Video Article

In Vivo Imaging Systems (IVIS) Detection of a Neuro-Invasive Encephalitic Virus

Allison Poussard*[1], Michael Patterson*[1], Katherine Taylor[1], Alexey Seregin[1], Jeanon Smith[1], Jennifer Smith[1], Milagros Salazar[1], Slobodan Paessler[1]

[1]Experimental Pathology, University of Texas Medical Branch

*These authors contributed equally

Correspondence to: Slobodan Paessler at slpaessl@utmb.edu

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Abstract

Modern advancements in imaging technology encourage further development and refinement in the way viral research is accomplished. Initially proposed by Russel and Burch in Hume's 3Rs (replacement, reduction, refinement), the utilization of animal models in scientific research is under constant pressure to identify new methodologies to reduce animal usage while improving scientific accuracy and speed. A major challenge to Hume's principals however, is how to ensure the studies are statistically accurate while reducing animal disease morbidity and overall numbers. Vaccine efficacy studies currently require a large number of animals in order to be considered statistically significant and often result in high morbidity and mortality endpoints for identification of immune protection. We utilized *in vivo* imaging systems (IVIS) in conjunction with a firefly bioluminescent enzyme to progressively track the invasion of the central nervous system (CNS) by an encephalitic virus in a murine model. Typically, the disease progresses relatively slowly, however virus replication is rapid, especially within the CNS, and can lead to an often, lethal outcome. Following intranasal infection of the mice with TC83-Luc, an attenuated Venezuelan equine encephalitis virus strain modified to expresses a luciferase gene; we are able to visualize virus replication within the brain at least three days before the development of clinical disease symptoms. Utilizing CNS invasion as a key encephalitic disease development endpoint we are able to quickly identify therapeutic and vaccine protection against TC83-Luc infection before clinical symptoms develop. With IVIS technology we are able to demonstrate the rapid and accurate testing of drug therapeutics and vaccines while reducing animal numbers and morbidity.

Video Link

The video component of this article can be found at https://www.jove.com/video/4429/

Protocol

1. Animal Preparation

1. Animal arrival: Upon arrival to the animal biosafety level 2 (ABSL2) facilities, allow animals a minimum of 2 days to acclimate to their new environment. Following this rest period, inspect the animals to evaluate their health and overall physical appearance.
    1. When utilizing fluorescent reporters it is important to place all animal subjects on an alfalfa free diet to limit the amount of GI autofluorescence and background signal.

2. Shave animals: To improve the bioluminescent signal detected from the cranial region during imaging; shave the heads of all the animals. Anesthetize the animals with a mixture of oxygen gas and vaporized isoflurane. Once the animals are fully anesthetized, use electric clippers to shave their heads. Once finished with shaving, return the animals to their cages and observe for complete recovery from the effects of the anesthesia.
3. Bio Medic Data Systems (BMDS) transponder insertion (to take place following shaving of the mice while the animals are fully anesthetized): For a less invasive means to track temperature implant a preprogrammed BMDS wireless transponder subcutaneously in the dorsal region of each mouse. These transponders allow for wireless identification and temperature recording. Following implantation of the transponder, apply a veterinary-grade tissue adhesive to the wound.
4. Baseline collection: Before infection, record a baseline temperature and weight for all animals. Collect blood for plaque reduction neutralization test (PRNT) analysis through retro-orbital (RO) bleed while the mice are under anesthesia. Following blood collection, apply veterinary antibiotic eye ointment to reduce potential secondary bacterial infection. This baseline collection should be completed with consideration to the number of times the animals are placed under anesthesia due to stress on the mice. Future RO collections should be completed after imaging, while the mouse is still under anesthetic. The maximum blood collected from an RO should be around 200 µl.

5. Infection: Place mice under anesthesia as described previously. Inoculate through the intranasal route using a dose of $5x10^6$ to $5x10^7$ pfu TC83-Luciferase in a total volume of 40 µl diluted by Phosphate Buffered Saline (PBS) through intranasal infection. Following infection, place the mice within their housing cage and observe recovery.

2. Animal Imaging

Disclaimer: Keep mice within their housing unit, the biosafety cabinet (BSC), or the XIC containment box at all times. The approved BSCs for this protocol are a Class II Type B1 or B2.

