Whole-Body Imaging of Bacteria Expressing mKate2 Fluorescence

Ivan Vuletic[1, 2], Alison Ren[1], Jiaxuan Li[1], Yichen Ding[1], and Jun Li[2]
[1]Department of Biomedical Engineering/College of Engineering, Peking University, No. 5 Yiheyuan Road, Beijing 100871, China
[2]Laboratory Animal Center/Peking University, No. 5 Yiheyuan Road, Beijing 100871, China
Email: vuleticivan@hotmail.it; alisonren603@yahoo.com; {li.jiaxuan97, ycding1122}@gmail.com; likejun@pku.edu.cn

Abstract—**We established and validated a non-pathogenic bacteria to express a far red fluorescence protein mKate2 for in vivo studies in mice. Using the fluorescence reflectance imaging (FRI) system, the bacteria expressing mKate2 was tested non-invasively and in real-time in different mouse body compartments, including subcutaneous, abdominal, and gastrointestinal tract. Our results suggested that bacteria colonization could be clearly visible and successfully monitored over time in live animals. This method could be a prospective approach for further studying of either pathogenic or non-pathogenic bacterial infection, antibiotic therapy or bacteria as drug-gene delivery for tumor therapy in small animal model such as mice.**

Index Terms—**bacteria imaging, mKate2, fluorescence**

I. Introduction

The study of bacteria in preclinical research is widely accepted as a crucial doorway to a myriad of medical disciplines, including gastrointestinal health, infection disease, and tumor-gene therapy [1]-[4]. While monitoring the development of bacteria in vitro can provide us with basic knowledge of bacterial nature, such observational methods cannot accurately simulate the bacteria interactions with the live body environment (cells, immunologic response, etc.). Thus, it is crucial for us to conduct longitudinal studies in vivo via small animal models to minimize the current disconnect between preclinical and clinical studies.

In recent years, powerful whole-body imaging techniques have been developed that allow real-time monitoring of animal models without the need of surgery. These new technologies not only drastically reduce the number of small animals needed for research, but more importantly, they also open up the possibility of observing animal models without any external interference. Unlike most popular imaging modalities, optical imaging allows surveillance of small animals with minimal modifications. Functional modalities such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) require the injection of harmful radiotracers that can potentially disrupt the biological conditions of both the animal model and the cells, leading to less reliable experimental results. Moreover, these devices are not suitable for frequent real-time imaging due to their high radiation. On the other hand, optical imaging using luminescence proteins as markers does not demand the use of exogenous injections (not required for fluorescence reporter genes but however, required for bioluminescence probes) and has no radiation, allowing safe and accurate imaging of the animal models. Furthermore, optical imaging using Fluorescence Reflectance Imaging (FRI) or Fluorescence Molecular Tomography (FMT) with fluorescent protein markers is the most economical and efficient method for frequent non-invasive real time imaging [3]. However, the major drawback of these techniques is the need to overcome the strong effects of scattering, absorption, and auto-fluorescence in order to monitor bacterial behavior under deep-tissues.

In the present study we have established and validated a non-pathogenic *Escherichia coli* strain to express a far red fluorescence protein mKate2 in vivo, using immunocompromised nude mice as experimental model. mKate2 is a far red fluorescent protein that possesses high brightness and photostability along with low background signals, making it the superior choice for imaging compared to its more common counterparts with shorter wavelengths like GFP and RFP [5]. Due to its long emission wavelength and ability to penetrate deep living tissues, mKate2 can effectively reduce problems such as the scattering and absorption of light. However, compared to other very common bacteria-imaging reporter gene (LuxABCDE), mKate2 requires light excitation to be imaged, which leads to higher auto-fluorescence detection. By contrast, the mKate2 fluorescence imaging described here is faster (requires shorter data collection time) and can be easily applicable to active and non-active bacteria. By using whole-body FRI system, we were able to closely monitor the movement and colonization of bacteria non-invasively and in real-time, in different animal compartments, including the subcutaneous, the abdominal cavity and the gastrointestinal tract (GIT). The components and procedures for setting up the imaging equipment, the construction of the animal model and the injections operating methods are comprehensively presented in

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detail. Furthermore, according to our knowledge, this is the first time that mKate2 reporter gene was used for detecting a bacterial infection in a nude mice animal model.

