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TITLE:

Longitudinal Follow-up of Urinary Tract Infections and Their Treatment in Mice using Bioluminescence Imaging

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KEYWORDS:

bioluminescence imaging, urinary tract infections, bacterial infection, uropathogenic *E. coli* murine model, *lux* operon, photons

SUMMARY:

This manuscript describes the intravesical administration of uropathogenic bacteria with a *lux* operon to induce a urinary tract infection in mice and subsequent longitudinal *in vivo* analysis of the bacterial load using bioluminescence imaging.

ABSTRACT:

Urinary tract infections (UTI) rank among the most common bacterial infections in humans and are routinely treated with empirical antibiotics. However, due to increasing microbial resistance, the efficacy of the most used antibiotics has declined. To find alternative treatment options, there is a great need for a better understanding of the UTI pathogenesis and the mechanisms that determine UTI susceptibility. In order to investigate this in an animal model, a reproducible,

non-invasive assay to study the course of UTI is indispensable.

For years, the gold standard for the enumeration of bacterial load has been the determination of Colony Forming Units (CFU) for a particular sample volume. This technique requires *post-mortem* organ homogenates and serial dilutions, limiting data output and reproducibility. As an alternative, bioluminescence imaging (BLI) is gaining popularity to determine the bacterial load. Labeling pathogens with a *lux* operon allow for the sensitive detection and quantification in a non-invasive manner, thereby enabling longitudinal follow-up. So far, the adoption of BLI in UTI research remains limited.

This manuscript describes the practical implementation of BLI in a mouse urinary tract infection model. Here, a step-by-step guide for culturing bacteria, intravesical instillation and imaging is provided. The *in vivo* correlation with CFU is examined and a proof-of-concept is provided by comparing the bacterial load of untreated infected animals with antibiotic-treated animals. Furthermore, the advantages, limitations, and considerations specific to the implementation of BLI in an *in vivo* UTI model are discussed. The implementation of BLI in the UTI research field will greatly facilitate research on the pathogenesis of UTI and the discovery of new ways to prevent and treat UTI.

INTRODUCTION:

Urinary tract infections (UTI) are among the most common bacterial infections in humans. Almost half of all women will experience a symptomatic UTI during their lifetime[1]. Infections limited to the bladder can give rise to urinary symptoms such as increase in urinary frequency, urgency, hematuria, incontinence, and pain. When the infection ascends to the upper urinary tract, patients develop pyelonephritis, with malaise, fever, chills, and back pain. Furthermore, up to 20% of patients with UTI suffer from recurrent infections resulting in a dramatic decrease in antibiotic sensitivity[2–4]. In recent years, there has been a growing interest in novel therapies for the treatment and prevention of recurrent UTI. Despite a better understanding of the innate and adaptive immunity of the lower urinary tract and of the bacterial virulence factors necessary for invasion and colonization, no radical changes in the treatment regime have been translated to the daily urological practice[2]. In order to study UTI pathogenesis and susceptibility *in vivo*, a reproducible and non-invasive assay is indispensable.

Multiple animal UTI models have been described ranging from nematodes to primates, but the murine model is predominantly used[5,6]. This model consists of transurethral catheterization of (female) mice and subsequent instillation of a bacterial suspension, most commonly uropathogenic *Escherichia coli* (UPEC), directly into the bladder lumen[7]. After inoculation, the bacterial load has traditionally been quantified by determining colony forming units (CFU). This technique requires sacrificing animals to obtain *post-mortem* organ homogenates and serial dilutions, limiting data output and reproducibility. Moreover, longitudinal follow-up of the bacterial load in individual animals is not possible using this technique.

In 1995, Contag et al. suggested the use of bioluminescent-tagged pathogens to monitor disease processes in living animals[8,9]. Since then, bioluminescence imaging (BLI) has been applied to

numerous infection models such as meningitis, endocarditis, osteomyelitis, skin, and soft tissue infections, etc.[10–12]. In the murine UTI model, a UPEC strain with the complete *lux* operon (*luxCDABE*) from *Photorhabdus luminescens* can be used[13]. An enzymatic reaction is catalyzed by the bacterial luciferase which is dependent on the oxidation of long-chain aldehydes reacting with reduced flavin mononucleotide in the presence of oxygen, yielding the oxidized flavin, a long-chain fatty acid and light[12]. The *lux* operon encodes for the luciferase and other enzymes required for the synthesis of the substrates. Therefore, all metabolically active bacteria will continuously emit blue green (490 nm) light without the need for the injection of an exogenous substrate[12]. Photons emitted by *lux*-tagged bacteria can be captured using highly sensitive, cooled charge-coupled device (CCD) cameras.

The use of bioluminescent bacteria in a model for UTI allows for the longitudinal, non-invasive quantification of the bacterial load, omitting the need for sacrificing animals at fixed time points during the follow-up for CFU determination. Despite the wide range of possibilities, accumulating evidence for the robustness of this BLI technique in other fields and its advantages over classic models of UTI, it has not been widely implemented in the UTI research. The protocol presented here provides a detailed step-by-step guide and highlights the advantages of BLI for all future UTI research.

PROTOCOL:

All animal experiments were conducted in accordance with the European Union Community Council guidelines and were approved by the Animal Ethics Committee of KU Leuven (P158/2018).

1. Culturing bacteria (adapted from[7,13,14])

1.1. Preparation

1.1.1. Choose a luminescent UPEC strain that best fits the experimental needs.

NOTE: Here, the clinical cystitis isolate, UTI89 (*E. coli*), was selected because of its uropathogenic capacity in both humans and rodents, and its common use in murine UTI models[5,7,15]. The bioluminescent isogenic strain carrying the complete *lux* operon (*luxCDABE*) is further referred to as UTI89-*lux*. This operon also carries kanamycin sulfate (Km) resistance genes. Therefore, Km can be used to select bioluminescent colonies[13].