1. Inject all animals with 10 µl per gram of body weight of intraperitoneal (IP) luciferin at a concentration of 15 mg/ml.
    1. Mice receiving Ampligen are to be injected IP at a dose of 12.5 mg/kg body weight at -4 hr post infection or +48 hr post infection based upon their group.

2. Prior to injection with luciferin, separate the mice into groups of three to ensure a proper fit within the XIC-3 containment box.
    1. For all imaging, utilize ocular ointment/lubricant to prevent the drying of the cornea throughout the procedure.

3. Open the LivingImage software and press *Initialize* to prepare the imaging box; set auto saving, exposure time to auto (exposure time on auto is a new feature of the LivingImage software 3.2 and newer), view field to C, and filter to open; field of view is based upon the number of mice you are imaging; and filter can be optimized as needed for other imaging projects.
4. Confirm that the air charcoal filters have not expired, the oxygen tank is full, and the isoflurane reservoir is filled. Place small strips of electrical tape in the XIC-3 isolation box which assists with reducing animal movement during imaging.
5. Inject the mice with luciferin IP as described in section 2.1 and place within the Isoflurane induction chamber (with lid opened) for 3-5 min.
6. After 5 min, close the lid of the induction chamber and start the flow of isoflurane. The anesthetic is operated at approximately 3-5% with the oxygen flow rate set at approximately 2.0 L/min. Once fully unconscious wait an additional 30 sec and transfer the mice to the XIC-3 isolation box. Ensure the biosafety cabinet being utilized allows for the safe scavenging of isoflurane as this procedure will involve loss of isoflurane from the induction chamber (the IVIS unit contains its own Passive Fair canisters and the XIC-3 containment box directly connects to the in/out sockets to ensure contained anesthetic flow).
    1. Transfer the mice based upon chip/group number into the XIC-3 isolation box with the isoflurane connector attached and open. Position the mice so they are laid out in sternal recumbency with heads gently resting on the tape strips.

7. Wipe down the XIC containment box with ethanol, spray hands and bottom of the XIC-3 with cavicide, and transfer to the IVIS imaging chamber. Reconnect the anesthesia to the containment box once inside the imaging unit.
8. After 10 min following the injection of luciferin, image the mice through the living image software.
9. Following initial imaging with an open filter, initiate a DLIT 3-Dimensional imaging using the Sequence analysis and Image Wizard setup for bioluminescent firefly luciferase. This imaging process will take 4-5 min and should be completed at a field of view relevant for the number of mice desired.
    1. *Ex-vivo* analysis is possible following necropsy and collection of the brain. Place the brain on a sterile Petri dish, drip 50-100 µl of stock luciferin on top, and place within the imaging box.

10. Finalize a second open filter image at 15-17 min post infection upon completion of DLIT imaging. Ensure sequence analysis is off and the field of view and filter settings are returned to previous settings.

3. Return Mice and Data Analysis

1. Turn off the isoflurane vaporizer and transfer the XIC containment box back to the biosafety cabinet.
2. Place the mice back within their housing unit, close the top, spray the exterior with appropriate disinfectant and place back on the housing rack.
    1. Ensure mice have fully recovered from anesthesia.

3. Repeat the above procedure as the experiment requires until imaging has completed.
4. Disinfect the BSC, shut down the equipment, and transfer all data to an outside laboratory location for further analysis.
5. Analyze the data and generate 3-dimensional images based upon IVIS results utilizing the LivingImage software.
    1. Ensure the image is properly oriented and lighting/color balance is set. Within the Tool Palette the options include Image Adjust, Corrections, Image Information, and ROI Tools.
    2. Utilize ROI shapes to identify specific signal strengths based upon location.
    3. Reconstruct the surface topography to highlight the mouse body, initialize 3D DLIT reconstruction, and use linear fit to set the organ map to the topographic reconstruction.

6. Further viral titration analysis can be completed through collection of blood and organs. Titration in this study was completed through homogenization of brain tissue in modified eagles medium (MEM). The homogenate was serially diluted and we infected a 6 well plate of Vero cells for 1 hr. The cells were covered with a 1.5% agarose/2xMEM overlay and incubated for 2 days at 37 degrees. Cells were fixed with 10% formalin for 30 min and stained with crystal violet.

