Highly Sensitive Real-Time In Vivo Imaging of an Influenza Reporter Virus Reveals Dynamics of Replication and Spread

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The continual public health threat posed by the emergence of novel influenza viruses necessitates the ability to rapidly monitor infection and spread in experimental systems. To analyze real-time infection dynamics, we have created a replication-competent influenza reporter virus suitable for in vivo imaging. The reporter virus encodes the small and bright NanoLuc luciferase whose activity serves as an extremely sensitive readout of viral infection. This virus stably maintains the reporter construct and replicates in culture and in mice with near-native properties. Bioluminescent imaging of the reporter virus permits serial observations of viral load and dissemination in infected animals, even following clearance of a sublethal challenge. We further show that the reporter virus recapitulates known restrictions due to host range and antiviral treatment, suggesting that this technology can be applied to studying emerging influenza viruses and the impact of antiviral interventions on infections in vivo. These results describe a generalizable method to quickly determine the replication and pathogenicity potential of diverse influenza strains in animals.

Influenza A virus is a serious public health threat. Each year, 3 to 5 million people are infected with influenza virus, resulting in 250,000 to 500,000 deaths (1). Infections from seasonal influenza viruses cause upwards of ~24,000 to 36,000 deaths per year in the United States alone (2). Pandemic viruses have the potential for significantly higher mortality rates, with the 1918 outbreak estimated to have caused upwards of 50 million deaths (3). Reinforcing the immediacy of these concerns, the reassortant H7N9 virus recently isolated from patients in China currently displays a case fatality rate approaching 25% (4–6).

Animal models of infection are useful for determining the pathogenicity and transmission potential of emerging influenza viruses (7–9). Mice and ferrets are routinely used to study influenza virus infections, host defenses, and responses to antiviral therapies or vaccines. However, animal models of influenza virus infections are constrained by the inability to monitor viral dynamics in real time. Additionally, in mouse models viral load determination typically requires sacrificing the animal, precluding longitudinal assessment. In vivo imaging of bioluminescent reporter viruses is a powerful alternative that allows real-time detection of viral load and spread in the same animal over time (10). This strategy has been exploited in animal models for multiple viruses, including dengue virus (11, 12), herpes simplex virus 1 (HSV-1) (13), Sindbis virus (14), vaccinia virus (15), and Sendai virus (16).

Generation of a replication-competent influenza reporter virus is confounded by the complex architecture of the segmented genome: (i) all of the viral genes are critical in vivo, precluding simple replacement with the reporter; (ii) the compact genome does not tolerate large insertions, which can destabilize the genome and are lost over time (17–19); (iii) most insertions severely attenuate replication (17, 20, 21); and (iv) insertion of reporter gene sequences at either end of the coding region disrupts packaging signals essential for virion assembly that are present in both the untranslated region (UTR) and the viral open reading frame (ORF) (22, 23). Some of these limitations have been addressed by inserting foreign sequences into viral genes to create protein fusions or polyproteins (17, 20, 21, 24, 25). Alternatively, entire viral genes have been replaced with foreign sequence and reporter viruses were grown in specialized complementing cell lines (18, 19, 26, 27). In extreme cases, an additional genome segment carrying extra sequence was inserted into virions by re-engineering viruses to package nine viral RNAs, as opposed to the normal complement of eight (28). Whereas these reporter viruses have been used successfully for in vitro studies and most recently with limited sensitivity in animal infections (17, 20), the genomes containing the reporter are generally unstable and/or the reporter viruses are highly attenuated. As such, no reporter has yet been developed that closely mimics the native virus, especially in animal infections.

We have overcome these limitations by using the very small and extremely bright luciferase variant NanoLuc (NLuc) to develop a replication-competent reporter virus suitable for in vivo imaging. NLuc is a 19-kDa engineered luciferase that possesses ~150-fold-greater specific activity (i.e., light output) than both Renilla and firefly luciferases (29). We show that an NLuc reporter virus replicates with near-native properties in culture and in vivo. Moreover, the NLuc reporter virus possesses pathogenicity and lethality in mice that is indistinguishable from those of the parental virus. We used this virus to perform in vivo imaging and to track viral load and dissemination of influenza virus infections in the lungs of mice. We further show that the reporter virus recapitulates known restrictions due to host range and antiviral treatment, suggesting that this technology can be applied to studying emerging influenza viruses and the impact of novel antiviral interventions on infections in vivo. These findings suggest that bioluminescent imaging of NLuc reporter viruses can be used to rapidly assess the properties of emerging viruses and screen the efficacy of candidate vaccines or antiviral therapies.

