WHITE PAPER

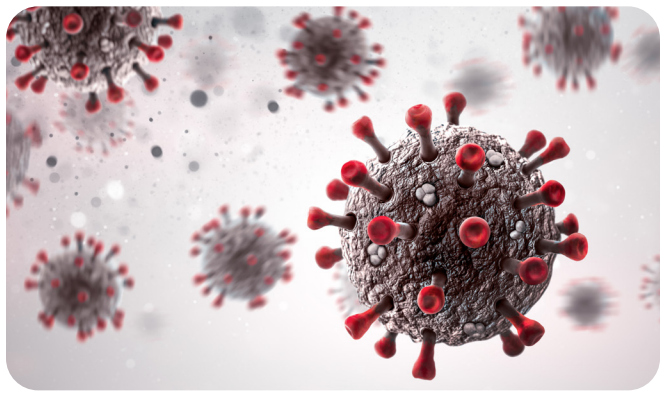
Non-invasive optical imaging for viral research and novel therapeutic and vaccine development

Viruses, described as “organisms at the edge of life”, have been responsible for some of the worst human pandemics in history. Smallpox alone is estimated to have killed more than 300 million people before its eventual eradication in 1978. Similarly, influenza has been a major determinant in shaping our future. The 1918 influenza pandemic was responsible for an estimated 100 million deaths. A single virus capable of devastating humankind globally in just two short years. Even today, The World Health Organization (WHO) estimates that annual global influenza epidemics result in about 3-5 million cases of severe illness with between 250,000 to 500,000 deaths.

The development of effective therapeutics and vaccines for treating viral infections is ever more important as the world’s population continues to grow at an alarming pace. Animal models for viral drug and vaccine development are often less than ideal, due in many cases to the host specific nature of a virus. Fortunately, non-invasive optical imaging methodologies are extremely flexible in their approach, allowing preclinical disease models to be developed that intuitively mimic what is seen in humans.

Respiratory viral disease models

Viral pathogens are the most common cause of respiratory infections. Causative agents include rhinoviruses, respiratory syncytial virus, influenza virus, parainfluenza virus, human metapneumovirus, measles, mumps, adenovirus, and coronaviruses (causing SARS-2003, MERS-2012, and COVID-19). Many of these viruses can be genetically engineered to incorporate an optical reporter (luciferase or



fluorescent protein), allowing their replication (infectivity) and dissemination within a small animal host to be visualized directly using one of Revvity’s IVIS® optical imaging platforms.

Figure 1 shows data from Czakó and colleagues (2017 - https://doi.org/10.1128/mBio.00714-17) using a NanoLuc engineered H1N1 influenza virus in a mouse model. The researchers demonstrated the application of non-invasive in bioluminescent imaging (BLI) to track the replication of the virus and evaluate the preclinical efficacy of candidate vaccines and immunotherapy. Sequential imaging revealed distinct spatiotemporal kinetics of bioluminescence in groups of mice passively or actively immunized by various strategies that accelerated the clearance of the challenge virus at different rates and by distinct mechanisms. Their findings support the potential of this optical imaging approach to enhance traditional preclinical efficacy evaluation of candidate vaccines and human monoclonal antibodies for the prevention and treatment of influenza.

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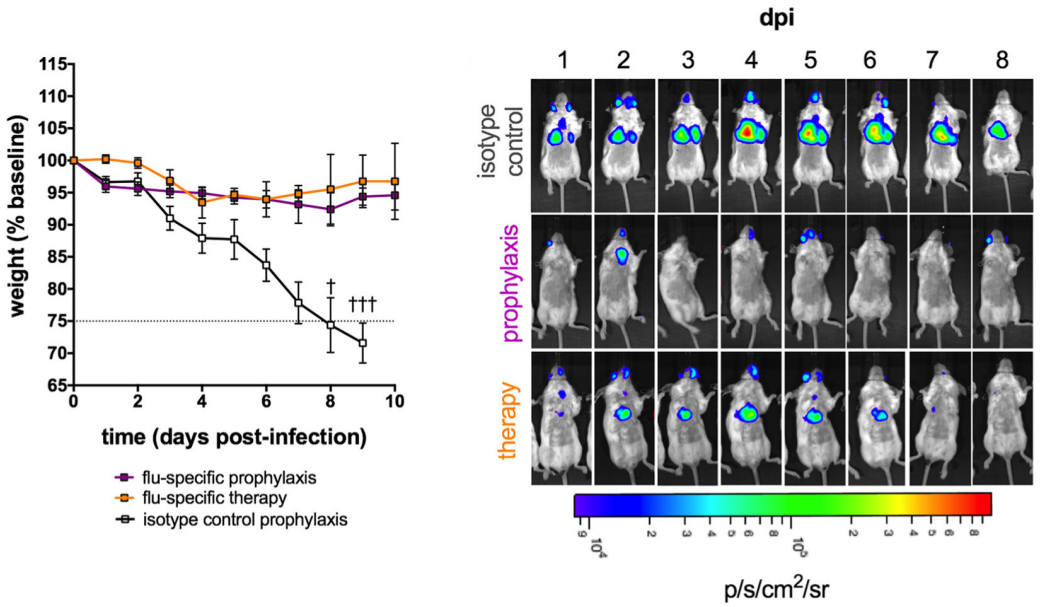

Figure 1. Bioluminescent in imaging of passively immunized mice. An influenza virus-specific hMAb (EM4CO4) was administered either prophylactically (24 h prechallenge) or therapeutically (72 h postchallenge). Mice that received prophylaxis with an equivalent dose of isotype control antibody (human IgG1κ) are shown for comparison. (Czakó *et al*, 2017. In imaging of influenza virus infection in immunized mice. mBio 8:714-17).

