

Video Article

Imaging *Mycobacterium tuberculosis* in Mice with Reporter Enzyme Fluorescence

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Abstract

Reporter enzyme fluorescence (REF) utilizes substrates that are specific for enzymes present in target organisms of interest for imaging or detection by fluorescence or bioluminescence. We utilize BlaC, an enzyme expressed constitutively by all *M. tuberculosis* strains. REF allows rapid quantification of bacteria in lungs of infected mice. The same group of mice can be imaged at many time points, greatly reducing costs, enumerating bacteria more quickly, allowing novel observations in host-pathogen interactions, and increasing statistical power, since more animals per group are readily maintained. REF is extremely sensitive due to the catalytic nature of the BlaC enzymatic reporter and specific due to the custom fluorescence resonance energy transfer (FRET) or fluorogenic substrates used. REF does not require recombinant strains, ensuring normal host-pathogen interactions. We describe the imaging of *M. tuberculosis* infection using a FRET substrate with maximal emission at 800 nm. The wavelength of the substrate allows sensitive deep tissue imaging in mammals. We will outline aerosol infection of mice with *M. tuberculosis*, anesthesia of mice, administration of the REF substrate, and optical imaging. This method has been successfully applied to evaluating host-pathogen interactions and efficacy of antibiotics targeting *M. tuberculosis*.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56801/>

Introduction

The slow growth rate of *M. tuberculosis* is a major roadblock in rapid diagnosis of tuberculosis^{1,2,3}. While culture based diagnosis takes weeks to produce results, acid-fast smear has diagnostic limitations⁴ in children⁵ and patients co-infected with human immunodeficiency virus^{6,7}. Optical imaging technologies have recently been recognized as an alternative to traditional diagnostic methods for tuberculosis^{8,9}. Fluorescence and bioluminescence can be used to optically image *M. tuberculosis* in live animals in real-time^{10,11,12,13,14,15,16,17,18,19}. Optical imaging has the benefit of a rapid and specific assessment of an infection with *M. tuberculosis*^{20,21,22}.

We outline details for optical imaging of *M. tuberculosis* in live mice using REF. This method is very specific and sensitive^{23,24} and, similar to other optical methods, is less expensive than other methods of tuberculosis (TB) imaging²⁵, including computed tomography (CT)²⁶, magnetic resonance imaging (MRI)²⁷, and F-fluorodeoxyglucose positron emission tomography/CT (F-FDG PET/CT)²⁸. REF utilizes custom fluorescent or bioluminescent substrates that upon cleavage by a bacterial enzyme, produce a fluorescent product^{8,29}. Hence, it has the advantage of not requiring a recombinant mycobacterial reporter strain^{30,31}. The FRET substrate described is comprised of a fluorochrome and a quencher connected by a β -lactam ring that is hydrolyzed by BlaC (β -lactamase), naturally constitutively expressed by tuberculosis-complex *Mycobacterium*^{8,32}. The bacteria directly generate signal due to REF catalytic activity that allows amplification by many orders of magnitude and sensitive detection of *M. tuberculosis*.

The REF substrate used in this study has excellent tissue penetration in live animals and reduced background due to its long wavelength. With this long-wavelength substrate it is possible to achieve a threshold of detection for *M. tuberculosis* of nearly 100 colony forming units (CFU) *in vitro* and <1000 CFU in the lungs of mice *in vivo* (whole animal)^{8,33}. REF can be used as a diagnostic tool for sputum, clinical materials and even directly in patients with micro endoscopic systems^{16,32,33,34} due its high sensitivity and specificity. REF can be applied to any tuberculosis clinical strain, because it uses a naturally produced bacterial enzyme, BlaC, for detection present in all strains. These characteristics make REF imaging a valuable tool in pre-clinical tuberculosis research in general to facilitate therapeutic and vaccine evaluation as well as analysis of pathogenesis, but may also ultimately be applied to diagnosis in tuberculosis patients.

