

Video Article

4D Multimodality Imaging of *Citrobacter rodentium* Infections in Mice

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Abstract

This protocol outlines the steps required to longitudinally monitor a bioluminescent bacterial infection using composite 3D diffuse light imaging tomography with integrated μ CT (DLIT- μ CT) and the subsequent use of this data to generate a four dimensional (4D) movie of the infection cycle. To develop the 4D infection movies and to validate the DLIT- μ CT imaging for bacterial infection studies using an IVIS Spectrum CT, we used infection with bioluminescent *C. rodentium*, which causes self-limiting colitis in mice. In this protocol, we outline the infection of mice with bioluminescent *C. rodentium* and non-invasive monitoring of colonization by daily DLIT- μ CT imaging and bacterial enumeration from feces for 8 days.

The use of the IVIS Spectrum CT facilitates seamless co-registration of optical and μ CT scans using a single imaging platform. The low dose μ CT modality enables the imaging of mice at multiple time points during infection, providing detailed anatomical localization of bioluminescent bacterial foci in 3D without causing artifacts from the cumulative radiation. Importantly, the 4D movies of infected mice provide a powerful analytical tool to monitor bacterial colonization dynamics *in vivo*.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50450/>

Introduction

Small animal models, in particular those utilizing mice, are routinely used to investigate bacterial pathogenesis or to test intervention strategies for infections, such as antibiotics, probiotics, prebiotics and vaccines¹⁻⁷. The main experimental readouts from small animal infections are pathogen load, spatial and temporal localization of the infection, and changes to the immune response of the infected organism. *In vivo* optical imaging is a valuable tool for infectious disease research and can be used to monitor multiple experimental readouts through the use of reporter genes (luciferase, fluorescent proteins, beta-lactamase, etc), fluorescent dyes, nanoparticles or chemiluminescent probes targeted to a protein, biological process, or microorganism⁶.

Bioluminescence imaging (BLI) is an optical imaging modality used to monitor the colonization of small animals, such as mice and rats, by pathogenic bacteria^{3,6,8,9}. Mice are infected with recombinant bacteria expressing a luciferase, such as the *luxCDABE* operon from *Photobacterium luminescens*. These bacteria can then be detected through their light production using a CCD based *in vivo* imaging system^{3,6,9}. Importantly, only metabolically active micro-organisms are bioluminescent (BL), meaning only viable bacterial cells are detected by this methodology^{10,11}. Using 2D BLI, the location of the BL source is inferred from the surface of the animal where the signal is emitted⁸. The exact anatomical localization of the BL foci *in vivo* has to be determined through the *ex vivo* analysis of organs^{3,6,9}. In contrast, composite 3D diffuse light imaging tomography (DLIT) can be used to compile a quantitative 3D reconstruction of the BL source¹². DLIT is performed by collecting BL images taken using defined narrow band-pass optical filters and subsequently inputting them into a diffuse optical tomography 3D reconstruction algorithm^{1,7,12,13}.

Currently, multi-modality imaging is the only methodology available to get true non-invasive anatomical localization of bioluminescent foci *in vivo* without the need for *ex vivo* analysis. Recently, we used a combination of DLIT co-registered with μ CT imaging to evaluate *Citrobacter rodentium* (*C. rodentium*) colonization dynamics following prophylactic treatment with a probiotic bacterium⁷. *C. rodentium* is a murine specific enteric pathogen used to model human infection with enteropathogenic and enterohemorrhagic *Escherichia coli*¹⁴. *C. rodentium* infection causes colitis, typically associated with mild weight loss, diarrhea, polarized Th1 immune response and distinct pathological changes, including colonic crypt hyperplasia and attaching and effacing lesion formation¹⁴. In addition to this, *C. rodentium* pathogenesis has been thoroughly studied using BLI and its colonization dynamics in C57BL/6J mice are well documented, making this bacterium an ideal model microorganism for use with multi-modality imaging^{3,4,7}.

This protocol is the first to outline a methodology for integrated DLIT- μ CT imaging of a bacterial infection using a single multimodality imaging platform, the IVIS Spectrum CT, and the generation of a 4D movie showing the true dynamics of this infection non-invasively.

