

## Short Communication

# Imaging of Pulmonary Granulomas Using a Photon Imager

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**SUMMARY:** To clarify the location of pulmonary granulomas in vivo, we prepared a *Mycobacterium tuberculosis* H37Rv mutant in which the gene for a green fluorescent protein (GFP) (GFP-H37Rv) was introduced. Five weeks after aerosol infection with GFP-H37Rv, the infected lungs from guinea pigs and mice were subjected to imaging using a photon imager. Pulmonary granulomas more than 1 mm in diameter were localized clearly by the photon imager. Therefore, if a method for binding a dye (GFP, fluorescein isothiocyanate [FITC], etc.) specifically to *M. tuberculosis* can be developed, it will be possible to visualize granulomas using a photon imager.

When tubercle bacilli enter lung alveoli, they eventually induce granulomas unless they are killed. Such granulomas are similar to carcinomas in that they are solid and spread to other organs hematogenously. Therefore, for the diagnosis of granulomas, it is useful if their locations within organs can be pinpointed. As a first step toward this goal, we prepared H37Rv in which the gene for a green fluorescent protein (GFP) was introduced (GFP-H37Rv). Briefly, a BCG hsp60 promoter-GFP mut 3.1 was prepared by using the pCR 2.1-BCG hsp60 promoter and pGFP mut 3.1 (Clontech Labs., Inc., Pal Alto, Calif., USA). Then, a pHSP 60 promoter-GFP mut 3.1 was constructed by utilizing pHSP 60 promoter-GFP mut 3.1 B and pGFM-12 (kindly supplied by Dr. C. Loch) (1). *Mycobacterium tuberculosis* H37Rv strain (ATCC25618) was then transformed with the pHSP 60 promoter-GFP mut 3.1 M to obtain a stable GFP-H37Rv mutant. This mutant was shown to remain stable for a year after subcutaneous administration to C57BL/6 mice. There was no statistically significant difference in in vitro growth between H37Rv and the GFP-H37Rv mutant (Fig. 1).

Next, the GFP-H37Rv mutant was smeared on glass slides and fixed with 4% paraformaldehyde for 30 min. The slides were then observed using a confocal laser microscope (Digi-

tal Eclipse C1; Nikon Optical Co., Tokyo, Japan). As shown in Fig. 2, clustered GFP-H37Rv tubercle bacilli emitted intense green fluorescence. We then used the RAW264.7 mouse macrophage cell line to determine whether the GFP-H37Rv



Fig. 2. GFP-H37Rv mutant emitting green fluorescence. After the GFP-H37Rv mutant had been smeared on glass slides and fixed with 4% paraformaldehyde for 30 min, it was observed by confocal laser microscopy.  $\times 600$ .

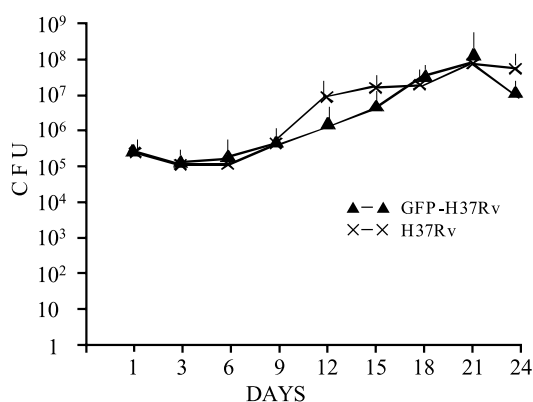


Fig. 1. Growth curve of GFP-H37Rv and H37Rv (parental strain).



Fig. 3. GFP-H37Rv mutant in phagosomes of the RAW264.7 murine macrophage cell line. After the GFP-H37Rv mutant had been added to RAW cells (multiplicity of infection, 50:1) they were fixed with 4% paraformaldehyde for 60 min, and observed by confocal laser microscopy.  $\times 600$ .

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mutant maintained its green fluorescence intracellularly. The GFP-H37Rv mutant was added to a culture of RAW264.7 cells (multiplicity of infection, 50:1) and cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum overnight. After fixation with 4% paraformaldehyde for 60 min, the cells were observed using a confocal laser microscope. As shown in Fig. 3, the GFP-H37Rv mutant in macrophages still emitted intense green fluorescence.

