Journal of Visualized Experiments

Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59763R2
Full Title:	Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis
Keywords:	Tumor growth, Tumor angiogenesis, Breast cancer, Tumor-bearing mice, HSV-ttk, Bioluminescence imaging, Firefly luciferase, Renilla luciferase
Corresponding Author:	Zongjin Li Nankai University Tianjin, Tianjin CHINA
Corresponding Author's Institution:	Nankai University
Corresponding Author E-Mail:	zongjinli@nankai.edu.cn
Order of Authors:	Kaiyue Zhang
	Chen Wang
	Ran Wang
	Shang Chen
	Zongjin Li
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Tianjin, Tianjin, China

TITLE:

Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis

AUTHORS:

Kaiyue Zhang[1], Chen Wang[1], Ran Wang[2], Shang Chen[1], Zongjin Li[1*]

[1]Nankai University School of Medicine, Tianjin, China
[2]State Key Laboratory of Medicinal Chemical Biology and College of Pharmacy, Nankai University, Tianjin, China

zhangkaiyue177@163.com
wangchen_echo@163.com
546814212@qq.com
chenshang2010@163.com
zongjinli@nankai.edu.cn

Corresponding Author:
Zongjin Li, MD, PhD
zongjinli@nankai.edu.cn

KEYWORDS:

Tumor growth, Tumor angiogenesis, Breast cancer, Tumor-bearing mice, HSV-ttk, Bioluminescence imaging, Firefly luciferase, Renilla luciferase

SUMMARY:

In this protocol, we build a tumor-bearing mouse model to real-time monitor tumor progression and angiogenesis by dual bioluminescence imaging.

ABSTRACT:

Angiogenesis, as a crucial process of tumor progression, has become the research hotspot and the target of anti-tumor therapy. However, there is no good model for tracing tumor progression and angiogenesis simultaneously in a visual and sensitive way. Bioluminescence imaging displays its unique superiority in living imaging due to its advantages of high sensitivity, strong specificity, and accurate measurement. Here, we present a protocol to build a tumor-bearing mouse model by injecting a renilla luciferase-labeled murine breast cancer cell line 4T1 into the transgenic mouse with angiogenesis induced firefly luciferase expression. This mouse model provided a valuable tool to monitor tumor progression and angiogenesis in real-time by dual bioluminescence imaging simultaneously in a single mouse. This model may be widely applied in anti-tumor drug screening and oncology researches.

INTRODUCTION:

Angiogenesis is an essential process in the cancer progression from small, localized neoplasms to larger, potentially metastatic tumors[1,2]. The correlation between tumor growth

and angiogenesis, therefore, becomes one of the emphases in the field of oncology research. However, traditional methods of measuring morphologic change fail to monitor tumor progression and angiogenesis simultaneously in living animals with a visualized approach.

Bioluminescence imaging (BLI) of tumor cells is a particularly appropriate experimental method to monitor tumor growth because of its non-invasion, sensitivity, and specificity characteristics[3-6]. BLI technology is based on the principle that the luciferase could catalyze oxidation of the specific substrate while emitting bioluminescence. The luciferase expressed in the implanted tumor cells reacts with the injected substrate, which can be detected by a living imaging system and signals will indirectly reflect the change of cell number or cell localization in vivo[6,7].

Except for the tumor growth, tumor angiogenesis, the critical step in cancer progression, can also be visualized through BLI technology using Vegfr2-Fluc-KI transgenic mice[8-10]. The vascular endothelial growth factor (Vegf) receptor 2 (Vegfr2), one type of receptors of Vegf, is mostly expressed in the vascular endothelial cells in adult mice[11]. In Vegfr2-Fluc-KI transgenic mice, the DNA sequence of firefly luciferase (Fluc) is knocked into the first exon of the endogenous Vegfr2 sequence. As a result, the Fluc will be expressed (which appears as BLI signals) in a manner that is identical to the angiogenesis in mice. To grow beyond a few millimeters in size, the tumor recruits new vasculatures from existing blood vessels, which highly express the Vegfr2 triggered by growth factors from tumor cells[1]. This opens the possibility of using Vegfr2-Fluc-KI transgenic mice to non-invasively monitor tumor angiogenesis by BLI.

In this protocol, we created a tumor-bearing mouse model to monitor the tumor progression and angiogenesis in a single mouse through firefly luciferase (Fluc) and renilla luciferase (Rluc) imaging, respectively (**Figure 1**). We created a 4T1 cell line (4T1-RR) that stably express Rluc and red fluorescent protein (RFP) to trace cell growth by Rluc imaging. To further investigate the dynamic changes of angiogenesis in the progression and regression of tumor, we created another 4T1 cell line (4T1-RRT) expressing suicide gene herpes simplex virus truncated thymidine kinase (HSV-ttk), Rluc and RFP. By administration of ganciclovir (GCV), the HSV-ttk expressing cells will be selectively ablated. Based on these cell lines, we built a tumor-bearing model in Vegfr2-Fluc-KI mice which served as an experimental model bridging tumor progression with tumor angiogenesis in vivo.

PROTOCOL:

Experiments must comply with national and institutional regulations concerning the use of animals for research purposes and permissions to carry out experiments must be obtained. The treatment of animals and the experimental procedures of the present study adhere to the Nankai University Animal Care and Use Committee Guidelines that conform to the Guidelines for Animal Care approved by the National Institutes of Health (NIH).

1. LV-Rluc-RFP (RR) and LV-Rluc-RFP-HSV-ttk (RRT) lentiviral packaging and production

NOTE: The pLV-RR carries the gene sequences of renilla luciferase (Rluc) and red fluorescent protein (RFP) under the promoter EF1α, whereas the pLV-RRT carries the gene sequences coding Rluc, RFP, and herpes simplex virus truncated thymidine kinase (HSV-ttk) (**Figure 2**).

1.1. Seed 1 x 10^6 per well 293T cells into 6-well plate and culture overnight in a humidified incubator with 5% CO_2 at 37 °C with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

1.2. Prepare the liposome suspension: mix 7.5 µL of liposome and 0.25 mL of minimal essential medium (MEM) into a 1.5 mL tube following incubation for 5 min at room temperature to disperse liposome equably.

1.3. Prepare the DNAs solution (DNAs-RR): separately, add pLV-RR vector and helper plasmids into 0.25 mL of MEM in a 1.5 mL tube as described in **Table 1**.

1.4. Obtain the liposome/DNAs-RR compound: gently add the DNAs-RR solution into prepared liposome suspension drop by drop and incubate for 20 min at room temperature to make DNA bond to the lipid membrane.

1.5. Replace the medium of the 293T cells with 1 mL of DMEM containing 10% FBS and add the liposome/DNAs-RR compound to the medium of the 293T cells gently.

1.6. After incubating in a humidified incubator with 5% CO_2 at 37 °C for 12-16 h, replace the liposome/DNAs-RR compound containing medium of the 293T cells with 2 mL of DMEM containing 10% FBS and 100 U/mL penicillin–streptomycin.

1.7. Continue culturing the 293T cells in the humidified incubator for 48 h after transfection. Then, collect the supernatant of the 293T cells and centrifuge the medium at 300 x *g* for 5 min to pellet the 293T cells. Transfer the lentivirus-RR (LV-RR)-containing supernatant into 1.5 mL sterile polypropylene storage tubes and store at -80 °C.

NOTE: A Biosafety Level 2 (BSL-2) facility is required in order to work with recombinant lentivirus.

1.8. Repeat steps 1.1-1.7 and use pLV-RRT vector instead of pLV-RR vector in step 1.3 to obtain the lentivirus-RRT (LV-RRT). Store the LV-RRT at -80 °C.

NOTE: The non-purified lentiviral stock may inhibit cell growth in some cases. Lentiviral stock may need to be purified. The lentiviral stocks containing LV-RR or LV-RRT particles should be divided into 1.5 mL tubes (1 mL per tube) for storage to avoid multiple free-thaw cycles.

2. LV-RR and LV-RRT lentiviral transduction for gene expression in 4T1 cells

2.1. Seed 4T1 cells into a 6-well plate (5 x 10^5 cells/well) and culture with Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS overnight in a humidified incubator with 5% CO_2 at 37 °C.