II. Materials and Methods

A. Bacteria Strains, Cancer Cell Lines, Plasmids and Experimental Animals

All reagents were purchased as follows: BL21-DE3 bacteria strains (Invitrogen), Kanamycin (HyClone), pmKate2-N vector (Evrogen) and isopropyl b-D-thiogalactoside-IPTG (Amresco). PET-28a(+) vector was kindly gift from Dr. Shang Shujiang (College of Life Science, Peking University).

B. Experimental Animals

BALB/c female nude mice, 6 weeks old, weighing 20-25g, were obtained from Vital River Laboratories, China. All the animals were housed in an environment with temperature of 24 ± 1 ℃, relative humidity of 50 ± 1% and a light/dark cycle of 12/12 hr. Before each experiment, mice were fasted for 12hrs, allowing the mice to freely drink. Moreover, all animals were handled according to the ethical guidelines of the Peking University Animal Research Committee. Surgical procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University.

C. Construction and Induction Method of Bacteria Expressing an mKate2 Reporter Gene

mKate2 fluorescent BL21-DE3 E.coli strains was generated. Briefly, a 700 base pair coding region encompassing the mKate2 gene was amplified by PCR from a pmKate2-N vector plasmid and consequently digested with HindIII and Not1 and ligated to a PET-28a(+) inducible plasmid, in order to generate a Pet28a-mKate2 expression vector. Pet28a-mKate2 was then transformed into BL21-DE3 E.coli strain using the standard heat-shock method, in order to generate a novel BL21-mKate2 strain.

In order to measure the BL21-mKate2 expression of the mKate2 protein, a SDS-page assay was used. Briefly, 3mL of bacteria was first cultured overnight in lysogeny broth (LB) medium with kanamycin, with additional shaking at 37 ℃. The overnight cultures were then diluted 1:100 with LB medium with an addition of 1mM IPTG and incubated at 37 ℃ for 1h to 6h. The protein expression of mKate2 was measured each single hour using SDS-page gel.

D. In Vivo Experiments: Subcutaneous, Abdominal and GIT Imaging of BL21-mKate2

For all in vivo experiments, bacteria were first cultured overnight in LB medium with kanamycin, with additional shaking at 37 ℃. The overnight cultures were then diluted 1:100 with LB medium with an addition of 1mM of IPTG and incubated at 37 ℃ for an additional 4h.

For the subcutaneous model, bacterial colonization was tested in the subcutaneous region of nude mice. Briefly, 10^7 BL21-mKate2 in 100μL of PBS were injected in the left lower flank using 1mL 27G latex-free syringes. The bacteria were monitored non-invasively and in real-time over 7days post gavage using a fluorescence detection method.

Secondly, the bacteria infection was tested in the abdominal and GIT compartments. For the abdominal experiment, 10^6 BL21-mKate2 in 200μL of PBS were injected using 1mL 27G latex-free syringes in the lower abdominal region and the mKate2 fluorescence was detected in the time range from 0 min to 120 min post gavage. For the GIT testing, 10^6 BL21-mKate2 were administered in 200μL per mouse by oral gavage and the mKate2 fluorescence was detected in the time range from 0 min to 140 min post gavage.

E. FRI System

The whole imaging system was composed of four parts: a tungsten-halogen based excitation module, an adjustable animal holder, a high sensitivity charged couple device (CCD) camera based detection module and a gas anesthesia component (Fig. 1). Briefly, each mouse was subject to isofluorane anesthesia procedures and placed in the animal bed in the ventral position. High resolution images (512x512 pixels) were taken with a sensitive CCD camera (iKon 934M, Andor) using a filter set (ext. FF01-562/40-25; em. FF01-641/75-25, Semrock).

Fig1.