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MATERIALS AND METHODS

Plasmids, cells, and antibodies. The virus rescue plasmids pTM-pol/WSN-All (a kind gift from Y. Kawaoka [30] and pTM-ΔRNPs [31]) express viral RNAs (vRNAs) from a genomic sequence of an influenza virus (A/WSN/33 [WSN]) flanked by a polymerase (pol) I promoter and terminator. pBD-PB1, -PB2, -PA, and -NP contain bidirectional pol I and pol II cassettes to express both vRNA and mRNA [32, 33]. NanoLuc (NLuc) sequences were amplified from pNL1.1 (Promega). p3X-IT expresses PB1 with a tandem affinity purification (TAP) tag, PB2, and PA. NP was expressed from pCAGGS-WSN-NP [30]. The vRNA-luciferase reporter plasmid pH21-vNA-NLuc contains the firefly luciferase-coding sequence in the antisense orientation flanked by untranslated regions of the vNA gene segment from the A/WSN/33 isolate and was previously described [34].

A549, 293T, MDBK, and MDCK cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Calu-3 cells were grown in DMEM/F12 supplemented with 20% FBS (HyClone). Infections were performed in media supplemented with 0.3% bovine serum albumin (BSA), 25 mM HEPEs, 1X penicillin/ streptomycin, and 0.25 to 0.5 μg/ml N-tosyl-l-phenylalanine chloromethyl ketone (TPCK)-trypsin. All cells were grown at 37°C in 5% CO₂. PA antibodies were raised in rabbits (kindly provided by T. Parlow [34]).

Creation of PA-NLuc constructs. PA fusions were created in pBD-P-A by Gibson Assembly (NEB) as diagrammed (Fig. 1A). Briefly, the NLuc coding sequence and stop codon were placed downstream of the PA sequence to create a contiguous ORF. Translation of this sequence appends NLuc to the C terminus of PA. Native packaging sequences were restored by repeating the terminal 30, 40, or 50 nucleotides (nt) of the ORF (including the stop codon) after the NLuc stop codon adjacent the native UTR. Where indicated, sequence encoding the “self-cleaving” 2A peptide from porcine teschovirus was placed between the PA sequence and the NLuc sequence [35]. Direct repeats were removed from the reporter gene by introducing 18 silent mutations in the wobble positions at the 3‘ end of the PA ORF.

Polymerase activity assays. 293T cells were reverse transcribed in triplicate with TransIT-2020 (Mirus) with vectors expressing PB1, PB2, NP, and the indicated PA proteins in addition to the reporter pHH21-vNA-NLuc. Cells were lysed 24 to 36 h posttransfection, and luciferase activity was measured with a luciferase assay system (Promega). Western blotting was performed on lysates to confirm equivalent levels of transfection and expression.

Virus rescue, titration, and multicopy replication. Virus was rescued by reverse transfection of a 293T/MDBK coculture using TransIT-2020 (Mirus). To obtain native WSN, cells were reverse transcribed with pTM-pol/WSN-All, p3X-IT, and pCAGGS-WSN-NP. PA fusion viruses were obtained by transfection with pTM-ΔRNPs, pBD-PB1, -NP, -PB2 (or PB2 K627E), and the desired pBD-PA-NLuc fusion. Wild-type (WT) and PB2 K627E PA-2A-NLuc50 viruses were plaque purified. Viruses were subsequently amplified in MDBK cells or embryonated eggs, and titers were determined by plaque assay performed on MDCK cells. Plaque sizes measured with ImageJ. Multicycle replication assays were performed on lysates to confirm equivalent levels of transfection and expression.

Nano-Glo viral titer assay. A Nano-Glo viral titer assay was developed to establish NLuc activity as a proxy for traditional forms of viral titer (e.g., PFU/ml or tissue culture infective dose [TCID₅₀/ml]). MDCK cells were grown to confluence in a white 96-well plate and infected in at least triplicate with 20 μl of viral supernatant for 1 h. Cells were washed to remove the inoculum (and any residual NLuc produced in previous infections) and incubated for 8 or 16 h. NLuc activity was measured in situ with the Nano-Glo assay system (Promega), and luminescence was detected with a plate reader (Tecan). For antiviral treatment, cells were incubated with 100 μg/ml ribavirin for 2 h prior to infection; ribavirin was then present throughout the infection.

