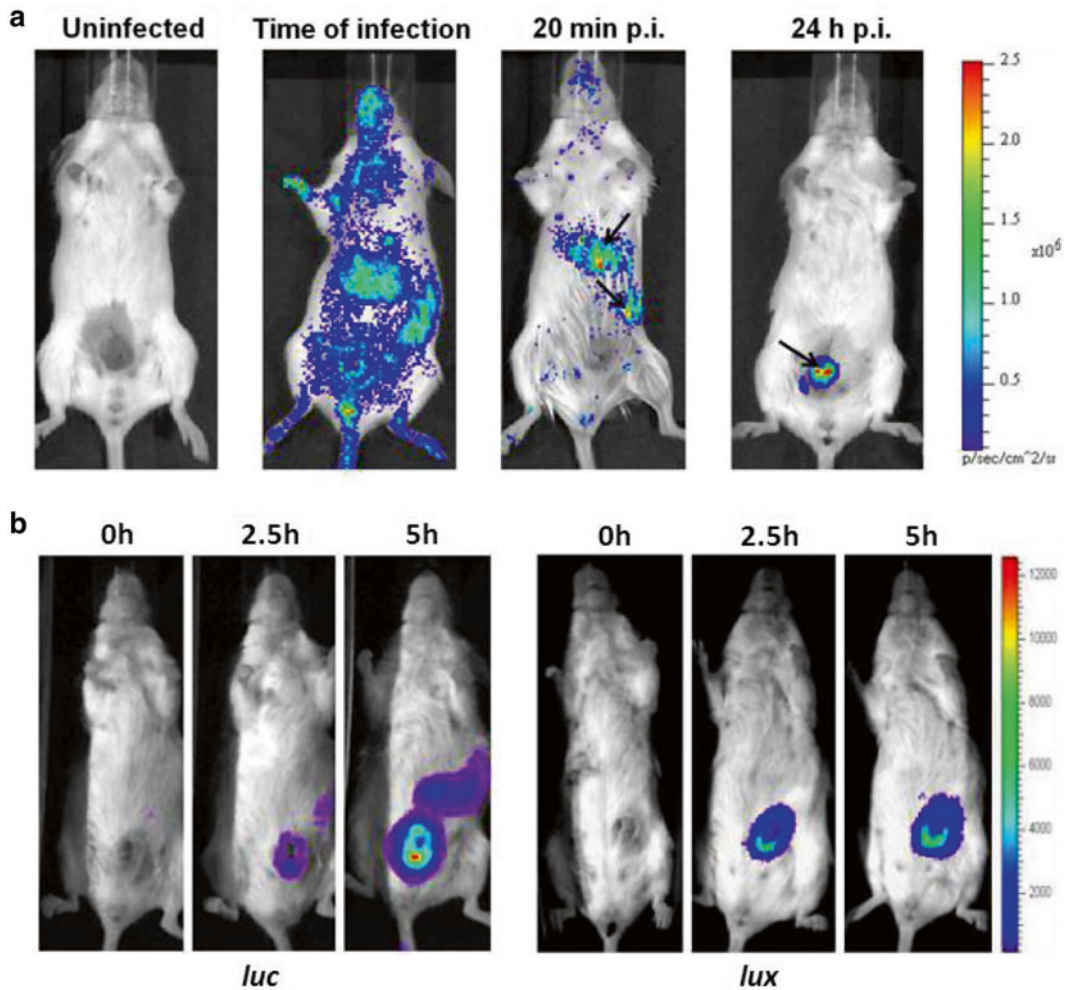
### 3.4 In Vivo Image Acquisition

1. Infect the tumor-bearing mice with the previously prepared inoculum by the desired route.
2. Five minutes before imaging, inject mice intravenously or intraperitoneally with luciferin.
3. Anesthetize the mice and place them into the IVIS imager, carefully making sure the mouse nose is firmly attached into the nose cone to ensure oxygen and anesthesia supply.
4. Adjust IVIS settings to “luminescent” and choose correct *field of view* (FOV) according to the number of mice that should be imaged, acquisition time, f-stop, and binning according to the strength of the expected signal.
5. Acquire image (Fig. 2).

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## 4 Notes

1. The recipient bacterial strain should carry an antibiotic-resistance gene. This should NOT be an ampicillin- nor a kanamycin-resistance gene as they are both on the carrier and/or helper plasmid. An antibiotic-resistance gene as a marker of the recipient strain allows selection for clones that have successfully integrated the transposon (Subheading 3.1, step 13).



**Fig. 2** Bacterial infections with *lux*- and *luc*-carrying *Salmonella typhimurium* in tumor-bearing mice. CT26 tumor-bearing mice were infected with *S. typhimurium* SL7207 carrying different constructs. The *black arrow* points at the subcutaneous tumor growing on the abdomen of the mice. **(a)** Constitutive *lux* expression was used to show *Salmonella* accumulation at the time of intravenous infection, 20 min postinfection (p.i.) and 1 day after infection (reproduced from [1]). **(b)** Inducible expression can be realized using *luc* (left three panels), or *lux* (right three panels) under control of an l-arabinose promoter [5]. Mice have been infected 3 days before inducing luminescence expression. Induction is achieved by intraperitoneal injection of 120 mg l-arabinose. Images were acquired at the indicated time points (reproduced from [5])

2. All SM10 $\lambda$ pir strains have to be propagated at 28 °C in order to prevent induction of the endogenous bacteriophage, the activation of which would result in killing of the bacterial cells. Once the construct is integrated into the chromosome of the recipient, this strain can be grown at 37 °C again.

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## References

1. Leschner S, Westphal K, Dietrich N, Viegas N, Jablonska J, Lyszkiewicz M et al (2009) Tumor invasion of *Salmonella enterica* serovar Typhimurium is accompanied by strong hemorrhage promoted by TNF-alpha. *PLoS One* 4:e6692
2. Meighen EA (1991) Molecular biology of bacterial bioluminescence. *Microbiol Rev* 55:123–142
3. Vieira J, da Pinto SL, Esteves da Silva JC (2012) Advances in the knowledge of light emission by firefly luciferin and oxyluciferin. *J Photochem Photobiol B* 117:33–39
4. Bao Y, Lies DP, Fu H, Roberts GP (1991) An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. *Gene* 109:167–168
5. Loessner H, Endmann A, Leschner S, Westphal K, Rohde M, Miloud T et al (2007) Remote control of tumour-targeted *Salmonella enterica* serovar Typhimurium by the use of l-arabinose as inducer of bacterial gene expression in vivo. *Cell Microbiol* 9:1529–1537