We next attempted to determine the fate of the GFP-H37Rv mutant in vivo. Permission for animal experimentation was given by the Animal Experiment Committee of The Tuberculosis Research Institute. Female guinea pigs and BALB/c female mice were infected with the GFP-H37Rv mutant ( $1 \times 10^6$  CFU) by an airborne infection apparatus (Model 099CA424; Glas-Col, Inc., Terre Haute, Ind., USA). The concentration was calculated to result in the uptake of around 200 viable bacilli by guinea pig lungs and around 70 viable bacilli by mouse lungs after inhalation exposure for 90 min under the experimental conditions employed (2). Five weeks later, the lungs were removed and fixed with 4% paraformaldehyde for 2 days. The guinea pig and mouse lungs were scanned for green fluorescence using a  $\Phi$  imager (photon imager; Biospace Mesures, Paris, France). This imager is

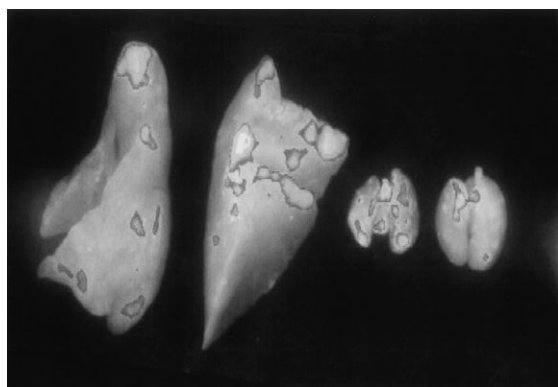


Fig. 4. Pulmonary granulomas visualized by a photon imager. Guinea pigs and BALB/c mice were infected with *M. tuberculosis* Kurono strain by aerosol infection. Five weeks after infection, lung tissues from guinea pigs (large) and mice (small) were visualized by a photon imager. Granulomas of various sizes were localized in the lungs.  $\times 10$ .

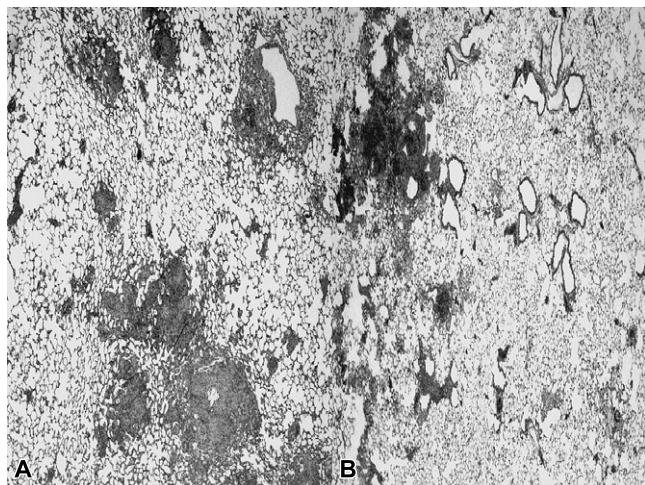


Fig. 5. Histopathology of infected lung tissues of guinea pigs (A) and mice (B). Pulmonary granulomas of various sizes are shown in this picture. Hematoxylin & eosin stain.  $\times 20$ .

based on a 3rd generation GaAs intensified charge-coupled device (ICCD) camera that allows real-time photon counting over a wide spectral range (400-900 nm). This imager amplifies every photon up to  $10^6$  light spots in a detector using an ICCD chip. The detection conditions of this imager were as follows: spatial resolution, equivalent to  $1,080 \times 1,440$  pixels CCD; dynamics, 2,000 counts/pixel/min; excitation, 485 nm; emission, 535 nm. As shown in Fig. 4, signals with green fluorescence were recognized to varying degrees in both guinea pig and mouse lungs. No signal was detected in exudative inflammation. Fig. 5 shows that the signals corresponded to granulomas (proliferative inflammation) of various sizes. The granulomas contained significant numbers of tubercle bacilli (GFP-H37Rv mutant) as evaluated by Ziehl-Neelsen staining for acid-fast bacilli (data not shown).

The spatial visualization technique (tumor imaging) is commonly utilized in the diagnosis of lung cancer (3-8). So far, however, there has been no research report on pulmonary granuloma imaging. The present study showed that it is possible to detect pulmonary granulomas by green fluorescence emission. This system detects signals from cyanin, fluorescein isothiocyanate (FITC), and rhodamine as well as GFP. If a technique for binding a dye specifically to tubercle bacilli could be developed, it would be possible to visualize granulomas (proliferative inflammation) in other organs as well as the lungs.

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