Protocol

1. Mice Preparation

1. Purchase or breed enough 18-20 g C57BL/6J mice required for the study.
2. If mice are purchased from an external supplier, following transport to the facility, habituate mice for 1 week on autoclaved food and water to stabilize the gut microbiota.
3. Weigh, ear notch, and separate the required number of mice per cage.
4. The day prior to infection, before performing any anesthesia, check that there is adequate oxygen and isoflurane for the duration of the procedure. If necessary, add more isoflurane or replace the oxygen cylinder. Subsequently, check the weight of the anaesthetic scavengers, if they have gained more than 50 g since their first use, discard them and replace with fresh scavengers.
5. Anaesthetise mice using 3% isoflurane in the XGI-8 Anaesthesia System and depilate using VEET for 7 min.
6. **Notes:**
 - Thorough depilation is essential, especially in black mice as melanin in the fur significantly attenuates the bioluminescent signal^{3,15}.
 - Depilation of mice usually lasts 8-10 days before the fur starts to regrow. To perform 4D multimodality imaging over longer periods, depilate mice when required so that the area of interest is nude for the imaging sessions.
 - Mice are housed in a Biosafety Level 2 (BSL-2) containment facility.

2. Bacterial Cell Preparation

1. Prepare enough Luria Burtani broth and agar plates supplemented with Kanamycin [$50 \mu\text{g ml}^{-1}$] required for the study and store at 4°C until required.
2. The day before the infection thaw a cryovial of *C. rodentium* strain ICC180 and immediately add one cryobead to 15 ml LB broth supplemented with Kanamycin [$50 \mu\text{g ml}^{-1}$] and culture overnight at 220 rpm, 37°C . As a control for medium contamination, prepare an additional uninoculated 15 ml LB broth supplemented with Kanamycin [$50 \mu\text{g ml}^{-1}$] and culture at 220 rpm, 37°C .
3. The next morning (~16 hr), check the medium control for turbidity as a sign of bacterial growth. If the control is clear, transfer the ICC180 culture to a falcon tube and centrifuge at 4,000 rpm. Subsequently, wash the bacterial pellet in PBS. Then resuspend in 1.5 ml PBS (1/10th of the original volume of LB supplemented with Kanamycin) and mix thoroughly to create the bacterial inoculum.
4. To verify that ICC180 has been cultured properly and is bioluminescent prior to infection of the mice, image the inoculum in the IVIS Spectrum CT using BLI and the auto setting in the Living Image 4.3.1 wizard as described previously¹.

Note:

 - Strain ICC180 is a bioluminescent derivative of wild type *C. rodentium* ICC169². This bacterial strain has the *luxCDABE* operon including a Kanamycin resistance gene inserted into a pseudogene of *xyIE* in the chromosome².
 - Carry out all bacterial culturing using standard aseptic techniques and if required either purchase sterile consumables/reagents or autoclave prior to use.
 - In step 2.4, the bioluminescence reading (p/s/cm²/sr) can be used to estimate bacterial numbers prior to the infection of mice.