Similar influenza studies have also been conducted in ferret models using NanoLuc engineered virus (Karlsson *et al,* 2015 - https://doi.org/10.1038/ncomms7378). Ferret models of influenza are thought to be more representative of the human disease. However, the increase cost of these animals and the necessity for separate housing from mice, makes their use challenging.

An alternative methodology for monitoring the pathology of lung infections is to use optical reporters to visualize the inflammation caused by the virus. Figure 2 shows data from Lienenklaus and colleagues (2009 - https://doi.org/10.4049/jimmunol.0804277) where an IFN-β luciferase transgenic mouse model (interferon beta promoter driving luciferase expression) was used to monitor upregulation of this cytokine (interferon beta) in response to an influenza infection. Not only does this approach give an indirect indication of the degree of infection but allows important cytokine and chemokine pathways to be observed. Influenza and other respiratory viruses have been shown to cause cytokine storms in infected patients leading to exacerbation of inflammation and breathing difficulties. Moreover, epidemiological evidence from influenza outbreaks and pandemics reveals that morbidity and mortality are often higher for women than men. Sex differences in the outcome of influenza are age-dependent, often being most pronounced among adults of reproductive ages (18–49 years of age). Small animal models of influenza virus infection illustrate that inflammatory immune responses also differ between the sexes

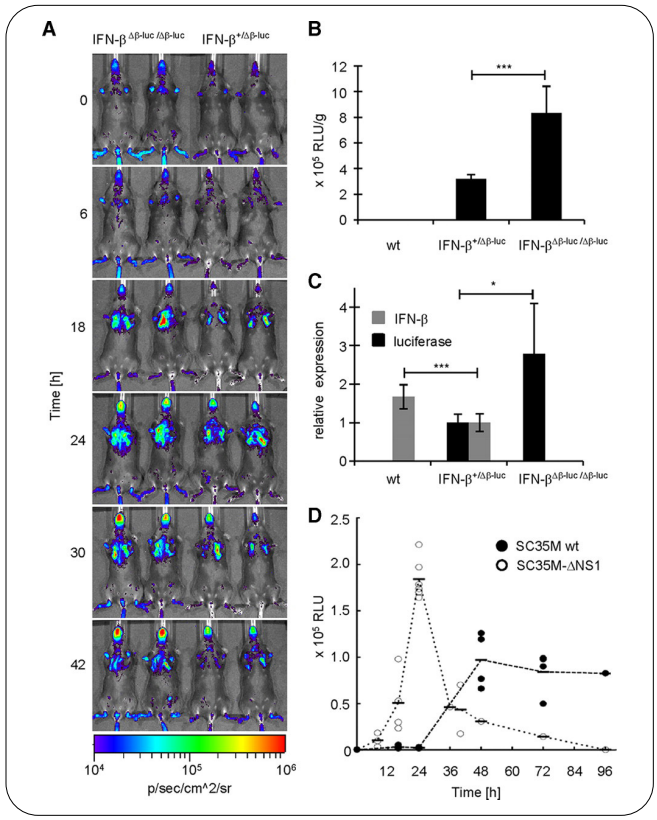

Figure 2. Luciferase gene expression in Δβ-luc reporter mice infected with influenza A virus. Kinetics of luciferase activity in IFN-β+/Δβ-luc and IFN-Δβ-luc/Δβ-luc mice infected with NS1-deficient variant of influenza A virus strain SC35M. (Lienenklaus *et al*, 2009. Novel reporter mouse reveals constitutive and inflammatory expression of IFN-β in . J Immunol 183: 3229-3236. Copyright 2009. The American Association of Immunologists, Inc.).

and impact the outcome of infection, with females generating higher proinflammatory cytokine and chemokine responses and experiencing greater morbidity and mortality than males (Klein *et al,* 2012 - https://doi.org/10.1189/jlb.0811427). All respiratory viruses are not the same, however. In contrast to influenza, SARS-CoV-2 appears to be significantly more detrimental to men, reportedly causing up to twice the mortality in this sex. Thus, being able to design animal models that individualize host-pathogen interactions is essential, making non-invasive optical imaging an ideal technology since both the virus and the host pathway can be tagged and monitored simultaneousl.

Proposed therapies for COVID-19 include use of IL-6 or IL-6-receptor blocking antibodies like tocilizumab (Actemra, Roche-Genentech), sarilumab (Kevzara, Regeneron), and siltuximab (Sylvant, EUSA Pharma) that are FDA-approved for non-viral therapies such as the management of cytokine release syndrome (CRS) in patients receiving CAR T cell therapy and rheumatologic disease. Luciferase engineered transgenic animal models, including IL-6 and other cytokine and chemokine pathways affected by viral infections, might prove extremely valuable for antiviral/anti-inflammatory drug discovery approaches for treating respiratory infections caused by both influenza and coronavirus.

