



Development of dual reporter imaging system for *Francisella tularensis* to monitor the spatio-temporal pathogenesis and vaccine efficacy

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Purpose: Study on the pathogen and the pathogen-related disease require the information at both cellular and organism level. However, lack of appropriate high-quality antibodies and the difference between the experimental animal models make it difficult to analyze *in vivo* mechanism of pathogen-related diseases. For more reliable research on the infection and immune-response of pathogen-related diseases, accurate analysis is essential to provide spatiotemporal information of pathogens and immune activity to avoid false-positive or mis-interpretations. In this regards, we have developed a method for tracking *Francisella tularensis* in the animal model without using the specific antibodies for the *F. tularensis*.

Materials and Methods: A dual reporter plasmid using GFP-Lux with putative bacterioferritin promoter (pBfr) was constructed and transformed to *F. tularensis* live vaccine strain to generate *F. tularensis* LVS (*FtLVS*)-GFP-Lux for both fluorescence and bioluminescence imaging. For vaccination to *F. tularensis* infection, *FtLVS* and lipopolysaccharide (LPS) from *FtLVS* were used.

Results: We visualized the bacterial replication of *F. tularensis* in the cells using fluorescence and bioluminescence imaging, and traced the spatio-temporal process of *F. tularensis* pathogenesis in mice. Vaccination with LPS purified from *FtLVS* greatly reduced the bacterial replication of *FtLVS* in animal model, and the effect of vaccination was also successfully monitored with *in vivo* imaging

Conclusion: We successfully established dual reporter labeled *F. tularensis* for cellular and whole body imaging. Our simple and integrated imaging analysis system would provide useful information for *in vivo* analysis of *F. tularensis* infection as well as *in vitro* experiments, which have not been fully explained yet with various technical problems.

Keywords: *In vivo* imaging, Vaccine, *Francisella tularensis*

Introduction

Biological phenomenon is the product of tremendous network consist with various number of complex pathway. To explain this phenomenon as a whole system, accurate analyses of spatio-temporal information of *in vivo* animal model is required. Real-time imaging of specific molecules in living cells or organisms provides more reliable and accurate information. Although the various mechanisms of life have been developed, the role of molecular imaging is increasingly emphasized in exploring this vital response, as the network between them is still questionable.

Infectious disease is one of the demanding fields for molecular imaging analysis.

Pathogenesis is a complex process, which is closely interplayed with various molecules of virulent factors and the immune systems, and varies with time point in each cell/or organ. However, these features can sometimes lead to misinterpretations related with limited spatio-temporal information *in vivo*. Spatio-temporal distribution of pathogens at the cellular or organism level provides valuable information to account for the detailed mechanisms of infection.

Discovery and development various fluorescent proteins opened a new era in cell biology by enabling researchers to visualize cellular molecules in living systems using optical imaging. By molecular cloning of fusing a fluorophore moiety with a wide variety of target proteins, fluorescence protein works as a successful reporter. Since fluorescence proteins require external excitation light source, luciferases can be used as alternatives of fluorescence proteins in limited light source because luciferases only need their substrate proteins to produce light. Various bacterial luciferases (Lux) from marine and terrestrial bacteria have been reported and cloned [1]. All Lux operons have a common gene organization of LuxCD-ABE, whereas significant differences exist in their sequences and properties with other Lux genes (I, R, F, G, and H). LuxAB encodes luciferase and LuxCDE encodes the fatty acid reductase complex responsible for synthesizing fatty aldehydes for the luminescence reaction. The Lux genes can be transferred into various different organisms to generate new luminescent species. Fusion of the Lux genes has also allowed the expression of luciferase under a single promoter in eukaryotic systems as well as bacteria. Unlike firefly luciferase, Lux system has its substrate and it can generate bioluminescence without adding exogenous substrate such as D-luciferin for firefly luciferase.

Previous studies of pathogenesis and immune-response were generally observed with fluorescence and bioluminescence imaging separately [2-5]. For *in vivo* tracking of pathogen in live animal, bioluminescence imaging is most frequently used. For visualization of involved molecule with high-resolution, *in vitro* fluorescent staining is the first choice. This method sometimes has difficulties when appropriate antibody for analysis does not exist [6,7]. In many cases of infectious disease study, especially for high-risk pathogen or newly identified bacteria, immuno-staining methods with antibody cannot be applied in tracing study because labeling living bacteria with antibody may retain their own physiology in the living organism. For this reason, it is difficult to study *in vivo* mechanism of several pathogens in live animals com-

pare to well-characterized disease such as cancer [8-10].

Francisella tularensis is the pathogen of high-risk infectious disease, tularemia. As extremely low dose (~10 colony-forming unit) of *F. tularensis* can lead to severe symptoms with rapid proceeding, vaccine are required for prevention of tularemia [11-13]. In spite of various effort for development, there is no official vaccine for tularemia approved by Food and Drug Administration [11-14]. Development of more efficient and trustable vaccine should be based on the molecular mechanism of pathogenesis and immune response, and molecular imaging methods can reveal the novel information on study of tularemia.

Constructions of either fluorescence or bioluminescence reporter plasmid for *F. tularensis* were reported [2-5] but those reporters produce relatively lower number of photons. For *in vivo* imaging, reporter plasmid should generate higher number of transcripts to produce higher number of photon and established plasmids are not suitable for *in vivo* imaging. In this regards, we decided to develop a new reporter construct for visualizing *F. tularensis* pathogenesis. We used conjugated GFP and Lux genes under the control of bacterioferritin promoter to produce strong reporter expression for real-time dual imaging.