1.1.2. Make a correlation curve for the CFU and optical density at 600 nanometers (OD 600 nm) for the chosen bacterial strain[7].

1.1.2.1. To do this, culture bacteria (using the protocol below) and make 8 different dilutions in phosphate buffered saline (PBS). Measure the OD at 600 nm for all these dilutions and plate them on Luria-Bertani (LB) plates to determine the CFU.

1.1.2.2. Plot the OD 600 nm values and the CFU values to determine the correlation and to obtain

a standard curve.

NOTE: For future experiments, measure the OD at 600 nm and use this standard curve to get an instant estimation of the CFU in a bacterial solution.

CAUTION: UPEC are pathogenic bacteria, ensure rooms are properly equipped and use personal protective equipment.

1.2. Three days before the instillation

1.2.1. Obtain single colonies by streaking out the glycerol stock of bacteria, using an inoculation loop, on LB plates supplemented with 50 µg/mL Km and culture overnight at 37 °C. Seal these plates with a paraffin film to store at 4 °C up to 1 week.

1.3. Two days before the instillation

1.3.1. Fill a sterile 14 mL polystyrene round bottom tube with dual-position snap caps with 5 mL of LB broth supplemented with 50 µg/mL Km. Pick a single bacterial colony with an inoculation loop and add this to the LB broth. Vortex for 10 s to ensure proper mixing.

1.3.2. Culture statically with the snap cap in the open position at 37 °C overnight.

NOTE: Static growth of *E. coli* promotes the expression of type 1-pili, which are critical for the adherence to and invasion of urinary epithelial cells.

1.3.3. Prepare a sterile Erlenmeyer or culture flask.

1.4. One day before the instillation

1.4.1. After the incubation, vortex the tube for 10 s to ensure proper homogenization of the bacterial culture. Make a sub-culture in the Erlenmeyer by adding 25 µL of the bacterial suspension to 25 mL of fresh LB medium without antibiotics.

1.4.2. Close the Erlenmeyer and culture statically at 37 °C overnight.

1.5. On the day of instillation

1.5.1. Vortex the Erlenmeyer for 10 s to ensure proper mixing.

1.5.2. Pour the culture from the Erlenmeyer into a 50 mL culture tube and centrifuge at 3,000 x *g* and 4 °C for 5 min. Decant the supernatant and resuspend the bacterial pellet in 10 mL of sterile PBS and vortex again for 10 s.

1.5.3. Choose the experimental concentration of the inoculum (CFU) and use the standard curve

obtained in step 1.1.2 to determine the corresponding OD 600 nm.

1.5.4. Add 1 mL of the resuspended bacterial culture into 9 mL of sterile PBS and check the OD at 600 nm. Adjust further by adding either sterile PBS or concentrated bacterial solution, until the desired OD 600 nm is reached[7].

NOTE: For example, for UTI89-*lux* adjust the culture by adding PBS until the OD 600 nm reaches 0.45, corresponding to 2 x 10^7 CFU/50 µL. To verify the number of viable CFUs, make 10-fold serial dilutions and plate 50 µL of the fifth and sixth 10-fold dilutions on LB plates in triplicate to obtain less than 300 colonies. Count the obtained colonies the next day after overnight incubation at 37 °C. OD 600 nm gives an instant read-out but use CFU as quality control.

2. Inoculation of the animals (adapted from[7,16])

2.1. Preparation of the animals

2.1.1. Choose the desired mouse strain(s) depending on the research question and availability of knock-out lines, experimental details, and differences in UTI susceptibility[5,17]. Keep in mind that transurethral catheterization is easier in female mice. Do not use animals younger than 8 weeks, as they are immunologically immature. Here, 12-week-old female C57Bl/6J mice were used.

2.1.2. Order animals well in advance and let them acclimate, ideally for 7 days. Group house animals in individually ventilated cages, under standard 12 h light/dark conditions.

2.1.3. Shave the abdominal region of the animals to limit the loss of signal. Do not use hair removal cream, as it can burn the skin of the animals quickly. Restrain the animal by tightly holding the scruff and hind limbs with the non-dominant hand while shaving with the dominant hand. Alternatively, shave under isoflurane anesthesia.

NOTE: Animals were shaved 2 days prior to imaging, considering the animals will groom the shaved area even further.

2.1.4. Provide water and standard food *ad libitum* throughout the experiment. However, deprive animals from water 2 h prior to instillation to minimize the bladder volume during instillation.

2.1.5. Mount a sterile 24 G angiocatheter tip on a 100 µL syringe and fill the syringe with the bacterial solution prepared in step 1.5.3.

NOTE: To determine the background luminescence, obtain a baseline image prior to the instillation (see step 3, below).

2.2. Instillation of the animals

2.2.1. Place the animals in an induction chamber and anesthetize them using inhalation of isoflurane with pure oxygen as a carrier gas (induction at 3% and maintenance at 1.5%).

2.2.2. Place one animal on a working surface in the supine position and maintain a stable isoflurane anesthesia using a nose cone during the instillation. Apply the eye-ointment.

2.2.3. Expel the residual urine by applying gentle compression and making circular movements on the suprapubic region. Clean the lower abdomen with 70% ethanol prior to instillation.

2.2.4. Lubricate the catheter tip with normal saline. Put the index finger of the non-dominant hand on the abdomen and push it gently upwards. Start the catheterization of the urethra in a 90° angle (vertically) and once resistance is encountered, tilt it horizontally before inserting it further (0.5 cm).