3. Infection of Mice with Bioluminescent *C. rodentium* and Assessment of Bacterial Colonization

1. Prior to infection, check that there is adequate oxygen and isoflurane for the duration of the procedure. If necessary, add more isoflurane or replace the oxygen cylinder. Subsequently, check the weight of the anaesthetic scavengers, if they have gained more than 50 g since their first use, discard them and replace with fresh scavengers.
2. Anaesthetise mice with 3% isoflurane using an XGI-8 Anaesthesia System to provide humane restraint.
3. Mix the bacterial inoculum thoroughly and draw 0.2 ml (approximately 5×10^9 cfu ICC180) into the syringe.
4. Remove mice from the anesthesia system one at a time and scruff them by firmly grasping the skin behind the neck with your thumb and forefinger.
5. Push the gavage needle towards the roof of the mouth, over the tongue, and down the esophagus and inject the bacterial inoculum into the stomach.
6. Following oral gavage of the mice, monitor their gait and breathing for signs of distress which can be a sign that the bacterial inoculum has been delivered into the lungs. Euthanize any mice that have been inoculated in the lungs.
7. Gavage uninfected mice with 200 μl PBS as a vehicle control, as described in steps 3.1-3.6.
8. To confirm that the oral gavage was performed correctly, image infected and mock infected mice in the IVIS Spectrum CT using BLI and the auto setting in the Living Image 4.3.1 wizard as described previously¹.
9. Determine the numbers of viable bacteria in the inoculum by serial dilution in PBS and spotting in triplicate onto LB agar supplemented with Kanamycin [$50 \mu\text{g ml}^{-1}$].
10. Calculate the number of ICC180 cfu/ml by finding a dilution where there are approximately 5-50 colonies and count the number of colonies from each spot at that dilution and record the dilution that the colonies were counted on. Subsequently, calculate the mean number of cfu (from the three spots) and multiply this value by the dilution factor 50 (each spot is 20 μl of the original 1 ml sample) and the dilution the colonies were counted on, giving cfu/ml.
11. Monitor colonization of the mice daily by collecting feces from each mouse into a pre-weighed Eppendorf tube (weighed on a fine balance) and dilute 1/10 in PBS based on the weight of the sample (0.1 g ml^{-1}).
12. Determine the numbers of viable bacteria in the feces by serial dilution in PBS and spotting in triplicate onto LB agar supplemented with Kanamycin [$50 \mu\text{g ml}^{-1}$].

13. Calculate the number of viable bacteria per gram of feces by following step 3.9, then multiply the number of cfu/ml by the faecal dilution factor in PBS (step 3.10) of 0.1 g ml^{-1} to give cfu/g.
14. To determine that mice were inoculated with a pure ICC180 culture and that all of the colonies are bioluminescent. Take the agar plates from Step 3.8, assess the bacterial colony morphology either by eye or pick a representative colony from the plate and analyze by light microscopy. If necessary, bacterial identification can be performed by strain specific colony PCR to assess culture purity. Subsequently, image the plates in the IVIS Spectrum CT using BLI as described in step 3.7 and check that 100% of the colonies on the plate are bioluminescent.

Notes:

- In step 3.4, when inserting the gavage needle, if you feel resistance do not force the gavage needle, as this causes the inoculum to go into the lungs. Instead, re-insert the needle and gently try again.
- Step 3.1 is optional and mice can be oral gavaged without anesthesia depending on the institutes animal welfare policy.
- Stools must be plated out the day that they are collected. *C. rodentium* will grow even if left at 4 °C overnight in PBS and will cause the quantification of cfu/g stools to be incorrect.

4. Daily Composite 3D Diffuse Light Imaging Tomography with μ CT Imaging (DLIT- μ CT) of Infected Mice

Animal welfare considerations: DLIT- μ CT involves DLIT optical imaging integrated with a fast, low radiation dose μ CT scan (~23 mGY for a two mouse scan, ~53 mGY for a single mouse scan) of an immobilized animal. This dose accumulates with each imaging session, so the aim is keep the dose as low as possible (and always well below the $LD_{50/30}$) while still accomplishing the study. In some cases, if there is no sign of infection in a conventional BLI scan of mice, the μ CT scan can be avoided to further minimize dose. Even though dose is kept as low as possible, in prolonged studies if there is any concern about radiation exposure, mice will be culled at the first sign of detrimental symptoms or at the end of the μ CT imaging period.