2.2. Remove the medium from the culture plate and replace it with 1 mL fresh RPMI 1640 medium as well as 1 mL of lentiviral stock (LV-RR or LV-RRT) to each well. Add 8 μg/mL polybrene and gently blend the medium containing lentiviral particles by pipetting up and down.

NOTE: Please be aware that the medium contains lentiviral particles, which could transduce human cells.

2.3. Spin transduction in a centrifuge at 1,000 × *g* for 60 min at room temperature helps increase of transduction efficiency. After centrifugation, culture 4T1 cells for 4-12 h and maintain in a humidified incubator with 5% CO_2 at 37 °C.

NOTE: For some cell lines, polybrene may be toxic for long-term culture. Therefore, the incubation time for transducing different cells may be changeable. Check the cell status multiple times to find appropriate incubation time.

2.4. Refresh the medium of transduced 4T1 cells with 2 mL of RPMI 1640 medium containing 10% FBS and 100 U/mL penicillin–streptomycin to remove lentiviral particles and polybrene.

3. Drug screening and identification of LV-RR and LV-RRT transduced 4T1 cells

3.1. Select transduced cells with medium containing blasticidin (BSD) according to the BSD-resistance gene carried by LV-RR or LV-RRT as the following steps described.

NOTE: Alternatively, the transduced cells which are RFP-positive could be selected by flow cytometry according to the RFP gene carried by LV-RR or LV-RRT.

3.2. 48 h after transduction, passage 4T1 cells at the ratio of 1:3 to 1:4 with selection medium (RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin–streptomycin, and 5 μg/mL BSD). Change medium every 2 or 3 days.

NOTE: The optimal BSD concentration may vary from cell line to cell line. Therefore, a pilot experiment of kill curve should be performed to determine the optimal concentration of BSD before initial experiment.

3.3. 7 days post drug screening, observe the LV-RR transduced 4T1 cells (4T1-RR) and LV-RRT transduced 4T1 cells (4T1-RRT) under the fluorescence inverted phase contrast microscope. Count the number of RFP^+ 4T1 cells and all 4T1 cells in three fields of vision to estimate the RFP-positive ratio, respectively (**Figure 2**).

NOTE: Alternatively, the RFP-positive ratio of transduced 4T1 cells could be identified by flow cytometry.

3.4. Measure the renilla signals of 4T1-RR cells and 4T1-RRT cells by using a living imaging system to detect the linear relationship between cell numbers and renilla signals (**Figure 3**).

3.5. Expand BSD-screened 4T1-RR and 4T1-RRT cells with selection medium at split ratios between 1:3 and 1:4 and store the cell line stocks in liquid nitrogen.

4. Vegfr2-Fluc-KI mice and tumor-bearing mouse model

NOTE: The transgenic Vegfr2-Fluc-KI mice, 6-8week, female, are used in this experiment to non-invasively monitor angiogenesis in vivo by BLI.

4.1. Culture 4T1-RR cells and 4T1-RRT cells in 60 mm Petri dishes in a humidified incubator with 5% CO_2 at 37 °C, respectively. When the cells are at 80% confluence, remove the medium and rinse with phosphate buffered saline (PBS).

4.2. Remove the PBS and add an additional 2 mL of 0.25% trypsin-0.53 mM EDTA solution respectively. Keep the dish at room temperature (or at 37 °C) until the cells detach.

4.3. Add 5-10 mL of fresh medium containing 10% FBS, aspirate and dispense cells to resuspend 4T1-RR and 4T1-RRT cells into 15 mL centrifuge tubes, respectively. Count two kinds of 4T1 cells by using a counting chamber and prepare the cell suspensions at a concentration of 1 x 10^6 per 100 µL in RPMI 1640 medium.

4.4. Anesthetize the Vegfr2-Fluc-KI mice with 1-3% isoflurane in 100% oxygen at anesthesia induction chamber with a flow rate of 1 L/min. Monitor the toe pinch response of the mouse to confirm the status of anesthesia. Then, apply ophthalmic ointment to the eyes of mouse to prevent dehydration.

4.5. Remove mouse from chamber and position in nosecone. Entirely remove the hair of the shoulder of mouse by using electric shaver and hair removal cream, which could provide a good view of surgical field and avoid blocking the BLI signals in following-up experiments.

4.6. Subcutaneously inject 4T1-RR cells (1 x 10^6 cells at a 100 µL total volume) and 4T1-RRT cells (1 x 10^6 cells at a 100 µL total volume) in left and right shoulders of each mouse, respectively (record as Day 0). Place mice in recovery area with thermal support until fully recovered.

4.7. After implantation of 4T1-RR and 4T1-RRT cells, touch the tumor masses to check that the mice are tumor-bearing every day (**Figure S1**). At day 7 post-implantation, intraperitoneally inject 50 mg/kg ganciclovir (GCV) to the tumor-bearing mice twice a day

until the end of experiment.

NOTE: Before this experiment, the cytotoxic of GCV on 4T1-RRT cells should be detected. The killing efficiency of GCV could be evaluated by cell counting assay with different concentration of GCV (**Figure S2**).

4.8. On the day 0, 3, 7, 14, and 21 after 4T1 implantation, monitor the tumor growth and angiogenesis of tumor-bearing mice and assess by both Rluc and Fluc imaging (**Figure 4**).

5. Dual bioluminescence imaging of tumor (Rluc) and angiogenesis (Fluc)

5.1. Open the living imaging system, initialize the living imaging software, and then initialize the system.

NOTE: The system initialization will take few minutes to cool down the charge-coupled device (CCD) camera to -90 °C before able to start imaging. The temperature will turn green when the CCD camera is cooled.

5.2. Use the following camera settings:
Check the Luminescence and Photograph.
Check Overlay.
Luminescence settings:
Exposure Time sets AUTO in normal conditions.
Binning sets to 8.
F/Stop sets to 1.
Emission Filter sets Open.
Photograph settings:
Binning sets to medium.
F/Stop sets to 8.
IVIS system settings:
Field of view: C=1 mouse view, D=5 mice view.
Subject height sets 1.5 cm.

5.3. Weigh and record the mice and calculate the volume of coelenterazine (CTZ; 2.5 mg/kg) and D-luciferin (150 mg/kg) needed.

5.4. Anesthetize tumor-bearing mouse by 1-3% isoflurane in 100% oxygen at anesthesia induction chamber with a flow rate of 1 L/min. Monitor the toe pinch response of the mouse to confirm the status of anesthesia. Then, dispense a drop of lubricating eye ointment onto both eyes to avoid corneal damage.

5.5. Inject 2.5 mg CTZ (3.33 mg/mL) per kilogram body weight into the retrobulbar of the mouse (e.g., for a 20 g mouse, inject 15 μL to deliver 50 μg of CTZ) by using an insulin syringe needle.

5.6. Move the tumor-bearing mouse into the camera chamber with its nose in the anesthesia cone gently and acquire several pictures of the mouse dorsal immediately to get the Rluc signals from 4T1 cells until the BLI signals fade away.

NOTE: The half-life of CTZ is very short and the signals of Rluc drop precipitously ~30 seconds. To ensure any residual Rluc signal has dissipated and the interval between Rluc and Fluc imaging should be more than 10 min.

5.7. Intraperitoneally inject 150 mg/kg D-luciferin (30 mg/mL) using an insulin syringe needle (e.g., for a 20 g mouse, inject 100 µL to deliver 3 mg of D-luciferin). Keep the mouse at room temperature for 10 min before Fluc imaging.

5.8. Move this mouse into camera chamber with its nose in the anesthesia cone again and acquire several pictures of the mouse dorsal to get the Fluc signals from angiogenesis.

NOTE: The Fluc kinetic monitor should be performed for each mouse until the signals reach the maximum and then fade.

5.9. Repeat the procedures steps 5.4-5.8 for each mouse.

5.10. After imaging, maintain the mice in a warm environment until animals wake up.

5.11. At the desired time point (day 3, 7, 14, and 21), repeat above procedures (step 5.3-5.10) to detect the tumor progression and tumor angiogenesis over time.