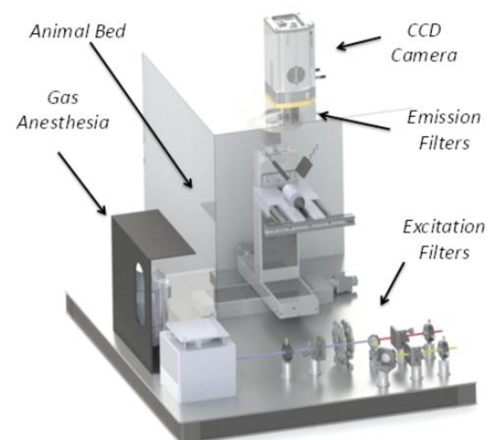

Figure 1. Fluorescence reflectance imaging system (FRI)

F. Data Processing

During the imaging, the signals were detected without averaging. The fluorescence was induced by the illumination source. For appropriate detection of mKate2 signal, 10s exposure time was used for the subcutaneous, abdominal and GIT imaging and 1s exposure time was used for the in vitro bacteria cell imaging. The raw data was acquired under the control of baseline clamp and processed by Andor SOLIS. The brightness and contrast of images were processed by ImageJ. All images were processed in pseudo-color labeled in rainbow.

III. Results

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A. Induction and Expression of mKate2 in Vitro

The mKate2 gene was induced by adding 1mM IPTG in the bacteria LB culture broth. The protein expression was measured after single time-point (from 1h to 6h) using SDS-page gel (Fig. 2A). Those results have shown good gene expression starting from the 3rd hour post IPTG addition. Moreover, lower gene expression was detected in the first two hours post IPTG addition.

Secondly, the mKate2 showed good sensitivity by the FRI system (Fig. 2B) and fluorescence microscope (Fig. 2C), respectively. Also, the Fig. 3 show excellent correlation between different bacteria BL21-mKate2 cells concentration and mKate2 fluorescence intensity detected by the FRI (Fig. 3).

Fig2.

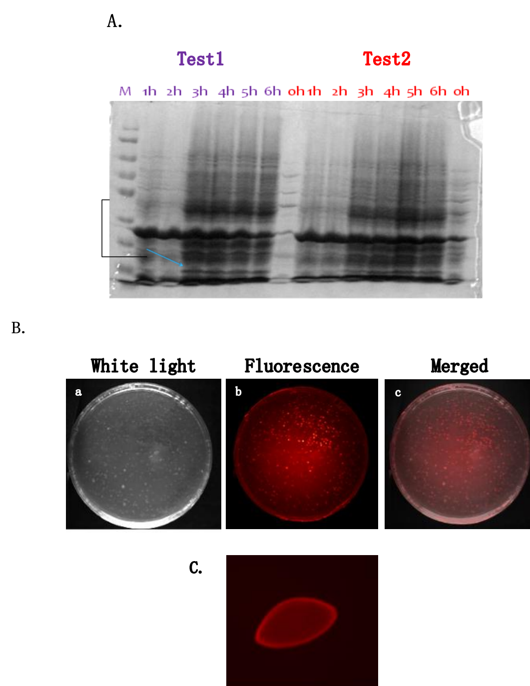

Figure 2. BL21-mKate2 induction and expression in Vitro A) SDS-page results from test1(violet) and test2(red) following 6h post IPTG addition. B) viable BL21-mKate2 clones in 100mm dish imaged with the FRI device a) white light image b) fluorescence image, and c) merged image. C) single viable BL21-mKate2 clone detected by the fluorescence microscope.

B. Whole-Body Imaging of Bacteria Expressing mKate2

Bacteria expressing mKate2 fluorescence was tested in different animal body compartments using an optical imaging device developed by our research group (Fig. 1). First, we tested the bacterial colonization and their fluorescence expression in the subcutaneous region. The injected bacteria showed persistence and good fluorescence emission over 7 days post gavage (Fig. 4). This result suggests that induced bacteria expressing mKate2 can be visible non-invasively and in real-time in the subcutaneous region over longer time without additional injection of IPTG. However, based on our results, we noticed some decreasing of mKate2 fluorescent signal after day two post cell injection (Fig. 4c and d), possibly due to bacteria migration and spreading to the abdominal cavity (Fig. 4d).