1. Prior to imaging, initialize the IVIS Spectrum CT and check that the X-ray safety interlocks are working and that the heated stage is at the correct temperature (37 °C) for imaging. Subsequently, check the level of isoflourane in the anesthesia system and the weight of the anaesthetic scavengers. If necessary, add more isoflourane and if the scavengers have gained more than 50 g since their first use, discard them and replace with fresh scavengers.
2. Set up the Spectrum CT so that the imaging parameters that run the automatic exposure feature in Living Image 4.3.1 are optimized for imaging bacterial luciferase. Select Edit > Preferences > Acquisition and Auto Exposure Window. Then select > Range Values > Exp. Time (seconds) and set the > Max. to 300s. Finally, select > Target Count (minimum) > Luminescent and set to 10,000 counts.
3. Insert the one mouse imaging platform into the Spectrum CT and plumb it into the anesthesia.
4. Switch on the X-ray safety interlock and initialize the IVIS.
5. Once the IVIS Spectrum CT has been initialized, anaesthetise the mice with 3% isoflourane using an XGI-8 Anaesthesia System to provide humane restraint.
6. To determine whether it's necessary to perform a DLIT- μ CT scan, pre-image the mice using BLI as described previously¹, in the one mouse imaging platform. If a robust signal is obtained the mouse is suitable for DLIT imaging. Leave the anaesthetised mouse in the one mouse imaging platform after the BLI scan ready for DLIT- μ CT.
7. Use the Imaging Wizard in the Living Image software 4.3.1 to set-up the DLIT- μ CT scan. The Imaging Wizard automatically determines the FOV, exposure time, F-stop and binning for the DLIT and the FOV, exposure time, filter sets and binning for the μ CT imaging to give the best signal to noise ratio. When prompted by the Imaging Wizard, include only the 560, 580, 600, and 620 nm emission filters and select the one mouse μ CT scan. Then click > Acquire to start the imaging.
8. Return the anaesthetised mouse to its cage after imaging, remove the one mouse imaging platform from the Spectrum CT and disinfect using 1% Tri-gene. If needed, replace the foam pad that the mouse is positioned on to minimize the risk of cross-contamination between mice.
9. Image mice daily up to day 8 post infection (P.I.) using DLIT- μ CT, as described in steps 4.1-4.8, to generate the longitudinal imaging data required to make a 4D movie of infection.

Note:

- In step 4.7, we recommend the use of the 560-620 nm emission filters to image bacterial bioluminescence. Although the peak of bacterial bioluminescence is around 485 nm, due to tissue optics (the significant attenuation of blue/green photons by murine tissue), it is important to use the orange/red photons for 3D reconstructions as they facilitate the calculation of signal depth.
- The 'auto' exposure settings in Living Image 4.3.1 for DLIT need to be adjusted to optimize the amount of photons captured and the maximum imaging time used for each filter set.
- It is only necessary to perform the BLI pre-imaging described in step 4.6 when the researcher is not sure if there is a detectable bioluminescent signal, for example when using a new infection model.

5. 3D Reconstruction of DLIT- μ CT Imaging Data

1. Open Living Image software 4.3.1 and select > Browse and open the folder containing the DLIT- μ CT files.
2. Open the DLIT- μ CT file in the Living Image Browser, for example *C. rodentium* Day 1 P.I. by clicking Load.
3. In the Tool Palette select > Surface Topography > Nude mouse, adjust the threshold, and then click > Generate Surface. If the surface reconstruction is successful a surface outline will be displayed in the 3D view window.
4. To view the rendered μ CT scan in the 3D view window, hide the surface outline (Click > 3D optical tools and unselect > Display Surface Object), or decrease the surface opacity (Click > 3D optical tools and adjust the > opacity slider bar).
5. To provide detailed anatomical localization of the 3D DLIT reconstruction it is necessary to alter the 3D volumetric data (μ CT scan) so that only the mouse's skeleton is visible and that it has the optimum contrast. Click > 3D Multimodality tools and select > Logarithmic Histogram,

- Gradient Illumination, Quality and Denoise. Finally, adjust the Histogram slider to the right and watch the 3D volumetric data change contrast. Keep adjusting the data until only the bones are clearly visible. If desired, change the color for the transfer function points on the histogram.
- Next select > DLIT 3D Reconstruction in the Tool Palette and make sure that only the 560, 580, 600, and 620 nm filter sets, which are required for imaging bacterial luciferase *in vivo*, have been selected. Click > Start. The Data Preview Window menu will now appear showing the spectrally filtered BL signals from the mouse that was imaged in 2D. These signals are automatically thresholded by the software, but can be adjusted manually if necessary using tabs at the bottom of the menu. The threshold determines the minimum data intensity (represented as a percentage of the maximum signal in the image) which will be included as data in the reconstruction.
 - Next select > DLIT 3D Reconstruction in the Tool Palette and check that the optical properties used for the DLIT reconstruction are correct. Select > Properties tab and make sure that the settings for Tissue Properties is set to mouse tissue, Source Spectrum is set to Bacteria.
 - Click > Reconstruct to perform the DLIT reconstruction.
 - To generate the DLIT- μ CT 3D movie click > Tools > 3D animation and select > Preset Animations > CCW Y-axis and > Total Duration > 10 sec (25 frames per second). Once the settings for the 3D animation are correct press > Record and save the DLIT- μ CT 3D reconstructions as .avi files labeled with experiment, group and time point in a separate folder.