As an alternative approach to using luciferase engineered transgenic animals, which may not be readily available in the desired genetic background of an animal, Ansaldi and colleagues (2011 - https://doi.org/10.1371/journal.pone.0025093) demonstrated (Figure 3) that in DNA delivery methodologies could be utilized to semi-stably transform the lung tissues of mice with cytokine/chemokine responsive elements fused to firefly luciferase reporter genes. Proof-of-principle studies using LPS as a stimulant for inflammation clearly showed this approach to be robust and sensitive to drug efficacy applications.

In response to the spate of epidemic and pandemic coronavirus outbreaks (SARS-2003, MERS-2012 and COVID-19), Bao and colleagues (2020 - https://doi.org/10.1101/2020.02.07.939389) generated a transgenic mouse model that incorporates human angiotensin converting enzyme 2 (ACE2), the cell entry receptor of SARS-CoV. It is likely that this and similar transgenic small animal models will be used as a test platform for future coronavirus studies, including the development of novel therapeutics and vaccines. Additionally, future models may incorporate transgenic optical reporter approaches similar to those shown.

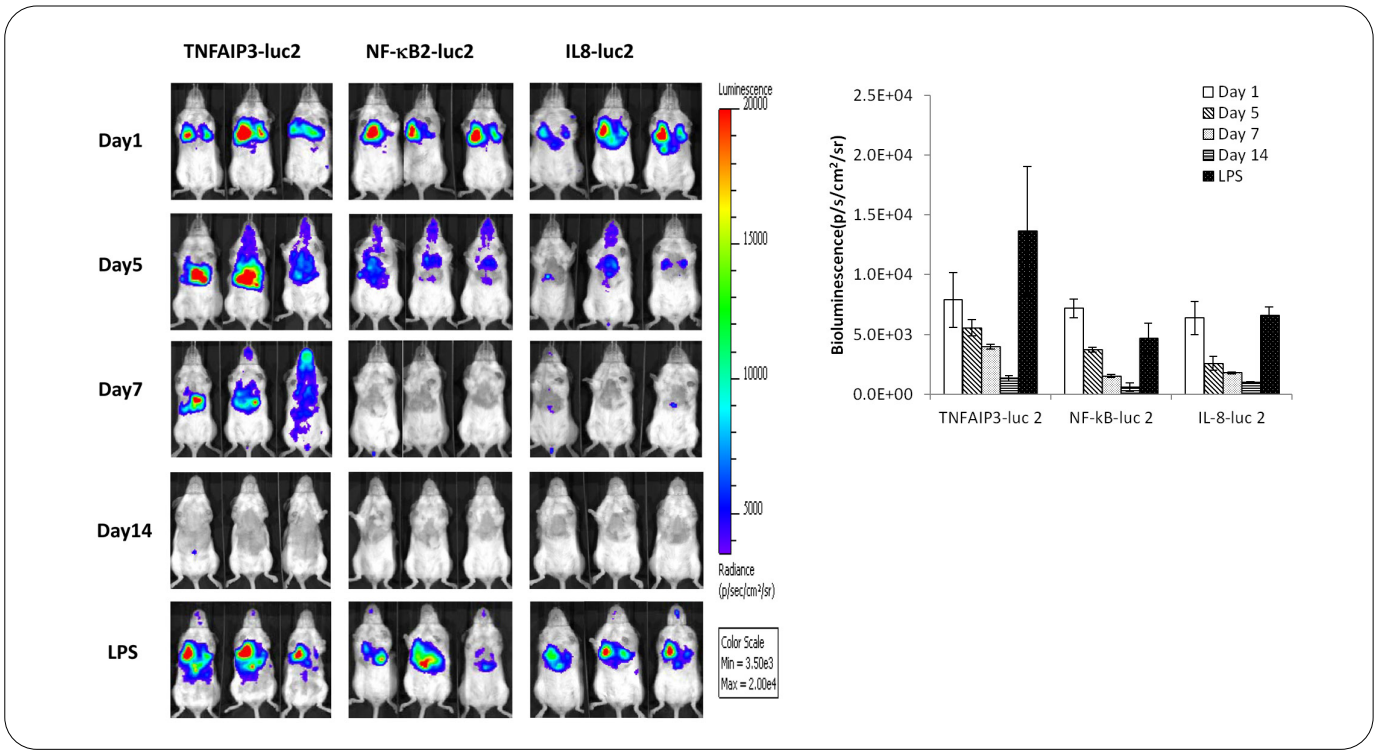

Figure 3. In gene delivery of NF-kB reporters to the lung and response to LPS treatment. Albino C57BL/6 mice (n = 3 per group) were injected intravenously with NF-kB reporter DNA using JetPEI. Mice were imaged following i.p. injection of luciferin at 1, 5, 7, and 14 days following transfection. After the imaging on the day 14, mice were intratracheally challenged with LPS at 1 mg/kg and re-imaged after 3 hours (Ansaldi *et al*, 2011. Imaging pulmonary NF-kappaB activation and therapeutic effects of MLN120B and TDZD-8. PloS One 6:e25093).