Protocol

Animal studies were performed in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee of Texas A&M University. Review and approval from a biohazard safety officer or committee at your institution may be required.

Caution: All the procedures require BSL3 containment. The personnel are required to wear personal protective equipment at all times. All manipulations are performed inside the Biosafety Cabinet (BSC) and all sharps are disposed of in sharps containers. Work surfaces and BSC are cleaned with buffered phenol and 70% ethanol before initiating work and after work. Procedures would normally be done at biosafety level 3 with *Mycobacterium tuberculosis*, but for illustration and filming purposes, the authors demonstrate these procedures at biosafety level 2 with less virulent bacteria.

1. Strains and culture conditions

Note: *M. tuberculosis* strain CDC1551 is used in this study, but any *M. tuberculosis* strain can be used in the same manner.

1. Grow the bacteria in M-OADC-TW (7H9 broth supplemented with 0.5% glycerol, 10% oleic acid dextrose complex without catalase, and 0.05% Tween-80) medium standing at 37 °C to an OD₆₀₀ of 0.5 (~ 0.2 x 10⁷ CFU).
2. Dilute the culture in M-OADC-TW (series of 1:10 dilutions of the bacteria). Plate the dilutions of the bacteria (10⁵, 10⁶, 10⁷, 10⁸) in triplicate onto selective 7H11 plates to allow determination of CFU.
3. Incubate the plates for four weeks at 37 °C or until colonies can be accurately counted.
4. Centrifuge the bacterial inoculum at 8,534 x g for 5 min to obtain a pellet. Wash the pellet once with 10 mL of saline (0.9% NaCl) and resuspend the pellet in 15 mL of saline (0.9% NaCl).

2. Aerosol infection of mice using a Madison Chamber

1. Allow mice to acclimate to the new surroundings for a week.
2. Weigh the mice prior to loading them into the chamber.
3. Plug three cords into the power strip: the main chamber power, the vacuum pump, and the air compressor, in that order.
4. Carefully unscrew the glass jar. Bring the glass jar to the biosafety cabinet and add the challenge inoculum suspended in 15 mL of saline (0.9% NaCl) to achieve ~ 10⁴ - 10⁶ CFU of bacteria in the lungs.
5. Close the lid of the glass jar in the biosafety cabinet. Attach the jar to the nebulizer unit and adjust the vertical stainless steel tube so that the lower (intake) end is about a quarter of an inch below the level of the fluid in the jar.
6. Load all the animals needed for the experiment (the chamber can hold up to 90 mice) into the chamber and close all the latches on the door.
7. Check the main (room) air flow meter. Ensure that the center of the float (ball) runs about 50 L/min as measured on the scale on the left.
8. Press Start button on the control panel of the chamber. Set the airflow rate through the compressor air flow meter (the smaller meter in the left) as 4 L/min on the scale. Check visually to ensure that the challenge inoculum is being nebulized.
9. After 15 min, when the red light on the front of the control panel appears and an audible signal indicates the end of the run, press Reset button on the lower right corner of the control panel to reset the timers.
10. Hold down the small red button on the door of the chamber to release the vacuum.
11. Open the chamber door and remove the animals. Place the mice back into their cages.
12. Remove the glass nebulizer jar and place in a sealed, leak-proof transport container. Place the container inside the bio-safety cabinet and discard the challenge suspension into a designated waste container.
13. Place the used nebulizer jar inside a biohazard bag and seal the bag for transport to the autoclave.
14. At the end of the infection procedure, spray the inside of the chamber with buffered phenol and 70% ethanol and allow the chamber to sit for 10 min.
15. Then, wipe down all accessible interior surfaces very thoroughly. Dispose of all contaminated paper towels, etc. in the biohazard trash. Autoclave the trash, waste container and used nebulizer jars.
16. Place the animals back in the containment room until the imaging time-point.
17. On the day of imaging, transfer the animals in a secondary container to the imaging room.