Mouse infections. Female BALB/c mice (Jackson Laboratory) (4 to 6 weeks old) were inoculated intranasally with the indicated amount of virus in 25 μl under light isoflurane anesthesia. Body weight was monitored daily. Mice losing >20% of their original body weight were humanely euthanized. Viral load in lung homogenates was determined by TCID₅₀ and the Nano-Glo viral titer assay. In vivo imaging was performed with an IVIS 200 imaging system on anesthetized mice. Nano-Glo reagent was diluted 1:20 in PBS, and 100 μl was injected retro-orbitally. Image acquisition and analysis were performed with Living Image software (PerkinElmer). Flux measurements were acquired from regions of interest automatically gated to the signal contours. All data in composite images utilized the same scale. Mouse experiments were performed under protocols approved by the University of Wisconsin—Madison Institutional Animal Care and Use Committees.
Statistical Analysis. Data represent means ± standard deviations (n ≥ 3) unless otherwise noted. Curve fitting was performed in Excel, and goodness of fit is depicted by R². Where indicated, a comparison between groups was performed by a two-tailed Student’s t test. Significance is defined as P < 0.05 and is indicated when necessary with an asterisk (*).

RESULTS

Development of a bioluminescent influenza reporter virus. Creation of an influenza reporter virus requires that the inserted reporter gene neither interfere with functions of the native viral proteins nor disrupt the packaging signals of the native gene segment. The polymerase subunit PA has previously been shown to tolerate fusion of exogenous protein sequence to its C terminus without disrupting polymerase function (37). In addition, a minimal packaging sequence has been identified in the PA gene (23).

Based on this information, we attempted to make a replication-competent reporter virus by creating a series of fusion proteins with NanoLuc (NLuc) appended to the C terminus of PA. Reporter constructs were created in a WSN background (Fig. 1A). Where indicated, the PA fusions were further modified by inserting the self-cleaving 2A peptide from porcine teschovirus to create discrete PA and NLuc proteins from a polyprotein precursor (35).

As packaging sequences have been identified in the end of the PA ORF (23, 38, 39), we also duplicated increasing portions of the PA ORF downstream of the NLuc stop codon.

To confirm the functionality of PA in the fusion proteins, polymerase activity assays were performed in 293T cells. PA-NLuc fusions were expressed with the remaining polymerase subunits PB1 and PB2, the viral nucleoprotein (NP), and a firefly-based luciferase reporter construct. Firefly luciferase and NLuc utilize different substrates and are thus suitable for multiplexing (29).

All PA fusions, regardless of the inclusion of the 2A peptide or repeated packaging sequences, supported robust polymerase activity within ~2-fold of the native PA level (Fig. 1B). Western blotting confirmed relatively equivalent levels of expression of all PA proteins (Fig. 1B). Blotting also showed effectively complete separation of PA and NLuc polypeptides when the 2A peptide was present. PA-NLuc fusions without the 2A peptide also produce small amounts of protein with a molecular mass similar to that of native PA, suggesting the presence of breakdown products or premature terminations. Based on these results, PA-NLuc genes containing the 2A peptide were used in subsequent experiments to minimize the possibility that the fusion would interfere with other PA functions, such as trafficking and assembly.

WSN-based reporter viruses were rescued with PA-2A-NLuc genes containing restored packaging signals (PA-2A-NLuc30, -40, and -50). Our initial experiments demonstrated that viruses with only 30 or 40 nt of repeated packaging signals displayed obvious attenuation compared to strain WSN (data not shown); therefore, we focused on virus with 50 nt of repeated packaging sequence, which we termed PA-2A-NLuc50 (PATN). A high-throughput Nano-Glo viral titer assay was developed to rapidly detect luciferase activity as a consequence of viral infection. The sensitivity and dynamic range of the assay were determined by performing infections with known amounts of input PATN virus. MDCK cells were infected in a 96-well format with reporter virus for 16 h and subjected to a Nano-Glo viral titer assay. The Nano-Glo viral titer assay detected robust luciferase activity, with a linear range over 4 logs of input virus (Fig. 2A). The assay was extremely sensitive, as infection with 0.1 PFU of virus routinely produced a signal greater than 5-fold above the background (P < 0.005). This raises the possibility that the Nano-Glo viral titer assay may be more sensitive than plaque assays. Additionally, the Nano-Glo viral titer assay may detect infection by the recently proposed “semi-infectious” particles that express viral proteins but cannot otherwise form a plaque (40), thus increasing sensitivity relative to that of plaque assays. Further experimentation is required to make a definitive determination.

