APPLICATION NOTE

Imaging oncolytic virus infection in cancer cells.

Author

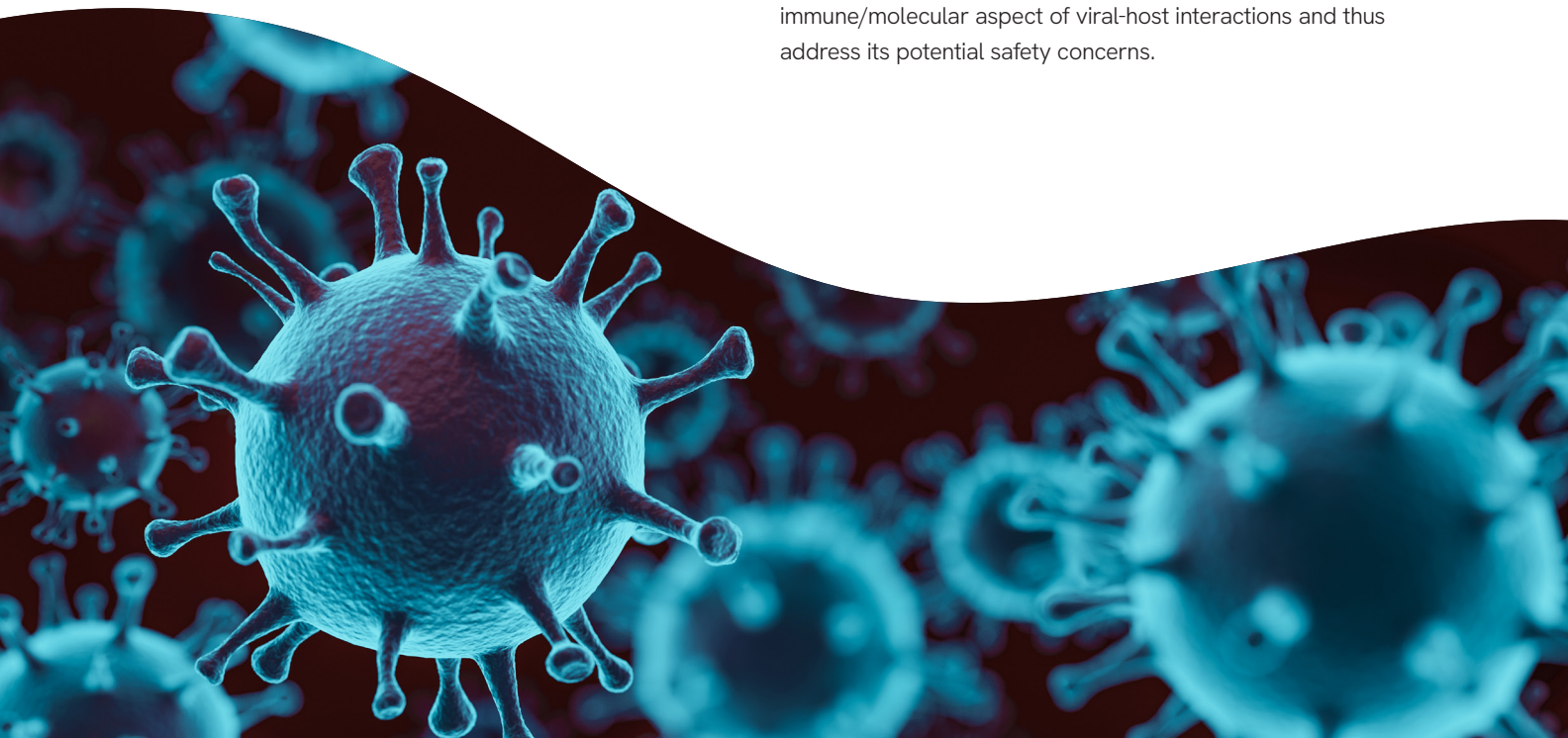
Jen-Chieh Tseng

Revvity, Inc.
Hopkinton, MA

Abstract

The ultimate goal of a successful cancer treatment is to achieve selective targeting and effective killing of cancer cells without undesired harm to the surrounding normal tissues. Aside from the traditional small-molecule chemotherapeutics or targeted therapy agents that have been widely used in the clinic for decades, a new type of cancer therapeutics based on oncolytic viruses has recently gained attention in the field of research. Oncolytic viruses are genetically modified viruses capable of delivering therapeutic gene payload to cancer cells. A critical step for oncolytic virus development is to evaluate effectiveness against cancer and address safety concerns in living subjects. To illustrate this point, this application note provides an overview on using IVIS® optical imaging to assess and quantify oncolytic viral infection in living tumors and the subsequent virus-host interactions in real-time.