Materials and Methods

Animal experiments

Pathogen free BALB/c mice, 4 weeks old, were purchased and maintained in animal-bio safety level-2 facility. All experiments were accomplished under the provision of Institutional Animal Care and Use Committee of Korea National Institute of Health (KNIH).

Generation of *F. tularensis* LVS (*FtLVS*)-GFP-Lux

pKK214-GFP plasmid, isolated from *FtLVS*-GFP bacteria, *FtLVS*-GFP which is kindly gifted from Umea University, was used as a backbone vector for pKK214-GFP-Lux construct. For increasing sensitivity of reporter signals, putative bacterioferritin promoter in *FtLVS* was amplified, and substituted with GroEL promoter of pKK214-GFP plasmid using *PacI/PstI*. Lux operon was amplified from pXen 13 plasmid vector (Caliper Life Science, Hopkinton, MA, USA) and inserted to the *EcoRI* site, next to the M2-GFP gene in pKK214-GFP plasmid.

F. tularensis LVS (*FtLVS*, ATCC 29684), gifted from Division of Zoonoses, KNIH, was transformed with pKK214-GFP-Lux plasmid and *FtLVS*-GFP-Lux was isolated by using 2 µg/mL

of tetracycline. All experiments using *FtLVS* and its derivatives were performed in bio safety level-2 facility accordance with bio-safe guideline of KNIH.

Lipopolysaccharide purification from *F. tularensis* LVS

Lipopolysaccharide (LPS) from *FtLVS* was purified using LPS extraction kit (Intron Biotechnology, Seongnam, Korea), followed to instructions from the manufacturer. Briefly, 5 mL of cultured *FtLVS* bacteria were harvest by centrifugation. Pellet of harvested bacteria was re-suspended with 1 mL of lysis buffer and vortexed vigorously. Then, 200 μ L of chloroform (Sigma, St. Louis, MO, USA) were added, vortexed vigorously and incubated at room temperature for 5 minutes. Mixture was centrifuged for 10 minutes, and supernatant was transferred to new tube. Supernatant was added with 800 μ L of purification buffer and incubated at -20°C for 10 minutes. Mixture was then centrifuged to get the LPS pellet. Pellet was washed with 70% EtOH, and dissolved with distilled water by boiling for 2 minutes.

LPS vaccination and *FtLVS*-GFP-Lux infection

Mice were vaccinated with 20, 100, and 500 ng of LPS purified from *FtLVS* by intra-peritoneal injection. For control group, phosphate buffered saline (PBS) was injected as a control for LPS vaccination. Three weeks after vaccination, vaccinated/control mice were infected with 10^2 - 10^6 colony forming unit (CFU) of *FtLVS*-GFP-Lux and subjected to following analysis.

Measurement of fluorescence and luminescent signals

FtLVS-GFP-Lux were cultured in Luria-Bertani broth supplemented with 10% IsoVitalEx enrichment (Becton Dickinsons and Company, Sparks, MD, USA), harvested and serially diluted with PBS. Each sample was loaded in 96-well plate and subjected to measurement. Fluorescent signal was measured using SpectraMax M2 (Molecular Devices, San Jose, CA, USA) with 485 nm of excitation and 535 nm of emission filter. Luminescent signal was measured using Fluoroskan Agent FL with luminescence filter (Thermo Fisher Scientific, Miami, FL, USA).

Flow cytometry analysis

Each organ enucleated from mice were mashed for extraction of single cells. Each population of cells was re-suspended with PBS and examined using Cytomics FC500 (Beckman Coulter Inc., Brea, CA, USA) for measuring of green fluorescent signal and analyzed with CXP Software (Beckman Coulter Inc.).

Time lapse microscopy

Spleens enucleated from mice were mashed for harvesting of splenocytes. Isolated splenocytes were washed with PBS and incubated with red blood cell lysis buffer (Sigma) for removal of red blood cells. Then cells were re-suspended with RPMI without phenol-red, supplemented with 10% fetal bovine serum and subjected to time lapse microscopic analysis using BioStation IM (Nikon Corp., Tokyo, Japan). Splenocytes were cultured in 35 mm clear cover glass-bottom petri-dishes (SPL Life Sciences, Pocheon, Korea) at 37°C with 5% CO₂ concentration during image acquisition. Images were recorded at every 15 minutes for 72 hours with bright-field and fluorescent filter (excitation, 485 nm; emission, 510 nm). Snapshots matched with each time point were constructed as movie using BioStation IM software (Nikon Corp.).

Bioluminescence imaging

In vivo bioluminescence imaging was acquired and analyzed using In-Vivo Xtreme and accompanied analyzing software (Bruker Corp., Bremen, Germany). Analysis and acquisition were performed according to instructions from the manufacturer's manual. Before imaging, mice were anesthetized using arvertin (10% of 2,2,2 Tribromoethanol, Sigma). Luminescent signals from the mice were acquired for 20 seconds with a binning of 4×4 and 1.1 of fStop value. Bioluminescent signals were recorded as photons/sec/sr/cm² and represented as pseudo-color image matched with signal intensity (red, least intensity; violet, most intensity; black, saturated).

