Video Article

Bioluminescent Bacterial Imaging *In Vivo*

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URL: http://www.jove.com/video/4318
DOI: doi:10.3791/4318

Keywords: Immunology, Issue 69, Molecular Biology, Cancer Biology, Genetics, Gene Therapy, Cancer, Vector, Lux, Optical Imaging, Luciferase

Date Published: 11/4/2012

Citation: Baban, C.K., Cronin, M., Akin, A.R., O'Brien, A., Gao, X., Tabirca, S., Francis, K.P., Tangney, M. Bioluminescent Bacterial Imaging *In Vivo*. *J. Vis. Exp.* (69), e4318, doi:10.3791/4318 (2012).

Abstract

This video describes the use of whole body bioluminesce imaging (BLI) for the study of bacterial trafficking in live mice, with an emphasis on the use of bacteria in gene and cell therapy for cancer. Bacteria present an attractive class of vector for cancer therapy, possessing a natural ability to grow preferentially within tumors following systemic administration. Bacteria engineered to express the lux gene cassette permit BLI detection of the bacteria and concurrently tumor sites. The location and levels of bacteria within tumors over time can be readily examined, visualized in two or three dimensions. The method is applicable to a wide range of bacterial species and tumor xenograft types. This article describes the protocol for analysis of bioluminescent bacteria within subcutaneous tumor bearing mice. Visualization of commensal bacteria in the Gastrointestinal tract (GIT) by BLI is also described. This powerful, and cheap, real-time imaging strategy represents an ideal method for the study of bacteria *in vivo* in the context of cancer research, in particular gene therapy, and infectious disease. This video outlines the procedure for studying *lux*-tagged *E. coli* in live mice, demonstrating the spatial and temporal readout achievable utilizing BLI with the IVIS system.

Video Link

The video component of this article can be found at http://www.jove.com/video/4318/

Protocol

1. Tumor Induction

1. For routine tumor induction, the minimum tumorigenic dose of cells suspended in 200 µl of serum-free culture medium was injected subcutaneously (s.c.) into the flank of infection free 6-8 week old female Balb/C or athymic MF1-nu/nu mice n=6 (Harlan, Oxfordshire, UK) (1×10^6 4T1 cells) using a 21-gauge syringe needle. The viability of cells used for inoculation was greater than 95 % as determined by visual count using a haemocytometer and Trypan Blue Dye Exclusion (Gibco).
2. Following tumor establishment, tumors were allowed to grow and develop and were monitored twice weekly. Tumor volume was calculated according to the formula $V=(ab^2)$ Π/6, where *a* is the longest diameter of the tumor and *b* is the longest diameter perpendicular to diameter *a*.

2. Bacterial Preparation

1. The bacterial strain used in this protocol was *E. coli* K-12 MG1655, a non-protein-toxin-expressing strain, harboring a *luxABCDE*-encoding plasmid that enables the bacteria to be detected by BLI. *E. coli* MG1655 containing the integrated *luxABCDE* was grown aerobically at 37 °C in LB medium (Sigma-Aldrich, Ireland) supplemented with 300 µg/ml erythromycin (Em). The bioluminescent derivative of MG1655 was created using the plasmid p16S*lux* which contains the constitutive P_{HELP}*luxABCDE* operon [1].
2. For preparation for administration to mice, cultures were incubated in LB medium at 37 °C in a shaker at 200 rpm to mid-log phase (optical density at 600 nm). Bacteria were harvested by centrifugation (6,000 × g for 5 min), washed with PBS (Sigma), and diluted in PBS 1×10^7 colony forming units (cfu) /ml for IV administration, or 1×10^{10} for gavage.

3. Bacterial Administration

1. Mice were randomly divided into experimental groups when tumors reached approximately 100 mm^3 in volume. For intravenous administration, restrained mice each received 10^6 cells in 100 µl, injected directly into the lateral tail vein using a 28G syringe needle. The viable count of each inoculum was determined by retrospective plating.

