Review Article

Development and application of bioluminescence imaging for the influenza A virus

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Abstract: Influenza A viruses (IAVs) cause seasonal epidemics and intermittent pandemics which threaten human health. Conventional assays cannot meet the demands for rapid and sensitive detection of viral spread and pathogenesis in real time cannot be used for high-throughput screens of novel antivirals. Bioluminescence imaging (BLI) has emerged as a powerful tool in the study of infectious diseases in animal models. The advent of influenza reverse genetics has enabled the incorporation of bioluminescent reporter proteins into replication-competent IAVs. This review briefly describes the current development and applications of bioluminescence in the study of viral infections and antiviral therapeutics for IAVs. BLI is expected to substantially accelerate the basic and applied research of IAV both *in vitro* and *in vivo*.

Keywords: Bioluminescence imaging (BLI); luciferase; replication-competent; influenza A virus (IAV)

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Introduction

Influenza virus is an enveloped virus that belongs to the family Orthomyxoviridae. It contains a single-stranded negative sense segmented genome (1). Based on the antigenic specificity of nucleoprotein (NP) and matrix protein (M), influenza viruses are classified into three types: A, B and C. Influenza A virus (IAV) is the major type that circulates in a variety of animals, such as birds, horses, dogs, pigs, and humans. IAV is further classified into 18 HA (H11–H18) and 11 NA (N1–N11) subtypes based on the serological properties of the primary viral surface proteins, hemagglutinin (HA) and neuraminidase (NA) (2,3). IAV remains a major threat to public health because of both seasonal epidemics and pandemics, which can occur periodically (4-6). A better understanding of the fundamental biology of IAV will accelerate the development of novel effective antiviral strategies to counter this reoccurring pathogen.

Animal models, especially mouse models, are essential for studying influenza virus infection, host responses, antiviral therapeutics and vaccines (7-10). Conventional assays that analyze IAV infection and the effects of antivirals require euthanization of animals at multiple time points and quantification of the viral titer at various anatomic sites. These experimental paradigms are often time and labor. Furthermore, these methods cannot meet the demands for real-time monitoring of the spatial and temporal progression of infection in the same living animal and cannot be used for high-throughput screening of antivirals *in vitro*.

In the past decade, bioluminescence imaging (BLI) has become a powerful tool for studying viral pathogenesis and the host immune response as well as for evaluating

the efficacy of antiviral strategies (11-14). In this review, we present current trends in the design and development of a replication-competent IAV carrying a bioluminescent reporter and its application.

Characteristics of bioluminescent proteins

Bioluminescent proteins are luciferase enzymes that catalyze light-producing chemical reactions in living organisms. Although many luminescent species exist in nature, only a few are commonly used in biomedical research: Photinus pyralis (firefly) luciferase (FLuc); sea pansy Renilla reniformis luciferase (RLuc); marine copepod Gaussia princeps luciferase (GLuc) (15-17); and a novel small luciferase enzyme called NanoLuc engineered from the deep-sea shrimp Oplophorus gracilirostris (18,19).

Firefly luciferase was the first luciferase reporter system that was used in mammalian cells (20,21). FLuc is 61 kDa in size and emits yellow to green light at a wavelength of 562 nm, which is less-readily absorbed by tissues than the blue light (480 nm) emitted by RLuc and GLuc. The substrate of FLuc, D-luciferin, has favorable pharmacokinetics and sufficient bioavailability because it uses adenosine triphosphate (ATP), O_2 and Mg^{2+} as cofactors. D-luciferin can be injected intraperitoneally and achieve a peak concentration within 10 min that remains stable for approximately 30 min in mouse models (22-24). Moreover, intranasal luciferin administration can also improve the bioluminescent signal in mice (25). D-luciferin can cross the blood-brain and placental barriers and has a wide distribution in animals (26). These properties of the firefly luciferase system have made it by far the most commonly utilized bioluminescent reporter for *in vivo* imaging.