Results

Construction of a plasmid with dual reporter and generation of reporter expressing *F. tularensis*

Plasmids containing fluorescence and bioluminescence reporter genes (pKK214-GFP-Lux) with constitutive bacterioferritin (Bfr) promoter was established for transforming *FtLVS* (Fig. 1A). Dual reporter expressing *FtLVS* was successfully generated (*FtLVS*-GFP-Lux) (Fig. 1B). To increase the imaging sensitivity, we introduced Bfr promoter to pKK214 vector. We compared the activity between pGloEL promoter and putative Bfr promoter in pKK214s for dual reporter expression. Both fluorescence and bioluminescence signals from transformed cells with plasmids under the control of Bfr promoter were higher than that of the cells with pGloEL, the original promoter of pKK214-GFP (Fig. 1C). Therefore, we used *FtLVS*-GFP-Lux with Bfr promoter to investigate spatiotemporal trac-

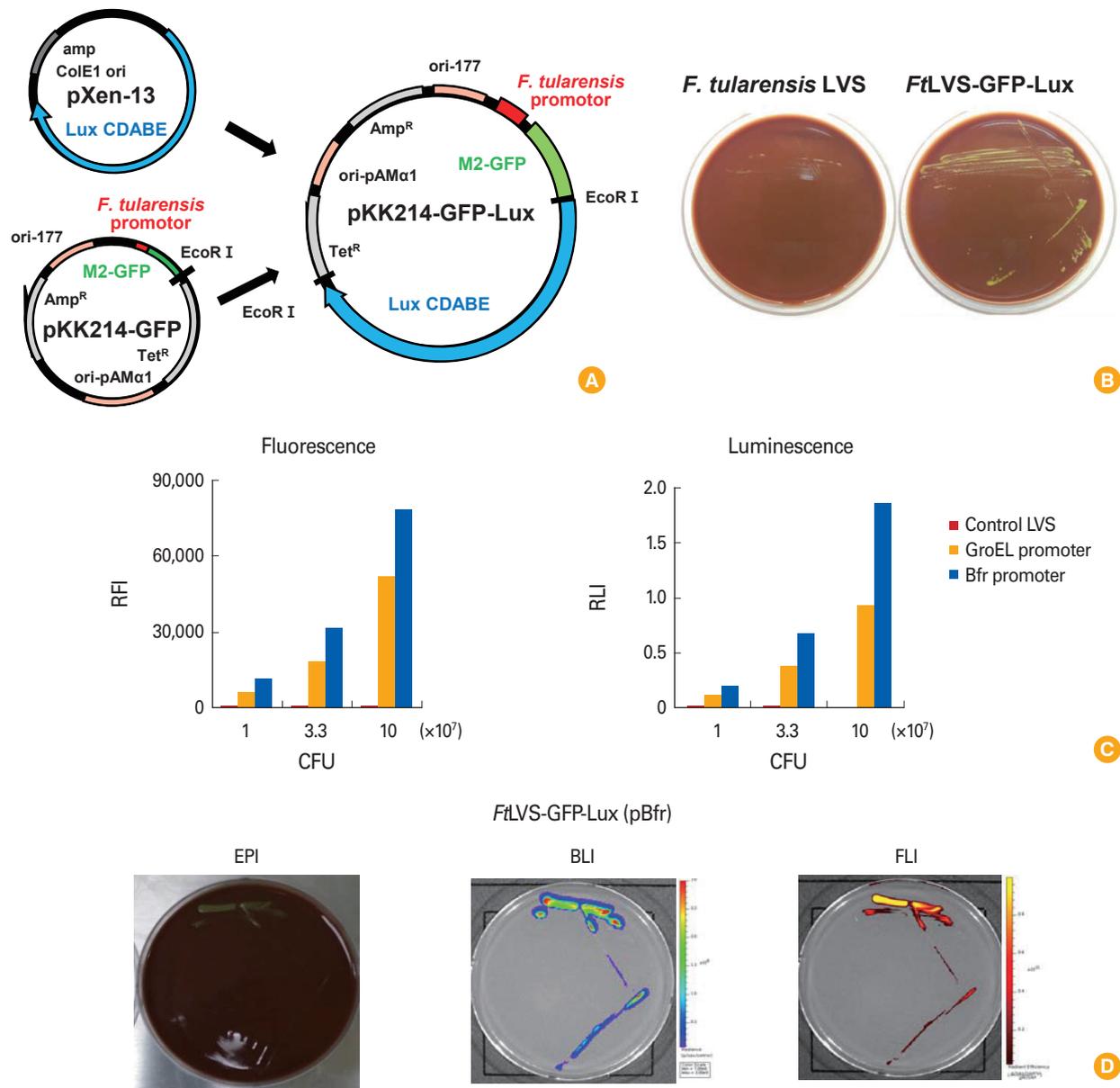


Fig. 1. Construction of a plasmid with dual reporter and generation of reporter expressing *Francisella tularensis*. (A) Design of a dual reporter construct. (B) Generation of reporter expressing *FtLVS*. (C) Comparison of reporter activity under the different promoter. (D) Plate images of *FtLVS*-GFP-Lux with putative Bfr promoter. RFI, relative fluorescence intensity; RLI, relative luciferase intensity; CFU, colony forming unit; BLI, bioluminescent imaging; FLI, fluorescent imaging; *FtLVS*, *F. tularensis* LVS; Bfr, bacterioferritin.

ing of *F. tularensis* in the mouse model.