Although there are many different types of oncolytic viruses each having a different tumor-targeting mechanism, this application note highlights pre-clinical imaging results of Sindbis oncolytic virus to present a general approach when designing IVIS imaging studies for oncolytic virus research. Bioluminescent Sindbis pseudovirus (Sindbis/Fluc) was used to show successful tumor delivery and infection in several mouse tumor models. Since Sindbis/Fluc carries a bioluminescent firefly luciferase reporter gene, its infection and anti-cancer efficacy can be readily assessed by light production in tumors. The non-invasive aspect of IVIS imaging enables whole body imaging of viral infection and makes longitudinal imaging possible. Further, by imaging infection in transgenic/knockout mice with immune defects, this approach can be extended to study the immune/molecular aspect of viral-host interactions and thus address its potential safety concerns.



Of note, this IVIS imaging strategy is not limited to Sindbis research and can be easily applied to other virus species. The latter part of this application note discusses potential IVIS fluorescent imaging applications to visualize host biological changes in response to viral infection.

Introduction

Viruses are infectious agents of small size and simple composition, consisting of genetic material (DNA or RNA) packaged within a protein or membrane enclosure. They are naturally evolved gene delivery vehicles, and their survival depends on using viral genetic information to hijack host cell molecular machinery and exploit its resources for viral particle reproduction. Based on a variety of wildtype viruses, oncolytic viruses are man-made pseudoviruses capable of delivering non-viral genes. In particular, they are engineered to achieve specific targeting and killing of cancer cells. This concept first emerged at the beginning of the twentieth century after a patient with myelogenous leukemia underwent complete tumor remission following influenza infection. This led to the investigation of a wide range of human virus species for their oncolytic potential, including adenoviruses, herpes simplex virus (type 1), measles virus, vascular stomatitis virus and Sindbis virus. After decades of cancer research and advances in molecular virology, many types of pseudoviruses have been developed to ensure selective infection and/or replication in cancer cells.

Although almost all human viruses can cause pathogenic conditions and can be considered harmful, there is a general strategy to convert them into much safer pseudoviruses for gene therapy. A typical viral genome, regardless of DNA or RNA, contains two types of gene cluster: structural and non-structural genes. As the name implies, structural genes encode proteins such as the capsid and envelope proteins that are building blocks of virion particles. Conversely, non-structural genes encode virus-specific enzymes such as replicase and protease required for genomic DNA or RNA replication and viral protein production, respectively. Taking advantage of this unique arrangement, researchers can produce a pseudovirus by replacing the structural gene cluster with a payload gene of interest. Thus, the resulting pseudovirus is capable of infecting cells and expressing the payload gene after infection, but it lacks essential structural genes for subsequent virion production.

This general strategy has been successfully applied to several virus species, and many types of oncolytic pseudoviruses have been developed for exogenous gene delivery. Nevertheless, it still remains a challenge to study their performance in living animal models. As viruses are infinitesimally small, direct and non-invasive detection in real-time is very difficult. Fortunately, with a proper reporter gene as a payload, *in vivo* detection of a pseudovirus infection can be observed using a suitable molecular imaging techniques. For example, a firefly luciferase gene (Fluc) can be used as the reporter gene payload for detecting live infection events: Fluc expression resulting in efficient light production that can be detected through bioluminescence imaging. This approach makes it possible to longitudinally detect and quantify virus infection of host/tumor cells throughout the course of study in living subjects.

This application note shows how IVIS optical imaging was used to non-invasively study viral infection and virus-host interaction in real-time. In particular, oncolytic Sindbis virus was chosen for this purpose, since it has several unique features that make it an attractive oncolytic agent for cancer gene therapy and treatment. Originally identified in 1952, Sindbis virus is an arbovirus naturally transmitted via mosquito bites. Thus, Sindbis virus is capable of circulating in the blood stream for systemic delivery. For selective tumor targeting, Sindbis infection in mammalian cells is mediated by binding to the 67-Kda laminin receptor (LAMR) on plasma membrane. LAMR is known to be over-expressed in cancer cells and higher expression levels have been associated with poor prognosis in several human cancers. This particular fact gives Sindbis virus specific affinity to tumors. In mammalian cells, Sindbis infection is known to induce apoptosis, suggesting the virus could be used for eradicating cancer cells. For efficient gene delivery, a Sindbis pseudovirus system has been developed by replacing its structural genes. In this note, successful tumor delivery and infection were illustrated using a bioluminescent Sindbis pseudovirus carrying a firefly luciferase gene (Sindbis/Fluc). Several different types of mouse tumor models were used to demonstrate Sindbis' tumor targeting capability. Tumor bioluminescence closely correlates with viral infection levels and thus allows convenient and accurate assessment of Sindbis-related infection in living animals.