Note:

- It is important when performing 3D reconstructions of DLIT- μ CT data to use a PC with Living Image 4.3.1 and service package 1 installed and that the PC has a graphics card suitable for running the Multi-modality imaging software. This software can be downloaded, and GPU specifications are contained in the release notes (<http://www.caliperls.com/support/software-downloads.htm>).
- If the DLIT- μ CT 3D reconstructions appear backwards after being saved, your computer is running an old version of its graphics driver and this can be rectified by downloading an update from the manufacturers' website.
- In step 5, we present our preferred settings for generating 3D DLIT- μ CT reconstructions. However, there are multiple points during the 3D reconstructions where parameters that are not discussed in this protocol can be modified. For a comprehensive guide to 3D reconstructions using Living Image 4.3.1 please refer to the manufacturers' instruction manual.
- It is important to create the 3D reconstructions using identical settings in the multi-modality imaging tool so that they are comparable. In addition to this, when creating the DLIT- μ CT 3D reconstructions, it's important to make the videos using the same number of frames per second and total duration (length), otherwise the timing of the 4D video is not homogenous.

6. Generation of a 4D Movie of *C. rodentium* Infection

- Clearly label all of the DLIT- μ CT 3D reconstructions with the correct experiment, time point and group in an easily accessible folder.
- Create a new file in Windows Live Movie maker and insert the DLIT- μ CT reconstructions in chronological order from day 1 P.I. using the tools tab from the folder prepared in step 6.1.
- Add captions to the start of every video describing the time point, for example Day 1 P.I. by selecting the appropriate DLIT- μ CT video and then selecting Home > Add Caption. A text box appears on the screen, where the appropriate legend is added. Adjust the Font size, style, color, and justification as desired using identical tabs to Microsoft word in the software toolbar. Finally, move the caption to the top left hand corner of the video.
- Repeat step 7.3 for all of the DLIT- μ CT videos.
- Add a title page, for example *C. rodentium* infection days 1-8 by clicking Home > Title. A text box appears on the screen where the appropriate title is added to a blank slide. Adjust the Font size, style, color, and justification as desired using identical tabs to Microsoft word in the software toolbar.
- Save the project as a movie maker project *.mmp in an appropriate folder with the title of the movie. This step is essential if you want to amend the movie.
- Save the movie as a .wmv file. Click Movie maker menu > Save movie > for computer.
- Convert the movie maker file using a file converter to .avi or another suitable file type compatible with your computer.

Notes:

- It is recommended that the 4D movies are generated on a PC with Windows Live Movie Maker installed (<http://windows.microsoft.com/en-GB/windows7/products/features/movie-maker>) and a good multi-media program capable of playing .wmv or .avi files types.

Representative Results

Infection of C57BL/6J mice with 5×10^9 cfu *C. rodentium* is a well described bacterial infection model and results in a self-limiting gastrointestinal infection that peaks between days 6-8 post infection and lasts between 3 to 4 weeks^{2,14}. The infection is confined to the intestinal lumen by the murine immune system and as a consequence, bacteria are continually shed in the feces. Colonization of mice by *C. rodentium* can be monitored non-invasively by direct bacterial enumeration from feces, or bioluminescence imaging^{2,3}.

This manuscript outlines an optimized protocol for performing 3D and 4D multimodality imaging of *C. rodentium* within mice to monitor bacterial load and localization during infection. The results presented in **Figure 1** demonstrate the controls that are required for successful 4D imaging. Prior to the infection of mice it is essential to determine that the bacterial inoculum used is bioluminescent (**Figure 1A**) and that following the oral gavage of a mouse with bioluminescent *C. rodentium*, that the signal can be observed in the stomach of the animal and not in the lungs (**Figure 1B**)¹⁶.