NOTE: Never forcefully push the catheter, as this will cause harm to the urethra. Gentle turning motions can be helpful in catheter insertion. On the other hand, a lack of resistance usually indicates erroneous insertion into the vagina.

2.2.5. Perform a slow (5 µL/s) instillation of 50 µL of the bacterial inoculum (2 x 10^7 CFU).

NOTE: Higher volumes or faster instillation might cause reflux to the kidneys. During practice of the technique, blue ink can be used to evaluate reflux.

2.2.6. After the instillation, keep the syringe and catheter in place for a few more seconds and then slowly retract to prevent leakage. Record any irregularities such as a high amount of leakage or a bloody meatus and exclude animals, if necessary.

2.2.7. Position the animal in supine position at the nose cone of the imaging chamber and repeat the preceding steps 2.1.1–2.1.6 for all the remaining animals. Use one catheter per experimental group. Ensure anesthesia is continued and minimize the time between the first and the last animal.

2.2.8. If necessary, administer antibiotics or experimental drugs, prior to or after the imaging (step 3). For example, to administer enrofloxacin, add 40 µL of the enrofloxacin (100 mg/mL) solution to 3.96 mL of physiological saline to obtain a 1/100 dilution. Inject 100 µL/10 g subcutaneously at 9 am and 5 pm to administer 10 mg/kg of enrofloxacin twice daily for 3 days.

3. Bioluminescence imaging

3.1. Preparation and selection of imaging parameters

3.1.1. Open the BLI acquisition software (see **Table of Materials**) and click on **Initialize** in the imaging device (see **Table of Materials**) to test the camera and stage controller system and to cool the CCD camera to -90 °C.

NOTE: During this process, the door is locked, and the progress of the initialization can be followed on the control panel. A green light indicates that the temperature of -90 °C is reached. A warning will appear if imaging is attempted before completion of the initialization.

3.1.2. Ensure data is saved automatically: Click on acquisition **Auto-save to** and select the correct folder.

3.1.3. Select **Luminescence** and **Photograph**. Check the default luminescence settings: Set excitation filter to **Block** and emission filter to **Open**.

3.1.4. Set the exposure time to **Auto** when taking the first image, especially when expecting a dim signal to ensure an adequate number of photon counts. For *in vivo* measurements and bright signals, set the **Exposure Time** to ~30 s. If the image is saturated, a warning will appear. If this happens, reduce the exposure time.

3.1.5. Select the medium **Binning, F/stop** 1 and choose the correct field of view (**FOV**) (D for 5 animals).

3.1.6. Set the **subject height** to 1 cm when imaging mice.

3.1.7. Click on **Add** three times in the **Acquisition Control Panel** to obtain a sequence of three images, as technical replicates.

3.2. Imaging

3.2.1. Place mice in the imaging chamber in the supine position and use the manifold nose cone to maintain anesthesia (isoflurane 1.5%) throughout the experiment. Image up to 5 mice simultaneously and separate the animals using the light baffle to prevent reflection.

3.2.2. Close the door and click on **Acquire** to start the imaging sequence.

3.2.3. Fill in detailed information about the experiment (wild type and knockout animals, treatments, day of imaging, etc.). The imaging settings, such as exposure time, are saved automatically.

3.2.4. Remove mice from the imaging chamber and return them to their cage. Check for the full recovery after anesthesia. Within minutes, the animals should be fully awake and explorative. Do not provide analgesia as this might interfere with the UTI course.

3.2.5. Return the cages to the ventilated racks until the next imaging cycle. After completion of the experiment, euthanize the animals by CO_2 asphyxiation or cervical dislocation. Do this before the recovery of isoflurane anesthesia to minimize distress.

3.3. Analysis of the images

3.3.1. Start the imaging software and load the experimental file by clicking on **Browse**.

3.3.2. Use the **Tool Palette** to adjust the color scale of the image. Standardize the visual aspect of the results by using the same settings for all the images, i.e., use a logarithmic scale with a minimum of 10^4 to a maximum of 10^7 photons. These adjustments do not affect the raw data but only the graphical presentation.

3.3.3. Use the **ROI tools** to draw a region of interest (ROI) on the image. Ensure the ROI is large enough to cover the complete area and use the same dimensions for all the images. Here, a square ROI of 3.5 cm x 4.5 cm was placed over the lower abdomen.

3.3.4. Click on ROI measurement to quantify the light intensity (counts or total photon flux). Export these data and use the average of the technical replicates.

NOTE: Counts represent the number of photons detected by the camera and should only be used for the image quality control. To report data, use total photon flux. Total photon flux is more physiologically relevant as it represents the light emitted from the surface and it is a calibrated unit, which is corrected for the exposure time (per second).

REPRESENTATIVE RESULTS:

***In vivo* BLI correlates with CFU of the inoculum at time of instillation.**

To evaluate the detection limit of BLI *in vivo* and the correlation with CFU of the inoculum, mice were infected with different concentrations of UTI89-*lux* and PBS as a negative control. Before instillation, uninfected animals were scanned to determine the background luminescence. Subsequent images were obtained immediately post-instillation (**Figure 1A**). After the instillation of UTI89-*lux*, bioluminescence was robustly detectable above 2 x 10^4 CFU and a linear correlation between the CFU of the inoculum and the bioluminescence was established (**Figure 1B**). In contrast, total photon flux was comparable to background signals for animals instilled with PBS and at the lowest bacterial concentration of 2 x 10^3 CFU.