2. For GIT colonization studies, 10^9 bacterial cells were orally administered in 100 µl per mouse by gavage, on three consecutive days. Pre-existing commensal bacterial levels were decreased prior to feeding by addition of 5 mg/ml streptomycin in mouse drinking water for 7 days prior to commencement of oral gavage [1].

4. BioLuminescence Imaging

1. 2D *in vivo* BLI imaging was performed using the IVIS100 (Caliper). At defined time points post bacterial administration, mice were anesthetized using Caliper's XGI-8 Gas Anesthesia System with 3% Isofluorane, and whole-body image analysis was performed in the IVIS 100 system for 2-5 min at high sensitivity.
2. For 3D imaging, anesthetized mice were placed in a mouse imaging shuttle inside of the optical imaging system for dorsal imaging (IVIS Spectrum, Caliper). To acquire images of the bacterial luciferase signal for 3D optical reconstruction, emission filter wavelengths ranging from 500-580 nm were used with bin 16 acquisition times of 3-4 min per filter to maximize the signal to noise ratio. As part of this image acquisition sequence, a structured light image was obtained to define a height map. This map was input diffuse light imaging tomography (DLIT) reconstructions algorithms that were used to form a 3D optical image using a non-negative least squares optimization [2].
3. Image Analysis: Regions of interest were identified and quantified using Living Image software (Caliper).

5. Representative Results

In this study, the non-pathogenic commensal bacteria *E.coli* K-12 MG1655 expressing the *luxABCDE* operon was IV administered to mice bearing s.c. 4T1 xenograft tumors. Bacterial *lux* signal was detected specifically in tumors of mice post IV-administration (**Figure 2**). Culture recovery of bacteria from sample mice validates the existence of a linear relationship between viable bacterial numbers and the quantity of light detected (**Figure 3**). *In vivo* imaging of orally-administered commensal bacteria in the GIT is also achieved using 3D BLI.

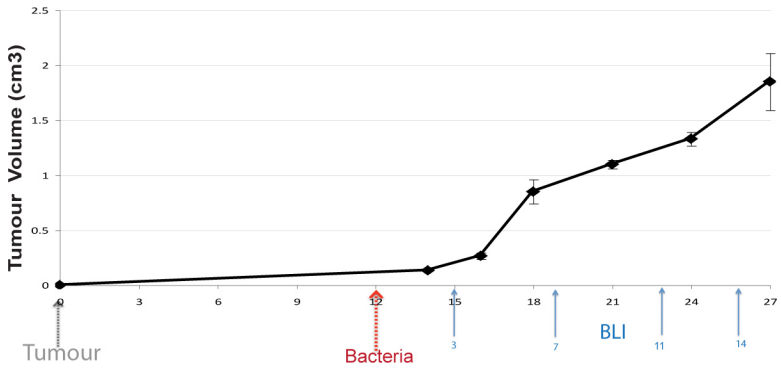

Figure 1. Protocol Timeline. Subcutaneous tumors are induced in mice, and bacteria administered upon tumor development (100 mm^3). Live mice are BLI imaged at various time-points post bacterial administration (arrows display typical times).