In addition to monitoring bacterial colonization using bioluminescence imaging, it's good practice to quantify bacterial numbers in feces; which is used as an indirect measurement of bacterial colonization of the gastrointestinal mucosa. **Figure 2** demonstrates the typical bacterial load in feces taken from 8 C57BL6/J mice that have been infected with $\sim 5 \times 10^9$ cfu ICC180 and monitored for 8 days P.I. Colonization increases from day 2 P.I. until day 6-7 where the infection peaks at $\sim 5 \times 10^{10}$ cfu/g, which is in line with previous reports^{2,3,17}.

To emphasize the importance of evaluating the spread of infection in a single mouse, **Figures 1b** and **3** as well as **Video 1** have been generated from the same mouse following *C. rodentium* infection, as described above. Daily DLIT- μ CT was used to evaluate the spatial distribution of

bioluminescent bacteria within this mouse, using the skeleton as an anatomical reference. **Figure 3** demonstrates the DLIT- μ CT reconstruction of a C57BL/6J mouse infected with ICC180 at 3 key time points P.I. At day 3 P.I. small bioluminescent foci can be observed in the colon, which exhibit a moderate increase in bioluminescence intensity by day 5 P.I. with little change in spatial distribution. At day 7 P.I. we observed a significant increase in bioluminescence and the bioluminescent foci spread across the entire colon.

Video 1 is a compilation of 3D DLIT- μ CT reconstructions from days 1-8 P.I. with ICC180 and illustrates *C. rodentium* within the proximal gastrointestinal tract between days 1-2 P.I. which spreads to the colon at day 3 P.I. From days 4-6 P.I. the bacterial numbers in the colon expand until peaking at day 7 P.I. where the bacterial foci covers the entire colon. At day 8 P.I. only two distinct bacterial foci are present in the proximal and distal colon. The ability to view the full 3D reconstruction at each time point in chronological order represents a powerful tool to analyse host pathogen interactions and is easier to interpret than a 3D still of the same dataset.

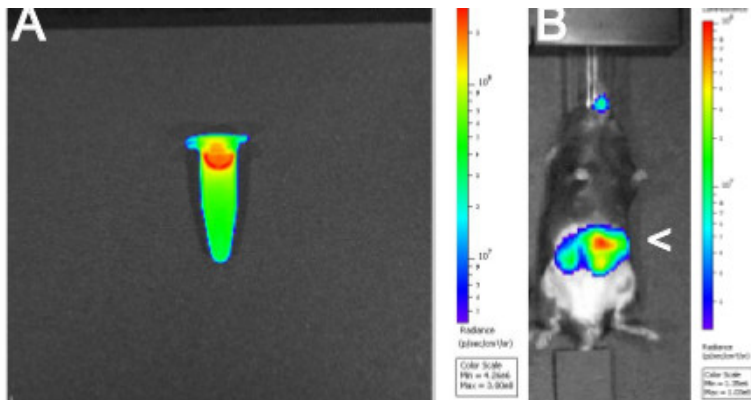


Figure 1. 2D bioluminescence imaging of **A)** Bioluminescent *C. rodentium* ICC180 inoculum and **B)** a mouse following oral gavage with 200 μ l of the inoculum. Arrowhead (\rightarrow) illustrates bioluminescent *C. rodentium* in the stomach.