Representative Results

With a genetically modified virus, TC83-Luciferase, we saw an increase in bioluminescent signal strength as the virus replication moves from the nasal region into the central CNS (**Figure 1**). Due to the high viral replication rate, we expect to see high levels of bioluminescent signal (**Figure 2A**) dependent upon the vector and the animal immune response to the vector. We expect this signal increase to continue to a peak, between days 5-7 post infection, in combination with viral replication peak within the CNS (**Figure 2B**). Following the signal and viral peak, we expect to

see a decrease in signal both due to a decrease in viral replication and due to a loss of the of the luciferase gene. With these signal values we expect to be able to quantify viral load based upon the strength of the signal, providing an *in vivo* method to accurately calculate progressively increasing viral load in a single animal specific to TC83-Luciferase.

Utilizing this model as a means to analyze vaccines and antiviral treatments, we expect to be able to detect a difference between efficacious treatments based upon a reduction in bioluminescent signal. In our case, against TC83-Luciferase, we can utilize IP Ampligen (a Toll-like receptor 3 agonist) or vaccination three weeks before infection, to significantly reduce viral infection and bioluminescent signal throughout the study (**Figure 3**). This decrease can be directly correlated to a decrease in viral load within the CNS (data not shown). As a way to fully develop and visualize the technology, IVIS software LivingImage is capable of making 3-dimensional images of the bioluminescent signal (**Figure 4**) which allows us to easily identify locations of high viral replication based upon tissue depth and composition.

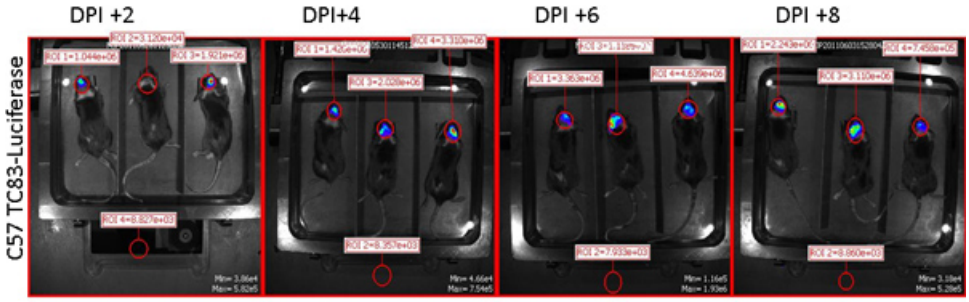

Figure 1. IVIS Imaging of TC83-Luciferase Three C57BL/6 mice were infected through intranasal challenge with TC83-Luciferase and imaged at 2, 4, 6, and 8 days post infection (DPI). Images were made following IP luciferin injection with an autoexposure length and open filter. Click here to view larger figure.