Results

In vivo imaging of oncolytic virus infection in tumors

Figure 1 summarizes the study design to evaluate Sindbis virus' tumor targeting capability in living animals. The goal was to demonstrate specific tumor targeting in living animals. To achieve this, a Sindbis pseudovirus carrying a firefly luciferase gene was used for IVIS imaging. Figure 1A illustrates a typical pseudoviral design of Sindbis virus. Wild type Sindbis virus has a positive-strand RNA genome with a 5′ cap and a 3′ poly-A tail. Its non-structural genes are on the 5′ side, followed by a subgenomic promoter (PSG) which is responsible for driving the structural genes downstream on the 3′ side. By replacing the structural gene cluster with a reporter gene, the resulting pseudovirus particle can be used to deliver a desired gene payload as its expression will be driven by a potent viral promoter. When a bioluminescent firefly luciferase gene (Fluc) is used, its successful infection and subsequent light production can be readily detected by the IVIS camera after concomitant luciferase substrate luciferin delivery.

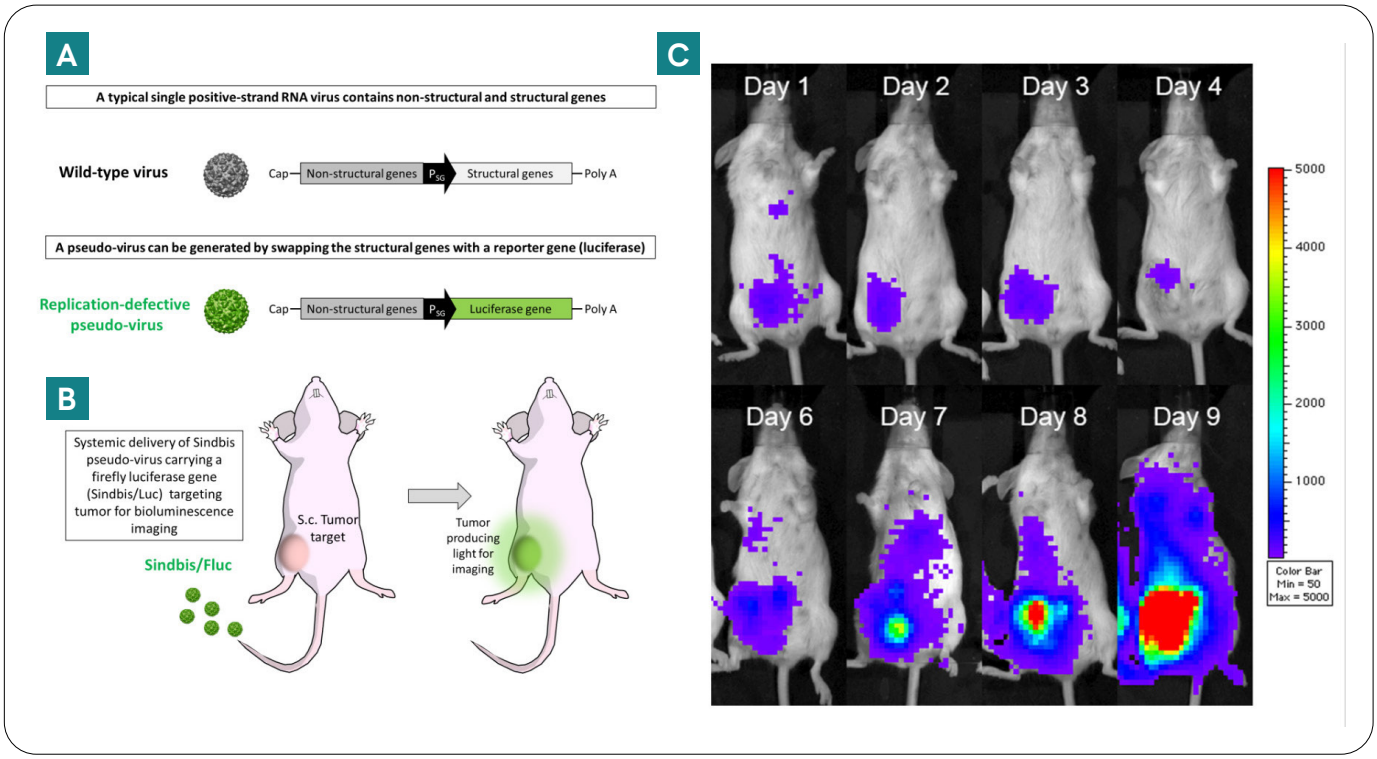


Figure 1. Study design and bioluminescence imaging using IVIS to visualize Sindbis infection in tumors.

Figure 1B outlines a simple study design to visualize Sindbis/Fluc infection in tumors. In this example, baby hamster kidney (BHK) cells were inoculated onto the mammary fat pads in immunocompromised SCID mice. BHK cells have a high level of LAMR expression and are highly sensitive to Sindbis infection. After tumor was established, mice received daily i.v. injections of Sindbis/Fluc starting on Day 0. Figure 1C shows longitudinal imaging results on a tumor-bearing mouse. Tumor-specific bioluminescence was observed as early as Day 1 and gradually increased during the first nine days of imaging. Luciferin was injected prior to each *in vivo* bioluminescence imaging (BLI) timepoint.