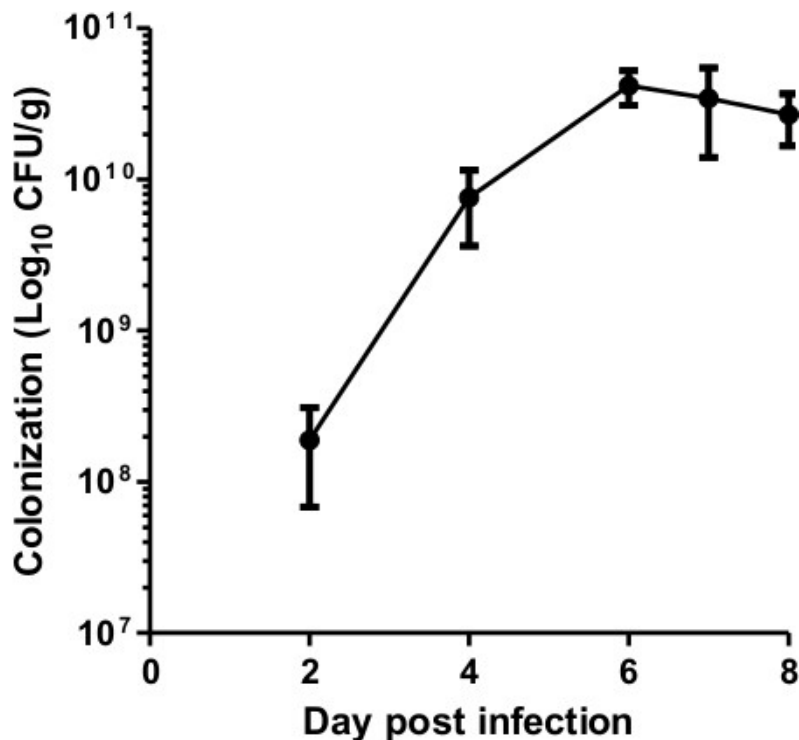


Figure 2. *C. rodentium* colonization dynamics. Quantification of *C. rodentium* colony forming units from feces for 8 days P.I.

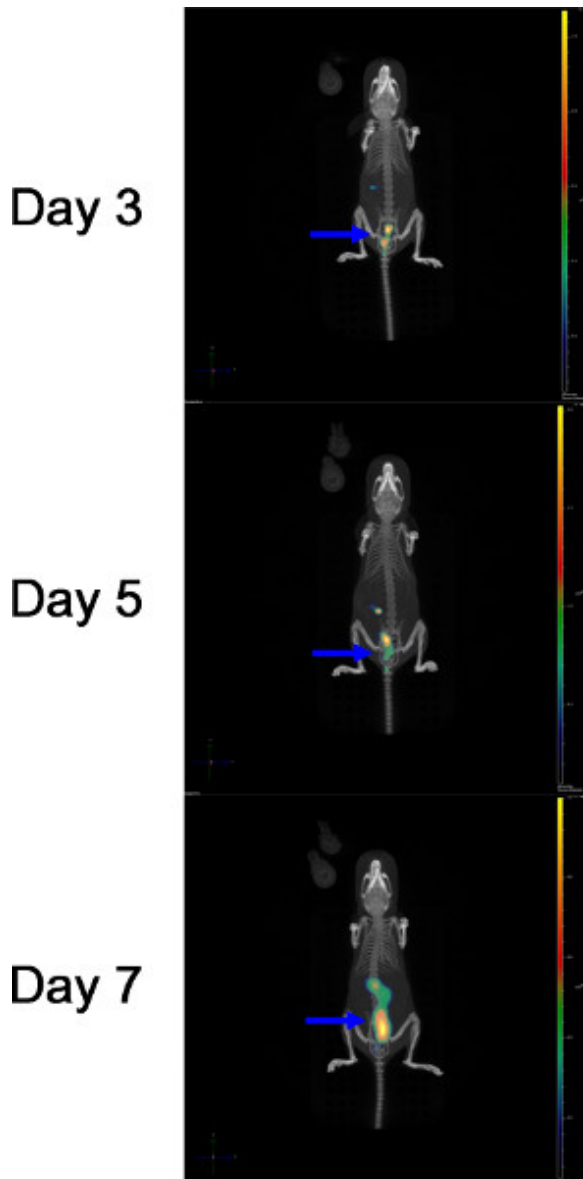


Figure 3. Diffuse light imaging tomography- μ CT scan of bioluminescent *C. rodentium* infection from one mouse monitored at day 3, 5 and 7 P.I. Arrowhead (>) illustrates colonic colonization. [Click here to view larger figure.](#)

Video 1. [Click here to view video.](#) 4D movie of *C. rodentium* infection from one mouse monitored from days 1-8 P.I.