RLuc and GLuc have smaller molecular weights than FLuc, 36 and 19.9 kDa, respectively (27-30). Both RLuc and GLuc use coelenterazine as substrates and produce an ATP independent bioluminescent reaction, which has flash kinetics and rapid onset and diminution of bioluminescence within 10 min in tissue culture and 1–2 min post intravenous administration in mice (31). The quantitative photons can be strongly affected by small variations in the time between substrate injection and imaging. Therefore, it is necessary to begin imaging rapidly at a fixed time after injecting coelenterazine. Increased background noise caused by oxidation in serum further reduces the bioavailability of this substrate. Despite these limits, the benefits of these enzymes are their relatively small size for flexible insertion into the viral genome, different optical properties and substrate, which allows discrimination from RLuc for multi-spectral imaging (32-34).

Recently, Hall *et al.* engineered a much smaller luciferase, 19.1-kDa NanoLuc luciferase (NLuc), from the deep-sea shrimp Oplophorus gracilirostris (18,19). The light output from the reaction of NLuc and its substrate furimazine, is 150-fold greater than both Renilla and firefly luciferase (18). However, like RLuc and GLuc, the blue-shifted light (460 nm) emitted by NLuc is readily absorbed by tissues, which hampers its utility in deep tissue studies.

Overall, BLI detects photons that are produced by the chemical reaction of luciferase enzymes and a defined substrate. High sensitivity, low background and real-time image analysis makes BLI a powerful tool in living animal studies. For BLI, FLuc is preferred over other luciferases due to its long wavelength, ease of use and effective diffusion of D-luciferin in animals. However, smaller luciferases have advantage in viruses that have the limited capacity of viral genomes, such as the influenza virus.

Generation of a replication-competent IAV carrying a luciferase reporter

The IAV contains eight negative-sense RNA segments in its genome (1). Unlike a positive-sense RNA virus, the naked genomic RNA of IAV cannot initiate viral replication. The vRNP complex, which is composed of viral RNA (vRNA), NP and polymerase proteins (PB2, PB1, and PA), is the minimal functional unit. Generation of IAV requires all eight functional vRNP complexes be delivered into the host cell nucleus to initiate the production of offspring infectious virions (1,35). The reverse genetics of IAV had not been well developed until the late 1990s (36,37). By using this technique, the influenza virus can be rescued from cloned cDNA. The most widely used eight-plasmid system retains bi-directional transcription of the viral cDNA template into both RNA pol I transcribed negative-sense viral RNA and RNA pol II transcribed positive-sense viral mRNA (38). The advantage of reverse genetics allows the incorporation of exogenous genes into the influenza viral genome, especially insertion of reporter genes. Since IAVs have a small genome, they are limited in their ability to accommodate relatively large reporter genes. Packaging signal sequences located at both the 3' and 5' ends of each viral RNA segment is another important factor that should be considered when designing complication-competent reporter influenza viruses (39-44). To date, multiple strategies have been employed to generate replication

competent influenza viruses carrying bioluminescent reporter genes.

Heaton *et al.* (45) chose PB2 as the target segment and inserted Gaussia luciferase sequences into the C-terminus of PB2 as a fusion protein, which was separated by a 2A peptide from the foot-and-mouse disease virus (FMDV) (46). For efficient packaging of the recombinant PB2-GLuc segment into the viral genome, they made silent mutations in the PB2 open reading frame (ORF) to eliminate the original packaging signals and then added a 120-nucleotide (nt) packaging signal after the GLuc insertion upstream of the 3' untranslated region (UTR). In chicken embryonated eggs, the rescued PR8-GLuc can be replicated to approximately 1×10^8 pfu/mL, which is 1 log lower in titer than that of the WT PR8. In BALB/c mice, the median lethal dose (LD_{50}) increased by 50 to 100 times compared to WT PR8. The inserted GLuc gene was demonstrated to be stable at least over four passages in eggs.