Specific targeting andWW infection in spontaneous tumors

Although the previous results shows strong affinity of Sindbis virus to BHK tumors, its preferential tumor infection may be due to underlying species difference between hamster (BHK tumor) and mouse (host). As discussed, BHK cells have a high level of LAMR expression and thus it is not surprising that the tumors are highly susceptible to Sindbis infection. To ensure the virus' selective affinity is truly due to their preference to cancer cells, a spontaneous and immunocompetent mouse tumor model was tested.

Figure 2A illustrates such imaging study design. In particular, the MSV-*RGR/p15*$^{+/-}$ transgenic mice are heterozygous for the Rgr oncogene and for the *Cdkn2b* gene, also known as *p15(Ink4b)*. These mice typically develop spontaneous fibrosarcoma in the paws or tails. Because these tumor are not artificially implanted or injected, they closely mimic the physiological development of cancer. In this particular example, a mouse with a tumor growing on its right hind foot received daily i.p. treatments of Sindbis/Fluc for three consecutive days prior to bioluminescence imaging. All viral injections were done in the peritoneal cavity to be as distant as possible from the tumor site.

Figure 2B shows the imaging results after three consecutive Sindbis daily treatments. The photograph image shows a fibrosarcoma tumor on the right hind foot, while the image overlay with bioluminescence signals clearly shows tumor-specific targeting. As the Sindbis/Fluc pseoduvirus particles accumulated in the fibrosarcoma tumor, specific bioluminescence signals in the foot tumor indicated the virus is capable of targeting tumors that arise spontaneously while avoid normal tissues. Notably, in this particular model, only mouse cells are involved whether they are normal or tumor. Of note, the immune system seems not to diminish its ability to reach and infect tumor cells during the first couple days of treatments.

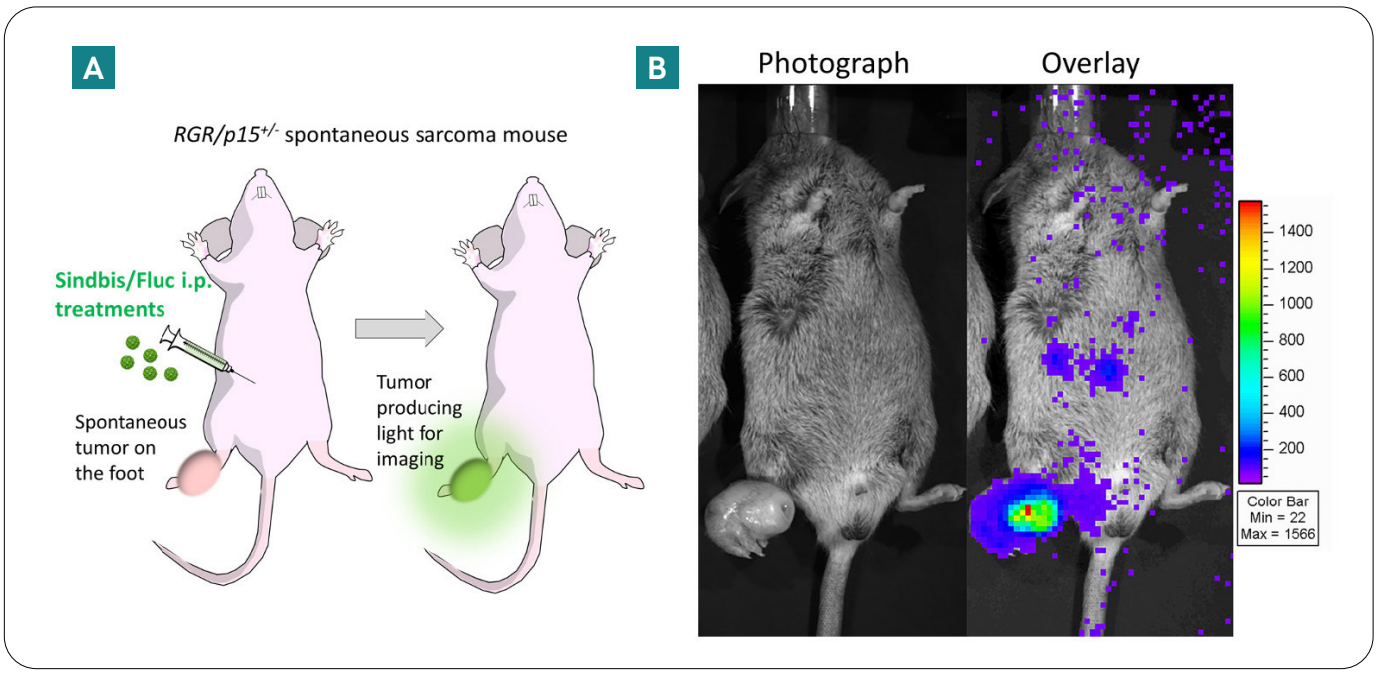


Figure 2. Sindbis virus specifically infects spontaneous fibrosarcoma in a transgenic mouse. Imaged on the IVIS system.