Next we analyzed the fluorescence and bioluminescence intensity from *FtLVS*-GFP-Lux to quantify the number of *F. tularensis*. Both fluorescence and bioluminescence signals from *FtLVS*-GFP-Lux increased with the number of *F. tularensis* represented by CFU (Fig. 2A). In addition, signals from *FtLVS*-GFP-Lux infected mouse also increased with the number of *F. tularensis* at the same time point after infection (Fig. 2B). We also monitored distribution of *F. tularensis* in the or-

gan of infected mouse. Though GFP signals from the injected *FtLVS*-GFP-Lux (intravenously, 10^2 CFU/mouse) were not observed in the whole body of a mouse at 24 hours after infection (Fig. 2A), GFP signals were observed in the thymus, lung, liver, spleen and intestine at 24 hours after infection when we open the body of an infected mouse. For flow cytometry, we isolated cells from each *F. tularensis* (*FtLVS* or *FtLVS*-GFP-Lux) infected organs. When we compared the GFP signals from *FtLVS* or *FtLVS*-GFP-Lux infected organs,

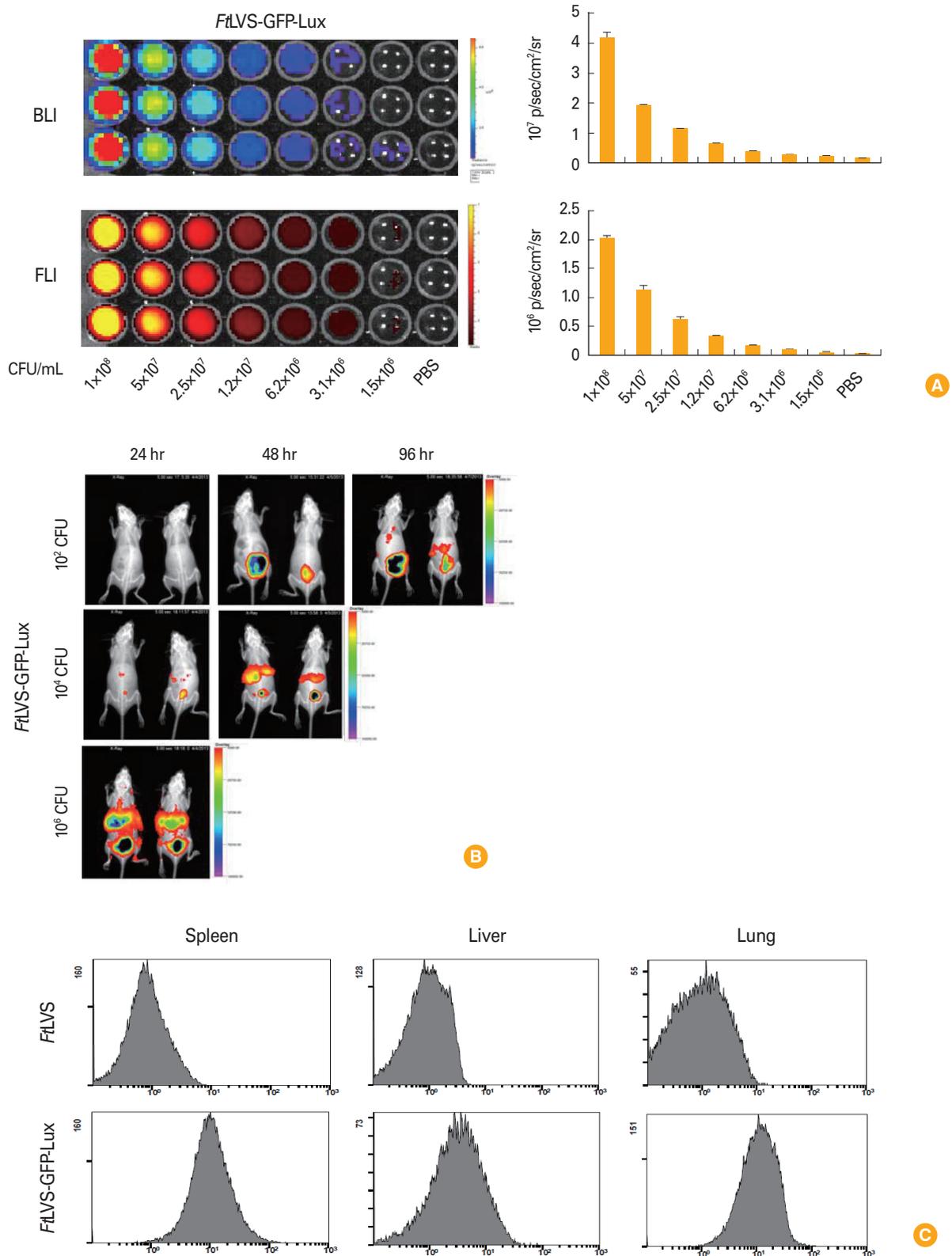
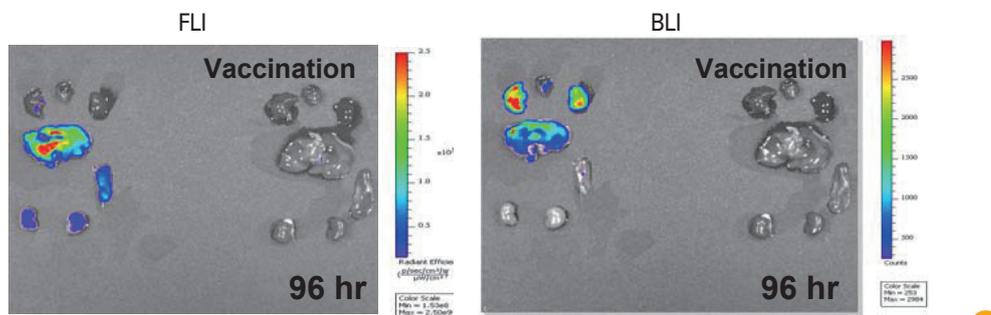
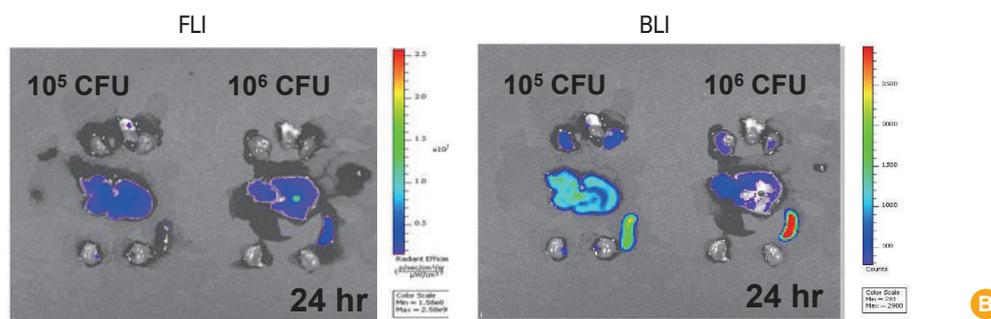
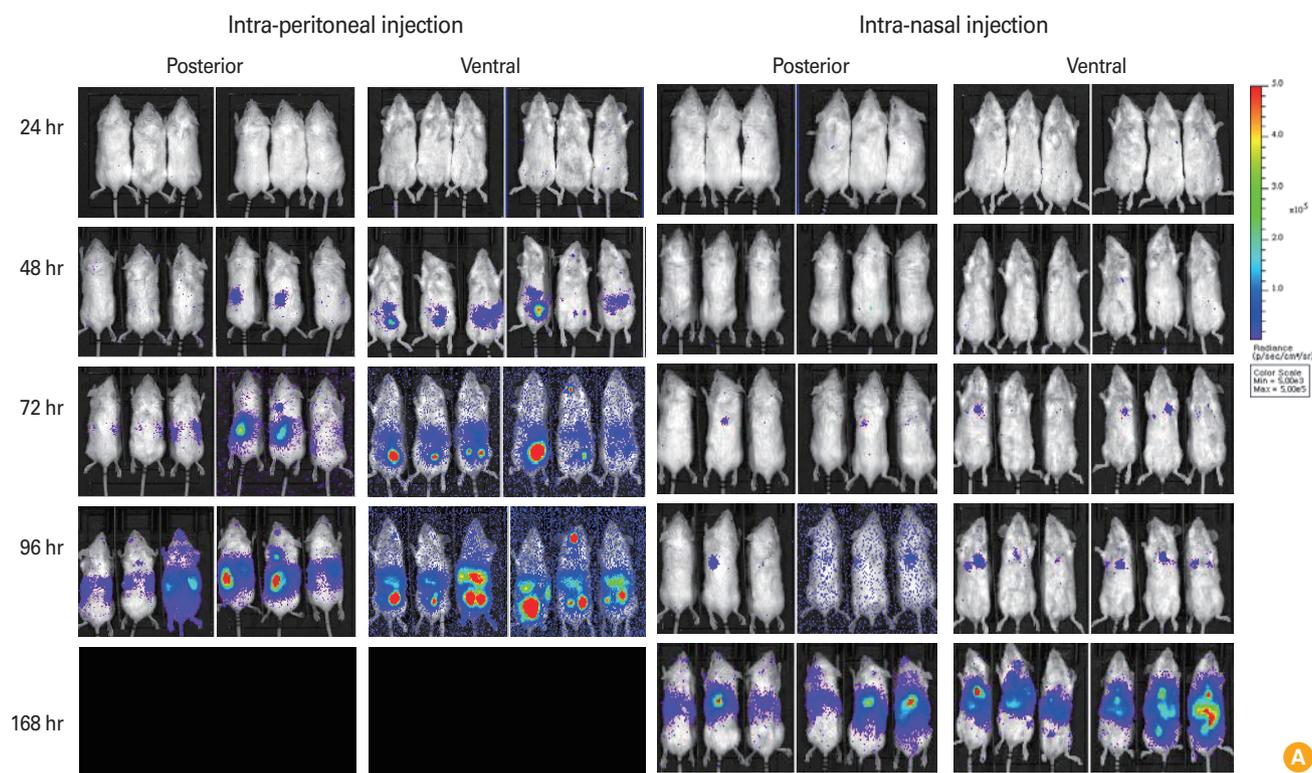