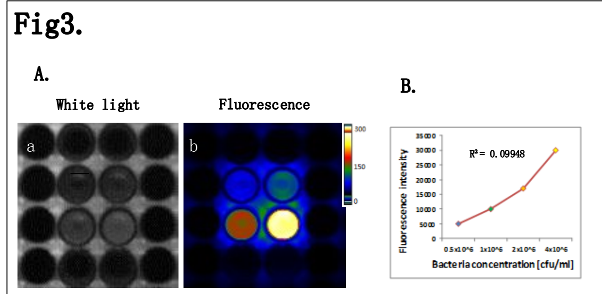

Figure 3. Correlation between different bacteria cells concentration and their mKate2 signal, detected using the FRI system. A) Four different cell concentrations (blue: 0.5x10^6, green: 1x10^6, orange: 2x10^6 and yellow: 4x10^6) were plated in 4 wells of a 96well dish. The images were detected a) under white light, and b) using mKate2 ext. and em. filters. B) Plot correlation between 4 different bacteria cell concentration and mKate2 signal detected. The four bacteria concentration points are indicated in color (from blue to yellow) and can be correlated with images represented in Figure3A-b.

Fig4.

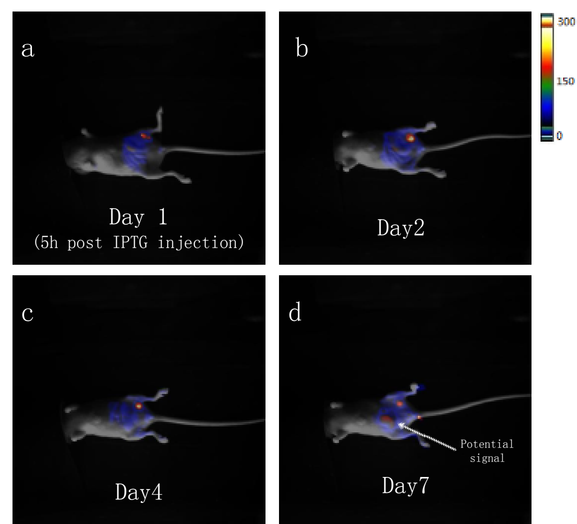

Figure 4. Subcutaneous imaging of BL21-mKate2 over different time points a) Day1 post gavage b) Day2 post gavage c) Day3 post gavage and d) Day7 post gavage. On Day7 the “potential signal” indicates the potential migration of the bacteria in the abdominal area.

Secondly, the bacterial colonization was tested in the abdominal cavity (Fig. 5). After the cells were carefully injected in the abdominal area, single images were taken over different time-points (Fig. 5A). These results suggested that bacteria expressing mKate2 can be clearly and successfully monitored in the peritoneum area non-invasively and in real-time, using a fluorescence imaging device. Moreover, the highest fluorescence point detected (higher point of the scale bar value) of each image was captured, demonstrating the spreading of bacteria from a larger area (Fig. 5A-b and Fig. 5A-c) to a single location point (Fig. 5A-d).

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Fig5.