We constructed a IAV-luc by cloning the codon-optimized gene encoding Gaussia luciferase into the C-terminal end of the full-length NA-coding sequence; the gene was linked via a 2A autoproteolytic cleavage sequence from a porcine teschovirus (47,48). The growth of IAV-luc was approximately 2 logs and 1 log lower than the replication titers in Madin-Darby canine kidney (MDCK) and in eggs, respectively. Mice infected with 10^6 pfu of IAV-luc demonstrated similar weight loss and lethality to infection with 10^3 pfu of parental PR8 virus. Both bioluminescence detection and sequencing analysis showed that the chimeric NA-GLuc segment could be stably maintained in the viral genome for five passages in eggs.

Tran *et al.* (49) developed a reporter virus that encodes the small and bright NLuc appended to the C terminus of a PA segment in the background of the WSN virus. The PA fusions were further modified by inserting the 2A peptide form porcine teschovirus to create discrete PA and NLuc proteins from a polyprotein precursor. PA-2A-NLuc50, which had 50 nt of a repeated packaging sequence downstream of NLuc, was demonstrated to be able to replicate in culture and in mice with near-native properties, and the reporter construct was stably maintained.

The eighth RNA, the NS segment, encodes NS1 as well as the NEP/NS2 protein from a spliced mRNA. Reuther *et al.* (50) converted the NS segment into three ORFs encoding NS1, the RLuc/GLuc reporter and NEP/NS2, by two separate porcine teschovirus 2A peptides. Although the resulting virus-encoded luciferases revealed impaired viral growth compared to wild-type virus in infected cells, they stably expressed the reporter gene for up to four passages in human A549 cells. In their study, they discussed that larger reporter genes, such as firefly luciferase (about 2 kb) or β-galactosidase (about 3 kb), could not be inserted into the IAV genome, suggesting that there is a length restriction when selecting a reporter to generate a replication-competent reporter virus.

Unlike the above strategies of bioluminescent reporter viruses containing a fusion protein in one gene segment, Sutton *et al.* (51) rearranged both the PB1 and NS segments and used a 2A peptide to enable auto-cleaved expression of NS2 downstream of PB1 and expression of GLuc downstream of the full-length NS1 gene in the background of a 2009 pandemic virus strain. Although both amantadine-resistant and -sensitive GLucCa04 were significantly attenuated compared to the parental strain, expression of GLuc could be used as an indicator of amantadine sensitivity and anti-viral efficacy.

Applications of bioluminescent reporter influenza viruses

Visualization of a real-time IAV infection in living animals

As mentioned above, several research groups have successfully rescued replication-competent bioluminescent reporter IAVs. The benefits of using these reporter viruses is their ease of use, rapid tracking and ability to quantify viral replication in living mice at multiple time points without the traditional animal sacrifice and cumbersome virus titration of tissue samples. Furthermore, whole animal imaging allows investigators to identify unexpected sites of infection that might be missed by analyzing only selected tissues. Following intranasal infection of the influenza reporter viruses, luciferase enzymes can be expressed along with the viral replication in host cells. After injection of substrate to the infected animal, the light (photons) emitted by the luciferase-substrate reaction can be detected by using a very sensitive charge-coupled device (CCD) camera system. Image acquisition and bioluminescence measurements are controlled by computer analysis of emitted photons, allowing relative quantification of data.

Several groups have imaged IAV infection in living mice (45,47,49-51). Bioluminescence was detected 1–2 days post-infection in the chest and nasal passage of infected mice, indicating the initiation of infections. The intensity of photons increased and peaked over the course of infection