5.12. Analyze the Rluc and Fluc signals data to investigate the relationship between the tumor growth and angiogenesis in tumor progression.

NOTE: The regions of interest (ROI) which cover the BLI signal site are used to analyze the data. Measure the total radiance (Photons) of ROI in the unit of Photons/seconds/cm^2/steradian (p/s/cm^2/sr) for every timepoint.

5.13. Analyze the Rluc and Fluc signals of ROI by using graphics software (**Figure 4**).

REPRESENTATIVE RESULTS:

In this experiment, we built a breast cancer mouse model using 4T1 cells to investigate the relationship between tumor growth and tumor angiogenesis (**Figure 1**). Firstly, we packaged two lentivirus which carried gene sequences expressing Rluc/RFP (LV-RR) and Rluc/RFP/HSV-ttk (LV-RRT) respectively as previously reported[7]. Then, two different 4T1 cell lines, named 4T1-RR and 4T1-RRT, were created by transducing LV-RR and LV-RRT respectively. After drug screening for 3 days, the 4T1-RR and 4T1-RRT were observed under a fluorescence microscope to detect the transduction efficiency. As the fluorescence imaging shown, more than 99% of the of 4T1-RR or 4T1-RRT cells were RFP positive, which hinted the 4T1-RR and

4T1-RRT cell lines were established by LV-RR and LV-RRT transduction (**Figure 2A,B**). Meanwhile, we found there was no difference in cell morphology and growth between wild-type 4T1 and 4T1-RR or 4T1-RRT during the culture time. In summary, we successfully built 4T1-RR and 4T1-RRT cell lines without influencing the cellular states. Subsequently, bioluminescence imaging (BLI) of 4T1-RR and 4T1-RRT cells was captured to detect the Rluc signals. The BLI images revealed that both 4T1-RR and 4T1-RRT cells emitted strong bioluminescent signals of the same strength (**Figure 3A**). Besides, the linear relationships between Rluc signals and cell numbers were observed in both 4T1-RR (R^2=0.9974) and 4T1-RRT cells (R^2=0.9989), which suggested the Rluc signals could be used to mirror the tumor growth in vivo (**Figure 3B**).

On this basis, using the transgenic Vegfr2-Fluc-KI mice, we built a tumor-bearing mouse model to investigate the angiogenesis as the breast cancer grows. As a result of knocking Fluc sequence into the first exon of the Vegfr2 sequence in murine, the Fluc could be expressed (which appears as bioluminescent signals) in a manner that was identical to the angiogenesis in mice during the tumor progression. After subcutaneous injection of 4T1-RR and 4T1-RRT cells, cell growth was monitored by Rluc signals in the presence of CTZ at day 0, 3, 7, 14, and 21 (**Figure 4A**). At the same time, angiogenesis induced by tumor growth was evaluated by Fluc signals in the presence of D-luciferin in the same mouse. At day 7 after implantation of 4T1-RR and 4T1-RRT, we administrated GCV to the tumor-bearing mice, which lead 4T1-RRT cells to die. The BLI images revealed that Rluc signals of 4T1-RR and 4T1-RRT cells increased at the same rate before GCV treatment, however, Rluc signals of 4T1-RRT cells sharply decreased post GCV treatment while the Rluc signals of 4T1-RR still increased gently. Obviously, a significant relativity existed between Rluc signals and the tumor size (**Figure S1**). Meanwhile, according to the Fluc images, the Fluc signals increased in keeping with the Rluc rise and decreased following the Rluc decline (**Figure 4B**). These results suggested there was a direct correlation between tumor angiogenesis and tumor growth. The death of tumor cells induced by drug GCV could lead to inhibition of tumor angiogenesis (**Figure 4C**). To demonstrate that the Fluc signal was indeed detecting the angiogenesis within the tumors, we sacrificed the animals after finishing imaging at day 21 to get the histological evidences of vasculature. According to the images of anti-VEGFR2 immunostaining, the microvascular structures in 4T1-RR tumor tissue were significantly more than in 4T1-RRT tumor tissue, which were consisted with the Fluc signals (**Figure 5**). In summary, this dual bioluminescence imaging strategy could be used to monitor the tumor progression and tumor angiogenesis as well as assess the anti-tumor effects of different drugs on tumor growth and angiogenesis in tumor microenvironment.

Figure & Table Legends

Figure 1. Schematic map of dual bioluminescence imaging of tumor growth and angiogenesis. The 4T1 cells transduced by LV-RR and LV-RRT were implanted in Vegfr2-Fluc-KI transgenic mouse. During the growth of tumor, BLI of Rluc and Fluc were performed respectively in a single mouse to reflect the tumor growth and angiogenesis status at the same time.

Figure 2. Transduction efficiency of 4T1-RR and 4T1-RRT cells identified by fluorescence imaging. (A) The diagrammatic drawing of pLV-RR showed the Rluc and RFP sequences were expressed under the promoter EF1α. The bright and fluorescent images of one field of view revealed that 4T1-RR cells were RFP positive. **(B)** The diagrammatic drawing of pLV-RRT showed the single promoter EF1α activated Rluc, RFP, and HSV-ttk genes. The bright and fluorescent images of one field of view revealed that 4T1-RRT cells were RFP positive. Scale bar, 200 μm.

Figure 3. Bioluminescence imaging of transduced 4T1-RR and 4T1-RRT cells. (A) Bioluminescence imaging of 4T1-RR and 4T1-RRT cells in the presence of CTZ. **(B)** The measured Rluc signals of 4T1-RR and 4T1-RRT cells kept a linear relationship with cell numbers.

Figure 4. Visualization of the dynamic processes of tumor growth and angiogenesis in a living animal. (A) Flow Diagram of experiment and dual BLI detection of Rluc and Fluc. **(B)** Representative Rluc images of tumor progression and Fluc images of angiogenesis during the tumor development in a transgenic mouse. **(C)** Measurement of Rluc signals demonstrated that the implanted tumor cells grew fast while 4T1-RRT cells were significantly regressed after GCV administration. **(D)** Quantification of Fluc signals showed angiogenesis happened after tumor cell implantation following a parallel trend with the tumor growth and death induced by GCV.

Figure 5. VEGFR2 immunostaining of 4T1-RR and 4T1-RRT tissues at day 21. Representative images of tumor tissues sections stained for VEGFR2 (green) at day 21. The nuclei were counter-stained with DAPI (blue). The scale bar represented 100 μm.

Figure S1. The curve of tumor size during tumor progression in vivo. The tumor size of 4T1-RR and 4T1-RRT cells increased after implantation, but the tumor size of 4T1-RRT cells started decreasing post GCV treatment.

Figure S2. The cytotoxic effect of GCV on 4T1-RRT cells. The 4T1-RRT cells died with the elevated concentration of GCV.

Figure S3. The BLI image of 4T1-RR cells in the lung. After tail vein injection of 4T1-RR cells, the Rluc signal of cells was detected by BLI.

Table 1. **The transfection conditions of lentiviral packaging system for producing LV-RR and LV-RRT viral stocks in 293T cells. (A)** The pLV-cDNA vector was pLV-RR and pLV-RRT respectively. **(B)** The Gag-Pol + Rev expression vector could be pCMV-deltaR8.91 (TRC) or psPAX2 (Addgene). **(C)** Gag-Pol expression vector could select ant one of pMDLg/pRRE (Addgene), pLP1 (Invitrogen), and pPACKH1-GAG (SBI). **(D)** Rev expression vector should be one of pRSV-REV (Addgene), pLP2 (Invitrogen), or pPACKH1-REV (SBI). **(E)** VSV-G expression vector could select pMD.G (TRC), pMD2.G (Addgene), pCMV-VSV-G (Addgene), pVSV-G (SBI), or pLP/VSVG (Invitrogen). In this protocol, we used the 3-plasmid system including psPAX2,

pMD2.G and pLV-RR or pLV-RRT.