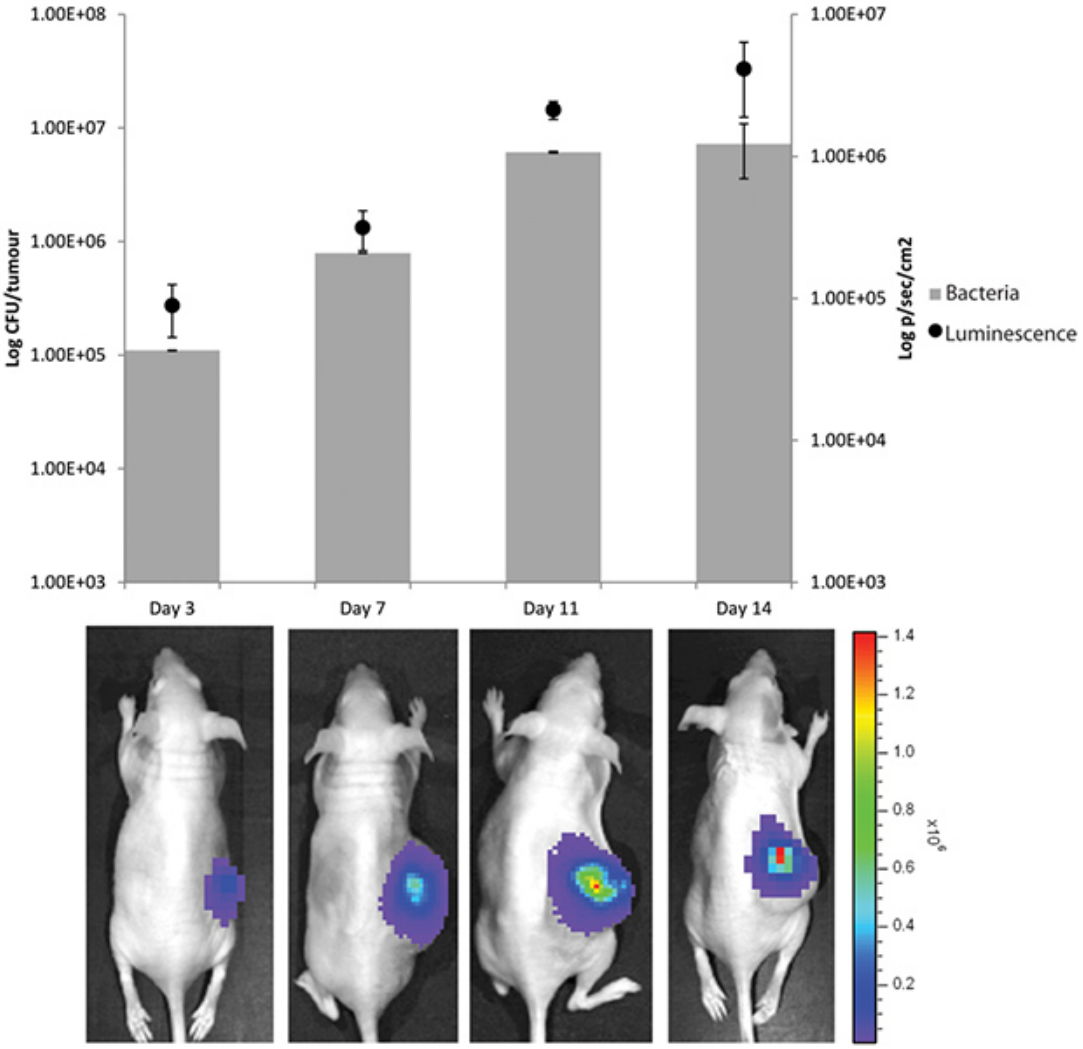

Figure 2. Administration of *E. coli* MG1655 *luxABCDE* to tumor bearing mice. Subcutaneous 4T1 tumors were induced in MF1 *nu/nu* mice and *E. coli* MG1655 *luxABCDE* administered upon tumor development. Each animal received 10^6 cells injected directly into the lateral tail vein. Mice were imaged at four time points during the study (black dots z-axis and images) with subsequent recovery of viable bacteria (cfu) from tumors of sample sacrificed mice (bar graph). Increase in bacterial numbers and plasmid gene expression specifically in tumors was observed over time (representative mouse illustrated per time point). Click here to view larger figure.