BLI as a tool for longitudinal follow-up during treatment

To determine whether the curative effect of a validated antibiotic treatment can be demonstrated using longitudinal BLI, 20 animals were infected with an inoculum of 2 x 10^7 CFU/50 µL, and 10 of these animals were treated with enrofloxacin 10 mg/kg subcutaneously twice daily during the first 3 days post-infection. Both the natural evolution in the infected but untreated control group and the effect of antibiotic treatment on the infection kinetics in the treated group could be visualized in detail (**Figure 2**). An immediate decrease in bacterial load, as measured by total photon flux, was seen after the first dose of enrofloxacin. None of the treated

animals had a subsequent rise in bacterial load. The inter-subject variability in the infected but untreated control group was higher and the decrease in bacterial load was slower. At day 10, 8 out of 10 animals of the control group had returned to a BLI signal comparable to their pre-instillation background. The overall bacterial load for each animal was calculated using the area under the curve (AUC) of the log-transformed total photon flux. A significant difference was observed between animals treated with enrofloxacin and untreated animals over the time course of 10 days. These results demonstrate that BLI is a powerful tool to evaluate the effect of a potential treatment on the disease course of a UTI.

FIGURE AND TABLE LEGENDS:

Figure 1: *In vivo* BLI correlates with CFU of the inoculum at the time of instillation. (**A**) Representative images obtained with *in vivo* BLI of mice infected with increasing concentrations of inoculum (2×10^3–2×10^8 CFU) imaged immediately after bacterial instillation in the bladder. CFU = Colony Forming Units. (**B**) Quantification of bioluminescence emitted over the bladder region (total photon flux) compared to the concentration of the inoculum (CFU). BLI signal was robustly detected above 2×10^4 CFU ($p = 0.0002$ compared to background and $p = 0.015$ compared to the PBS control, unpaired *t*-test). Pearson correlation for the inoculi ranging from 2×10^3–2×10^8 CFU was $r = 0.9995$ ($p < 0.0001$). BG = background, PBS = Phosphate Buffered Saline, CFU = Colony Forming Units.

Figure 2: BLI as a tool for longitudinal follow-up during treatment. (**A**) Individual traces of the total photon flux for animals infected with 2×10^7 CFU of UTI89-*lux* and treated with enrofloxacin 10 mg/kg twice daily for 3 days (blue) versus an untreated control group (black), n = 10 per group. BG = background. (**B**) Analysis of traces from animals shown in panel A, reporting the AUC of log-transformed total photon flux for both the enrofloxacin (blue) and control group (black). N = 10 per group, mean ± SD, unpaired *t*-test $p < 0.0001$. AUC = Area under the curve.

DISCUSSION:

Advantages of BLI compared to CFU counts

Longitudinal data

A major disadvantage of the traditional method of counting CFU to quantify microbial burden is the requirement of *post-mortem* organ homogenates, providing only one cross-sectional data point per animal. Conversely, BLI enables non-invasive longitudinal follow-up of infected animals. The animals can be imaged 2 to 3 times a day, providing detailed insight into the kinetics of the infection. Additionally, repeated measures of the same animal drastically reduce the number of animals needed for adequately powered experiments. Furthermore, researchers can focus on the inter-individual variability and prior to examining or treating animals with a novel agent, they can select animals with prespecified bacterial loads by using the real-time data. On the other hand, using the AUC of an experiment allows researchers to focus on the overall bacterial load and not on a single cross-sectional value. The added value of longitudinal and real-time data in the UTI setting cannot be overestimated.

Identification of dissemination of the infection

The spatial resolution of the images obtained with BLI is adequate to identify other sites that are colonized by *lux*- or *FLuc*-tagged pathogens[18]. For UTI, ascending infections to the kidneys or dissemination to the blood are highly relevant findings. In this experimental setting, any spread of infections induced by UTI89-*lux* beyond the lower urinary tract was not observed. This was expected considering this strain predominantly causes cystitis.

Data reproducibility and comparison

The reproducibility of experiments is higher when using BLI compared to CFU counts. Classically, triplicates of serial 10-fold dilutions of homogenates are plated and only those plates with 30 to 300 CFU are then counted to calculate the total number of CFU. This method is cumbersome, prone to high variability, results in many futile plates, and is extremely prone to human errors. Furthermore, there is no consensus on how to report the CFU, namely, per gram bladder tissue, per bladder, or per 1 mL homogenate. This renders the comparison of the results of different groups extremely difficult and could be ameliorated by the use of BLI and total photon flux.

Considerations and limitations of the method

Besides the classical considerations related to BLI *in vivo* such as absorption of photons by fur or hemoglobin, there are some considerations to be made when correlating BLI and CFU counts at the time of sacrifice.

First, discrepancies between both methods at the time of sacrifice are encountered[19], especially when antibiotic therapies are used. Therefore, the correlation between BLI and CFU counts at the time of sacrifice can be suboptimal and is not necessarily relevant. For BLI, the total photon flux can be lower than expected, because challenged bacteria can redirect their metabolism toward recovery and repair processes, rather than light emission. Additionally, the BLI measurement captures a snapshot of the bacteria and their metabolism in real-time *in vivo,* resulting in lower BLI signals if bacteria are in a dormant or elongated state at the time of imaging. On the other hand, for CFU determination, drug-challenged bacteria react in an all-or-nothing manner after being removed from their infectious habitat (i.e., a biofilm) and plated. Once plated onto agar, the challenged bacteria may be irretrievably injured and unable to develop into observable colonies, resulting in lower CFU counts[20]. Additionally, challenged bacteria can enter a viable but non culturable state (VBNC). They remain metabolically active and viable but are no longer culturable on standard laboratory media. Bacteria that enter the VBNC state or that are organized in biofilms can be detected easily with BLI, while enumeration of CFU requires additional processing[20–22]. In conclusion, bioluminescence and viable counts measure different aspects of cell physiology and neither should be regarded as the definitive indicator of cell viability[20].