DISCUSSION:

In this protocol, we offered a non-invasive dual BLI approach for monitoring tumor development and angiogenesis. We firstly developed the BLI report system containing the HSV-ttk/GCV suicide gene for tracking tumor progression and regression in vivo by Rluc imaging. Meanwhile, tumor angiogenesis was assessed by using Vegfr2-Fluc-KI mice via Fluc imaging. This tumor-bearing mouse model could provide a practical platform for continuous and non-invasive tracking tumor development and tumor angiogenesis by dual BLI in the single mouse with better relevance, reproducibility and translatability.

Angiogenesis which concerns long-term tumor progression, and thereby, is of high importance[1]. It is necessary to study the relationship between tumor progression and angiogenesis. An increasing number of anti-angiogenesis strategies have been investigated for cancer treatment, which rely on the visualized monitor approaches for accurately assessing the treatment outcomes. Besides, the neovascularization of tumor tissue after traditional radiotherapy and chemotherapy is another hot area of oncology research[12-14]. All of these studies require an animal model that can monitor tumor growth and angiogenesis in real time. The pathological changes of tumor tissues in traditional animal models are usually depended on histopathological examination which have to sacrifice the animals. Our dual BLI mouse models help address the problems of the bigger error range and the cost from sacrifice of animals before.

In this dual BLI mouse model, the most critical step is using two kinds of luciferases including Fluc and Rluc to respectively tracing cells and angiogenesis at the same time. The substrate specificity of these two luciferases makes it possible to perform two kinds of BLI in a single host. Besides, half-life of coelenterazine, the substrate of Rluc, is very short, which results in the Rluc signals fading away quickly without influencing the next Fluc signals detection[15]. Hence, in the operating process, the Rluc imaging should be implemented before Fluc imaging on account of the longer half-life of D-luciferin, the substrate of Fluc. In addition, figuring out the incubating time of the substrates is the key to acquiring perfect BLI images. Metabolism of substrates would change the concentration of the substrates in vivo, leading to the variation of BLI signal intensity.

Owning to the advancements of technology, many other imaging modalities for in vivo tracking certain cellular and subcellular events have been applied in preclinical and clinical researches, such as fluorescent imaging, magnetic resonance imaging (MRI), and positron emission tomography (PET)[16,17]. Comparing with these imaging strategies, bioluminescence imaging with high sensitivity, strong specificity, and accurate measurement shows its unique superiority in the field of living imaging studies[15]. The Rluc imaging we employed allows tumor growth and anti-tumor effect of the HSV-ttk/GCV prodrug system to be visualized dynamically in a living animal. Except for monitoring subcutaneous tissues, we have used Rluc to trace cells in lungs by BLI technology in our other research. After tail vein injection of 4T1-RR into a mouse, we moved this mouse into the living imaging system to detect the Rluc

signals after administration of CTZ. The image of Rluc signal showed that injected cells were mainly located in the lung (**Figure S3**). As mentioned above, the Rluc report gene could trace various cancer cells in different locations, which can be appreciated the full utilization of this mouse model in cancer biology.

In addition to these advantages, BLI technology could be used to sense the expression levels of specific molecules. Previously, fluorophores report genes which were expressed under relevant promotors were used to measure the vessel development in subcutaneous tumor. During the tumor progression, the vascular structure and molecule could be observed through the surgically implanted window chambers in mice. However, this method still has the limitations including unavoidable invasion, fluorophore quenching, and strong background noise. The tumor-bearing mouse model established in the Vegfr2-Fluc-KI mouse creates a non-invasive observation of the expression level of Vegfr2, which is the most important molecule in tumor angiogenesis. Meanwhile, the BLI images display great specificity without noise. So, the dual BLI mouse model may have a broader application in studying the potential molecular mechanisms in tumor progression and regression.

Although, BLI technology, based on expression of Rluc with emission at 480 nm and Fluc with emission at 562 nm, has been adopted in a number of in vivo models of disease. The wide-spread use of BLI technology in vivo has been restricted because of the low sensitivity of bioluminescence at wavelengths below 600 nm in detecting deep tissue. This is caused by the absorption and scattering of light, which decreases the detectable signal by up to ten folds per centimeter of tissue. To address this question, some researchers have focused their studies on the red-emitter variants of Fluc that emit light above 600 nm[18]. Because the absorption and scattering of light could be remarkably reduced by using these variants of Fluc [18,19], the applications of luciferase variants will help this protocol extend to a larger field of oncology researches.

DISCLOSURES

The authors have nothing to disclose.

ACKNOWLEDGEMENTS

This research was supported by National Key R&D Program of China (2017YFA0103200), National Natural Science Foundation of China (81671734), Key Projects of Tianjin Science and Technology Support Program (18YFZCSY00010), Fundamental Research Funds for the Central Universities (63191155). We acknowledged the Gloria Nance's revisions, which were valuable in improving the quality of our manuscript.

REFERENCES

1 Folkman, J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285**, 1182-1186, doi:10.1056/NEJM197111182852108 (1971).

2 Kerbel, R. S. Tumor angiogenesis. *The New England journal of medicine* **358**, 2039-2049, doi:10.1056/NEJMra0706596 (2008).

3 Hosseinkhani, S. Molecular enigma of multicolor bioluminescence of firefly luciferase.

Cellular and molecular life sciences : CMLS **68**, 1167-1182, doi:10.1007/s00018-010-0607-0 (2011).
4 Nakatsu, T. *et al.* Structural basis for the spectral difference in luciferase bioluminescence. *Nature* **440**, 372-376, doi:10.1038/nature04542 (2006).
5 McMillin, D. W. *et al.* Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. *Nature medicine* **16**, 483-489, doi:10.1038/nm.2112 (2010).
6 Madero-Visbal, R. A., Hernandez, I. C., Myers, J. N., Baker, C. H. & Shellenberger, T. D. In situ bioluminescent imaging of xenograft progression in an orthotopic mouse model of HNSCC. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **26**, 17006 (2008).
7 Wang, R. *et al.* Molecular Imaging of Tumor Angiogenesis and Therapeutic Effects with Dual Bioluminescence. *Current pharmaceutical biotechnology* **18**, 422-428, doi:10.2174/1389201018666170523165053 (2017).
8 Rivera, L. B. & Bergers, G. CANCER. Tumor angiogenesis, from foe to friend. *Science* **349**, 694-695, doi:10.1126/science.aad0862 (2015).
9 Zhang, K. *et al.* Enhanced therapeutic effects of mesenchymal stem cell-derived exosomes with an injectable hydrogel for hindlimb ischemia treatment. *ACS applied materials & interfaces* **10**, 30081-30091, doi:10.1021/acsami.8b08449 (2018).
10 Du, W. *et al.* Enhanced proangiogenic potential of mesenchymal stem cell-derived exosomes stimulated by a nitric oxide releasing polymer. *Biomaterials* **133**, 70-81, doi:10.1016/j.biomaterials.2017.04.030 (2017).
11 Lee, S. *et al.* Autocrine VEGF signaling is required for vascular homeostasis. *Cell* **130**, 691-703, doi:10.1016/j.cell.2007.06.054 (2007).
12 Dewhirst, M. W., Cao, Y. & Moeller, B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nature reviews. Cancer* **8**, 425-437, doi:10.1038/nrc2397 (2008).
13 Wigerup, C., Pahlman, S. & Bexell, D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. *Pharmacology & therapeutics* **164**, 152-169, doi:10.1016/j.pharmthera.2016.04.009 (2016).
14 Wong, P. P. *et al.* Dual-action combination therapy enhances angiogenesis while reducing tumor growth and spread. *Cancer Cell* **27**, 123-137, doi:10.1016/j.ccell.2014.10.015 (2015).
15 Mezzanotte, L., van 't Root, M., Karatas, H., Goun, E. A. & Lowik, C. In vivo Molecular Bioluminescence Imaging: New Tools and Applications. *Trends in biotechnology* **35**, 640-652, doi:10.1016/j.tibtech.2017.03.012 (2017).
16 Du, W., Tao, H., Zhao, S., He, Z. X. & Li, Z. Translational applications of molecular imaging in cardiovascular disease and stem cell therapy. *Biochimie* **116**, 43-51, doi:10.1016/j.biochi.2015.06.021 (2015).
17 Liu, J. *et al.* Synthesis, biodistribution, and imaging of PEGylated-acetylated polyamidoamine dendrimers. *Journal of nanoscience and nanotechnology* **14**, 3305-3312, doi:10.1166/Jnn.2014.7995 (2014).
18 Branchini, B. R. *et al.* Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments. *Analytical biochemistry* **534**, 36-39, doi:10.1016/j.ab.2017.07.001

(2017).
19 McLatchie, A. P. *et al.* Highly sensitive in vivo imaging of Trypanosoma brucei expressing "red-shifted" luciferase. *PLoS neglected tropical diseases* **7**, e2571, doi:10.1371/journal.pntd.0002571 (2013).