Viral hemorrhagic fever models

Viruses are the most abundant and the most diverse organisms on earth, ranging in size from the tiny Porcine Circovirus with an average capsid size of 17 nm (genome of 1.7 thousand base pairs) to the colossal Mimivirus at 750 nm (genome of 1.2 million base pairs). Moreover, viruses can be either DNA or RNA, single or double stranded – as described by Sir Peter Medawar "a piece of bad news wrapped up in a protein". Some of the most devastating viral pathogens known to man are those that cause hemorrhagic fever, the BSL-3 and -4 viruses, like Yellow Fever virus, Lassa virus, Marburg virus and Ebola virus. These viruses can be devastating, with mortality rates as high as 90%. The impact of the 2014 Ebola Viral Disease (EVD) epidemic on West Africa and the world was significant. A total of 28,616 cases of EVD and 11,310 deaths were reported in Guinea, Liberia, and Sierra Leone. Thankfully, there were only an additional 36 cases and 15 deaths that occurred when the outbreak spread outside of these three countries.

Non-invasive optical imaging has been widely accepted and adopted by researchers dealing with both BSL-3 and -4 pathogens, predominantly because this technique allows longitudinal imaging/monitoring of the animals without sacrifice and unnecessary exposure of the researcher to the microorganism. Additionally, the evolution of molecular biology has allowed for the development of attenuated strains and pseudo viruses that can be studied in BSL-2 laboratories, making this imaging technique considerably more amenable for essential drug and vaccine development studies.

Such a study using a pseudo Ebola virus-like particle, engineered to contain firefly luciferase, has been conducted by Li and colleagues (2016 - https://doi.org/10.1128/JVI.01239-16). Figure 4 shows a series of BLI figures generated with mice infected by this engineered virus and imaged in an IVIS SpectrumCT system, which has the added advantage of being able to generate three-dimensional reconstructions of the animal and its disease foci. 3D reconstructions identified the liver as the primary target organ, where the bioluminescent signal was intense in both the left and right lobes, corresponding to data in both non-human primates and human clinical studies.

Viral encephalitis models

Encephalitis is inflammation of the brain. There are several causes, but the most common is viral infection. Although the incidence of viral encephalitis is quite low

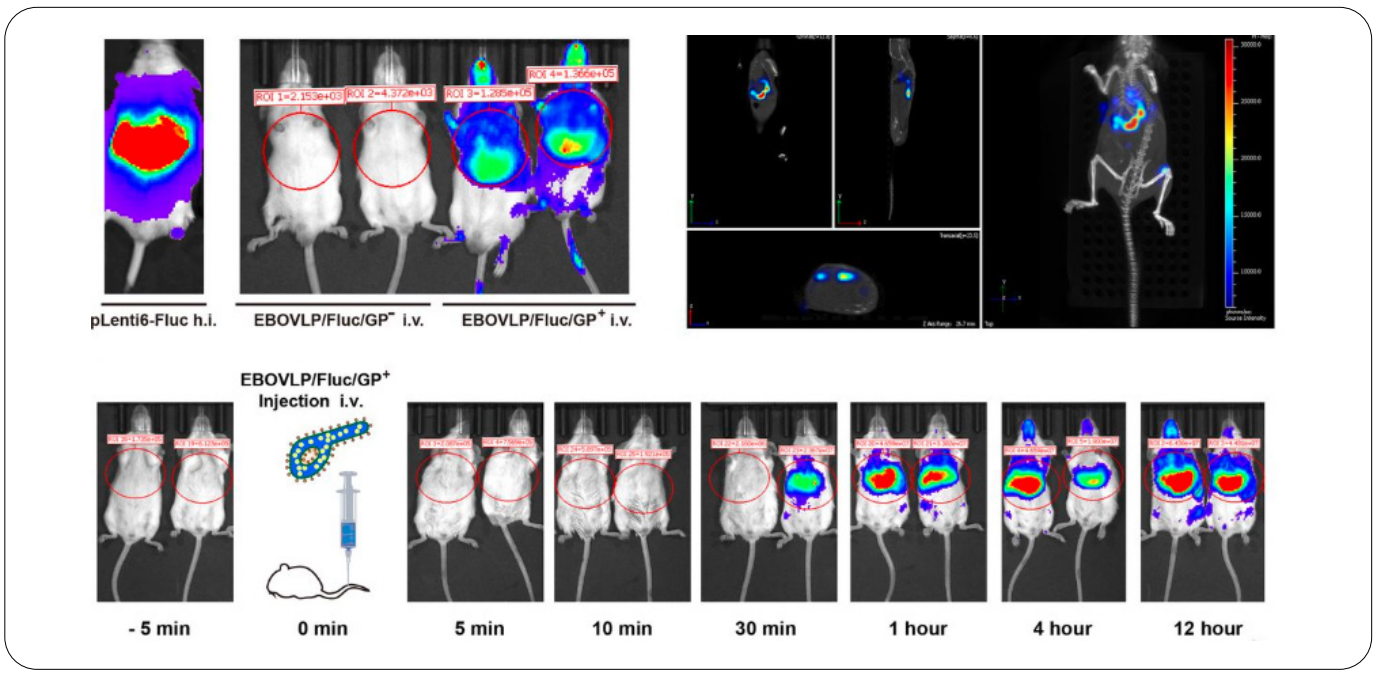

Figure 4. Six-week-old BALB/c mice were challenged by intravenous (i.v.) injection with VP40-normalized EBOVLP/Fluc/GP+ or EBOVLP/Fluc/GP+. PBS was given as a negative control, while hydrodynamic injection (h.i.) of the pLenti6-Fluc plasmid was set as a positive control. In imaging was performed 12 h postinoculation. BLI from EBOVLP/Fluc/GP+-inoculated mice was performed at 12 h. (Li *et al* 2016. An Ebola virus-like particle-based reporter system enables evaluation of antiviral drugs in under non-biosafety level 4 conditions. J. Virol. 90: 8720-8728.