Specific targeting of metastatic ovarian cancer in the peritoneal cavity

With the success of targeting localized solid tumors, the researchers then turned their attention to metastatic cancers. In the following example (Figure 3), an advanced mouse ovarian cancer model was used to evaluate Sindbis' capability to target small, disseminated, and microscopic ovarian cancer nodules in the peritoneal cavity. The model was established by i.p. inoculation of mouse MOSEC ovarian cancer cells into C57BL/6 mice (Figure 3A). Four weeks after cancer cell inoculation, tumor-bearing mice received a single Sindbis/Fluc i.p. treatment. As reference, a tumor-free control mouse was also i.p. treated with Sindbis/Fluc and imaged the day after. Figure 3B shows the imaging results.

Interestingly, tumor-free control mouse (left panel) showed no detectable Sindbis/Fluc infection while the tumor-bearing mouse (right panel) showed significant and wide-spread infection BLI signals in the peritoneal cavity.

To validate specific Sindbis/Fluc tumor infection, various organs in the peritoneal cavity were harvested and imaged *ex vivo*. A good correlation of positive Sindbis infection and presence of tumor nodule was observed (Figure 3B, red arrows). This result was further validated by conventional histological, microscopic imaging (data shown in the original publication Ref 2).

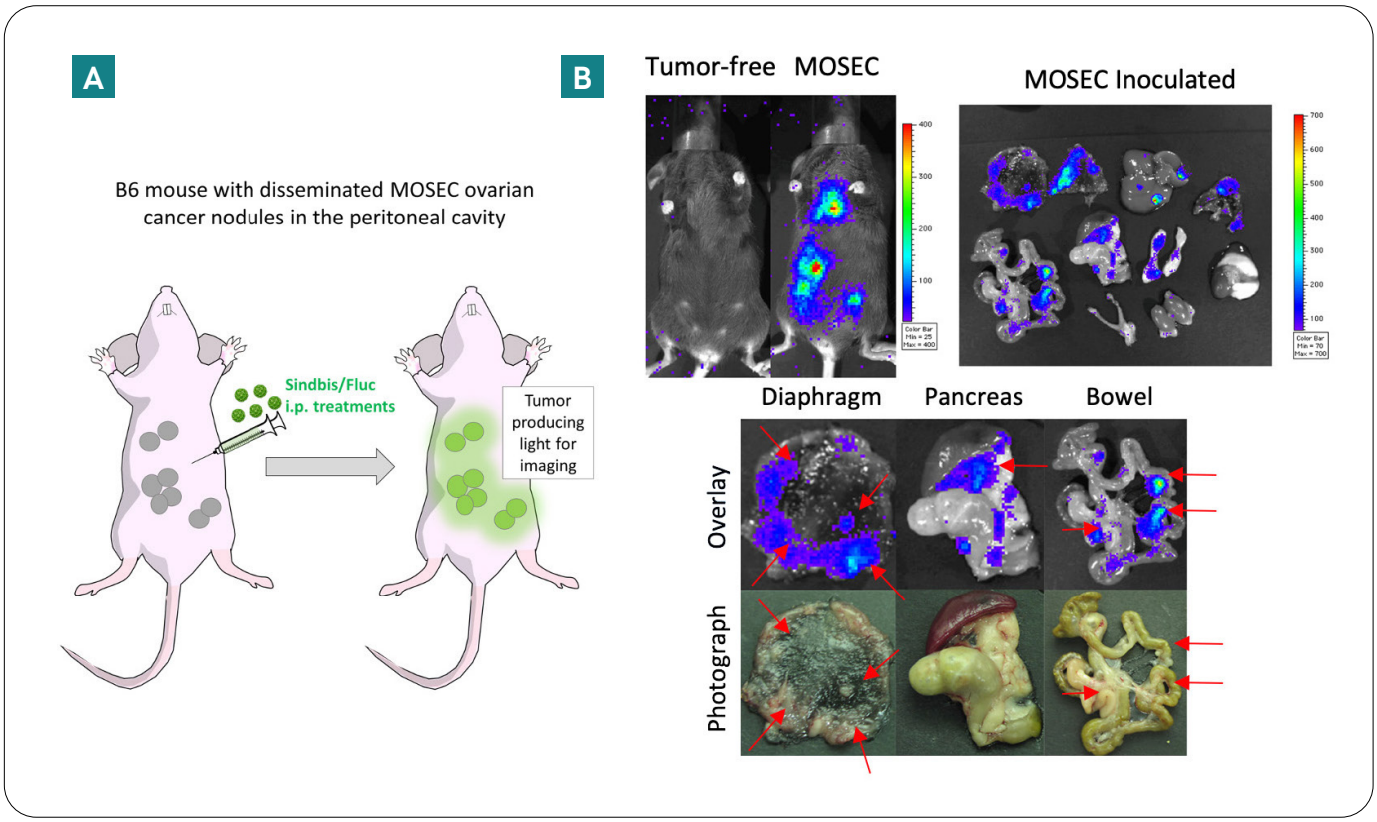


Figure 3. Sindbis specifically targets small, metastatic ovarian cancer in the peritoneal cavity.