In addition, while plaque formation may require 48 to 72 h, the Nano-Glo titer assay quantitatively measures viral levels in as little as 8 h (data not shown). The Nano-Glo titer assay was repeated in the presence of ribavirin to confirm that the luciferase output was a product of viral gene expression and not the result of NLuc carried forward in the viral supernatant or fortuitously packaged into virions. Ribavirin treatment reduced NLuc activity to below the level of detection at low doses of input virus and reduced activity by 2 to 3 logs compared to that seen with untreated samples at higher doses (Fig. 2A). Ribavirin does not directly inhibit NLuc activity (data not shown). Thus, the Nano-Glo titer assay is a sensitive, replication-dependent measure of input virus levels. Our results with ribavirin further show that this assay is highly responsive to therapeutic interventions and thus may be suitable for rapid screening of antiviral compounds or antibodies.

NLuc activity in the Nano-Glo titer assay was directly related to the amount of input virus, suggesting that NLuc activity could be used to measure titers in samples with unknown amounts of virus. To determine the utility of the reporter gene as a readout of viral level, PATN was used to initiate a multicycle infection in human Calu-3 lung cells. Titers were determined by both plaque and Nano-Glo assays. Viral titers determined by standard plaque assay paralleled the NLuc activity detected in our Nano-Glo titer assay (Fig. 2B). These data also established a linear relationship between PFU and relative light unit (RLU) measurements throughout the time course (R² = 0.990; Fig. 2B, inset). These results demonstrate that the Nano-Glo titer assay serves as a direct measure of viral titer similar to plaque assay results.

Previous influenza reporter viruses were unstable and rapidly lost the reporter coding sequence, possibly due to the presence of direct repeats in the reporter gene (17, 19). Consequently, 18 silent mutations were introduced into the 3’ end of the PA ORF to remove the direct repeat arising from duplication of the packaging signal (Fig. 1A). This construct with codon-swapped mutations was used to create the PA-SWAP-2A-NLuc50 (PASTN) virus. The properties of the PASTN reporter virus in culture relative to the parental virus were determined in multicycle replication assays. MDBK cells were infected with either WSN or PASTN, and viral titers were determined by plaque assay (Fig. 2C). PASTN reached high titers, replicating with kinetics similar to native WSN kinetics. PASTN titers were ~0.5 log lower than the parental strain titers throughout the time course, although the difference in titers was statistically significant only at 24 and 39 h postinfection (lpi) (P < 0.05) (Fig. 2C). Similar patterns of replication were observed during WSN and PASTN infections in human lung A549 cells (data not shown). We also amplified PASTN in embryonated eggs as this is a common source of viral stocks. PASTN grew to average titers of 1.1 × 10⁷ PFU/ml, which was slightly less than but not significantly different from those determined for WSN, which grew to average titers of 6.1 × 10⁷ PFU/ml (n = 3 eggs, P = 0.32). Plaque size was measured during the course of these experiments. Plaques for PASTN were less sharply defined and ~80% of the di-
PA-2A-NLuc permits exquisitely sensitive detection of an influenza reporter virus. (A) Nano-Glo viral titer assay performed with defined amounts of input virus. Where indicated, cells were pretreated with 100 μg/ml ribavirin, which was then present throughout the assay. A trend line (gray dashed line) was fitted to untreated samples. *, P < 0.005; n = 4 ± sd. (B) Multicycle replication in human Calu-3 lung cells infected at MOI = 0.001. Viral titers were determined by both a plaque assay and a Nano-Glo viral titer assay (n = 3 ± sd). The limit of detection for luciferase activity is indicated as a black dashed line in panels A and B. To demonstrate the relationship between the two titer measurements, RLU was plotted as a function of PFU (inset). (C) Multicycle replication kinetics of native and PASTN viruses in MDBK cells infected at MOI = 0.01. Viral titers were determined by a plaque assay. Titers of WSN and PASTN at 24 and 39 h were significantly different (*, P < 0.05; n = 3 ± sd). (D) The PASTN gene is stable in vivo and through multiple passages in culture. RT-PCR was performed on RNA recovered from viral stocks (passage 0 [P0]), mouse lung homogenate from 5 dpi, or virus following serial passage in MDBK or Calu-3 cells. The PCR product was analyzed by agarose gel electrophoresis. Expected product sizes for native PA and the full-length reporter gene are indicated.