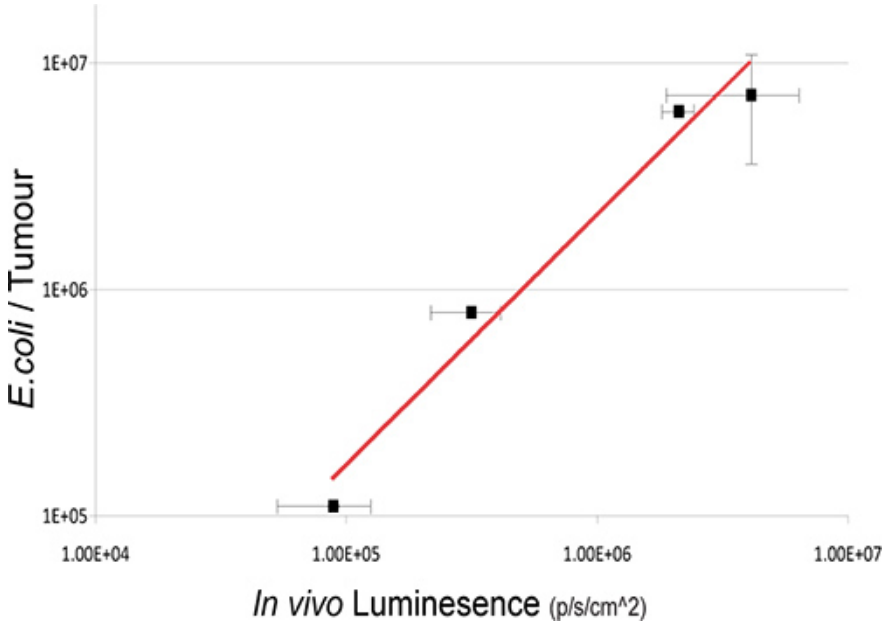

Figure 3. Relationship Between Intratumoral Bacterial Numbers And Bioluminescence. Viable bacteria in tumors were enumerated by *ex vivo* bacterial culture from tumors subsequent to BLI at various time-points post IV administration. Log values of bacterial numbers (cfu) relative to *in vivo* bioluminesce units are graphed. A robust correlation between bacterial counts and bacterial bioluminescence signals is observed R^2=0.9717[1].

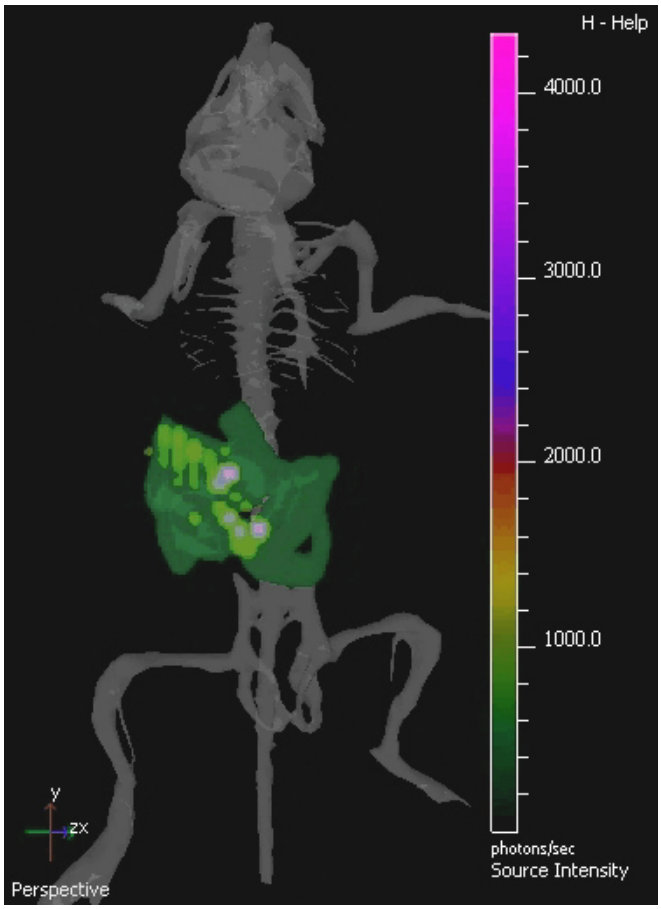

Figure 4. 3D IVIS Image Of Murine Gastrointestinal Tract Colonized By *E. coli* MG1655. The GIT of mice was colonized by oral administration of 10^9 cfu of *E. coli* for three consecutive days. A sample isolated image from 3D tomography of the colonized mouse is shown.