Quantitative imaging of sindbis treatment efficacy

As Sindbis infection is known to induce apoptosis and kill the infected mammalian cells, the next step was to determine if repeated Sindbis treatments have any anti-cancer efficacy. Figure 4A illustrates a study design using the human ES-2/Fluc ovarian cancer cells which, after i.p. inoculation, were capable of establishing tumor nodules on the surface of various organs in the peritoneal cavity. As the cell carries the reporter firefly luciferase gene, its growth and spreading can be easily monitored using IVIS imaging. The bioluminescence imaging (BLI) signal levels directly correlated with tumor burden in the peritoneal cavity. In addition, to enhance its tumor suppression capability, immune-stimulating IL-12 or IL-15 gene was introduced into the Sindbis pseudovirus construct. As a reference, a pseudovirus carrying the bacterial β-galactosidase LacZ gene was used. In this particular study, ES2/Fluc cells ($1.5x10^6$) were i.p. inoculated into female SCID mice on Day 0 and daily i.p. Sindbis treatments began the Day after (Day 1).

Figure 4B shows the BLI imaging results of ES2/Fluc tumor load on Day 13 (after 12 consecutive treatments) and quantitative representation of BLI tumor signals for up to Day 20. Control mice did not receive any pseudovirus treatment. Total whole body photon counts were determined by IVIS imaging on days 1, 5, 13 and 20 to assess disease progression of ES2/Fluc metastases. Without any anti-cancer cytokine, the Sindbis/LacZ pseudovirus is sufficient to suppressed cancer growth when compared with the untreated control mice. The use of either IL-12 or IL-15 cytokine gene further enhanced the tumor suppressing activity of Sindbis virus. In both cases, more than 95% growth suppression was observed in cytokine viruses within five days of treatment.