Figure 1

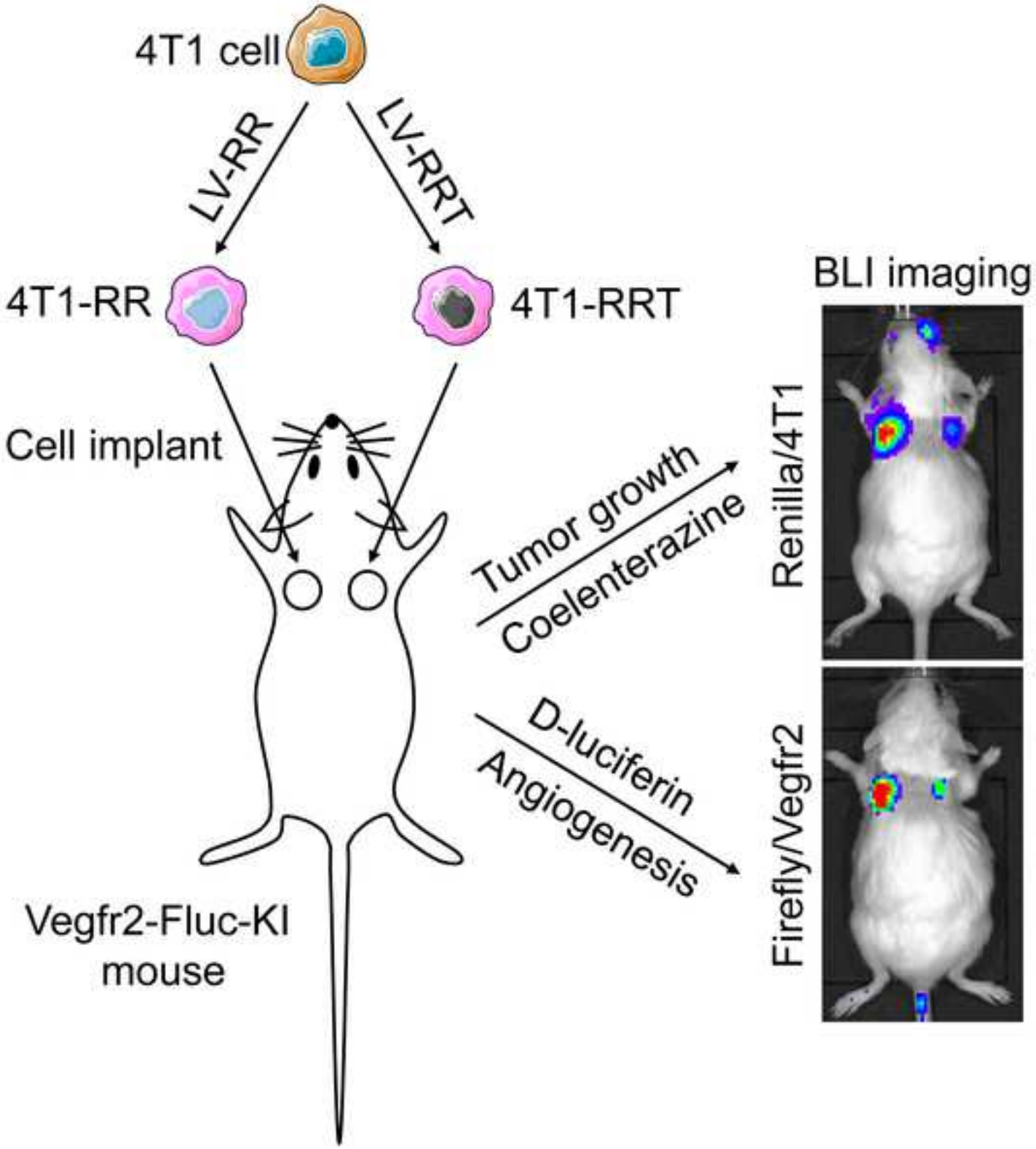


Figure 2

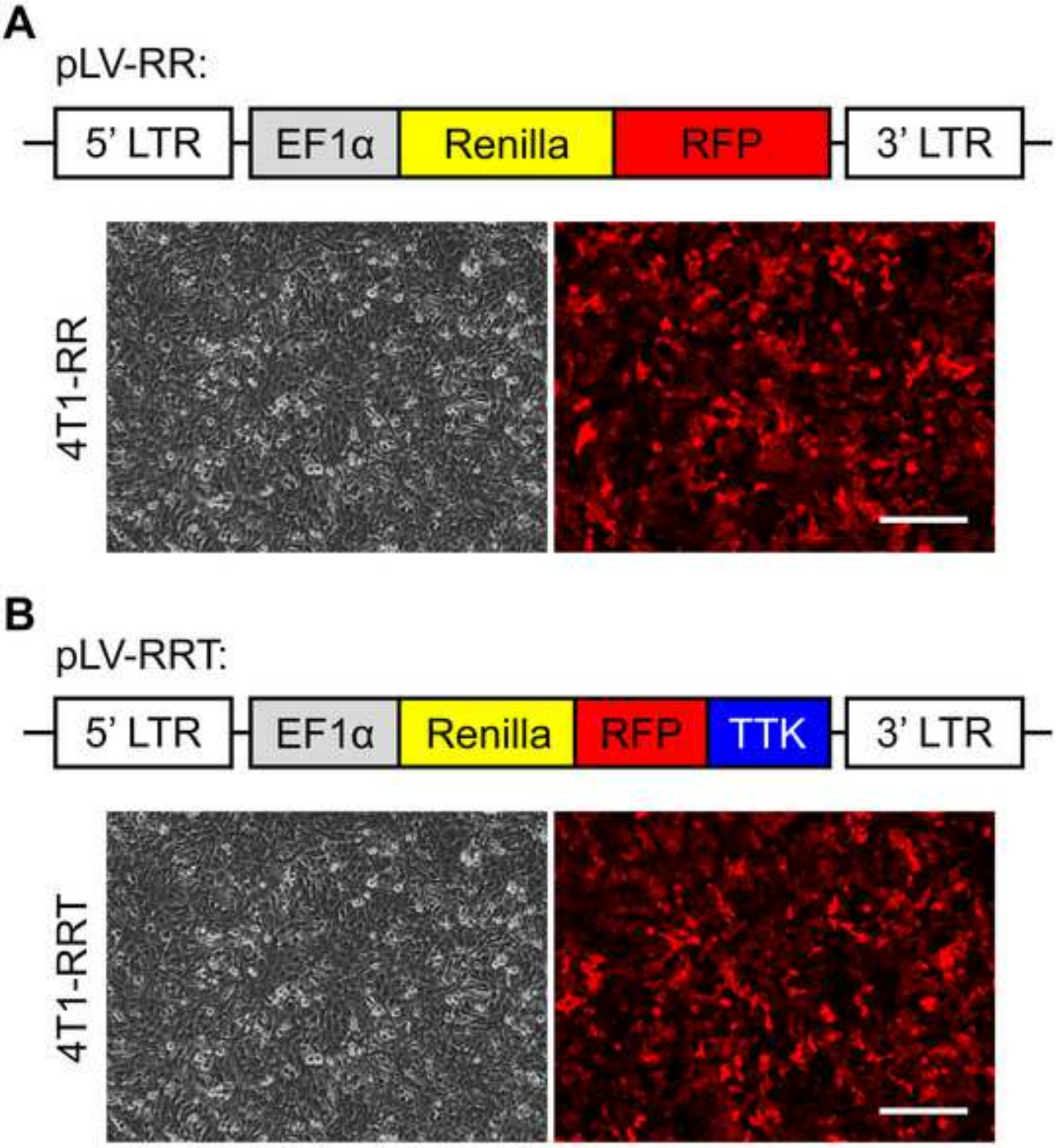


Figure 3

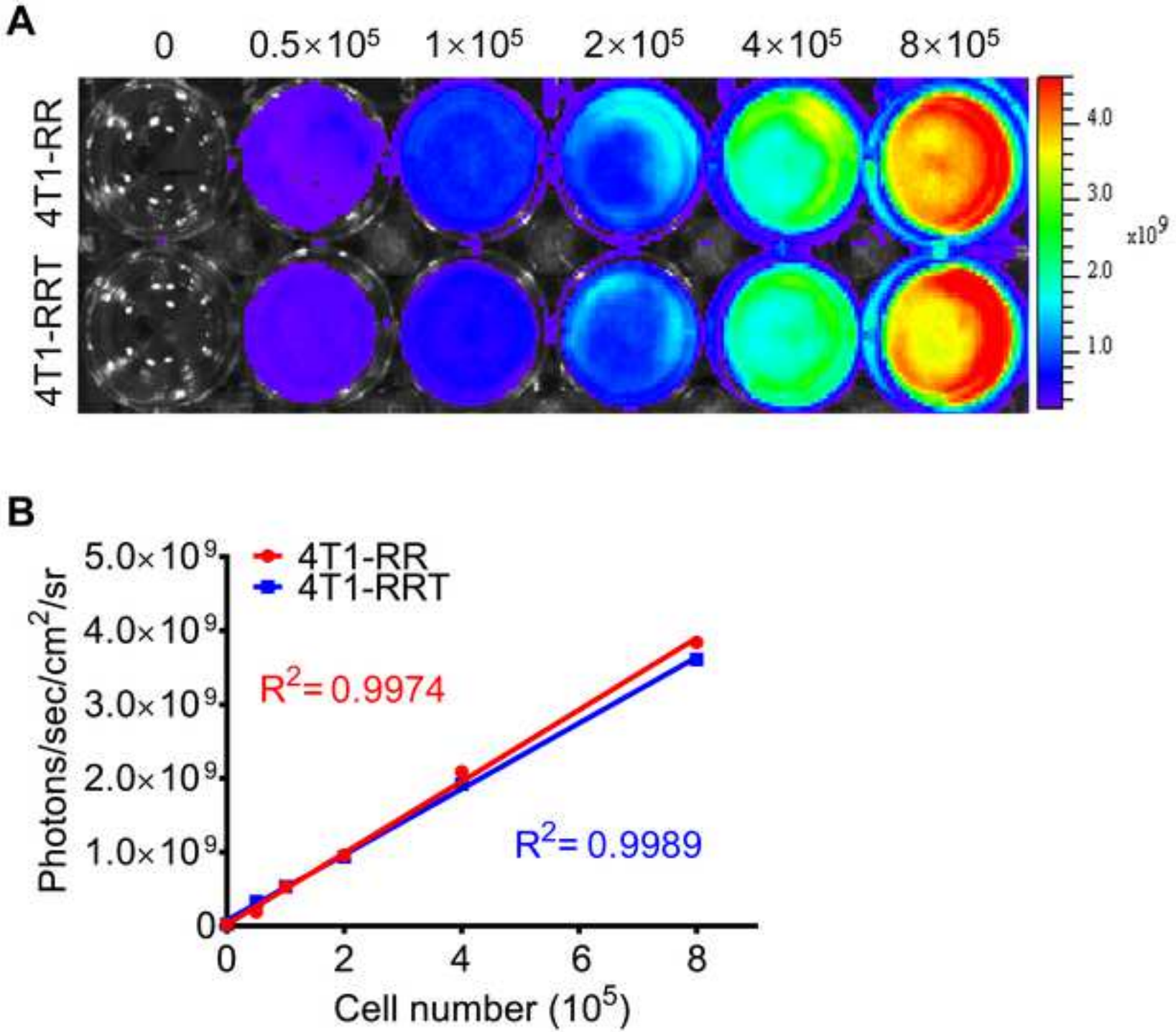


Figure 4

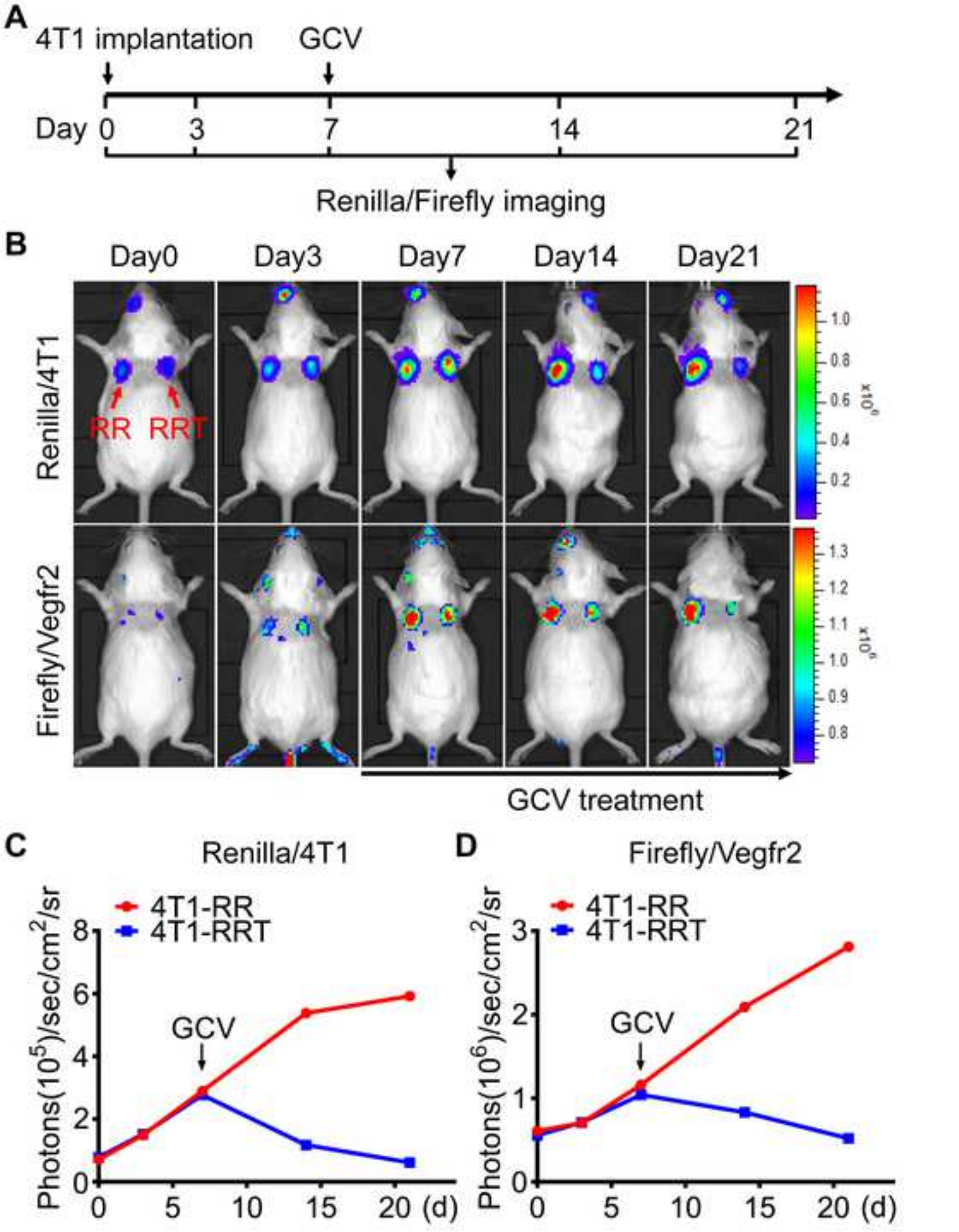
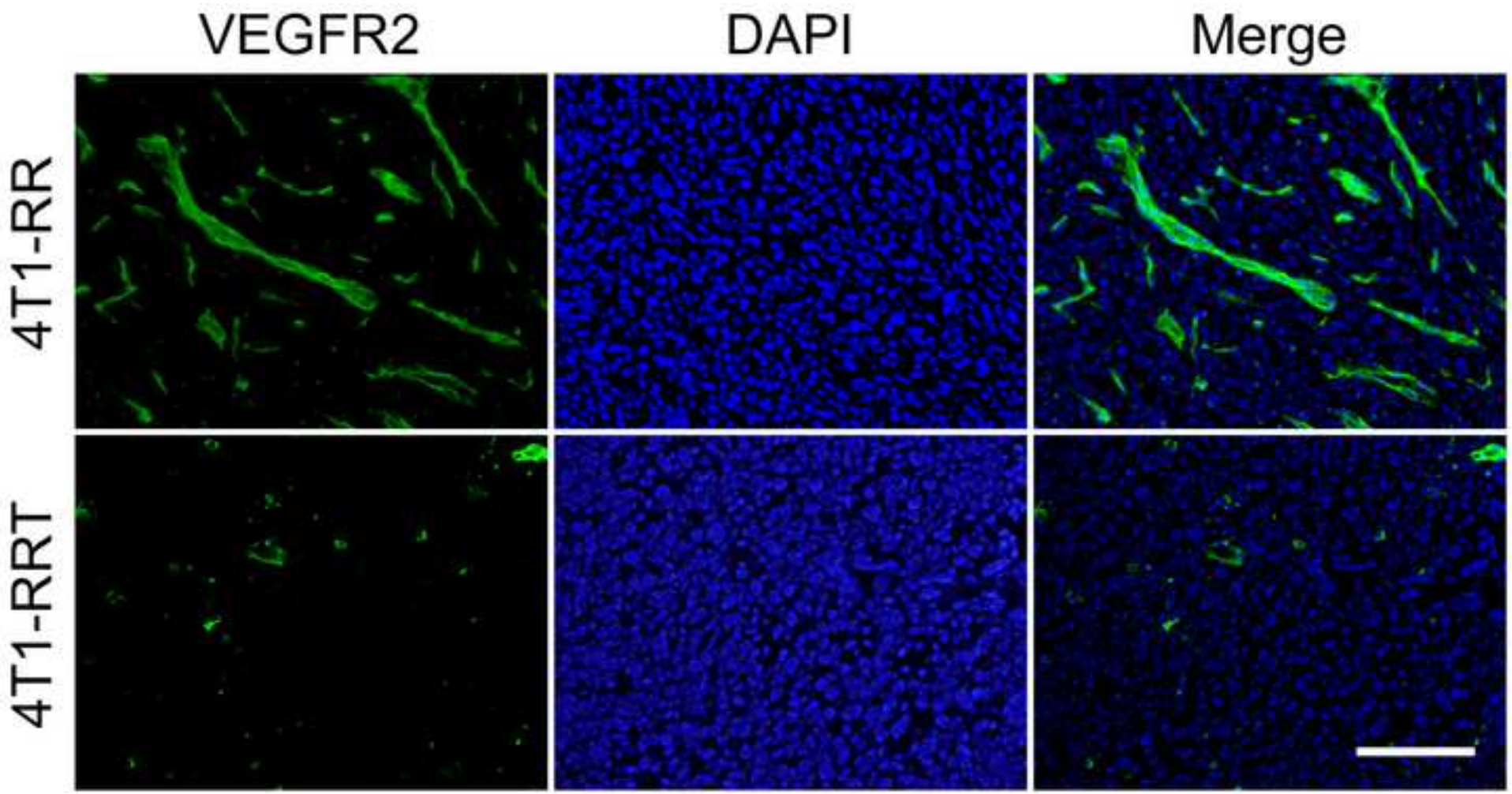


Figure 5

Click here to access/download;Figure;Fig 5.tif



LV-RR and LV-RRT Packaging Conditions

Components	MEM medium	3-plasmid system	4-plasmid system
pLV-RR/pLV-RRT vector[A]	0.25 mL	1.5 μg	1.5 μg
Gag-Pol + Rev expression vector[B]		1.0 μg	
Gag-Pol expression vector[C]			0.75 μg
Rev expression vector[D]			0.3 μg
VSV-G expression vector[E]		0.5 μg	0.45 μg
Liposome	0.25 mL	7.5 μL	7.5 μL

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% Trypsin-0.53 mM EDTA	Gibco	25200072	
1.5 mL Tubes	Axygen Scientific	MCT-105-C-S	
15 mL Tubes	Corning Glass Works	601052-50	
293T	ATCC	CRL-3216	
4T1	ATCC	CRL-2539	
60 mm Dish	Corning Glass Works	430166	
6-well Plate	Corning Glass Works	3516	
Biosafety Cabinet	Shanghai Lishen Scientific	Hfsafe-900LC	
Blasticidine S Hydrochloride (BSD)	Sigma-Aldrich	15205	
Cell Counting Kit-8	MedChem Express	HY-K0301	
CO_2 Tegulated Incubator	Thermo Fisher Scientific	4111	
Coelenterazine (CTZ)	NanoLight Technology	479474	
D-luciferin Potassium Salt	Caliper Life Sciences	119222	