3. Animal anesthesia

1. Anesthetize the mice with isoflurane using a customized gas anesthesia system.
2. Weigh each of the two charcoal filter canister located on top of the anesthesia unit.
3. Replace with a new canister if the weight is 50 g above the initial weight.
4. Check the vaporizer unit to ensure sufficient isoflurane for the procedure.
5. Place the nose cone holder inside the imaging chamber. Place the number of nose cones required for the procedure and seal the remaining openings with the nose cone blockers.
6. Turn on the oxygen supply from the high-pressure cylinder and set it at 55 psi.
7. Turn on the evacuation pump located in front of the anesthesia unit and set it to 8 L/min.
8. Turn on the oxygen toggle located in front of the anesthesia unit.
9. Turn on the gas flow to the anesthesia induction chamber to set the flow of gas at 1.5 L/min. Turn the gas flow off.
10. Turn on the gas flow to the imaging chamber to set the flow of gas at 0.25 L/min. Turn the gas flow off.
11. Turn on the isoflurane vaporizer and set it at 2 - 2.5%. Adjust the isoflurane level according to the number and weight of animals being used for the experiment by rotating the dial on the isoflurane vaporizer (2 - 2.5% for a mouse weighing ~20 g; 4% for a guinea pig weighing 300 g).
12. Place the mice in the anesthesia chamber and close the lid. Turn on the gas flow to the anesthesia induction chamber.

13. Leave the mice in the anesthesia induction chamber for 5 - 10 min until completely anesthetized.
14. Once the mice are anesthetized, apply an optical ointment to the eyes to protect them while imaging.
15. Place the mice in ventral or sternal recumbency such that their noses are placed in the nose cone to facilitate anesthesia of mice during the imaging procedure.

4. Reporter enzyme fluorescence (REF) imaging

1. Inject the substrate (20 μ M, 2.5 μ L/g of weight) by intraperitoneal injection into the infected as well as the control mice. The mice are under anesthesia inside the imaging chamber for less than 1 min.
2. Start the imaging system.
3. Initialize the system by clicking on Initialize. Wait until the temperature bar turns to green.
NOTE: The imaging chamber consists of a heated platform to maintain body temperature of the animal while imaging.
4. For imaging acquisition setting, select Fluorescent | Trans-illumination for whole animal or Epi-illumination for lung tissues, Structure and Overlay in Acquisition Control Panel.
5. Set Field of view to B for single mouse and lamp level to high.
6. Set exposure time to auto, medium binning, 2 - 3 for the f/stop, and excitation filter at 745 nm and emission filters from 780 nm to 840 nm.
7. For sequence setup, click on Sequence Setup, select 9 - 12 Trans-illumination points in the lung area.
8. Click on Acquire for image acquisition.
9. Place the mouse back into the cage post-imaging.
10. Monitor the mice until fully recovered or sacrifice for quantifying CFU if imaging at a time-point of the experiment. Perform a modified Karnofsky score to monitor the well-being of mice post-imaging.

5. Analysis of REF imaging

1. Open the imaging software.
2. Load image files by clicking Browse icon.
3. For spectral unmixing, click on Spectral Unmixing in the tool palette and click the spectra required to unmix. Click on start unmixing.
4. For optical surface reconstruction, click on Surface Topography.
5. Select orientation to dorsal, subject to fur mouse and, click Generate surface.
6. Draw Crop image and set Threshold image for a region of interest. Click Done.
7. For organ registration, go to the registration tab in 3D Optical Tools dropdown menu.
8. Register organs of interest in an organ atlas.
9. Adjust organ size or location to the generated surface topography using transform tool on/off located on the panel-Registration Tools.
10. Go to Fluorescent tomography (FLIT) 3D reconstruction, and select analyze tab. Select the images to be included for analysis, image type to Normalized Transmission Fluorescence (NTF) efficiency, click Start.
11. Check on select all and reconstruct in Data Preview tab.
12. For fluorescence quantification, go to Region of Interest (ROI) Tools, add ROI cube to the organ of interest and adjust cube size to cover the organ of interest. Click on measure 3D ROIs.
13. In the ROI measurements window, go to 3D ROI measurements, select data type to source voxels and measurements unit to $\text{pmolM}^{-1}\text{cm}^{-1}$.
14. Save and/or export the result of FLIT 3D reconstruction analysis into a figure or data file.