Moreover, the definition of bacterial load as measured by BLI or CFU counts is different due to differences in sample processing. When using CFU counts, only bacteria that reside within the bladder wall are included in the homogenate and counted. By contrast, BLI also captures photons emitted by bacteria in the urine and the urethra. In our opinion, the latter is a physiologically more correct representation of the total bacterial load as it includes all anatomically relevant

compartments (bladder, urine, and urethra).

In contrast to CFU counts, BLI requires the generation of genetically altered luminescent bacteria. Depending on the insertion site, it is plausible that the introduction of the *lux* operon in the genome can alter the virulence potential of the bacterial strain. Therefore, when developing a new bioluminescent strain, its virulence and growth characteristics should be compared to the parental strain[10,13]. However, recent advances in genome editing allow for efficient and scarless tagging of all major bacterial pathogens, thereby limiting the impact on virulence or treatment responses. Moreover, genetic engineering opens possibilities toward conditional expression of bioluminescence reporter genes and, therefore, *in vivo* measuring of relevant bacterial subpopulations, gene expression, etc[23,24].

Finally, access to the advanced imaging device and acquisition software for *in vivo* BLI might be a limiting factor compared to the basic equipment required for CFU enumeration. However, collaborations with imaging cores can minimize the expenses and investments necessary for BLI.

Considerations specific to the implementation of BLI in an *in vivo* UTI model

Animal model

The advantages of a murine UTI model are three-fold: It allows monitoring of disease progression in a mammalian urinary tract, the animals and their maintenance are relatively inexpensive, and mutant lines are available. On the other hand, induction of the infection usually entails transurethral catheterization and subsequent instillation of a high dosage of bacteria directly into the bladder lumen, thereby bypassing the natural process of ascension via the urethra[5].

Animal housing

Animals can be group housed during the experiment. We have examined the occurrence of transmission between animals of the same cage, by introducing sentinel animals into cages with infected animals. None of the sentinel animals had a detectable bacterial load, measured with both CFU and BLI.

Bladder volume

When combining the murine UTI model with BLI, researchers should keep the unique anatomical properties of the bladder in mind. The bladder is a hollow organ that contains a varying volume of urine. During UTI, the presence of bacteria-infested urine could theoretically influence the total photon count depending on the degree of bladder filling. However, standardization of the bladder volume is impractical and could intervene with the experimental design. Furthermore, in our experience, differences in bladder volume do not result in statistically significant differences in total photon flux.

Sample temperature

In vitro BLI (or as a simpler and cheaper alternative, measuring bioluminescence in a luminometer) can also be used to determine bacterial load on homogenates, urine, or bacterial suspensions containing UTI89-*lux*. However, the temperature of the sample is important as the emission of photons results from an enzymatic reaction and thus requires metabolic activity and

the presence of oxygen. Drastic changes in temperature during organ harvesting should be avoided by allowing the sample to calibrate on the heated (37 °C) stage 5 min prior to imaging.

Potential applications and importance

In summary, the cumbersome method of enumeration of CFU was limiting the reproducibility and efficacy of research in the UTI field. The experimental setup with BLI will advance *in vivo* UTI research on bladder physiology, UTI pathogenesis, and susceptibility by enabling longitudinal follow-up, while reducing the number of animals needed for these types of studies.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Fig1