Real-time in vivo imaging of influenza virus infection. In vivo imaging of reporter viruses has proven useful in the study of replication dynamics and spread for many viral systems, including Sindbis virus (14), dengue virus (11,12), HSV-1 (13), and vaccinia virus (15). To apply this approach to influenza virus, we used PASTN in an attempt to visualize influenza virus replication in the mouse model. BALB/c mice were infected with reporter virus and subjected to longitudinal weighing and in vivo imaging (Fig. 3A). Bilateral multifocal bioluminescence was detected as early as 2 dpi, indicating the initiation of infections in both the right and left lobes of the lung. Light output, measured as flux, increased over the course of infection and peaked on day 6 when the animal reached the experimental endpoint. The increase in flux accompanied spread of virus throughout the lungs and weight loss in the mouse, demonstrating that the NLuc virus was capable of supporting a pathogenic infection. Whole-body imaging has the potential to detect viral spread without a priori knowledge of tissue tropism. While WSN has the potential to initiate neurotropic infections, primarily by intracerebral inoculation (41), bioluminescence was not detected in the brain and virus was not recovered from the brain in any of our experiments. Whether bioluminescent imaging of this reporter virus will quantitatively detect extrapulmonary replication remains to be determined.

For the reporter virus to be most useful in animal models, it
must replicate with properties similar to those of the parental strain. The replicative capacity and pathogenicity of PASTN in mice were thus directly compared to those of WSN, the parental virus from which it was derived. Cohorts of mice were infected with $10^4$ PFU of PASTN or WSN and weighed daily. In addition, one set of mice infected with PASTN was imaged each day and euthanized to determine viral titers in the lung (Fig. 3C). Viral lung titers were also determined each day for a set of mice infected with WSN. Weight loss results were indistinguishable between PASTN and WSN throughout the infection time course (Fig. 3B), with WSN. Weight loss and bioluminescence was near background levels throughout the experiment. Nonspecific bioluminescence was occasionally detected in the eye at the site of substrate injection. Thus, the reporter virus faithfully recapitulates the known polymerase-mediated restriction due to host range and could therefore also be used with newly isolated influenza virus strains to determine their host range and the role of species-specific adaptive mutations.

In a final series of experiments, mice were infected with serial dilutions of PASTN to simultaneously measure the lethality and sensitivity of the reporter virus. Groups of four mice were infected with $10^5$, $10^3$, or $10^4$ PFU of PASTN and monitored daily for weight loss. Imaging was performed throughout. Weight loss and viability displayed a strong dose-dependent effect (Fig. 4A and B). All of the mice inoculated with $10^4$ PFU and a majority of those infected with $10^3$ PFU succumbed to infection, whereas half of the mice infected with $10^2$ PFU survived. These initial experiments suggest that the minimal lethal dose (MLD$_{50}$) of PASTN in BALB/c is $\sim 10^2$ PFU, in remarkable agreement with the reported MLD$_{50}$ for WSN of $10^3$ to $10^{3.4}$ PFU (27, 51–53). This is a significant improvement over other reporter viruses that show an MLD$_{50}$ 50 to 100 times higher than the parental MLD$_{50}$ (17, 20).

Longitudinal imaging of the same mouse from each group revealed that viral levels and dissemination in the lungs increased...
faster with higher doses, concomitant with weight loss (Fig. 5C). Notably, imaging of PASTN is sensitive enough to detect viral clearance of a sublethal infection (Fig. 5D). Combined, these data suggest that the PASTN reporter virus replicates with properties very close to those of the parental strain and, unlike previous influenza reporter viruses, shows very little attenuation in culture or in vivo. Further, bioluminescence detected by in vivo imaging of this extremely sensitive reporter virus measures viral localization, spread in the lungs, and levels in the infected animal.

DISCUSSION

The continual emergence of novel influenza viruses requires the ability to quickly assess their pathogenicity, pandemic potential, and susceptibility to antiviral interventions. Public health responses to outbreaks rely heavily upon experimental determination of viral pathogenicity and transmission capabilities. The rapid acquisition of these data has been hampered by the inability to monitor influenza virus replication in animals in real time. Here we describe a novel influenza reporter virus capable of simultaneously measuring viral load and dissemination in vivo. This virus permits longitudinal measurements in the same animal throughout the course of infection. The virus was engineered to maintain native protein expression and packaging signals. As such, the replication characteristics of the reporter virus are nearly identical to those of the native virus. Furthermore, the reporter virus enabled rapid detection of viral replication and titers in culture. Thus, our influenza reporter virus is a powerful tool to visualize infection in animal models and quantitatively measure replication in culture.