DMEM Medium	Gibco	C11995500BT	
Fetal Bovine Serum (FBS)	BIOIND	04-001-1A	
Fluorescence Microscope	Nikon	Ti-E/U/S	
Ganciclovir (GCV)	Sigma-Aldrich	Y0001129	
Graphics Software	GraphPad Software	Graphpad Prism 6	
Insulin Syringe Needles	Becton Dickinson	328421	
Isoflurane	Baxter	691477H	
Lentiviral Packaging System	Biosettia	cDNA-pLV03	
Liposome	Invitrogen	11668019	
Living Imaging Software	Caliper Life Sciences	Living Imaging Software 4.2	
Living Imaging System	Caliper Life Sciences	IVIS Lumina II	
MEM Medium	Invitrogen	31985-070	
Penicillin-Streptomycin	Invitrogen	15140122	
Phosphate Buffered Saline (PBS)	Corning Glass Works	R21031399	
Polybrene	Sigma-Aldrich	H9268-1G	
RPMI1640 Medium	Gibco	C11875500BT	
SORVALL ST 16R Centrifuge	Thermo Fisher Scientific	Thermo Sorvall ST 16 ST16R	
Ultra-low Temperature Refrigerator	Haier	DW-86L338	

XGI-8 Gas Anesthesia System	XENOGEN Corporation	7293

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis
Author(s):	Kaiyue Zhang, Chen Wang, Ran Wang, Shang Chen and Zongjin Li

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

[X] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

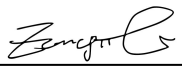
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Zongjin Li
Department:	
Institution:	School of Medicine Nankai University
Title:	PI
Signature:	
Date:	Jan 31, 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Nankai University School of Medicine

Zongjin Li, MD, PhD
Professor
Department of Pathophysiology
Nankai University School of Medicine

94 Weijin Road
Tianjin, China
Phone: +(86) 22-2350 9332
Email: zongjinli@nankai.edu.cn

Apr 7, 2019

Dear Editor,

We would like to re-submit a manuscript, manuscript ID: JoVE59763, entitled "Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis" for consideration in *JoVE*.

We appreciate the helpful comments and suggestions by the editors. Our manuscript has been revised according to the editorial comments. Detailed replies to editorial comments are listed below. **Changes to the manuscript are highlighted in *blue* font.**

Editorial Comments:

1. Please copy-edit the manuscript. There are many scattered typos throughout.

We are so sorry for our mistakes. We have checked and corrected the spelling mistakes in our manuscript. Besides, we re-wrote some sentence to make our protocol easier to read. All modifications are highlighted in *blue* font.

2. Please reference Figure 5 in the manuscript text.

As you required, we have referenced **Figure 5 in** our manuscript (line 328).