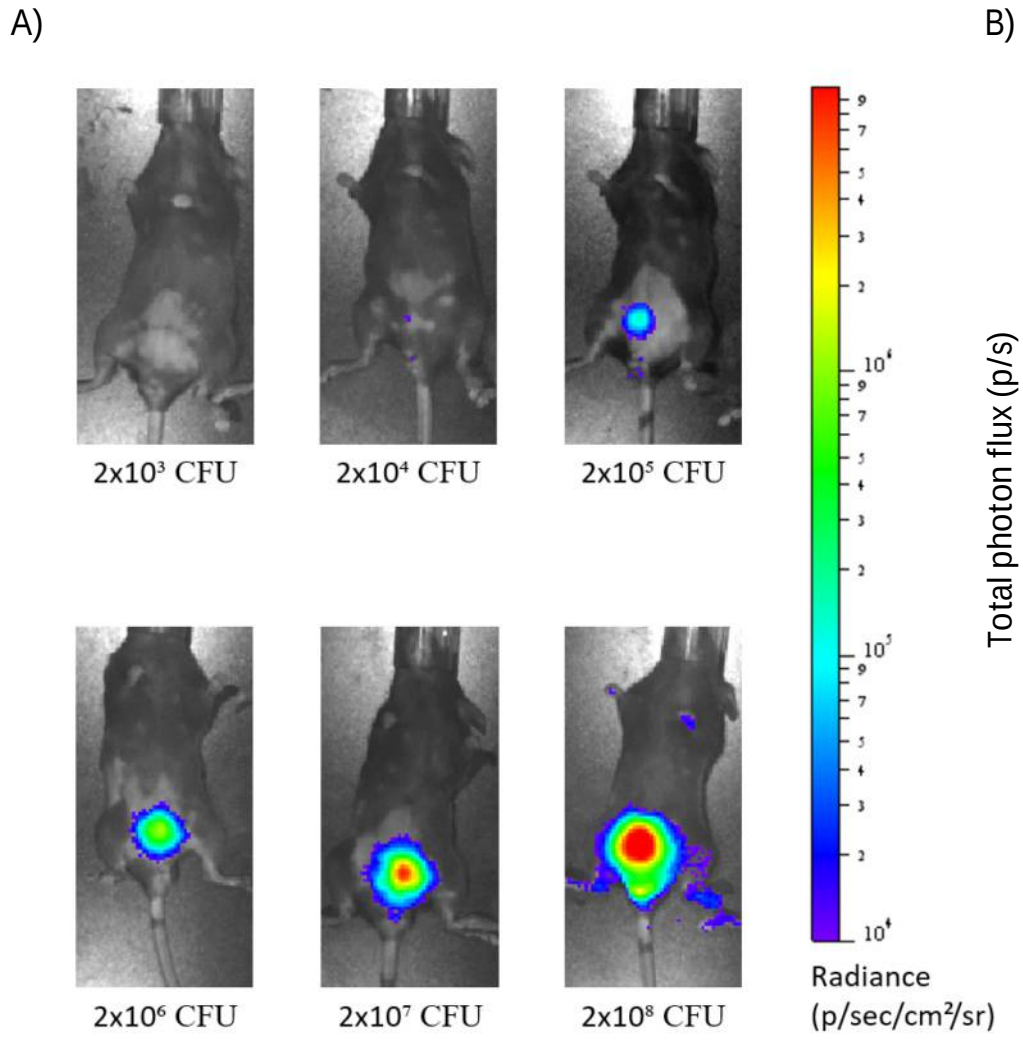

Fig2

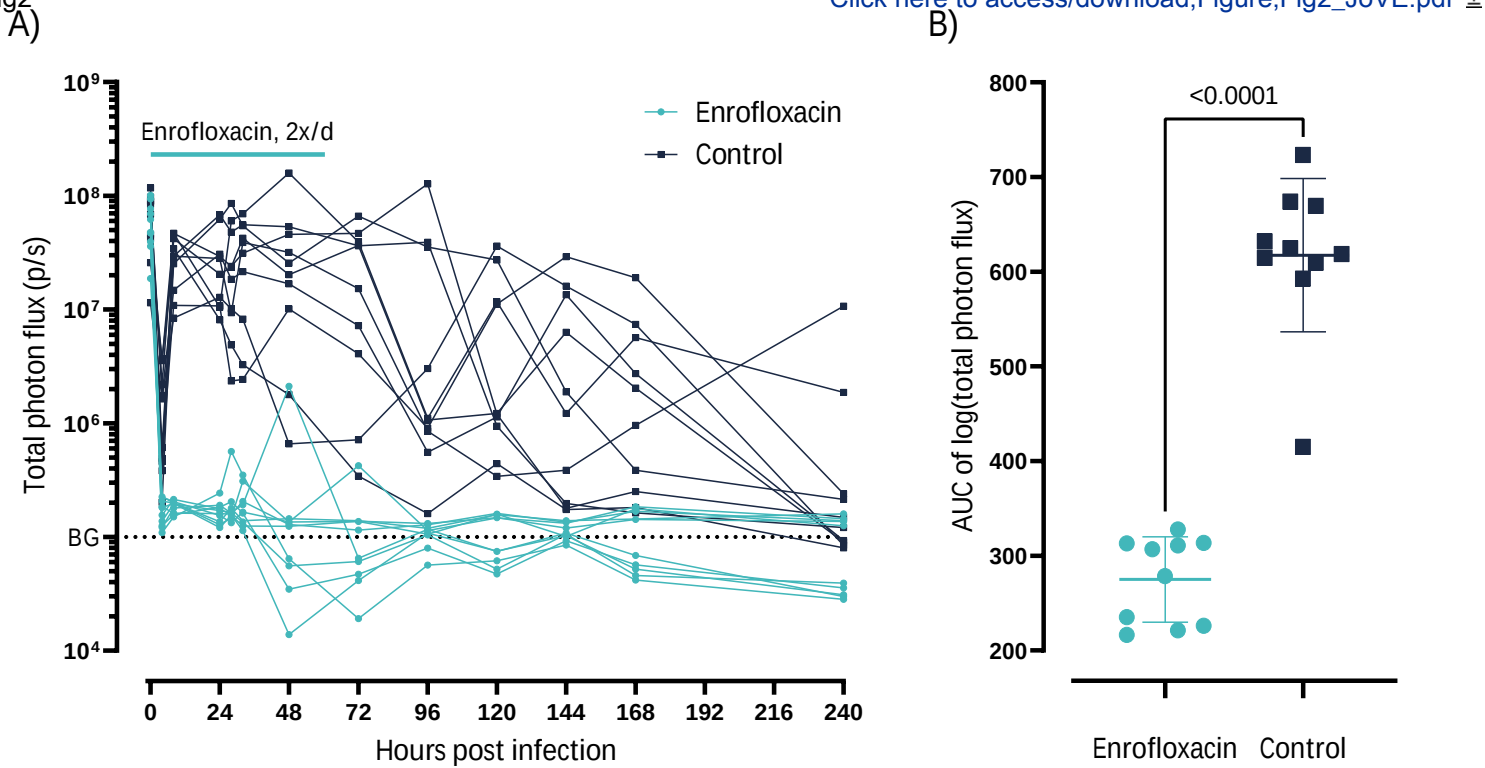

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_revised.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
96-well Black Flat Bottom Polystyrene Plate	Corning	3925	for *in vitro* imaging
Aesculap ISIS	Aesculap	GT421	hair trimmer, with GT608 cap
Anesthesia vaporizer	Harvard apparatus limited	N/A	https://www.harvardapparatus.com/harvard-apparatus-anesthetic-vaporizers.html
Baytril 100 mg/mL	Bayer	N/A	Enrofloxacin
BD Insyte Autoguard 24 GA	BD	382912	Yellow angiocatheter, use sterile plastic tip for instillation
C57Bl/6J mice	Janvier	N/A	
Centrifuge 5804R	Eppendorf	EP022628146	
Dropsense 16	Unchained Labs	Trinean	to measure OD 600nm
Dulbecco's Phosphate Buffered Saline, Gibco	ThermoFisher Scientific	REF 14040-083	
Ethanol 70% denaturated 5L	VWR international	85825360	
Falcon 14ml Round Bottom Polystyrene Tube, Snap-Cap	Corning	352057	
Falcon 50ml cellstart	Greiner	227285	
Hamilton GASTIGHT syringe, PTFE luer lock, 100 µL	Sigma-Aldrich	26203	to ensure slow bacterial instillation of 50 µL
Inoculation loop	Roth	6174.1	holder: Art. No. 6189.1
Iso-Vet 1000mg/g	Dechra Veterinary products	N/A	Isoflurane
IVIS Spectrum In Vivo Imaging System	PerkinElmer	REF 124262	imaging device
Kanamycine solution 50 mg/mL	Sigma-Aldrich	CAS 25389-94-0	
Living Imaging Software	PerkinElmer	N/A	BLI acquisition software, version 4.7.3
Luria Bertani Broth	Sigma-Aldrich	REF L3022	alternatively can be made
Luria Bertani Broth with agar	Sigma-Aldrich	REF L2897	alternatively can be made
Petri dish Sterilin 90mm	ThermoFisher Scientific	101VR20	to fill with LB agar supplemented with Km
Pyrex Culture flask 250 mL	Sigma-Aldrich	SLW1141/08-20EA	
Slide 200 Trinean	Unchained Labs	701-2007	to measure OD 600nm
UTI89-*lux*	N/A	N/A	Generous gift from Prof. Seed
Vortex	VWR international	444-1372	

Rebuttal Manuscript: Bioluminescence imaging for longitudinal follow-up of urinary tract infections and their treatment in mice.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

[Author response]: After implementing the requested changes, the manuscript was proofread thoroughly and remaining spelling or grammar issues were corrected.

2. Please provide an email address for each author.

[Author response]: The email addresses of all authors are as follows:

- Noémie Luyts: noemie.luyts@kuleuven.be

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- Wouter Everaerts: wouter.everaerts@kuleuven.be

3. Please define all abbreviations during the first-time use.

[Author response]: This was checked during proofreading and all abbreviations were defined at first-time occurrence in the text.

4. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

[Author response]: The text was revised and personal pronouns in the protocol were removed.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Falcon, VIS Spectrum In Vivo Imaging System, etc.

[Author response]: We thank the editors for pointing our attention to this. All commercial language was removed from the manuscript and replaced by generic terms if needed. However, step 3 of the protocol ("*Imaging"*) describes the use and specific settings for the IVIS apparatus and Living Image Software. We believe the commercial name of this device and software might be relevant to the reader since these instructions apply