(3.5 to 7.5 per 100,000 people), mortality can be extremely high especially in young children and seniors. Some forms of viral encephalitis are more severe than others, for example herpes simplex encephalitis (HSE) has a mortality rate of up to 30% even with specific anti-viral treatment, and 70-80% without the treatment.

Although the incidence of viral encephalitis from mumps and measles has decreases due to vaccination, EBV and CMV encephalitis are seen more frequently now because they occur in immunocompromised individuals, such as AIDS, transplant, and chemotherapy patients. Other important epidemiologic factors include the time of the year, geography, and animal or insect exposure. For instance, arboviruses such as eastern equine, western equine, St. Louis, Venezuelan equine, Zika, Chikungunya and West Nile cause disease during the summer months when mosquitos are active.

A large number of viruses known to cause encephalitis have been engineered with optical reporters, allowing their infections to be accurately tracked and monitored non-invasively in rodent models using optical imaging systems such as IVIS, including enteroviruses which impact an estimated 30-50 million people annually in the United States and more than a billion worldwide. The data in Figure 5 from Caine and Osorio (2017 - https://doi.org/10.1128/JVI.01759-16), shows direct monitoring of enterovirus in AG129 and BALB/c mice after infection with a NanoLuc engineered a mouse-adapted strain (mEV71-NLuc).

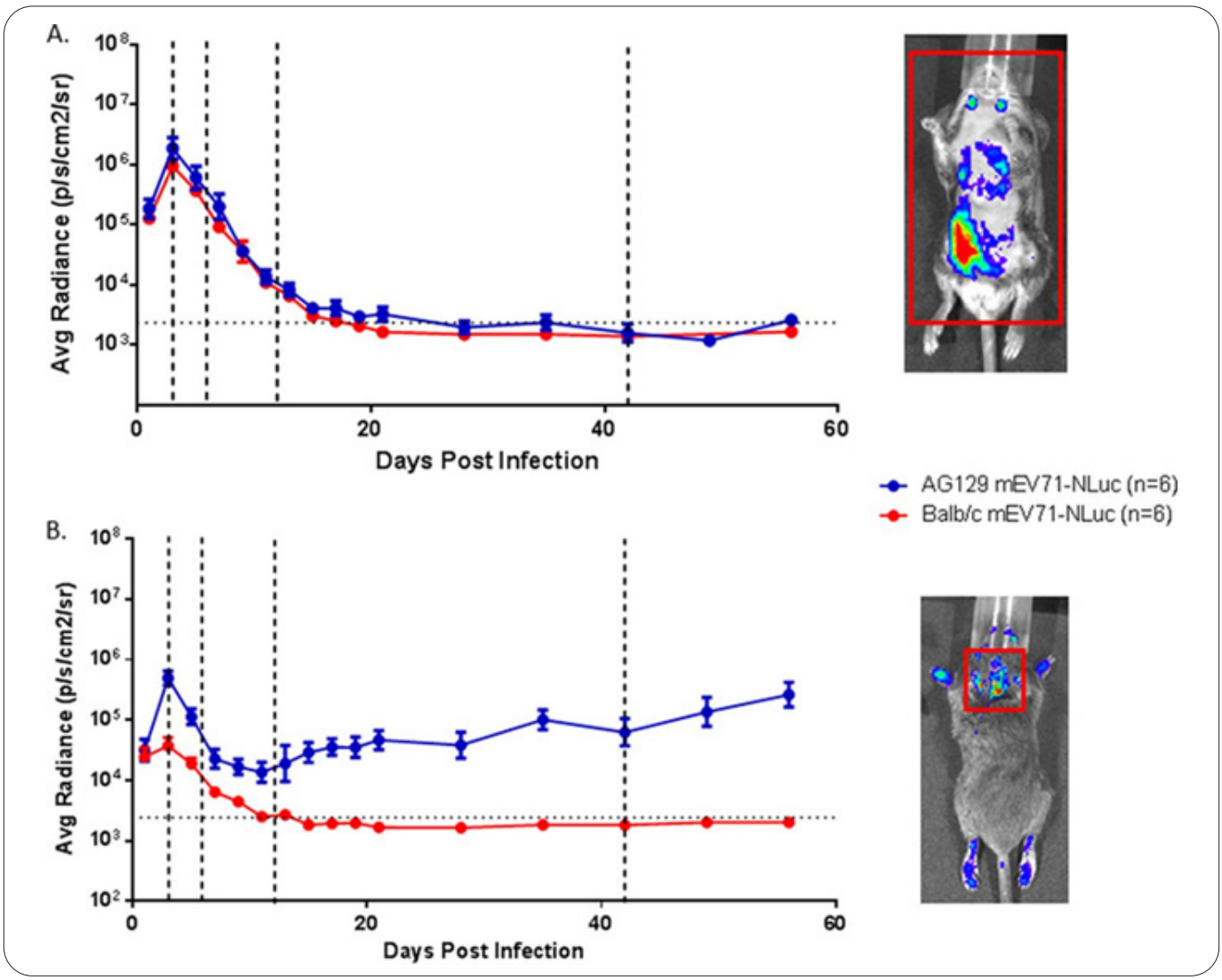

Figure 5. Average radiances of AG129 and BALB/c mice after infection with mEV71-NLuc. The average radiance was determined by placing a red box over a region of interest. (A) Radiance on the ventral side peaked at 3 dpi for both animal models. (B) Radiance continued to increase in the brain region of AG129 mice over the course of infection. (Caine and Osorio, 2017. In imaging with bioluminescent Enterovirus 71 allows for real-time visualization of tissue tropism and viral spread. J. Virol. 91: e01759-16).