3. Additional comments are in the attached manuscript.

A copy of the additional comments (highlighted in *grey*) followed by a detailed reply can be found below.

4. Please provide the legends for the supplemental figures in the manuscript text and upload the supplemental figures individually as image files.

According to your suggestions, we have separated the supplemental figures and their legends. The legends were provided after the legend for **Figure 5** in the manuscript. The supplemental figures were uploaded individually.

Sincerely,

Zongjin Li

Additional Comments:

3.1 Please reference Figure 1 before Figure 2. Figure 1 could be referenced in the Introduction.

According to your suggestions, we have referenced **Figure 1** in the Introduction. Thank you for your comment.

3.2 Liposome or Lipofectamine 2000? I changed it to liposome suspension (step 1.2?).

Thank you for your revision. The liposome suspension is what we want to describe in step 1.4.

3.3 Please be more specific here.

Sorry for our unclear description. The polybrene should be blend in medium by pipetting in step 2.2.

3.4 What happens after centrifugation? Aspiration?

Thank you for your question. As previously reported, co-centrifugation of cells and lentivirus particles could increase the transduction efficiency {Rouas, 2002 #24}. Centrifugation may increases the probability of contact between the lentivirus particles and the cells.

3.5 Please revise for clarity. The grammar is confusing. At the proportion?

We are so sorry for our mistake. The steps of sub-culturing 4T1 cells were re-organized in our manuscript.

3.6 What temperature? 5% CO_2 at 37 °C?

Sorry for our unclear statement. The cells were cultured in a humidified incubator with 5% CO2 at 37 °C. We have clarified this cultured condition of 4T1 cells in our manuscript.

3.7 How is sufficient depth of anesthesia confirmed? Is eye ointment used?

We are so sorry for our innocent omission. We have added the above procedures you mentioned in our manuscript.

3.8 How is this done? What is used?

Sorry for our unclear statement. The hair of mice was removed by using electric shaver and hair removal cream. We have re-written this step in our manuscript.

3.9 How much is injected? 1 x 10^6 cells per 100 µL? Or is this just the concentration? For how many mice? Step 4.6 mentions more than one mouse.

We appreciate your kind suggestions. There were 1 x 10^6 4T1-RR cells at a 100 µL total volume as well as 1 x 10^6 4T1-RRT cells at a 100 µL total volume which injected to each mouse in step 4.6.

3.10 How do the mice recover from anesthesia?

Sorry for our incomplete statement. The anesthetic animals should be returned to the cage in warm environment, and monitor frequently until the mouse wake up. We have added this step in our manuscript.

3.11 How is sufficient depth of anesthesia confirmed? Eye ointment?

We are so sorry for our innocent omission. We have added the above procedures you mentioned in our manuscript.

3.12 Please make into a sentence. How much of what is injected? Please provide all volumes and concentrations throughout.

We appreciate your comments. We have re-written this step to illustrate the volumes and concentrations of substrates we used in the manuscript. Thanks for your suggestions.

3.13 How much is injected?

Sorry for our unclear statement. We have added the volume and the concentration of D-luciferin in step 5.7.

3.14 What is being incubated? Substrate or subject?

Sorry for our unclear statements. We have re-written the sentence to describe that the mouse were kept at room temperature for 10 min.

3.15 Please specify the step numbers.

Thank you for your comment. We have added the step numbers that we mentioned in step 5.11.

Supporting Information

Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis

Kaiyue Zhang[1], Chen Wang[1], Ran Wang[2], Shang Chen[1] and Zongjin Li[1*].

[1]Nankai University School of Medicine, Tianjin, China;
[2]State Key Laboratory of Medicinal Chemical Biology and College of Pharmacy, Nankai University, Tianjin, China.

*Corresponding Author:
Zongjin Li, MD, PhD
Email: zongjinli@nankai.edu.cn
Tel: +86-22-23509332

Figure S1

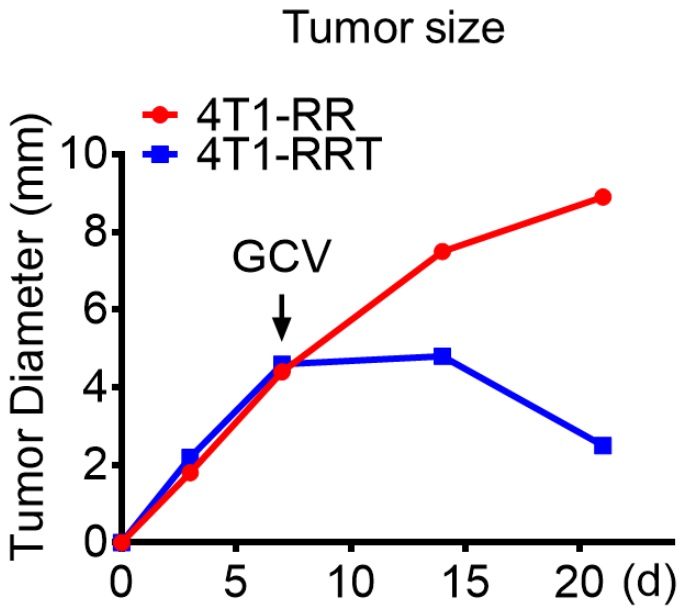


Figure S1. The curve of tumor size during tumor progression in vivo. The tumor size of 4T1-RR and 4T1-RRT cells increased after implantation, but the tumor size of 4T1-RRT cells sharply decreased post GCV treatment.

Figure S2

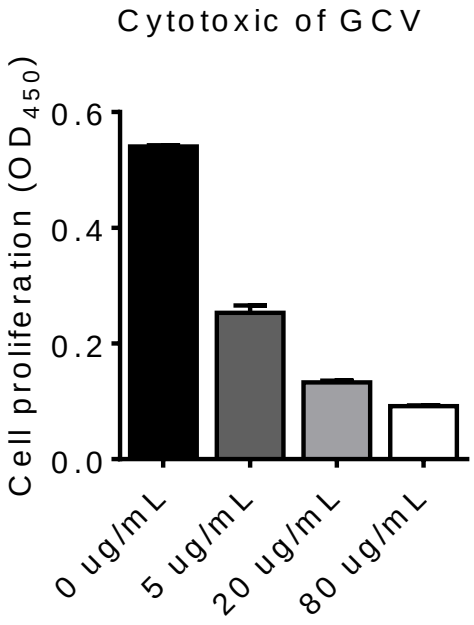


Figure S2. The cytotoxic effect of GCV on 4T1-RRT cells. With increased concentrations of GCV, the viability of 4T1-RRT cells was significantly inhibited.

Figure S3

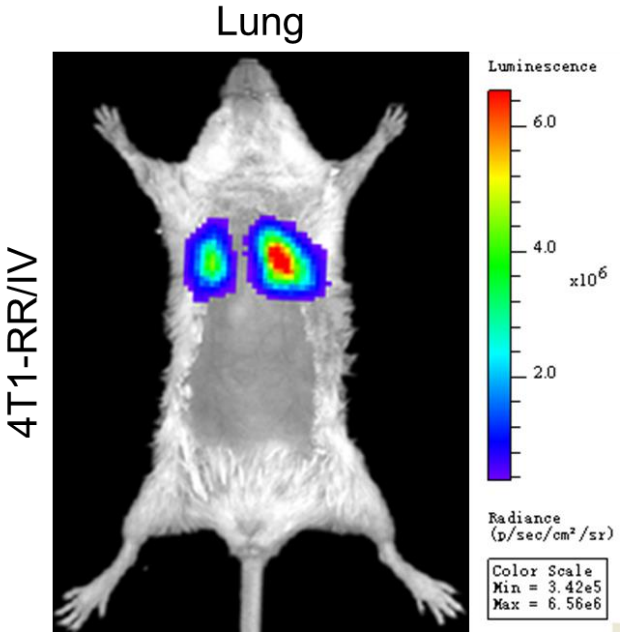


Figure S3. The BLI image of 4T1-RR cells in the lungs. After tail vein injection of 4T1-RR cells, the Rluc signal of cells was detected by BLI.