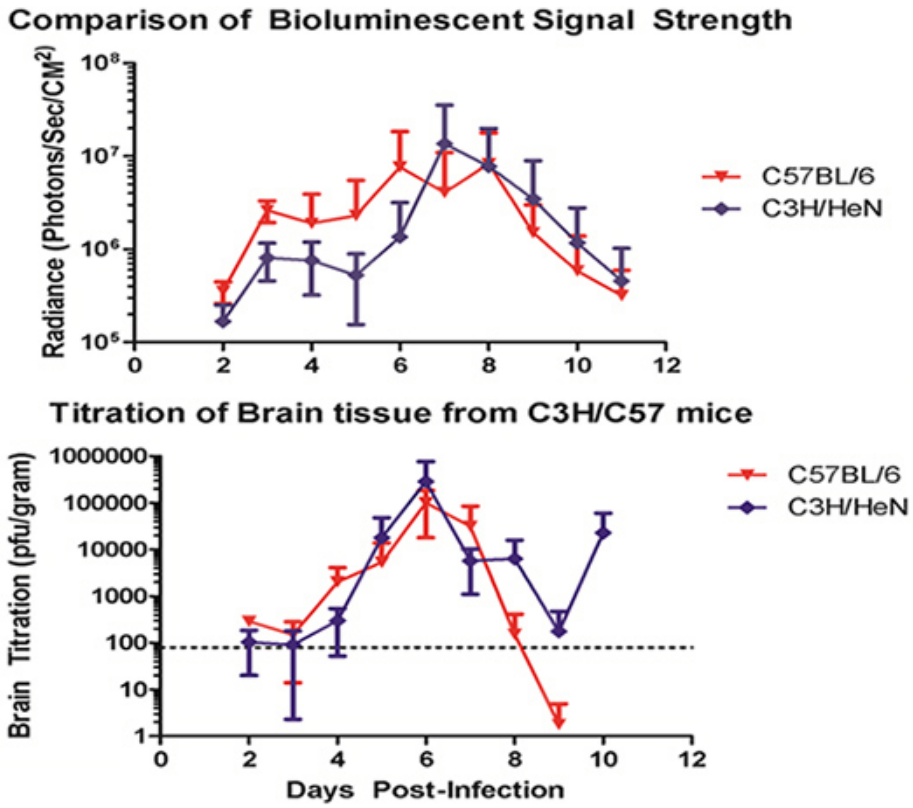

Figure 2. Both strains of mice had similar bioluminescent signal strength following IP injection of luciferin with a delay of 10 minutes post injection before imaging was completed (A). Viral load in the brain (pfu/gram tissue) shows a strong correlation throughout the infection to the biolumiescent signal (B).

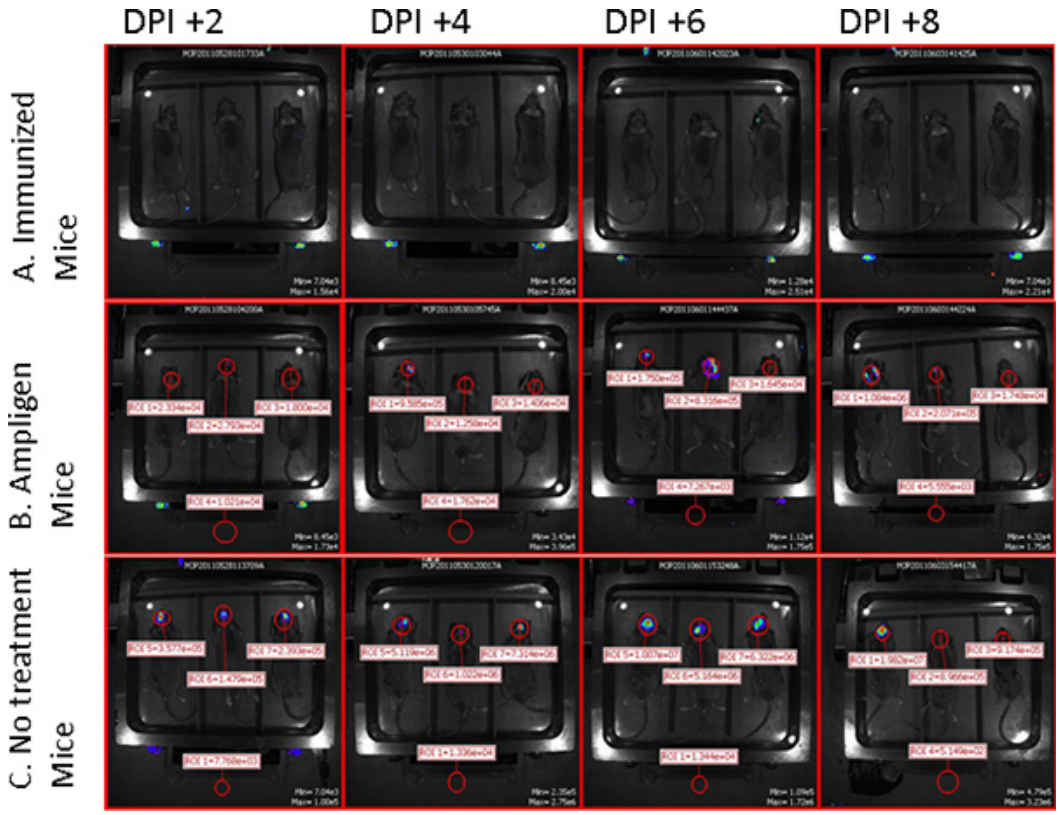

Figure 3. Treatment Comparison Utilizing IVIS. Treatment comarison following TC83-Luciferase infection in C3H/HeN mice. Mice immunized with subcutaneous TC83 23 days prior to challenge with TC83-Luciferase presented with no bioluminescent signal (A). Mice receiving IP Ampligen at -4 and +48 hr post infection (B) showed reduced levels compared to mice receiving no treatment (C).