An alternative BLI methodology for imaging this same enterovirus was developed by Guo and colleagues (2014 - https://doi.org/10.1016/j.antiviral.2013.11.002). In this approach, shown in Figure 6, a plasmid-based reporter was constructed to express the fusion

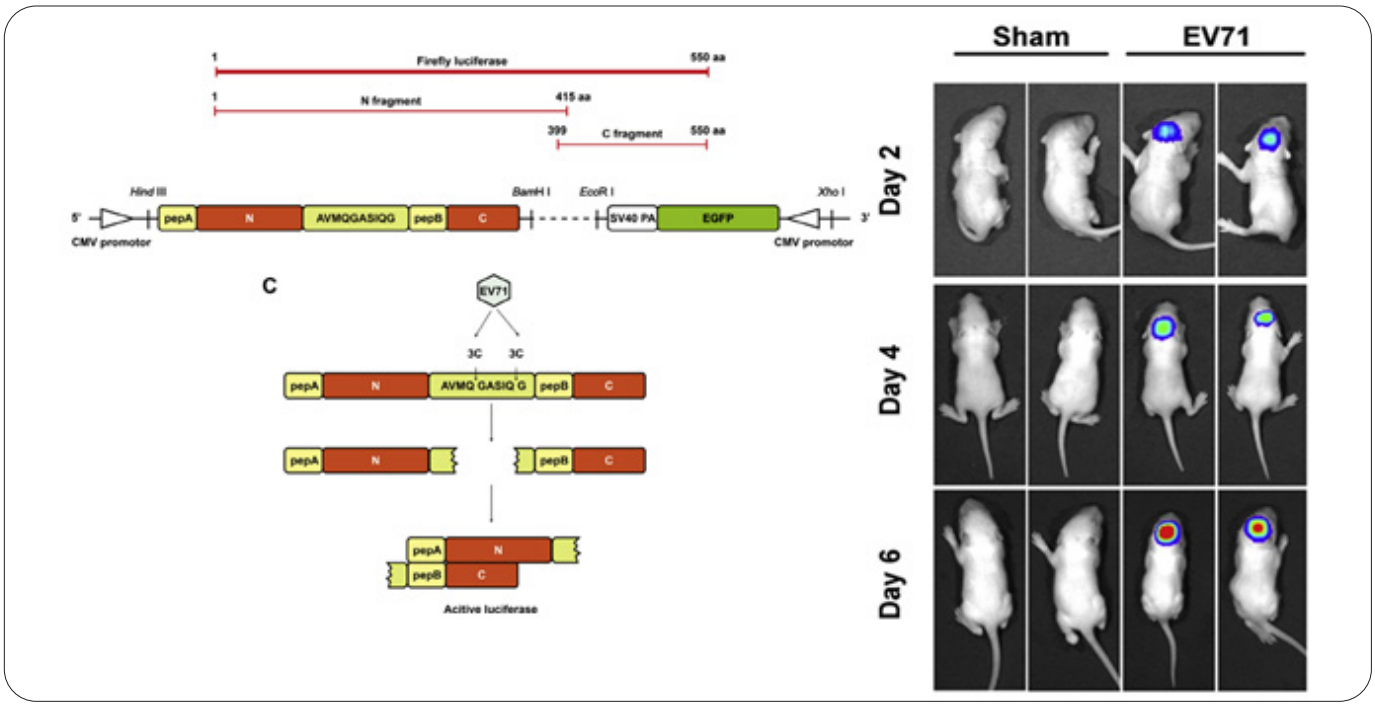

Figure 6. Three-day-old ICR mice were infected with 2 x 107 TCID50 of EV71 by intracerebral injection. The DNA of pAmN(Q/G)BC was intracerebrally inoculated into the mice 24 h before bioluminescence detection. (Guo *et al*, 2014. A 3Cpro-dependent bioluminescence imaging assay for in evaluation of anti-enterovirus 71 agents. Antiviral Res. 101: 82-92).