The NLuc reporter virus permits exquisitely sensitive detection of viral replication. The sensitivity and noninvasive longitudinal measurements made possible the imaging of infection and clearance of a sublethal dose of PASTN in BALB/c mice, a mouse background that is relatively resistant to influenza virus (54) (Fig. 5). In a mouse inoculated with the low dose of $10^2$ PFU, infection began primarily in the left lung. By 5 dpi, two foci appeared on the right side, indicating spread of virus to the lobes in the right lung. Flux decreased in the left lung by 6 dpi, suggesting that the infection had begun to resolve. Unexpectedly, flux measurements showed that large amounts of virus were again present in the left lung at 7 dpi, accompanied by an 8% drop in body weight. The infection then continued to clear and was undetectable at 13 dpi. Viral lung titers were determined at 14 dpi, and no virus was detected. While only a single example, this time course alludes to the influenza viral dynamics that in vivo imaging can detect, with the potential to elucidate mechanisms of dissemination in the lungs, immune escape, or emergence of antiviral resistance. It will be important to determine if this approach is viable in ferrets, the currently accepted best animal model of human influenza virus infection.

Our NanoLuc reporter virus complements two other recently described Gaussia luciferase (GLuc) viruses. GLuc was incorporated into the NS gene of a rearranged influenza virus genome engineered to create vaccine candidate viruses (21). Whereas versions of the rearranged virus stably expressed GLuc, the rearranged virus was severely attenuated in culture and displayed a complete loss of pathology in mice. While beneficial for vaccine safety, the dramatic reduction in viral growth suggests a limited utility of this rearranged genome as a reporter virus. GLuc was also incorporated into the viral genome of PR8 (20). Using an approach similar to the one described here, GLuc was encoded as a polyprotein on the PB2 segment. The PR8-GLuc virus supported in vivo imaging, although high doses of virus (15 to $1,500$ MLD$_{50}$) were used in the DBA.2 mouse, a background that is highly sensitive to influenza virus infection (54). In addition, the PR8-GLuc virus was highly attenuated, with an MLD$_{50}$ 50- to 100-fold higher than the parental PR8 MLD$_{50}$. This contrasts with the PASTN strain described here, which displays robust bioluminescence during even sublethal infections with 0.1 MLD$_{50}$ as well as pathology and mortality that are indistinguishable from those of the parental WSN. Nevertheless, both PR8-GLuc and PASTN have the potential to make significant contributions to understanding the spatiotemporal dynamics of influenza virus replication in vivo. Given the modular nature of the NLuc reporter construct, this approach should be broadly applicable to any influenza virus isolate and may also be amenable to other small reporters. To that end, we have already rescued a NLuc reporter virus encoding the RNP genes from the patient isolate A/Utah/01/2009 from the 2009 pandemic (data not shown). Thus, reporter viruses will be useful in monitoring replication in existing strains, and their ease of construction will make them particularly useful in rapidly examining the replication characteristics of emerging strains as well.

Positron emission tomography (PET), fluorescence, and bioluminescence elicited by firefly luciferase are all compatible with NanoLuc imaging. Thus, multiplexing influenza luciferase viruses...
with other in vivo imaging modalities could simultaneously measure viral replication and the host response. For example, previous experiments monitoring inflammatory responses to influenza virus infection via PET imaging could be coupled with PASTN to determine the impact of the viral load and the site of replication on the severity of the response (55). Similarly, recruitment of discrete immune cell populations to sites of infection can be monitored by infecting mice with PASTN and detecting immune cells that have been labeled ex vivo with tracking dyes (56) or by detecting fluorogenic probes that are selectively activated by specific immune cell populations (57). Alternatively, innate immune responses and viral replication could be measured concurrently by using PASTN to infect reporter mice that drive firefly luciferase from an NF-κB or IFN-β promoter(s), as NLuc and firefly luciferase exclusively use different substrates (29, 58, 59). Multiplexing could also be used to study the interplay between primary viral infections and the development of secondary bacterial pneumonia by performing coinfections with PASTN and bioluminescent Streptococcus (60). Thus, with near-native replication properties, a stable genome, and a very strong bioluminescent output, our influenza reporter virus provides a noninvasive spatiotemporal measure of viral replication that now enables in vivo studies of the interplay between infection, host response, and disease severity.

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REFERENCES