Fig. 2. Bioluminescence and fluorescence imaging of *FtLVS-GFP-Lux* and infected mouse. (A) Dose-dependent fluorescence and bioluminescence signals from *FtLVS-GFP-Lux*. (B) Dose-dependent fluorescent signals from *FtLVS-GFP-Lux* infected mouse model. Higher CFU of *FtLVS-GFP-Lux* infected mouse showed detectable fluorescent signal for *in vivo* imaging. (C) Flow cytometric analysis of cells from the organs of *FtLVS-GFP-Lux* infected mouse. *FtLVS*, *Francisella tularensis* LVS; CFU, colony forming unit; BLI, bioluminescent imaging; FLI, fluorescent imaging.

we could observe strong GFP signals from *FtLVS*-GFP-Lux infected organs such as liver, lung and spleen (Fig. 2C).

For spatio-temporal molecular imaging *F. tularensis*, we

monitored *FtLVS*-GFP-Lux (10^2 CFU/mouse) infected mouse with various infection route such as intra-peritoneal, and intra-nasal injection (Fig. 3A). We analyzed bioluminescence



Vaccination (10^3 CFU/mouse) 3 wks

Fig. 3. Spatio-temporal imaging of *FtLVS*-GFP-Lux infected mouse. (A) Spatio-temporal bioluminescence imaging of *FtLVS*-GFP-Lux (10^2 CFU/mouse) infected mouse by injection route. (B) Fluorescent and luminescent signals from each organ from LVS-GFP-LUX infected mice. (C) Monitoring vaccination effects of *FtLVS* (1×10^3 CFU) in each organ after challenging with *FtLVS*-GFP-LUX (3 weeks after vaccination, 1×10^6 CFU). *FtLVS*, *Francisella tularensis* LVS; CFU, colony forming unit; FLI, fluorescent imaging; BLI, bioluminescent imaging.

signals from infected mice for a week (168 hours) with noninvasive imaging. Though bio-distribution of *FtLVS*-GFP-Lux in the infected mice with all injection route were similar, proliferation of *FtLVS*-GFP-Lux in the intra-nasal injected mice were slower than that in the intra-peritoneal injected mice. In nasally injected mice, lux signals were observed in the lung first, and the signals spread throughout body later. In peritoneally injected mice, lux signals observed in the injection site and then spread to spleen, liver, and lung.

When we compared fluorescence and bioluminescence imaging in the organ of infected mouse, bioluminescence signals were more sensitive than fluorescence signals (Fig. 3B). For fluorescence imaging, external excitation light source is required and the tissue penetration depth of each light source is different. However, our *FtLVS*-GFP-Lux contains LuxCDE as well as LuxAB (luciferase) and does not require the external light source. This may relate with lower sensitivity of GFP fluorescence compared to Lux bioluminescence.

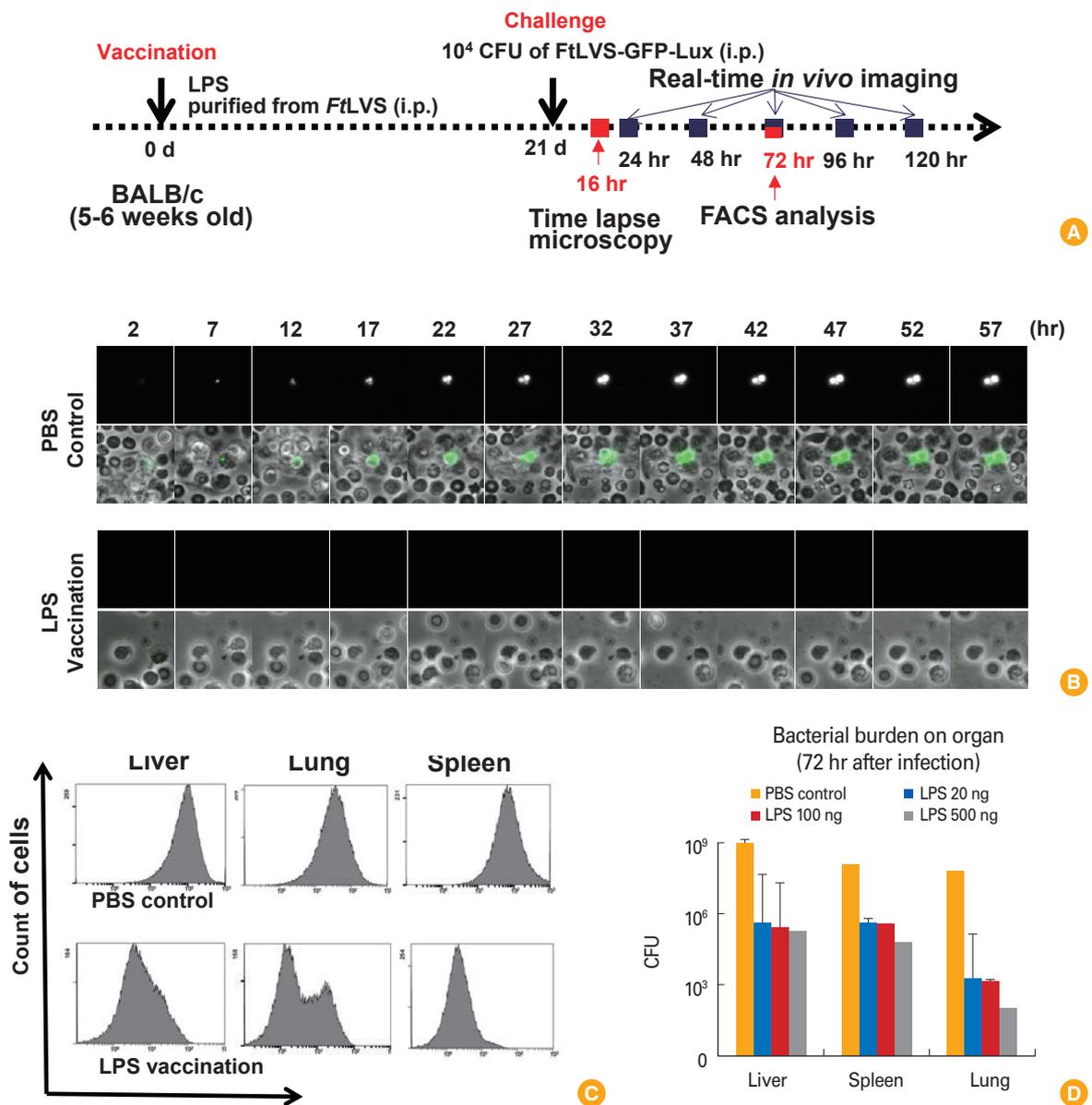


Fig. 4. *FtLVS*-GFP-Lux imaging after LPS vaccination. (A) Experimental plan to evaluate *FtLVS*-GFP-Lux imaging system. (B) Time-lapse imaging of splenocytes from non-vaccinated mouse and LPS vaccinated mouse. (C) Flow cytometry analysis of cells from organs of non-vaccinated mouse and LPS vaccinated mouse. (D) Bacterial burden on target organ after *FtLVS*-GFP-Lux challenge by LPS amount of vaccination.