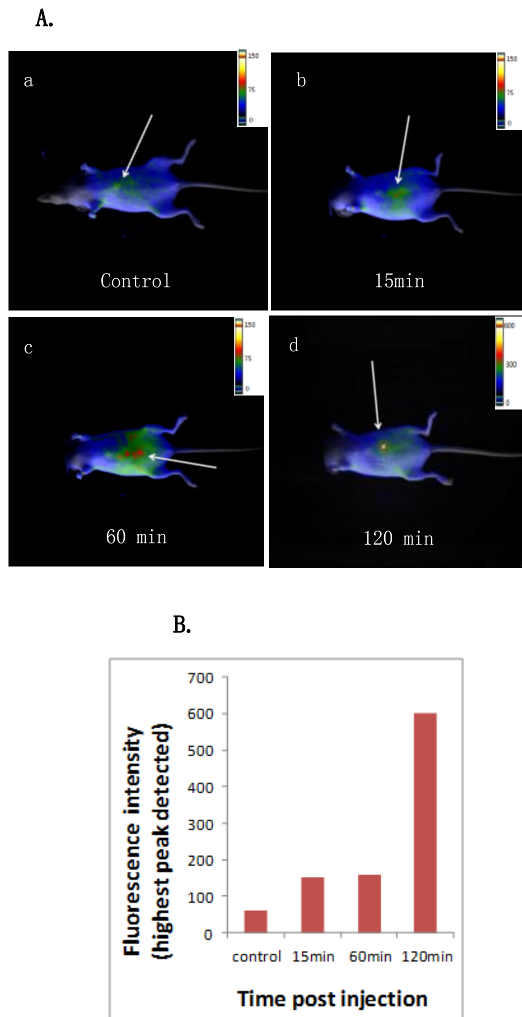

Figure 5. Abdominal imaging of Bl21-mKate2 A) Non-invasive imaging of BL21-mKate2 colonization in the abdominal region over different time points a) control-no cells injected b) 15 min after gavage c) 60 min after gavage and d) 120 min after gavage. B) Correlation between the highest fluorescence intensity peak detected (white arrow from Fig5A images) of each image and the time of fluorescence detection (time post cell injection-time after gavage).

Fig6.

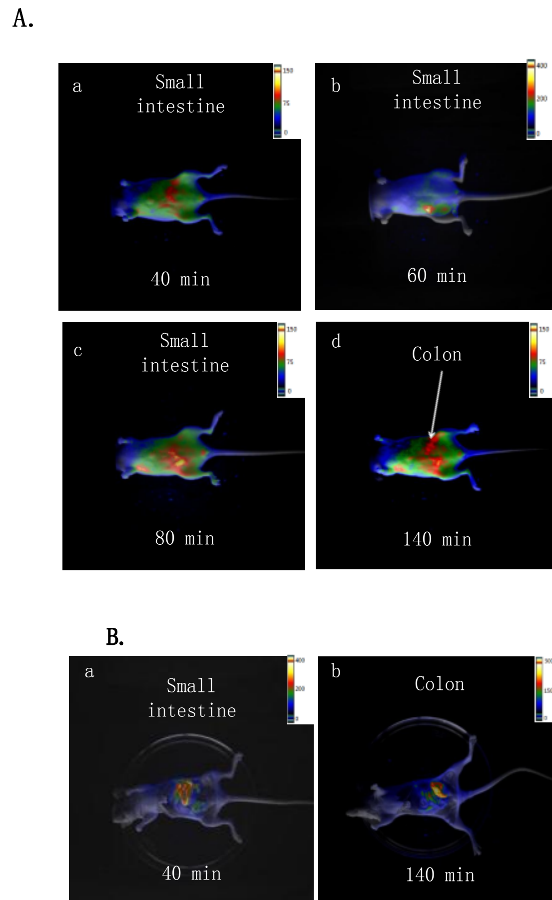

Figure 6. GIT imaging of Bl21-mKate2 A) non-invasive imaging of BL21-mKate2 colonization in the GIT region over different time and in different location areas: a) Bl21-mKate2 colonization of the small intestine 40 min after gavage b) Bl21-mKate2 colonization of the small intestine 60 min after gavage c) Bl21-mKate2 colonization of the small intestine 80 min after gavage d) Bl21-mKate2 colonization of the colon 140 min after gavage B) ex vivo imaging of BL21-mKate2 colonization of a) small intestine 40 min after gavage , and b) colon 140 min after gavage

Lastly, the bacterial colonization was tested in the GIT (Fig. 6). The cells were injected by oral gavage and the bacteria colonization was monitored in different time points and different GIT location areas (Fig. 6A). These results suggested that bacteria expressing mKate2 can be clearly and successfully monitored in the different GIT locations (bacteria travelling from the small intestine to the colon) non-invasively and in real-time, using fluorescence imaging. In addition, the fluorescence detection from BL21-mKate2 40 min after gavage and 140min after gavage was also tested ex vivo. Those results confirmed a perfect correlation between non-invasive images and the ex vivo result, demonstrating the localization of bacteria in the small intestine and in the colon region, respectively (correlation between Fig. 6A-a and Fig. 6B-a and between Fig. 6A-d and Fig. 6B-b)

IV. Discussion and Conclusion

The use of non-invasive real-time imaging of the mKate2 expressing bacteria has shown to be an excellent approach for bacteria detection and observation in vivo using powerful imaging devices such as FRI device. Our results suggest that bacteria expressing mKate2 fluorescence could be easily developed and clearly detected in different mice body compartments including subcutaneous, abdominal and gastrointestinal tract.