3D images show a digital mouse atlas of the skeleton to provide anatomical registration. *E. coli* MG1655 bioluminescence is visible in green at lower, and purple at higher levels.

Discussion

In the context of gene therapy, the use of biological agents for delivery of therapeutic genes to patients has shown great promise [3-5]. Like viruses, the innate biological properties of bacteria permit efficient DNA delivery to cells or tissues, particularly in the context of cancer. It has been shown that bacteria are naturally capable of homing to tumors when systemically administered resulting in high levels of replication locally, either external to (non-invasive species) or within tumor cells (pathogens). Cancer-specific bacterial replication was initially attributed to the hypoxic nature of solid tumors (low O_2 levels), with the anaerobic nature of hypoxic/necrotic regions within tumors promoting growth of anaerobic and facultatively anaerobic bacteria. More recently, factors such as the irregular, leaky blood supply and local immune suppression in tumors have been proposed to play a role. Most preclinical studies have utilized xenograft tumor models to investigate bacterial vector tumor colonization. Murine models of spontaneously arising tumors more closely resemble clinical reality in terms of vasculature (a potential variable in bacterial colonization of tumors), and bacteria have also been shown to colonize such models [6]. Various preclinical and clinical trials have shown the ability of different bacterial strains to transport and amplify genes encoding factors such as prodrug-converting enzymes, toxins, angiogenesis inhibitors, and cytokines specifically within tumors [4,7]. While bacterial tumor colonization has been shown to be independent of bacterial strain and tumor type [6], the choice of strain optimal for a particular model may vary - *e.g.* strict anaerobes may be more suitable for large necrotic tumors.

We have engineered a number of strains to express the *luxABCDE* cassette [1,8-11]. The protocol outlined in the video animation uses *lux*-tagged *E. coli* as an example. *E. coli* is part of the flora of the human GIT. Several studies have outlined the safety of IV administration of non-pathogenic *E. coli* strains to mice, and their ability to grow specifically within tumors [4,12]. *E. coli* MG1655 (as used in this study) also colonizes the mouse GIT to high levels [13].

The study of bacteria in small animal models is of high importance to a range of medical research fields; including infectious diseases, gut health and gene therapy. For example, adaptations of this protocol may be applicable to studies of bacterial infection tracking, biofilm formation etc. Other gene-based reporter systems have also been examined, including Positron Emission Topography (PET) scanning in combination with bacterial expression of thymidine kinase (tk), be it endogenous expression in *E. coli* or *S.* Typhimurium engineered to express the tk gene from Herpes Simplex Virus (HSVtk) [14]. Both fluorescent (Green Fluorescent Protein and its variants) and luminescent (*lux*) genes are available for bacteria. An advantage of using bacterial luciferase is that the *lux* cassette encodes the enzymes required for substrate biosynthesis, resulting in a directly imagable agent [15]. BLI is based on the detection of bioluminescent light from the subject through use of a cooled charged coupled device (CCD) camera. The relatively simple instrumentation and lack of requirement for radioactivity puts the technology well within the reach of the average laboratory. BLI displays many benefits when compared with other *in vivo* modalities. It is easy to use, inexpensive, rapid and facilitates imaging of multiple animals simultaneously, producing little background with high sensitivity.

Disclosures

No conflicts of interest declared.

Acknowledgements

The authors wish to acknowledge support relevant to this manuscript from the European Commission Seventh Framework Programme (PIOF-GA-2009-255466) and the Irish Health Research Board (HRA_POR/2010/138). *Lux*-tagged *E. coli* was a kind gift from Dr. Cormac Gahan, University College Cork.

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