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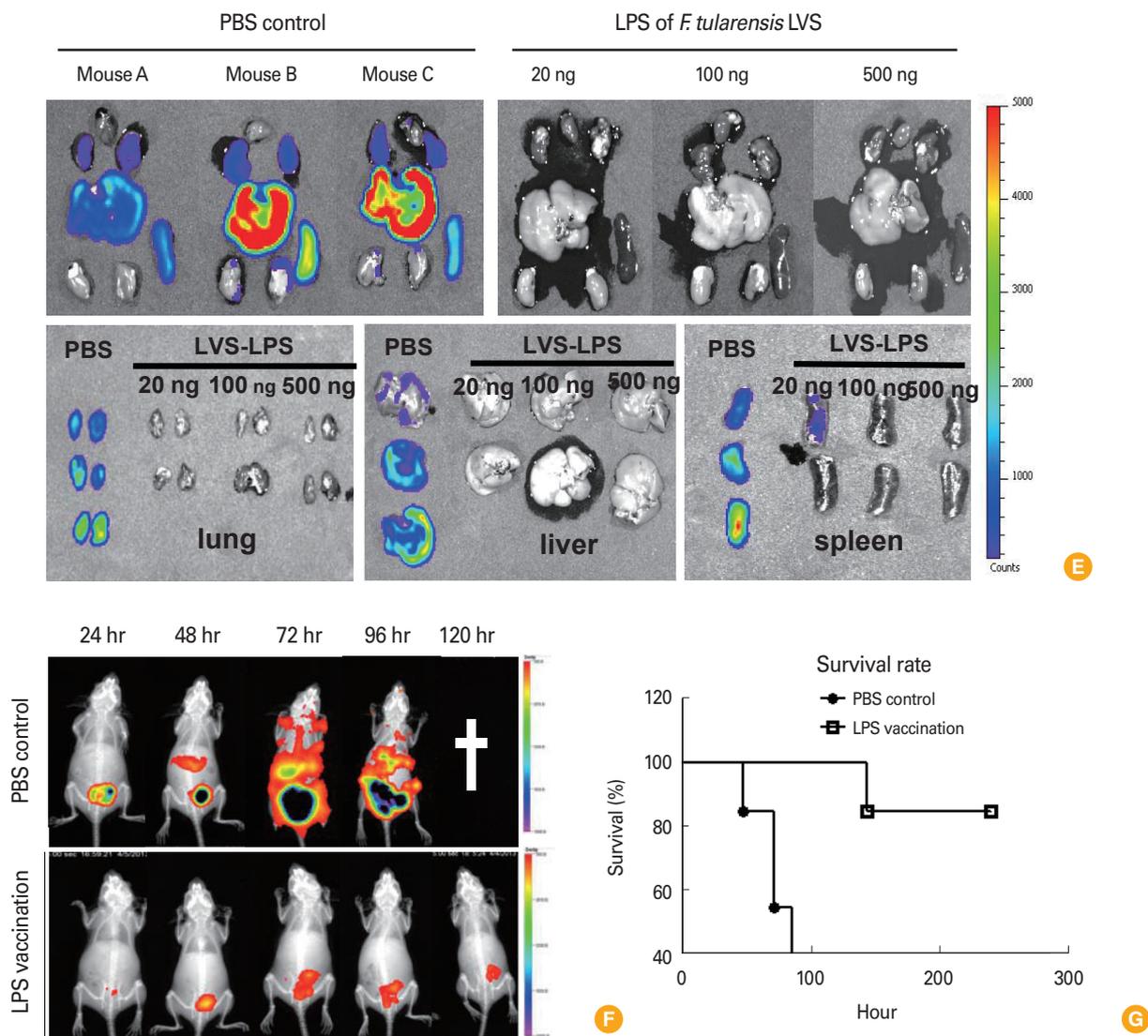


Fig. 4. (Continued) (E) *Ex-vivo* bioluminescence imaging of *FtLVS*-GFP-Lux infected mouse by LPS amount of vaccination. (F) Whole body bioluminescence imaging of *FtLVS*-GFP-Lux infected mouse with or without LPS vaccination. (G) Survival rate of infected mice with or without LPS vaccination. *FtLVS*, *Francisella tularensis* LVS; LPS, lipopolysaccharide; FACS, fluorescence-activated cell sorting; PBS, phosphate buffered saline; CFU, colony forming unit.

Since we use live vaccine strain, we tested the vaccination effect of *FtLVS* by monitoring *FtLVS*-GFP-Lux in the infected mice. *FtLVS* (1×10^3 CFU) were vaccinated to mice, challenged with *FtLVS*-GFP-LUX at 3 weeks after vaccination, and monitored the proliferation of *FtLVS*-GFP-LUX by fluorescence and bioluminescence. We isolated the organs from infected mice with or without vaccination, and monitored imaging signals (Fig. 3C). At 96 hours after challenge of *FtLVS*-GFP-LUX, vaccinated mouse clearly showed less fluorescence and bioluminescence signals in the target organ indicating less proliferation of *FtLVS*-GFP-LUX.