While subcutaneous fluorescence is already a well-known model for targeting both bacteria and tumor using fluorescence proteins with shorter wavelength (bellow 600) such as GFP [6], deeper compartments such as GIT often require fluorescence proteins with longer wavelength such as mNeptune or mKate2 (emission over 630nm) to maximally reduce problems with light scattering and autofluorescence. Over the past decade a great interest was shown toward the gastrointestinal

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bacteria development. Many researches in this field have been focusing on early bacteria colonization in the gut [7] and on how "good" or "bad" bacteria interact with other bacteria and cells, contributing to our health or disease, respectively [8]. However, the routes and mechanisms of those bacterial biological behaviors are still very unclear. Furthermore, more preclinical and clinical studies are necessary. Our results suggested that mKate2, which showed very good sensitivity and stability in deep tissue compartments by the FRI device, could be used as appropriate choice for studies on gut microbiome development using nude mice as animal model. Furthermore, this approach could be also an ideal model for studying both pathogenic and non-pathogenic bacterial infection, antibiotic therapy and tumor drug-gene delivery, in both subcutaneous and deep tissue organs. In addition, the combination of mKate2 with LuxABCDE imaging could be very useful for future multiple bacteria experiments using a single mouse as a control model.

This study has also demonstrated a good application of detecting fluorescent bacteria using the optical imaging device designed by our group. In addition, the far-red fluorescence protein mKate2 have a potential to be applied in a three-dimensional optical tomography system for quantitative bacteria deep tissue imaging experiments.

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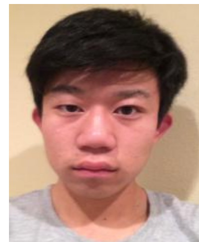
Ivan Vuletić was born in Croatia on July 1st, 1985. He obtained his BSc in Sanitary Biotechnology (2007) and MSc in Medical Biotechnology (2011) from University of Padua, Italy. During his MSc he spent 9 months at UCLA working on master's dissertation project. In 2012 he was awarded Chinese governmental scholarship for PhD in Biomedical Engineering lab at Peking University. His research interest is molecular imaging, using mice as animal model.

Alison L. Ren is a high school senior at Fairmont Preparatory Academy and will be graduating in May as part of the class of 2015. She is in the International Baccalaureate Diploma Program and also the Advanced Science and Engineering Program at her school. She is currently applying to universities and looking forward to a future in Biomedical Engineering. She has been involved in ongoing research at Peking University in Beijing, China since the summer of 2011.



Jiaxuan Li was born in Shandong, China on March 6th, 1997. He is currently attending the 12th grade at Fairmont Preparatory Academy in the state of California, USA. Jiaxuan will be graduating in May 2015 and is currently applying for various universities to pursue his passion in the field of engineering. Besides his participation in biomedical engineering research at Peking University during the summer 2014, Jiaxuan also enjoys competing in various rocketry and robotics events.

Yichen Ding is currently a Ph.D. candidate in the Department of Biomedical Engineering, Peking University, China; He obtained his BSc (2010) from the Department of Precision Instrument, Tsinghua University, China. His research interest is optical imaging.

Jun Li was born in China on July 1st, 1970. He obtained his BSc in Medical Science from Faculty of Preventive Medicine, Jilin University, China in 1993. In 2002 he obtained his MSc of Tumor Pathology and in 2006 his PhD in tumor pathology from Shantou University Medical College, China. In 2007, he was awarded postdoctoral position in a Laboratory of Nucleic Acid Technology, Institute of Molecular Medicine, Peking University, China. Currently he is working as Senior Engineer in molecular imaging lab, Laboratory Animal Center, PKU.

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