to this specific device only and could be different for other devices. We adapted the text by adding generic names and providing the brand names between brackets. If the editors prefer not to use the brand names, these can be removed from the text so only the generic descriptions remain.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

[Author response]: We have checked the protocol and rewritten it in the imperative tense where needed.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

[Author response]: We have checked the protocol and added more details to address the 'how' question.

8. 2.1.3: do you anesthetize the animal before shaving? If yes how?

[Author response]: This was clarified in the text. We do not anesthetize the animals but restrain them during shaving. This is feasible if you have a colleague that is used to handling animals or if you are able to restrain the animals with one hand. Alternatively, animals can be anesthetized shortly using Isoflurane, but we prefer not to anesthetize the animals.

9. Is there a time gap between step 2 and 3?

[Author response]: There is no time gap between inoculation and imaging. Animals are imaged immediately before (to obtain a baseline) and after inoculation with the bacteria. This provides proof that they were effectively infected with the bioluminescent strain.

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please do not go beyond this. Please refrain from using bullets, alphabets, or dashes.

[Author response]: The numbering was revised as asked. Throughout the text, dashes were removed, and adequate numbering implemented.

11. Please include a single line space and ensure that the highlighted section is no more than 3 pages including headings and spacings. Please ensure that highlighted section forms a cohesive story.

[Author response]: The protocol section now has single line space, and the highlighted section is no more than 3 pages including headings and spacings. Most of the parts that were not highlighted give extra background or are steps in preparation of what we want to be featured in the video.

Reviewers' comments:

[Authors]: We thank the reviewers for dedicating their time and effort to thoroughly and critically review our manuscript. We appreciate their insightful comments and believe addressing them has contributed to an improved overall quality, relevance and readability of the manuscript. Please find below a step by-step response to all the reviewer's questions and suggestions. Within the manuscript, all changes are applied using "track changes".
A detailed overview of all major and minor changes is provided below.

Reviewer #1:

Manuscript Summary:
This manuscript describes pathogenesis of UTI caused by UPEC using a bioluminescent mouse model with Enrofloxacin as a control drug. The manuscript is well written and easy to follow for researchers using bioluminescence (BLI) to study bacterial infections. The major advantages of BLI over traditional methods were well articulated and limitations also highlighted.

[Author response]: We thank the reviewer for this supportive comment.

Major Concerns:
No major concerns

Minor Concerns:
1. Line 193: Please indicate the Living Image software version used.

[Author response]: The Living Image software version used is 4.7.3, this was clarified in the text.

2. Line 259: The authors wrote: "these animals were treated with Enrofloxacin 10 mg/kg twice daily for 3 days....". Please indicate the route and the times Enrofloxacin was administered post-infection.