6. Quantification of bacteria by CFU

1. Euthanize the mice by intraperitoneal injection of 0.1 mL pentobarbital sodium (390 mg/mL).
2. Check for pedal reflex by squeezing the pads of the feet of mice to ensure there is no reflex reaction.
3. Explant the lung tissue from the mice using sterile forceps and scissors. Homogenize the lung tissue in 1 mL of 1x phosphate-buffered saline (PBS).
4. Make 10-fold serial dilutions of the lung homogenate in sterile 1x PBS.
5. For plating, spot three aliquots of 20 μ L each of the respective dilution on the agar plate.
6. Incubate the plates in an incubator at 37 $^{\circ}$ C and monitor for bacterial growth.
7. Count the colonies in each spot after the incubation time and express as CFU/ mL by correcting for volume and dilution using the following equation:

$$\text{CFU/ mL} = (\text{C/V}) \times \text{M}$$
 Where C = colony counts per spot, V = volume of sample inoculated on each plate (mL), and M = multiplication factor (reciprocal of the dilution used).
 NOTE: If 11 colonies are counted in one spot for a sample volume of 0.002 mL at a sample dilution of 10^{-4} , using the equation, the colony count will be calculated as

$$(11/0.02) \times 10^4 = 5.5 \times 10^6 \text{ CFU/ mL}$$

Representative Results

REF imaging of mice infected with *M. tuberculosis* along with uninfected control mouse are shown in **Figure 1A**. The infected mice produced a significantly ($P = 0.0057$) higher fluorescence signal from the lungs at 6 weeks post-infection compared to control upon substrate administration. A typical time course to study the therapeutic efficacy against *M. tuberculosis* using REF could be imaging at week 1, 2, 4, 6, 15, 24 post-infection. Fluorescence intensity is quantified using epi-illumination for lung tissues (**Figure 1B**). The consistently increasing signal from week 2 to week 6 in lungs of infected mice suggests that REF is successfully able to detect *M. tuberculosis in vivo*. A drop in the background signal of control mice at a later time point (**Figure 1B**) could be attributed to the increase in body mass and volume of mice over a period of 6 weeks thereby reducing the excitation wavelength penetration. 3D FLIT reconstruction of fluorescence sources in mice infected with *M. tuberculosis* is represented in **Figure 2A**. The image sequences are acquired at multiple trans-illumination points in mouse using the same excitation and series of emission filters. These image sequences are then used for 3D FLIT reconstruction for fluorescent source distribution within the animal subject. **Figure 2A-D** demonstrates the different directions (coronal, sagittal and transaxial) of mouse tomography with lung organ registration. NTF efficiency maps for checking the reconstruction quality are represented in **Figure 2E**. NTF allows subtracting the background light leakage from trans-illumination images through an extra image captured with neutral density filter. The image taken with the specific excitation filter (**Figure 2E-measured**) is normalized to the transmission image measured with the same emission filter and an open excitation filter (**Figure 2E-simulated**) to give the signal produced by the substrate alone. The similar measured and simulated NTF efficiency profile in both the horizontal (**Figure 2F**) and vertical (**Figure 2G**) profiles with nearly 0% percentage error (**Figure 2E-% error**) provides evidence of a good quality 3D reconstruction with reduced artifacts and improved signal localization and sensitivity.