Figure 4. 3-Dimensional reconstruction imaging. Utilizing 3-Dimensional reconstruction we visualized the peak location of TC83-Luciferase infection utilizing LivingImage's DLIT reconstruction tool. We are able to localize the depth and location of the primary signal within the CNS utilizing this technique.

Discussion

While this protocol covers the imaging aspects for *in vivo* analysis, it is important to recognize the bioluminescent vector as a key factor for future studies. Our utilization of TC83, an attenuated vaccine strain of VEEV, as a vector for expression of luciferase ensures that large quantities of the enzyme are being produced due to the high replication rate of the virus in the CNS as previously described[1-4]. While the addition of a second subgenomic promoter and the luciferase gene results in further attenuation of the recombinant virus compared to wild type TC83, this attenuation does not appear to alter the clinical course of the disease within the first 6 days of infection[5].We expect to see a loss of the luciferase gene through replication of the virus over time but again this is not seen until later in the development of disease. Development of other pathogenic vectors, viral and bacterial, confirms the potential and efficiency of IVIS and luciferase across multiple fields of pathogen research[6-9].

The imaging process itself has a few critical steps that must be completed before a full study can be implemented. An initial analysis of the bioluminescent strength of the vector should be finished in a pilot study containing a minimal group of animals. The expected location *in vivo* of luciferase accumulation should be known beforehand due to the vector utilized but the expected signal strength should be determined before initiating a large study. With the new software an exposure length analysis is not as necessary due to the auto exposure detection feature now built into the software but a time delay based upon the injection of luciferin will still need to be determined. Even with all of these signal calculations, bioluminescent detection can still be limited due to vector depth and overall animal pigment and fur. We strongly recommend the shaving of animals before imaging begins to gain the strongest signal possible.

Even accounting for vector attenuation and enzyme production, we believe we have designed an accurate and more efficient model utilizing luciferase then could be generated with a standard fluorescent molecule system for *in vivo* analysis. Bioluminescence is not limited by having an initiating light source, which initially must pass through tissue resulting in a loss of signal. It also does not have the high background auto fluorescent problems seen with mice on their fur and even from their diet. The risk of toxicity from the luciferin substrate is easily controlled through proper filtration and dosage, making bioluminescent just as safe for the animal. Both fluorescent and bioluminescent systems can drastically reduce animal usage due to *in vivo* analysis which will be important for future research due the changing political attitude towards animal research and further adherence to Hume's 3Rs (replacement, reduction, refinement) towards animal use[10].

For our application of viral pathogenesis and antiviral efficacy analysis, *in vivo* modeling is more efficient than current systems[11,12] for multiple reasons. We are able to detect specific key steps for disease development, CNS invasion[1] in the case of TC83, before any clinical disease develops. From initial invasion detection, we are able to repeatedly visualize in a single animal the spread and replication of the virus providing a more accurate means of studying disease progression[5]. The model we have designed is also safer for researchers due to the decreased requirement of sacrifice and organ collection, the capability to detect key disease progression points before clinical disease can lead to animal

irritability, and, specific to our system, we can complete these studies in an attenuated viral vector in the ABSL2 instead of the ABSL3 utilizing a select agent.

Future disease studies will increasingly utilize bioluminescent IVIS modeling. Advancements in modern molecular biology techniques and cloning allow the insertion of a luciferase gene quickly and cheaply into both viral and bacterial vectors. The ability to develop transgenic mice expressing a promoter dependent luciferase gene also provides another system for bioluminescent study. Finally, newly developed bioluminescent probes such as Calipers RediJect Inflammation probe reacts with myeloperoxidase allowing for *in vivo* visualization of phagocyte inflammation reaction. These new technologies combined with the increasing efficiency of the cameras and software makes bioluminescent IVIS a viable system for future research in many different fields of study.

Disclosures

No conflicts of interest declared.

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