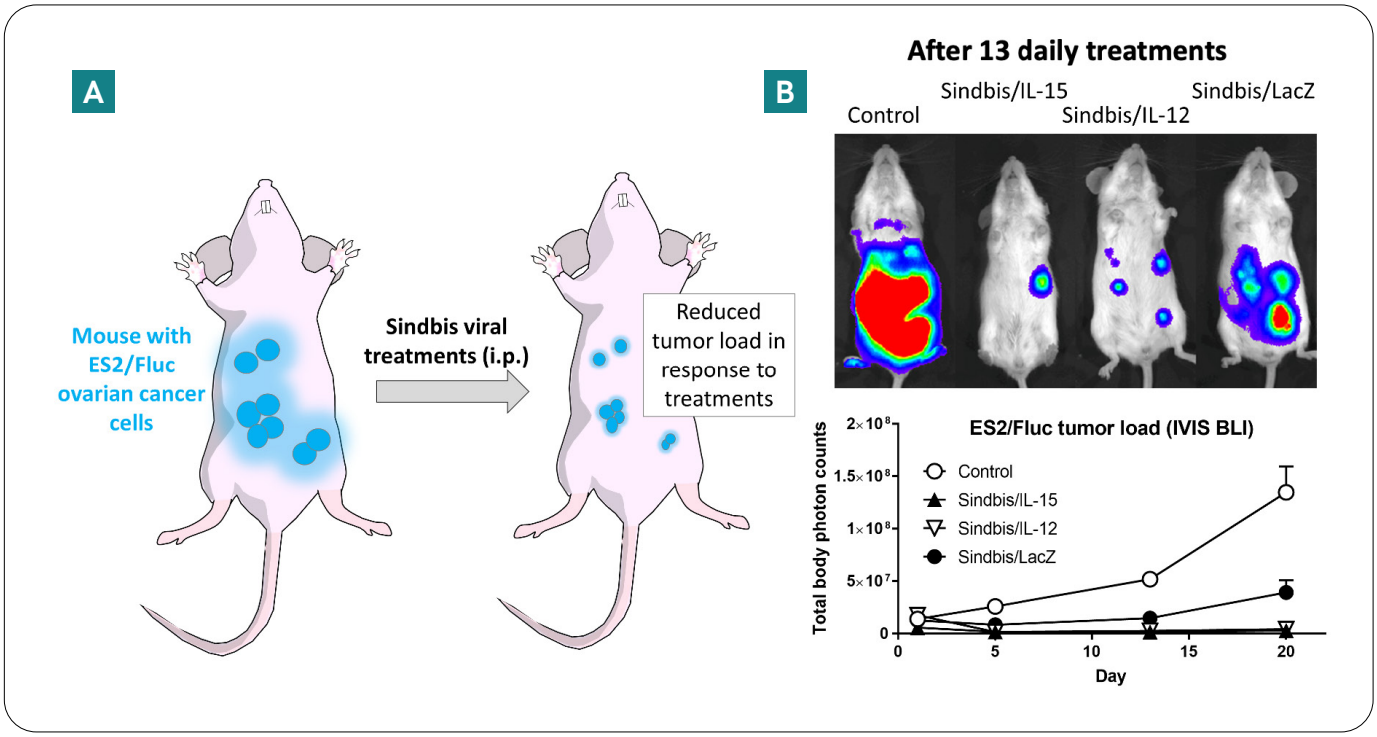


Figure 4. Repeated Sindbis treatments suppress ovarian cancer growth.

Type I Interferons protect liver from sindbis infection

For downstream clinical development, safety is of great importance and therefore warranted further investigation. In particular, the viral interaction with the host immune systems during repeated treatments was an important consideration. The data suggest that consecutive and repeated treatments of replication-defective Sindbis pseudovirus are safe for animals, and part of the reason could be the innate anti-viral immunity which protects normal tissue from infection. Since mammalian interferons (IFNs) play pivotal roles in antiviral responses, mice with genetic defects in either Type 1 (alpha/beta) or Type 2 (gamma) IFN pathways were used to determine if IFNs play any roles in protecting normal tissues from Sindbis infection.

The BLI imaging results in Figure 5 clearly indicates that the type 1 IFNs protect normal tissues from Sindbis infection. When Sindbis/Fluc was i.p. delivered into mice lacking either functional type 1 IFN-α/β receptor or its downstream transducer STAT1, significant liver infection was observed within 24 hours (Figure 5A). The liver infection was confirmed when the peritoneal organs were exposed and examined by IVIS BLI imaging (Figure 5B, red arrow). In some cases, a lower infection level present in the abdomen fat (Figure 5B, yellow arrow), irrelevant to its IFN-γ status. The imaging data indicate that type 2 IFN does not confer protection against Sindbis infection since no BLI signal was observed in the liver of IFN-γ knock-out mice (Figure 5C).