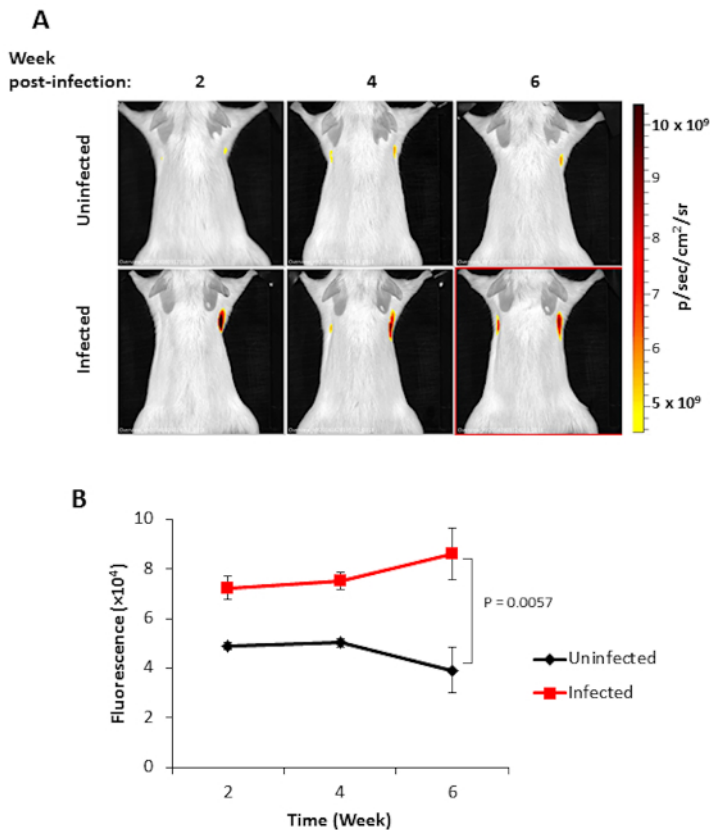


Figure 1. Imaging of *M. tuberculosis* with REF. (A) *in vivo* imaging of mice uninfected and infected with *M. tuberculosis* at 2, 4, and 6 weeks post-infection. The color bar represents the intensity of the fluorescent signal in photons per second per cm^2 from low (yellow) to high (red). (B) Quantification of fluorescence intensity from mice infected with *M. tuberculosis*. Fluorescence values for both control (black) and infected (red) are represented along with the standard error for each time point. The significance of results was determined by Students *t*-test, *p* values of <0.05 were considered significant. [Please click here to view a larger version of this figure.](#)