[Author response]: The necessary details were added to the manuscript. The animals were treated with Enrofloxacin 10 mg/kg subcutaneously twice daily during the first 3 days post-infection.

3. Lines 256-270: Was total cfu enumerated at the end of the experiment using the traditional cfu count and compared to total flux to examine if they correlate; i.e. in case of biofilm formation as seen with chronic UTIs?

[Author response]: For these specific animals, CFUs of the inoculum were enumerated after instillation to establish the correlation between BLI signal and inoculum size, but no CFU were enumerated after the experiment so no correlation was made between the total flux and CFU at endpoint. As we discuss (lines 326-343 in the discussion), post-mortem CFU counts do not always correlate with BLI signals and we agree with your suggestion that the formation of biofilms could indeed be a factor to account for here. Here, the catheter was removed immediately after instillation, so no biofilm was formed. Other more dormant states (QIR or IBC) of bacteria were not measured in this experiment. We have nevertheless extended our discussion to further clarify the factors at endpoint, such as biofilm formation. that may influence the interpretation of bioluminescence signal versus CFU enumeration at endpoint in different situations such as acute vs chronic UTI. . It would certainly be useful follow-up research to explore the potential of BLI to discriminate bacteria in biofilms from planktonic cells (such as done for C. albicans by the use of specific promotors [1]and discriminate culturable from nonculturable bacterial sources of bioluminescence, as seen in biofilms. This potential presents another advantage of bioluminescence imaging over CFU enumeration.

4. Lines 326-343: With reference to limitations of BLI, please write a sentence or two on how the formation of biofilms can affect the accuracy of photon measurements. I adittion, please discuss the effect that the viable but nonculturable (VBNC) state can have on BLI measurements

[Author response]: We thank the reviewer for this interesting comment. We have found that bioluminescence imaging is an attractive alternative to follow the evolution of biofilms longitudinally. Caution is warranted but BLI has the great advantage that the biofilm does not need to be disrupted to be enumerated and can therefore facilitate research. For VBNC states, similar remarks can be made (revised manuscript, discussion limitations). The bacteria that enter this state remain viable and metabolically active and will therefore emit photons, yet they are not enumerated when cultured on standard media. We are open to discuss this issue further with the reviewer.

Reviewer #2:

Manuscript Summary:

This manuscript describes the protocol for urinary track infections (UTI) in mouse model through bioluminescence imaging. In vivo imaging of mice showed that the bioluminescent bacteria could establish persistent and antibiotic resistant infections. Although the text and data are clearly organized, and the results evaluated are interesting, there are some recommendations for the manuscript that should be addressed.

[Author response]: We thank the reviewer for the supportive comments and insightful recommendations. We have addressed these as explained below.

Major Concerns:

1. The title of manuscript needs modification, "Bioluminescence imaging of urinary tract infections and their treatment for longitudinal follow-up in mice"

[Author response]: We have adapted the title.

2. Line 300, "multiple times a day" a day does not seem to fit since mice require anesthesia during each imaging process and multiple administration of anesthesia could be extremely stressful for mice.

[Author response]: We fully agree that minimizing stress caused by repeated imaging sessions is a very important point of attention that was lacking in the original manuscript. We have adapted the manuscript by giving a maximal amount of imaging sessions (2-3 times daily). To minimize stress, the mice are anesthetized very briefly, less than 10 minutes per imaging session, with a low dose of isoflurane, which has a quickly reversible effect. The remaining stress is minimal, but not absent. However, imaging the animals multiple times allows detailed insights in the early development of the infection and further longitudinal follow-up without sacrificing animals at each time point. We believe this major advantage of greatly reducing the total number of animals needed, outweighs the stress induced by repeated isoflurane anesthesia.

3. Line 203, it would be better to set first exposure time at "auto" and then define proper time period. The imaging first at auto will provide image, which may be then easily optimised to define imaging exposure time.

[Author response]: We thank the reviewer for this valuable suggestion. This was added in the text.

4. Line 326, "limitations of method", I would suggest to include the mention of advanced and expensive imaging tools are required for BLI. Moreover, BLI requires the generation of genetically luminescent bacteria (UTI89-lux). It may be expected that such genetically modified bacteria become mild or show different responses to injected antibiotics compared to same species as wild type.

[Author response]: We have adapted the manuscript. We agree that more expensive tools are necessary but collaborations with imaging cores at universities can circumvent this problem. Regarding the second comment, we support researchers to control the virulence after insertion of the lux-operon. However, recent advances in genome editing allow for efficient and scarless tagging of all major bacterial pathogens, thereby limiting the impact on virulence or treatment responses.

5. Fig. 1A, application of 2.108 CFU seems extremely high dose for the mice to survive more than 24 hours. I am not convinced if such a high dose is necessarily required at the beginning to monitor the course of infection or antibacterial therapy.

[Author response]: This is indeed a high dose; however, in literature doses of up to 10^9 or even 10^10 CFU per instillation are described. In our hands, none of the animals died when using 10^8 CFU. The specific reason to use this high concentration in the experiment depicted in figure 1 was to investigate the upper detection limit of the technique. The curve was still linear at this dose and saturation was not a problem. For figure 2 and all other *in vivo* experiments, a lower concentration of 10^7 CFU was used.

Minor Concerns:
Abstract line 45, "A step-by-step guide is provided", please place "is provided" at the end of sentence

[Author response]: This was adjusted in the manuscript.

References

1 Vande Velde, G., Kucharikova, S., Schrevens, S., Himmelreich, U. & Van Dijck, P. Towards non-invasive monitoring of pathogen-host interactions during Candida albicans biofilm formation using in vivo bioluminescence. Cellular Microbiology. 16 (1), 115-130, (2014).