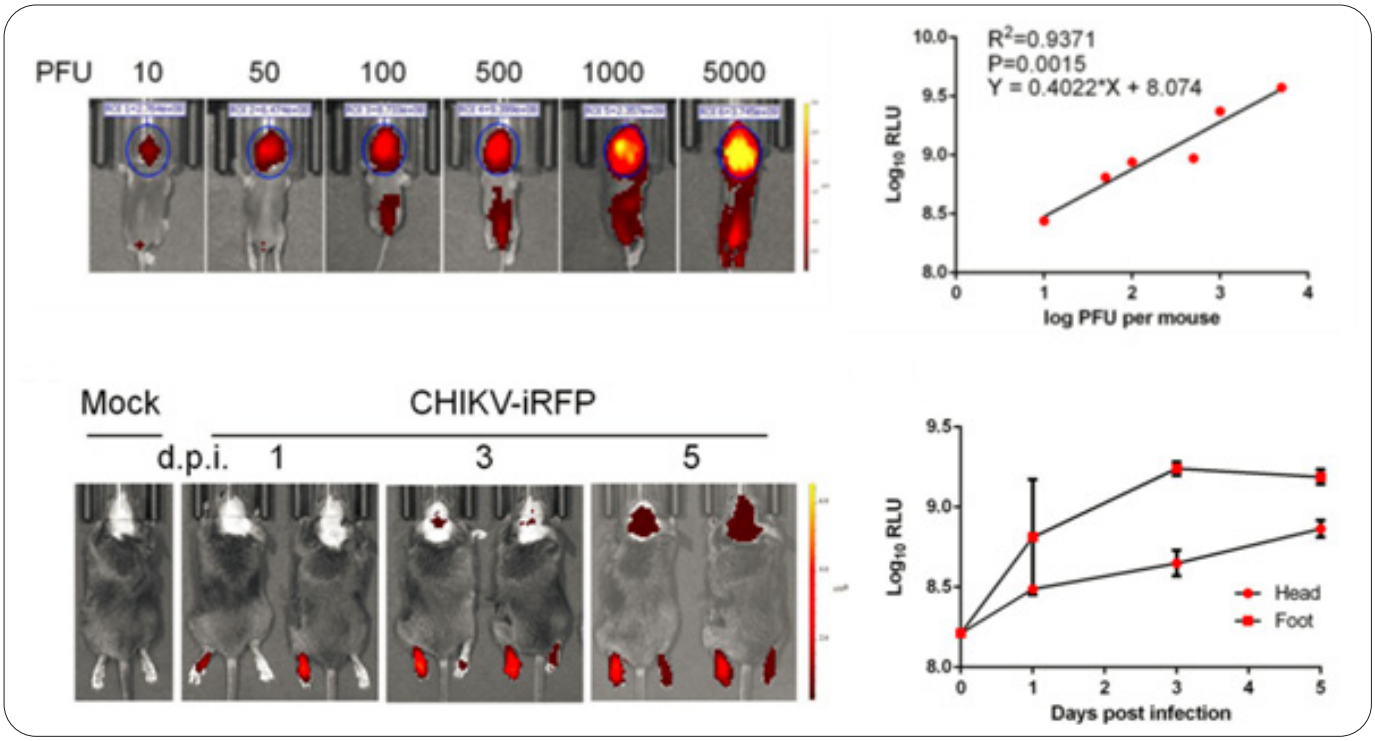

Figure 7. Neonatal BABL/c mice and IFNAR-/- A129 mice were highly susceptible to CHIKV-iRFP infection. Following intracranial inoculation, CHIKV-iRFP efficiently replicated and disseminated into whole body, resulting in rapid death in an age-dependent manner. Remarkably, upon footpad injection, CHIKV-iRFP readily disseminated from footpad to head. (Zhang *et al*, 2019. Visualization of chikungunya virus infection *in vitro* and in. Emerg Microbes Infect. 8:1574-1583).

protein AmN(Q/G)BC, a split firefly luciferase mutant, which can be specifically cleaved by EV71 protease 3Cpro. Upon cleavage, the splitting fusion protein restores luciferase activity.

Chikungunya virus (CHIKV), a mosquito-borne alphavirus, has become an important re-emerging pathogen with its rapid spread to many non-endemic areas. Like enterovirus, CHIKV infection can lead to severe encephalitis and death, especially in young babies and older adults. The lack of effective vaccines and antiviral agents is largely attributed to the elusive infection and dissemination dynamics *in vivo*. Figure 7 shows a study by Zhang and colleagues (2019 - https://doi.org/10.1080/22221751.2019.1682948) where a novel CHIKV reporter virus (CHIKV-iRFP) was designed and developed to encode a near infrared fluorescent protein (iRFP). Although not as sensitive as luciferase reporters for *in vivo* imaging, fluorescent reporters have the added advantage of being amenable to translational imaging (i.e., *in vitro* and ex vivo microscopy).

Fluorescent and bioluminescent imaging can be conducted sequentially in an IVIS system while the animal remains in the chamber anesthetized and immobilized. This could allow for two biological events or entities to be visualized and superimposed relatively seamlessly, such as imaging unique host pathogen interactions (e.g., iRFP labeled virus in a firefly luciferase labelled transgenic mouse).