***FtLVS*-GFP-Lux imaging after LPS vaccination**

For evaluation of our imaging analysis as an efficient monitoring system of vaccine development, we tested the vaccine efficacy of LPS for *F. tularensis* infection. General protection efficacy of LPS from *F. tularensis* was reported [11,12], but the detailed process of pathogenesis or vaccination was not fully investigated. With advantage of our dual-labeled *F. tularensis* monitoring system using *FtLVS*-GFP-Lux, we investigated the process of *in vivo* pathogenesis in mice with or without *F. tularensis*-LPS vaccination (Fig. 4A). Fluorescent signals from GFP from *FtLVS*-GFP-Lux were analyzed for time-lapse single cell imaging (Fig. 4B) and flow cytometry (Fig. 4C). Biolumi-

nescent signals from Lux of *FtLVS*-GFP-Lux were analyzed for non-invasiveness pathogen tracing (Fig. 4E, F).

For visualizing replication pattern of *F. tularensis*, splenocytes from non-vaccinated and LPS (20 ng/mouse) vaccinated mice were subjected to time-lapse fluorescent microscopy. *FtLVS*-GFP-Lux was rapidly replicated within splenocyte of non-vaccinated control mouse throughout observation, while GFP-expressing *FtLVS*-GFP-Lux cannot be replicated and disappeared within splenocytes of LPS vaccinated mice (Fig. 4B). Then, we analyzed more detailed event of *F. tularensis* infection using fluorescent signals from *FtLVS*-GFP-Lux in cellular level by measuring the portion of infected cell in each target organ using flow cytometric analysis. At 72 hours after infection, GFP signals were observed in the cells from liver, spleen and lung of control mouse indicating *FtLVS*-GFP-Lux infection, while GFP signals were not observed in the cells from LPS vaccinated mice (Fig. 4C).

To decide the optimized vaccination amount of LPS, bacterial burden was also calculated by counting the infected cells in the target organ of mice with or without LPS vaccination. LPS vaccination clearly reduced *F. tularensis* infection and higher amount (500 ng) of LPS showed more effective vaccination results (Fig. 4D). We also monitored the proliferation of challenged *FtLVS*-GFP-Lux in the organ of infected mouse with different amount of LPS vaccination (Fig. 4E). LPS vaccinated mice showed lower bioluminescence signals indicating less *FtLVS*-GFP-Lux proliferation.

In the whole body imaging, *FtLVS*-GFP-Lux in the control non vaccinated mouse was multiplied at infection site (peritoneal cavity for intraperitoneal injection) first and propagated to other sites 24 hours after infection (Fig. 4F, upper panel). Liver is the first target site of propagation and, lung and spleen is also revealed as target organ of bacterial invasion. On the other hand, only limited signals of *FtLVS*-GFP-Lux from LPS vaccinated mouse were detected at infection site (Fig. 4F, lower panel). LPS vaccination also clearly increased survival rate of infected mouse (Fig. 4G).

Our results successfully demonstrated the efficacy of *F. tularensis*-LPS vaccination to the *F. tularensis* infection by using various imaging methods at the cellular, organ, and whole body level. These indicate that our *FtLVS*-GFP-Lux system can be very useful for the investigation of *F. tularensis* pathogenesis with various analysis methods such as histology, flow-cytometry, *in vitro* and *in vivo* molecular imaging.

Discussion

To evaluate our bacterial reporter imaging as an efficient monitoring system for vaccine development, we developed a sensitive imaging reporter to visualize *F. tularensis* and tested the efficacy of LPS vaccine for *F. tularensis* infection. In previous studies, LPS purified from *F. tularensis* has meaningful efficacy as vaccine for *F. tularensis* LVS infection [11,12]. Our results from cellular, organ, and whole body analysis clearly confirmed that vaccination with *F. tularensis*-LPS efficiently prevent infection by *FtLVS*. These results suggest that our imaging analysis system with dual-labeled bacteria is valuable for study on infectious disease. Bioluminescent imaging capacitates real-time tracing of bacteria for analysis of pathogenic progress in whole organism level, and fluorescent imaging is able to be applied for the analysis of mechanisms using real-time single cell imaging, infected cell sorting, histology and other *in vitro*, *ex vivo* imaging.

Application field of our imaging analysis system is not restricted only in the study of *F. tularensis*, but our system can be expanded to general pathogens, especially in the case bacteria specific antibodies are not available. Any kinds of pathogens can be visualized and traced by substitution of appropriate replication origin and promoter for target bacteria. Furthermore, for several pathogens, attenuated bacteria with good vaccine efficacy are available [13,14], and our system can be used for spatio-temporal tracing of bacteria pathogenesis. In study of vaccination mechanism of attenuated bacteria or individual analysis of sub-species which cannot be distinguished by antibody, our system would be very useful. As all subspecies of *F. tularensis* share same activity of replication-origin and promoter, each subspecies would be labeled by substitution with luminescence/fluorescence of different wavelength.

Recently, several kinds of near infra-red (NIR) fluorescent protein was developed and applied to some biological experiment [15,16]. NIR fluorescent protein has relatively longer excitation and emission wave-length so can reduce the autofluorescence and blockage of light which is critical limitation of conventional fluorophores in application to non-invasiveness imaging. For future application, NIR can be another option to overcome the limitation of GFP.

Our dual imaging analysis system provides platform for *in vivo* study without specific antibodies for bacterial infectious disease in cellular and organism level. Moreover, there is large possibility of application using our system to understand certain biological events which specifically activated in certain

condition by changing with conditional promoter.

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