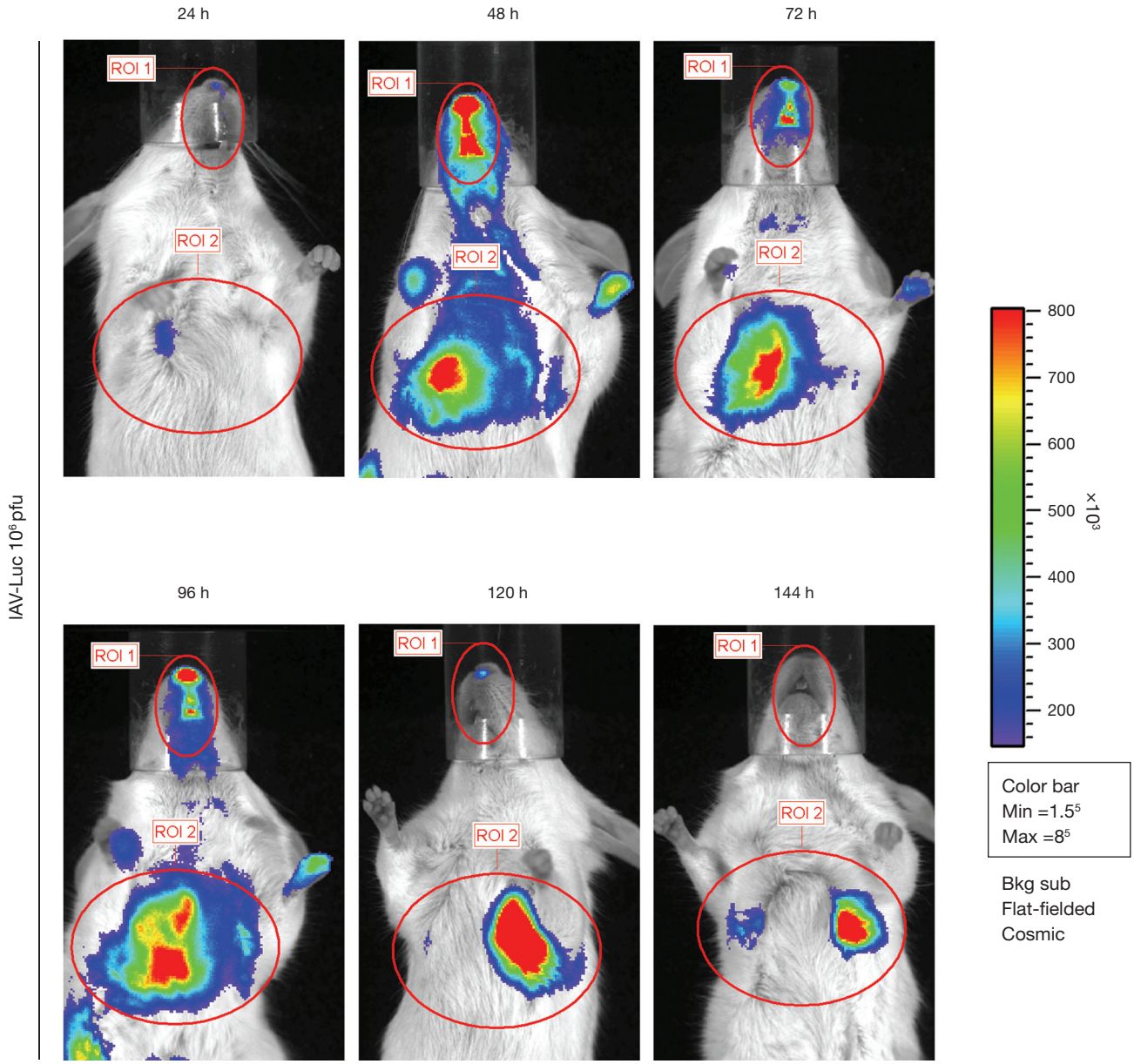

Figure 1 Bioluminescence imaging of mice infected with IAV-Luc virus. Balb/c mice (6–8 weeks old) were infected with 106 pfu of IAV-Luc. Mice were imaged by IVIS200 at 24 h intervals from 24 to 144 h post infection after injection of coelenterazine. IVA, influenza A virus; ROI, region of interest.

and then decreased corresponding to viral clearance by the host immune system. As shown in our previous study, mice were infected with IAV-Luc, the reporter influenza virus, which carrying Gaussia luciferase by nasal inoculation. Bioluminescence could be detected at 1 day after infection, peaked at 2 days and remained detectable for at least 6 days after initiating the infection (*Figure 1*) (47). A bioluminescent reporter virus could also be used to rapidly assess the ability of influenza viruses to replicate and/or transmit to a new host species by generating human strain- and avian strain-like reporter viruses and visualizing their infection in mice (49).