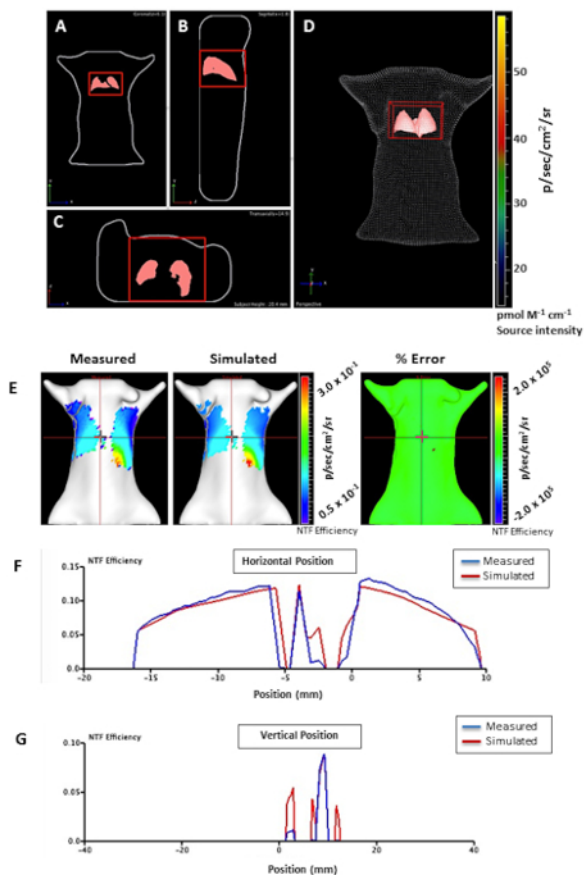


Figure 2. 3D FLIT reconstruction of fluorescence sources in mice infected with *M. tuberculosis*. Mouse tomography represented in different directions; A) coronal, B) sagittal, and C) transaxial with D) lung organ registration. The 3D region of interest (ROI) is represented as a red cube in the lung for source measurements. (E) NTF efficiency maps of the measured and simulated for checking the reconstruction quality. The measured and simulated NTF efficiency profile was compared, providing good quality of 3D reconstruction (similar measured and simulated NTF efficiency). F) Horizontal and G) vertical signal profiles representing measured (blue) and simulated (red) NTF efficiency curve. The horizontal and vertical red bars indicate the source position. The color bar represents the intensity of the fluorescent signal in photons per second per cm² from low (blue) to high (red). [Please click here to view a larger version of this figure.](#)

Discussion

When using imaging techniques, such as REF, there are key strategies that allow generation of robust and consistent data. Optical imaging produces scattered light in tissues which can impact the depth of penetration, since it is difficult to capture light emitted in all directions. Use of a near infra-red fluorophore (NIR) substrate for REF imaging having an excitation and emission wavelength in the Querange of 700 - 900 nm facilitates minimal absorption of the fluorescent signal by mammalian tissues. The custom designed substrate was constructed by linking a NIR fluorophore, IRDye 800Cw to a quencher, IRDye QC-1, by a lactam ring allowing fluorescence resonance energy transfer-based quenching. IRDye has excellent tissue penetration and light scattering characteristics and does not have any evident harmful effect on mammals³⁶, being cleared from the blood and organs by 24 h. Fluorescence signal significantly increases beginning 4 h after substrate administration, reaching maximal levels 6 h after administration.

The bacterial infectious dose, mode of administration of infectious dose and the substrate as well as time-points of imaging post infection by performing pilot studies should be standardized before embarking on large, complex, experiments. Pilot studies can greatly reduce time and cost when imaging a large number of animals because a standard procedure can be optimized prior to doing the key experiment. Bacterial loads should be determined in the organs/tissues of interest following whole body imaging using trans-illumination and *ex vivo* lung imaging using epi-illumination to validate the source of the signal and determine the quality of correlation with bacterial numbers present⁸. Pilot studies will provide insight into the threshold of detection, dynamic range of the technique as well as determination of the optimal experimental conditions for imaging.

The primary advantages of using REF imaging as compared to other fluorescent and bioluminescent strategies are its high sensitivity and ability to image natural *M. tuberculosis* strains. REF imaging utilizes the catalytically robust enzyme BlaC that is conserved in all *M. tuberculosis* clinical isolates and tuberculosis-complex strains. The great sensitivity of REF imaging is due to the rapid catalytic rate of BlaC in combination with retention of the cleaved fluorescent product by the host cell. Signal continuously increases as long as the substrate is available resulting in nearly limitless build-up of the signal within the infected cells and tissues. This increased sensitivity of REF imaging as compared to alternative approaches of imaging allows specific detection of *M. tuberculosis* both *in vitro* and *in vivo*^{8,23,24,29}.

REF imaging can be used for bacterial detection without genetic modifications enabling its direct application to any infection model, either laboratory animals^{8,37} or human clinical materials^{29,38}. REF can be used to detect and image a wide array of pathogens^{39,40}, since fluorogenic substrates can be developed for numerous enzymatic targets other than BlaC such as proteases, kinases, ureases, and β -galactosidases. However, careful thought should be given to the target in order to ensure it displays optimal characteristics for imaging. BlaC represents a good model enzyme for characteristics that will ensure successful application of this strategy. REF imaging provides an immediate read-out on bacterial load present in lungs during infections, which greatly speeds progress in study of tuberculosis pathogenesis, since determination of bacterial numbers normally requires three to six weeks, but even in more rapid-growing organisms this approach will save a great deal of time. REF could also be used to discriminate carcinomas from tuberculosis, a key problem in diagnosis of nodular lesions in patients^{41,42}. REF serves as a novel tool to accelerate translational tuberculosis imaging and can even be applied to humans, potentially allowing rapid prediction of therapeutic outcomes.

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