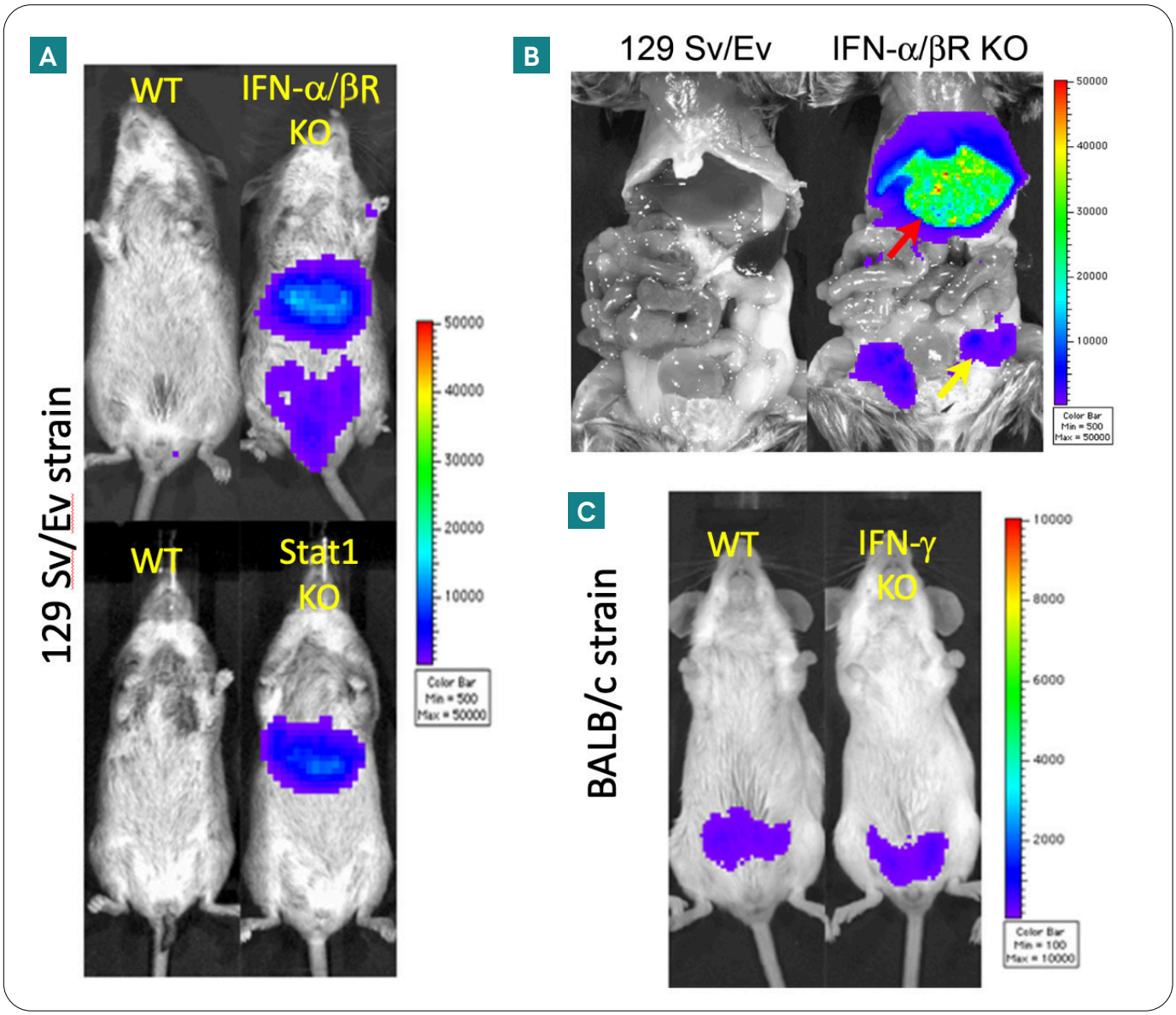


Figure 5. Type 1 interferon protect mouse liver from Sindbis infection.

Discussion

The imaging results presented in this application note demonstrate the value of optical imaging using the IVIS platform for visualizing virus infection non-invasively in living subjects. By imaging different types of tumor models and animals with well-characterized immune defects, these *in vivo* observations provide important insights into Sindbis' virus-host interaction and its therapeutic implications. Importantly, the bioluminescent imaging strategy illustrated here can easily be translated to other types of viruses, given most viruses' small genome sizes and recent advancements in genetic modification techniques. Furthermore, the IVIS imaging platform is versatile, and researchers are not limited to bioluminescent reporter genes when designing a pesudovirus imaging strategy. As most IVIS imaging systems are equipped with red and nearinfrared (NIR) fluorescence imaging capability, they can also be used to visualize fluorescent reporter expression in living animals. One advantage of fluorescence protein imaging is that it does not require any substrate to produce imaging signals. By choosing a suitable fluorescent reporter gene, specific viral infection and fluorescent protein expression in tumors can be readily visualized using the IVIS imaging system in study designs similar to those demonstrated in this application note. Table 1 lists the bioluminescent and fluorescent reporter genes that are compatible with the IVIS platform.

In addition to direct imaging of viral infection using bioluminescence imaging, IVIS systems can be used to study viral-host interaction by using a variety of NIR fluorescent imaging agents. Table 2 lists several NIR fluorescent imaging agents developed by Revvity that can be used in conjunction for imaging physiological changes and host responses during/after viral infection. In addition to complimenting bioluminescence imaging, a unique advantage of fluorescence imaging is the ability to multiplex more than one fluorescent probe providing a wealth of information on host's biological responses to viruses.

Table 1. Reporter genes suitable for IVIS imaging of infection/ replication events.

Bioluminescence	Firefly luciferase; Renilla luciferase; click beetle luciferase.	Highly sensitivity and with low background, but all require corresponding substrates for light production.
Fluorescence	Red fluorescent protein (RFP); DsRed and its variants (e.g. mFruits: mCherry, mOrange, mRaspberry).	Less sensitive and with higher background, but with no need for substrate to establish signals.

Table 2. NIR fluorescent imaging agents for imaging host responses to viral infection

Angiogenesis and vascular leakiness for virus delivery.	AngioSense 680 EX or 750 EX; AngioSpark 750; Superhance 750; TLectinSense 680.
Inflammation caused by virus or secondary infection.	ProSense 680, 750 EX or 750 FAST; MMPSense 680, 645 FAST or750 FAST; Cat B 680 FAST or 750 FAST; Cat K 680 FAST.
Apoptosis induced by viral infection or tissue damage.	Annexin *Vivo* 750.

References

1. Tseng JC, Levin B et al. Systemic tumor targeting and killing by Sindbis viral vectors. *Nat Biotechnol*. 2004 Jan;22(1):70-7.
2. Tseng JC, Hurtado A et al. Using Sindbis Viral Vectors for Specific Detection and Suppression of Advanced Ovarian Cancer in Animal Models. *Cancer Res*. 2004 Sep 15;64(18):6684-92.
3. Tseng JC, Zheng Y et al. Restricted Tissue Tropism and Acquired Resistance to Sindbis Viral Vector Expression in the Absence of Innate and Adaptive Immunity. *Gene Ther*. 2007 Aug;14(15):1166-74.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com