In addition to mice, Karlsson *et al.* utilized a reporter virus harboring Nanolight luciferase to investigate the dynamics

of influenza virus infection/transmission in ferrets (52). Bioluminescence was detected both in the upper and lower respiratory tracts of infected ferrets. The intensity of bioluminescence correlates well with the viral load in tissues. This system was then exploited to track airborne dissemination of influenza virus between infected and naïve ferrets. Similar to donor animals, bioluminescence was easily detected directly in the nasal wash of all contact ferrets. These results demonstrated the potential of BLI to assess the tissue distribution and transmissibility of infection in larger animal models.

Evaluation and screening of antiviral therapeutics

BLI enables easy and rapid quantification of virus replication, which makes it an efficient antiviral screening tool both *in vitro* and *in vivo*. Secreted expression of Gaussia luciferase into infected cell cultures is of significant advantage to develop high-throughput antiviral compound screening. Eckert and colleagues (53) demonstrated that the enzymatic activity of GLuc correlates with the viral titers produced by infected cells. For proof-of-principle, they established fast and sensitive assays to screen the antiviral activity of the neuraminidase inhibitor (NAI) zanamivir, host cell interferon-inducible transmembrane (IFITM) proteins 1–3 and a modulator for endosomal cholesterol. By using amantadine sensitive strains of GLucCa04 reporter viruses, the detected IC_{50} values of amantadine were consistent with the published values. Furthermore, Sens/GLucCa04 has the potential to accelerate *in vitro* antiviral screening in cells by shortening the incubation period to 16 hours and less than 10 min for luciferase detection (51).

The possibility of using BLI to evaluate the efficacy of monoclonal antibodies and antiviral serum was also tested in living mice (45,47). The ability of the neutralizing antibodies to alleviate influenza virus infection has been examined by passive transfer experiments (54). IVIS imaging showed that both the luciferase-positive area and signal strength of antibody-treated mice significantly reduced compared to the levels of control-treated mice. The viral titers of lung homogenate l also had good correlation with quantification of the photo flux. It is important to note that the imaging of mice can clearly differ between antiviral serum treated mice and untreated mice at a very early stage when there is no difference in body weight changes. These results confirmed that BLI analysis allows convenient and highly sensitive prediction of an antibody's therapeutic outcome *in vivo*.

The development of a high-throughput screening protocol for the identification of novel antivirals against influenza and other infectious diseases is urgently needed to treat emerging resistant mutants. Yan *et al.* established an efficient human cellular co-infection system of respiratory syncytial virus (RSV) carrying the firefly luciferase reporter combined with IAV harboring nano-luciferase (55). By using this system, they developed and validated a high throughput screening (HTS) assay for the simultaneous discovery of pathogen- and host-targeted hit candidates against either IAV or RSV. In a proof-of-concept screen of a compound library, this dual-pathogen protocol had high efficiency and a good cost.

Neutralizing antibodies play a major role in protecting against IAV infection and disease. The standard assay currently used to measure IAV neutralization is the microneutralization (MN) assay, which is divided into two parts: a virus neutralization assay and enzyme-linked immunosorbent assay (ELISA) to detect the presence of NP protein in infected cells (56). However, ELISA is time consuming and not readily adaptable to high-throughput technology. Attempts have already begun to develop a simple, rapid, high-throughput IAV MN assay by using bioluminescent reporter IAVs, which could be of great value to influenza vaccine development (51).

Conclusions

In summary, by developing and applying replication-competent reporter influenza viruses carrying the luciferase enzyme, BLI has been proven to be a powerful tool for studying and monitoring viral infections, screening antiviral compounds, and detecting specific or broadly reactive NAbs of IAV *in vivo* and *in vitro*. However, the small genome of IAV is much less tolerant of large foreign insertions. In this regard, developments of novel small luciferases or variants that produce a high yield signal near the infrared wavelength, and the better understanding of the regulation of viral genome replication and gene expression, are still needed to further improve bioluminescence technology imaging in IAV.

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Footnote

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