Discussion

The 4D movie of bacterial infection provides a useful tool to visualize and interpret large amounts of multi-modality imaging data quickly and easily. This technique facilitates the detailed analysis of how an infection spreads through an individual mouse and can be used to investigate how deletion of host or bacterial genes or particular intervention strategies effect bacterial load, distribution, and localization during a longitudinal study⁷. These videos also provide useful teaching aids and a means of disseminating information to the public.

There are several critical steps in this protocol that could affect the quality of the data obtained from DLIT- μ CT imaging and the ability to compile a 4D video of infection. The most important part of this protocol is the successful and homogenous infection of mice with *C. rodentium*. It is essential that the mice used for the study are between 18-20 g and that the bacterial inoculums are freshly prepared and approximately 5×10^9 cfu, as described previously^{2,3}. Prior to infection of the mice it is important to check that the inoculum is bioluminescent using the Spectrum CT and once the inoculum has been prepared, it must be continually homogenized before each mouse is gavaged to ensure that the mice receive similar infectious doses. The DLIT- μ CT imaging of mice has been optimized so that the auto exposure function in Living Image 4.3.1 software automatically determines the optimized imaging parameters for the signal to be well above the noise. However, the auto exposure function relies on user defined settings and parameters which need to be modified as described in the procedure. Failure to do this will result in poor images with a low number of photons collected that do not result in an obvious progression in the infection, as the Spectrum CT's factory settings for

autoexposure are programmed for imaging tumors expressing firefly luciferase. Reconstructions performed using 560-620 nm give the best agreement between simulated and measured data and, therefore, are the more reliable data to include in the reconstruction.

A limitation to the use of DLIT- μ CT is that ionizing radiation from the μ CT scan causes sub-lethal radiation damage that is cumulative over a longitudinal study¹⁸. Sub-lethal radiation exposure can weaken the immune response, cause DNA damage, and apoptosis in internal organs¹⁹. Ultimately, cumulative sub-lethal radiation damage can cause death if the LD₅₀/30 for ionizing radiation is exceeded, which is between 5 to 7 Gy depending on the mouse strain and age of the mice used^{18,20,21}. Although some of the molecular damage from ionizing radiation can heal, since the overarching principle is to estimate dose conservatively, this is not typically accounted for in study planning. Instead, the aim is to stay as far below these limits as possible while still accomplishing the study goals. This is particularly important in this study because of the normal immune response to the infection, the frequency of imaging, and the fact that transgenic, immuno-compromised, or heavily infected animals may be more susceptible to ionizing radiation.

When planning the experiment to generate a 4D movie of infection, it is important to consider the length of the experiment, the number of μ CT scans required during this period and the LD₅₀/30 for ionizing radiation for the mouse strain being used. Another potential limitation to DLIT- μ CT is the strength of the reporter expression within the bacterial strain being used, as this will affect bacterial detection limits and imaging times. It is highly recommended that researchers use validated bacterial strains that are fully virulent, but optimized for maximal *lux* operon expression, as demonstrated previously for BLI^{2,3}.

One caveat to the current design of the 4D imaging is that each movie is comprised of individual DLIT- μ CT scans which have different photon scaling. This can make the images difficult to interpret if the changes to the localization of the BL foci, or its intensity are subtle, or if there is one intense BL focus surrounded by multiple weak foci. Therefore, for longitudinal visualizations, it is important to keep the color bars consistent across the time points.

The concept of a 4D movie of infection can be applied to any suitably labeled bacterial pathogen. Future development of this technique will aim to use fluorescence imaging tomography (FLIT) as well as DLIT to facilitate the investigation of host responses to infection using a combination of bioluminescent bacterial pathogens and injectable fluorescent near infrared probes to investigate host responses to infection. In addition to this, in this protocol we only describe the use of bioluminescent bacteria to create 4D movies of infection. However, in some instances it may be necessary to use fluorescent labeled bacteria, for example tagged with iRFP, so that the bioluminescence reporter can be used for investigating host genetics during infection. Importantly, the use of multi-modality imaging combining DLIT/FLIT- μ CT will allow us to non-invasively investigate multiple parameters during a bacterial infection, which will contribute significantly to the reduction, refinement, and replacement of the use of animals in scientific research as outlined in the NC3R's initiative (<http://www.nc3rs.org.uk/>).

Disclosures

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