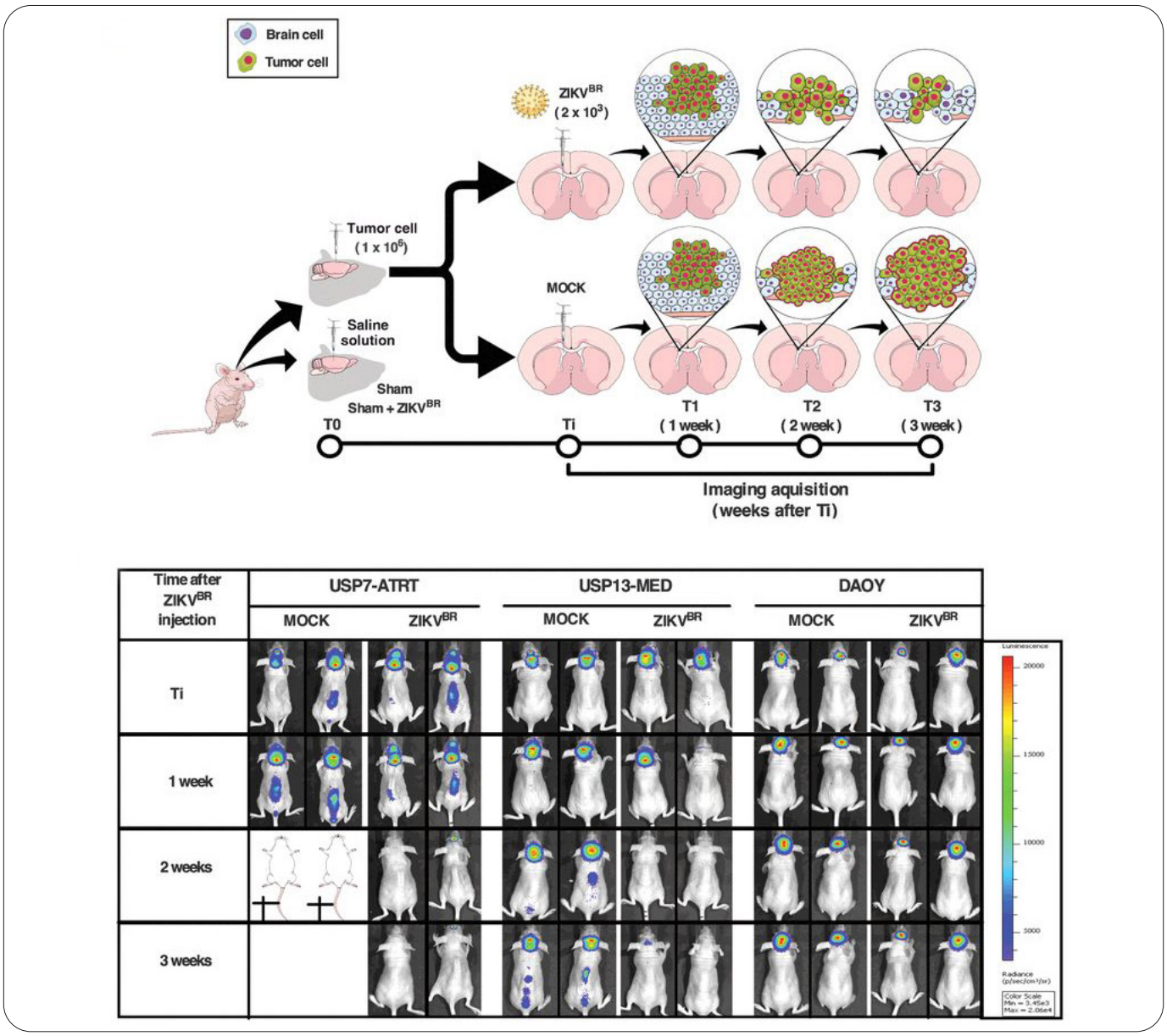

Figure 8. BLI of tumor development in control (mock) and ZIKVBR treated mice. In both USP7-ATRT and USP13-MED tumor bearing mice, the Brazilian Zika virus strain kills aggressive metastatic forms of these human CNS tumors and demonstrated potential as an oncolytic agent for cancer therapy. (Kaid *et al*, 2018. Zika virus selectively kills aggressive human embryonal CNS tumor cells *in vitro* and in. Cancer Res. 78: 3363-74).

In recent years, one of the most devastating viruses causing encephalitis and a plethora of neurological issues, is the Zika virus. This flavivirus that is again transmitted by mosquitoes, was first discovered in 1947. However, it was not until 2015 when it spread rapidly through South and Central America reaching epidemic proportions and infecting large numbers of pregnant women that the true nature of this disease was recognized. In children who had been infected during pregnancy, the prognosis is usually poor, with cognitive deficits and a psychomotor development delay.

From this dreadful disease however has come a glimmer of hope for cancer patients. Zika preferentially infects human neural progenitor cells and triggers cell apoptosis, and researchers have been monopolizing upon this pathology to treat brain cancers in mice. Using luciferase labelled stem-like cancer cells from aggressive human embryonal tumors of the CNS, Kaid and colleagues (2018 - https://doi.org/10.1158/0008-5472.CAN-17-3201), as seen in Figure 8, showed that Zika is capable of infecting and destroying tumors established from these labelled cell lines in the brains of mice.

Summary

Viral diseases have emerged and re-emerged throughout history, and as the human population continues to increase globally, so will the frequency of viral pandemics. Not only have Ebola and COVID-19 demonstrated most recently mankind's vulnerability to contagious diseases, but also our inability to respond adequately from a therapeutic standpoint. Scientific ingenuity is our best defense, and technologies such as non-invasive optical imaging are key to the development of new drugs and vaccines. As seen from the few research articles presented herein, this preclinical imaging technique is extremely versatile, allowing the most intricate host-pathogen interactions to be visualized and monitored. Moreover, as the IVIS platform has demonstrated with other diseases, such as cancer, non-invasive optical imaging can help unravel some of the most complex mechanisms of pathology and help identify lead therapeutic candidates.

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